YEAST ENOLASE: MECHANISM OF ACTIVATION BY METAL IONS

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I. INTRODUCTION

Enolase, or 2-phospho-D-glycerate hydrolyase (E.C.4.2.1.11), catalyzes the dehydration of 2-phosphoglycerate or, since the reaction involves a relatively small free energy change (approximately 1 kcal/mol),[1] the hydration of phosphoenolpyruvate. The enzyme itself, as an integral part of the glycolytic reaction sequence, is probably ubiquitous. Its property of major interest to biochemists, however, is that all known enolases exhibit an absolute requirement for a limited number of divalent cations for activity.[2-4]

While enolases have been purified (some to homogeneity) from many sources,[5] the best characterized and most extensively studied enolase is that from yeast. This is both because of its ease of purification in large quantities[6] and its high stability to most laboratory manipulations.[7,8] The properties of this enzyme have been discussed in several reviews,[5,9,10] the most recent of which was published in 1971. The most recent review[5] concentrated on comparative aspects of the structure and activity of enolases from various sources. This review will emphasize recent developments concerning the mechanism by which some metal ions produce enzymatic activity in the yeast enzyme, with brief mention of what appears to be a similar mechanism shared by a number of other metal ion dependent enzymes.

II. THE STRUCTURE OF YEAST ENOLASE

A. Homogeneity of the Enzyme — "Isozymes"

Multiple electrophoretic forms of enolases have been demonstrated in several species[5] and also occur in preparations of the enzyme from yeast ("the simplest eucaryote").[11] For example, Susor et al.[12] using isoelectric focusing techniques, showed that this enzyme, along with some others, consisted of forms with different isoelectric points. However, these are partly artifacts of the isolation conditions. Westhead and McLain showed that yeast enolase could be converted by prolonged autolysis or prior storage of the yeast to species which are separable by TEAE chromatography.[6] These had the same molecular weight, amino acid composition, and kinetic parameters, and were ascribed to deamidation of a single active ("A") form of the enzyme. Three enzymatically active forms of the enzyme with different isoelectric points have been separated using countercurrent distribution,[13] though the isolation conditions, which include dialysis of partially purified fractions, would appear to favor production of species with lower isoelectric points through deamidation.

Susor et al. also showed that even the presumably homogeneous and native "A" form had subspecies with different isoelectric points.[12] This finding was confirmed by Mann et

al.[14] using the same technique. However, the apparent heterogeneity of the A form originates in instability of the enzyme under some conditions. Recently it has been shown that the major form of the A enzyme, with pI = 6.05, slowly converts to active forms with more alkaline isoelectric points on isoelectric focusing.[15] The extent of the conversion is inversely proportional to the amount of protein focused and is a function of focusing time, so it was concluded that the conversion is related to subunit dissociation.* So the A form of the enzyme is inherently highly homogeneous, but forms more alkaline-focusing (and probably less stable) species on dissociating.

While deamidation and conformational rearrangements are factors in isozyme production, there has been a suggestion that the A and B forms separated using TEAE chromatography have inherently different amino acid sequences, specifically around the single cysteine residue.[16] The first 25 to 30 residues in both the A and B forms from brewer's and baker's yeast enolases are the same, however.[17]

It has been discovered recently that two genes code for enolase.[18] The gene products differ in amino acid sequence by 20 single residue substitutions, 13 of which occur in the first half of the molecule (see Section II.B). The substitutions would make the two proteins differ by a potential 5.5 charges (at pH 7). Still, the chemical sequence data on the A form of the enzyme, which corresponds to Holland et al's[18] p *eno* 46 form, show no sign of contamination with the other gene product, though according to Holland et al.[18] both genes are expressed, and indeed what we call the A form appears to be the less abundant form of the enzyme. There are, however, three more methionines per subunit in the other (p *eno* 8) form, and an examination of sulfur or methionine contents reported for the enzyme by Warburg and Christian[2] and by Malmstrom et al.[19] suggests that everyone has been working with the A form of the enzyme. For example, Warburg and Christian[2] reported 8 sulfurs per 67,000 g of enzyme, which would be 11.1/93,000 g. The sequence (below) shows 12 sulfur-containing residues — 2 cysteines and 10 methionines — in the A enzyme, and according to Holland et al.[18] the other form has 18 — 2 cysteines and 16 methionines. Incidentally, the sequences around the cysteines are the same, contradicting the suggestion of Oh et al.[16]

Since enolase is normally prepared using the first three steps of the method of Warburg and Christian,[2] this suggests that only the A form survives these first three steps in the purification procedure. The author's experience has been that major losses of activity occur in the third, ethanol step; about 60% of the activity precipitates in the 0 to 35% ethanol fraction.[20] Attempts to purify the enzyme in the 0 to 35% fraction have so far been unsuccessful. It is possible that this is where the two forms are separated.

The question arises as to the reason for two separate genes for this enzyme. The yeast in nature relies on glycolysis for its energy, its normal habitat being the skins of fruits.[18] It would seem reasonable if the second gene were a "backup" to prevent a single mutation from knocking out a crucial protein. Two other enzymes are found in high concentration in yeast, phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase, and duplicate genes appear to exist for them also.[21]

The data of Holland et al.[18] showed the two genes were not derived from mixtures of two varieties of yeast whose enolases had different sequences. Indeed, even the A enzymes from brewer's and baker's yeast are experimentally indistinguishable as far as amino acid composition[6] and amino-terminal sequence.[12] In further discussion of the properties of yeast enolase, however, we will be referring to the A form of the enzyme from baker's yeast unless otherwise stated.

* Endogenous magnesium ion, which reduces subunit dissociation (see Section II. D), is stripped from the protein on focusing.

B. Composition and Sequence

The enzyme appears to consist entirely of amino acids. The strongest evidence for that statement is that 95% of the sequence was established by chemical methods and in no instance was a modified (e.g., glucosylated) residue obtained.[17] Amino acid analyses showed no amino sugars were present,[22] and chemical analyses for carbohydrate showed no carbohydrate was present.[23] It should be noted that sialic acid was not measured. Some preparations of the enzyme contain a mysterious organic phosphate compound.[23] This may be removed by heat denaturation of the enzyme. It does not appear to have anything to do with enolase activity.

The amino acid composition of the A form of the enzyme from baker's yeast is given in Table 1,[17] along with the composition as calculated from the data of Malmström et al.[19] The compositions are in fair agreement, at least with respect to the methionine content (see above). The most notable features from the standpoint of determining the amino acid sequence are 14 arginines, 5 methionines, and a single cysteine.

The amino acid sequence of the enzyme is presented in Figure 1. This was determined both from the sequence of the gene[18] and by chemical methods.[17,24] Although there was some communication between the groups as the work progressed, the overall results were independently in good agreement. Each technique strengthened and complimented the other and the overall sequence of 436 residues is believed highly reliable.

For the sequence determination using chemical methods, cleavage at the single cysteine using nitrothiocyanobenzoic acid, at the 5 methionines using cyanogen bromide, and at the 14 arginines using trypsin (after acetylation of lysines) were employed.[17] For the determination from the DNA sequence,[18] the genes for the enolases were isolated using the messenger RNA for these enzymes. Isolation of the messenger RNA was described previously.[21] The isolated genes were copied after cloning in *E. coli* and the sequence of the copied DNA determined using restriction endonucleases.

The sequence appears to be unique.[17] The subunits, in other words, are identical, a conclusion supporting the results from peptide mapping experiments.[22] There is also no convincing evidence for microheterogeneity in the preparations employed in the sequence determination by chemical methods.[17]

Despite the length of the sequence, there is no obvious indication of gene duplication being involved in the evolution of the enolase molecule. While short sections of parts of the sequence — three to five residues — reappear further along the sequence, there is no clear pattern to this. In addition, there is no sign that the yeast enolase sequence is related to any other protein of known sequence.[17]

C. Physical Properties

1. Size and Shape

The molecular weight of yeast enolase was originally estimated at 67,000 on the basis of the mercury (II) to sulfur ratio in the crystalline enzyme[2] (see above). Later work[10,25] appeared to confirm this value, but it now appears that these later measurements were erroneous because of partial dissociation and because many of the measurements were made in concentrated (0.5 to 1 *M)* salt solutions.[25] Mann et al.,[14] from sedimentation equilibrium experiments, estimated the molecular weight of magnesium-enolase to be 88,000, close to that of other enolases,[5] and similar values have since been obtained in other laboratories (Table 2). The subunit molecular weight, from results of SDS-gel electrophoresis, was 52,000, suggesting a molecular weight of 104,000 for the dimer.[14] Osmotic pressure measurements gave 93,000 as the molecular weight of the enzyme.[26] The molecular weight of the dimeric enzyme with 2 mol of bound magnesium ion was 93,345, calculated from the amino acid sequence.[17] It is not surprising that osmotic pressure measurements should give a value closest to the "true" (sequence) value, since

Table 1
AMINO ACID COMPOSITION OF YEAST ENOLASE A AND SOME OF ITS PEPTIDES

	Residues/mole of subunit			Residues/mole of cyanogen bromide peptide					
				Peptide					
Amino acid	Malmstrom et al.[19a]	Brewer et al.[22a]	Sequence	M3 + M4	(%)	M6	(%)	M5	(%)
Tryptophan	4	4	5	1	0	0	0	3	4
Lysine	36	38	36	22	10	3	5	7	8
Histidine	10	11	11	6	3	3	5	1	1
Arginine	12	14	14	3	1	5	8	2	2
Aspartate	(53)	(47)	31	17	7	4	6	7	8
Threonine	21	19	20	7	3	4	6	5	6
Serine	36	29	31	16	7	3	5	5	6
Glutamate	(34)	(33)	25	8	3	7	11	5	6
Asparagine	(—)	(—)	19	13	6	3	5	2	2
Glutamine	(—)	(—)	9	4	2	2	3	3	4
Proline	12	14	15	9	4	1	2	3	4
Glycine	38	37	37	21	9	7	11	4	5
Alanine	59	51	55	32	14	6	9	13	15
Cysteine	0	1	1	1	0	0	0	0	0
Valine	32	36	35	18	8	4	6	5	6
Methionine	5	4	5	2	1	0	0	1	1
Isoleucine	20	22	22	10	4	3	5	8	10
Leucine	41	38	40	25	11	8	12	5	6
Tyrosine	11	8	9	6	3	0	0	1	1
Phenylalanine	17	15	16	8	3	3	5	4	5
Total	(441)	(421)	436	229		66		84	

[a] Corrected to a subunit molecular weight of approximately 46,500 (see text). Aspartate and glutamate values include amides.

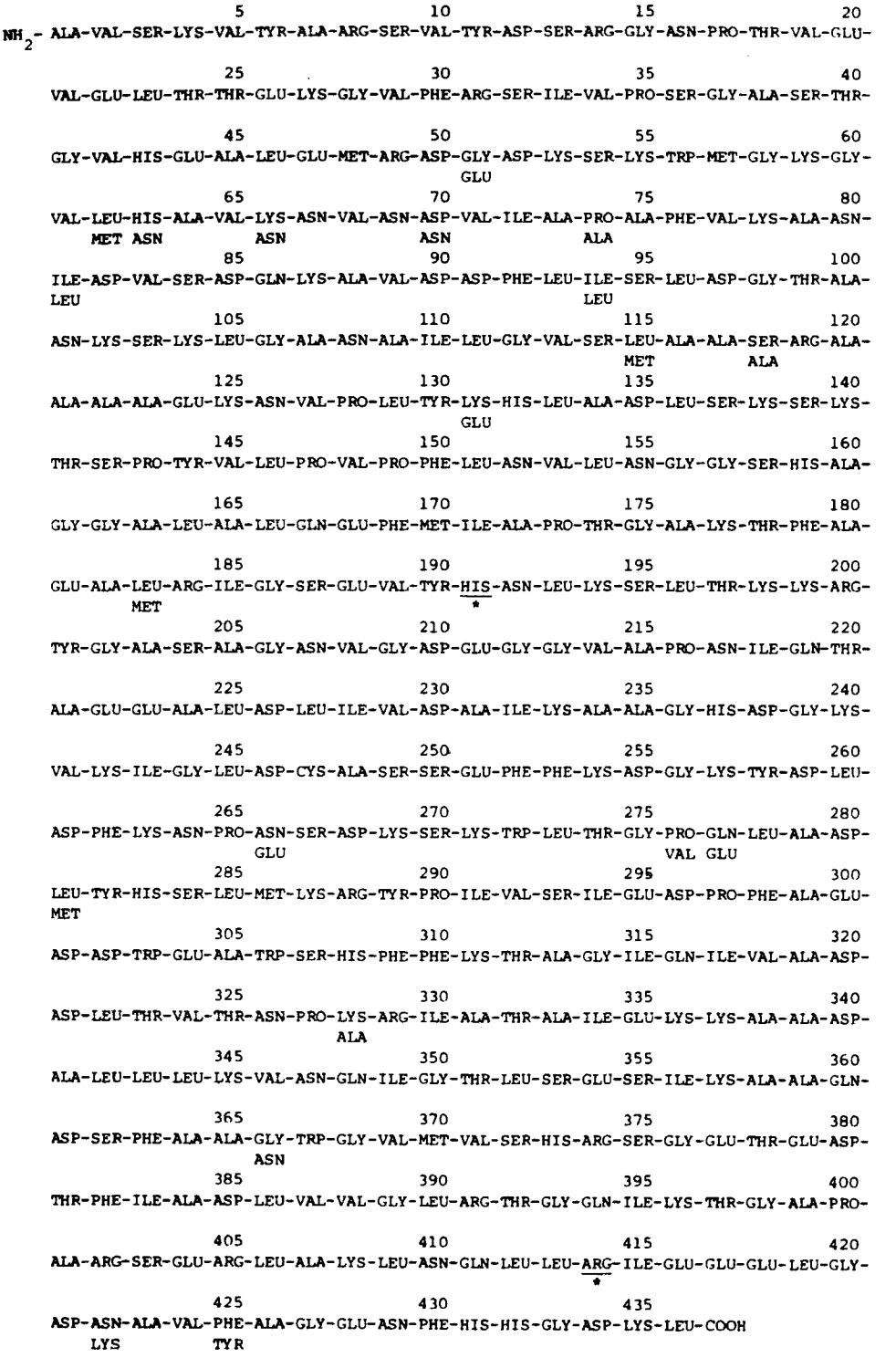


FIGURE 1. Amino acid sequence of yeast enolase. The underlined residues marked with an asterisk are active-site residues (see text). Residues given underneath the sequence of the A enzyme (p *eno* 46, according to Holland et al.'s[18] nomenclature) are from the isozyme that is apparently lost in the purification procedure (p *eno* 8). Note the greater methionine content and acidity of the latter protein. (Adapted from figures in Chin et al.[17] and Holland et al.[18])

the interaction of SDS with proteins cannot be expected to be entirely uniform, and since charge effects reduce molecular weights obtained from sedimentation equilibrium measurements.[27]

The effective size of a macromolecule in solution is given by its Stokes radius.[27] A value for the Stokes radius of the enzyme, calculated from the elution profiles from Sephadex presented by Keresztes-Nagy and Orman,[28] is also presented in Table 2. These measurements were made in 1 *M* KBr, using bovine serum albumin as the standard which was nearest in molecular weight. Keresztes-Nagy and Orman[28] estimated the molecular weight of yeast enolase to be close to that of bovine serum albumin (67,000) on the basis of the similarity of their elution volumes. Since these are actually measures of the Stokes radii of the molecules,[27] the fact that the actual molecular weight of the protein is above 93,000[17] suggests that yeast enolase is much more compact and symmetrical and/or less heavily hydrated than bovine serum albumin, at least in 1 *M* KBr.

The solvent does not appear to drastically affect the effective size of the enzyme. The Stokes radius can also be calculated from the molecular weight, sedimentation constant and partial specific volume.[27] The partial specific volume, calculated from the composition, is 0.742 cc/g[29] (Table 2). The values obtained for the sedimentation constant in three laboratories[8,30,31] in solutions of low ionic strengths (0.05 to 0.1) are also given. The Stokes radius calculated from these data is 36.2 Å, somewhat larger than the value in 1 *M* KBr, 33.8 Å.

The combined effects of asymmetry and hydration are given by the frictional ratio, the ratio of the measured hydrodynamic frictional coefficient to the theoretical one, calculated assuming the macromolecule is spherical and unhydrated.[27] The value of the frictional ratio calculated from the sedimentation constant, molecular weight, and partial specific volume is 1.20 (Table 2), consistent with a compact, symmetrical molecule of low or moderate hydration. The frictional ratio calculated from the intrinsic viscosity of yeast enolase[8] is also in fair agreement. Table 2 also gives the rotational relaxation time of dansyl-labeled conjugates of the enzyme, measured from their polarization of fluorescence.[30] From this, the ratio of the observed rotational relaxation time to that of the unhydrated spherical molecule is calculated and presented in Table 2, along with the theoretical ratio calculated from sedimentation data. They are again in fair agreement.

The hydration of a polypeptide can be calculated using the residue hydration values given in the review by Kuntz and Kauzmann.[32] These are based on NMR measurements of the amount of water associated with synthetic polypeptides that does not freeze. The calculated hydration of yeast enolase is 0.43 g/g, also given in Table 2. Since such calculations assume each polar residue is exposed to the solvent, values of hydration calculated in this fashion[32] tend to be overestimates, unless the protein is denatured.

The hydration can also be calculated from the measured sedimentation constant, intrinsic viscosity, and rotational relaxation time, assuming a spherical molecule. Although Kuntz and Kauzmann[32] have pointed out that the hydrations as defined by these methods would be different, we find a fair agreement between hydrations as calculated from the residue values (NMR measurements) and those calculated using the results of shear, translational, and rotational motion measurements assuming a spherical molecule. In addition, some preliminary electron micrographs of the enzyme obtained at the University of Georgia by Dr. Frank Mayer[33] show a "double dot" structure, which also suggests the dimeric enzyme has a low axial ratio. However, since there is evidence (see below) that some hydrophilic residues are not readily available to specific chemical modifying reagents, and since a protein cannot realistically be considered a smooth, impenetrable sphere, we believe yeast enolase to have only a relatively compact and symmetrical shape and a moderate hydration in solution.

Table 2
PHYSICAL PROPERTIES OF YEAST ENOLASE[17]

Molecular weight (amino acid sequence)	93,345
(Experimental values)	88,000; 104,000; 93,000
Sedimentation constant, $S^{\circ}_{20,w}$,S (Mg-enzyme)	5.90; 5.87; 5.85
Intrinsic viscosity, cc/g	2.9
Apparent axial ratio (electron microscopy)	1.0
Stokes radius (measured in 1 *M* KBr)	33.8Å
(Calculated from sedimentation data)	36.2Å
Calculated frictional ratio (sedimentation data), f/fo	1.20
(Intrinsic viscosity)	1.17
Rotational relaxation time, $\rho_{25,w}$, nsec	101 ± 19
Calculated ρh/ρo	1.35 ± 0.26
(Calculated from sedimentation data)	1.58
Calculated partial specific volume, cc/g	0.742
Calculated hydration (residue values), gH_2O/g protein	0.43
(Sedimentation value) g/g	0.54
Isoelectric point (apoenzyme)	6.05
Extinction coefficient, $A_{280,\ 1\ cm}$	8.95
Fluorescence emission maximum (apoprotein), nm	338
(Mg-enzyme), nm	335
Fluorescence quantum yield (apoprotein)	0.20
(Mg-enzyme)	0.23
α-Helix content, ORD value, %	18.5
Laser Raman value, %	<40

Note: The Stokes radius and ρh/ρo calculated from sedimentation data are assuming spherical symmetry. See text.

2. Secondary and Tertiary Structure

There is no information available about the structure from X-ray measurements on the enzyme, as no suitable crystals have yet been obtained despite decades of effort.

Westhead[8] examined the structure of yeast enolase — almost certainly the magnesium-enzyme — using ORD. He obtained values for a_o and b_o which suggested a low α-helix content, 18.5%. A more recent examination of the laser-Raman spectrum of the enzyme[26] suggested an α-helix content of less than 40%, and no β-pleated sheet structure. Magnesium ion had no effect on the Raman spectrum. Classification of the residues in the sequence according to their preferences for participation in α-helical or β-pleated sheet structures using the system of Levitt[34] indicated there were only a few sections of ten or more α-helix preferring or indifferent residues in a row.[17] These were all in the C-terminal half of the protein and amounted to about 14% of the total residues. There were only two segments of ten or more residues which favored (or were indifferent to) β-pleated sheet structures for a total of about 6% of the 436 residues. The agreement with the spectroscopic studies may be fortuitous, since Levitt[34] has noted that the preferences were very weak ones. However, the lack of extensive α-helical or β-pleated sheet structure is consistent with a compact, symmetrical structure.

One would expect a compact structure like the enolase molecule to be stabilized by strong internal interactions. Subtilisin treatment of the enzyme can be carried out so that several bonds are cleaved, without any effect on enzyme activity.[17] The two peptides produced by peptide bond cleavage at the single cysteine interact strongly, to the extent that 3*M* guanidine hydrochloride-3*M* sodium thiocyanate was employed to separate them.[24] The cyanogen bromide peptides interacted to the extent that the fourth peptide (M-4) was never obtained pure. The third, fourth, and fifth peptides formed a complex

which was stable to chromatography in 30% formic acid. If the carboxyl terminal fragment of the cysteineyl-cleaved enzyme was digested with cyanogen bromide, the fifth and sixth cyanogen bromide peptides formed a stable complex.[24] Examination of the amino acid compositions of these fragments shows that the fifth peptide may interact either with the sixth or with peptides three and four through some interaction involving isoleucine and leucine, since a surplus of one residue (isoleucine) in the fifth peptide is matched by a surplus of the other in peptides three plus four and peptide six (Table 1). It has been suggested that van der Waal's interactions, involving large hydrophobic side chains, are a major factor in protein structure.[35]

Krigbaum and Komoriya[35] presented statistical analyses of X-ray diffraction structures of proteins that suggested a gradient of polarity, with residues like tryptophan, tyrosine, and cysteine at or near the center of the molecules. Such evidence as is available for yeast enolase is not completely consistent with this picture.

The tyrosines and tryptophans at least do not appear to be in unusual environments. The laser-Raman spectrum of the enzyme also indicated that the tryptophans are exposed to the solvent and that the tyrosines are weakly hydrogen bonded.[26] This is itself not altogether consistent with the fluorescence emission spectrum of the enzyme (Table 2), which suggests that the tryptophans are in a moderately hydrophobic environment (about the polarity of dioxane) and that the tyrosines are strongly quenched.[30]

The single cysteine residue[16,39] reacts only very slowly with sulfhydryl reagents unless the enzyme is denatured with guanidine hydrochloride or urea. In the case of the native enzyme, addition of metal ion has no effect, but tris or imidazole stop the reaction,[39] perhaps restricting access of the residue to the solvent via a "pore". Reaction of the cysteine with a variety of reagents blocks recovery of enzymatic activity after denaturation with urea of guanidine hydrochloride.[16] However, the residue does not appear to be at the active site, so the reasons for these observations are not known.

The protein does appear to have many hydrophilic groups on its surface, since it is quite water soluble: even isoionic (deionized) enzyme can be concentrated to above 200 mg/mℓ.[20] At higher concentrations the solutions solidify to a clear plastic mass. This may be dissolved again without significant loss of activity.[20]

On the other hand, chemical modification studies have shown that only 1 or 2 of the 14 arginines react with 2,3 butanedione;[36,37] only 6 of 11 histidines react with diethylpyrocarbonate; and only 18 of the 36 lysine residues react with 2,4,6-trinitrobenzenesulfonate.[38] While there could be many possible reasons for these findings, the clear implication is that some hydrophilic residues may be "buried". This is consistent with the fact that the structural rearrangement undergone by the enzyme on isoelectric focusing (above) results in forms with more alkaline isoelectric points.[15] The measured isoelectric point of the native enzyme, 6.05, seems a bit low considering the amino acid composition.

D. Subunit Structure

Brewer and Weber[25] showed the enzyme was composed of two subunits of apparently equal molecular weight. This was demonstrated by means of sedimentation equilibrium experiments in 1 *M* KCl: the enzyme had a considerably higher molecular weight with excess magnesium ion present than in the presence of excess EDTA. The lower molecular weight was accompanied by a lower initial activity, and a similar effect could be seen in the absence of 1 *M* KCl, though the salt increased the extent of dissociation. The dissociation was readily and completely reversible by adding excess magnesium ion. In addition, enzyme covalently labeled with varying ratios of fluorescein could be diluted in 1 *M* KCl with unlabeled enzyme, then magnesium ion added, and the extent of energy transfer among fluorescein molecules measured from the polarization of the fluorescence. This also demonstrated the occurrence of subunit dissociation. The dimeric

structure of the enzyme was confirmed by Gawronski and Westhead.[40] They prepared hybrids of ^{14}C-acetic anhydride-labeled and native enzyme, dissociating the enzymes with 1 *M* KBr.

1. Kinetics of Subunit Association

The effect of magnesium ion addition and removal (with EDTA) has been examined using stopped-flow techniques.[41] In one study the difference spectral changes produced in partially dissociated (by 1 *M* KCl) enzyme by magnesium ion were used to follow the reaction. The metal bound to both monomers and dimers within the dead time of the stopped flow. Association of monomers followed and then activity was regained in a first-order reaction:

$$2E \overset{\text{fast}}{\rightleftharpoons} 2EMg \rightleftharpoons E_2Mg_2 \rightleftharpoons E_2^*Mg_2 \qquad (1)$$

$E_2^*Mg_2$ is active enzyme. The final, first-order reaction was called an "annealing" reaction. Similar reactions have been observed with other proteins.[42,43]

On mixing the magnesium-enzyme with EDTA, a reaction occurred within the dead time of the instrument. This was attributed to metal removal, but later work suggested that metal ion loss may be rate limiting (see Section V.D.1). Then a reaction was observed whose kinetics are consistent with a slow reaction followed by a faster one.[41] This was independent of whether or not subunit dissociation occurs, which indicates that the two reactions precede dissociation:

$$E_2Mg_2 \overset{\text{very fast}}{\rightleftharpoons} E_2'Mg_2 \overset{\text{slow}}{\rightleftharpoons} E_2' \overset{\text{fast}}{\rightleftharpoons} E_2 \overset{\text{fast}}{\rightleftharpoons} 2E \qquad (2)$$

The reactions subsequent to magnesium (II) loss would include one in which the substrate binding site is lost or altered; if magnesium (II) is present when dissociation occurs,[28,31] the subunits remain active (see Section II.D.2).

Because of the complex nature of the difference spectral change it was sometimes difficult to obtain quantitative estimates of reaction rates or even to interpret the reaction at all. Accordingly, these experiments were repeated and extended using the fluorescence of 1,8 anilinonaphthalene sulfonic acid (ANS) to follow the reactions.[44] ANS does not bind strongly to the enzyme, but the extent of binding increases when the enzyme dissociates, reminiscent of the situation with phosphofructokinase.[45]

There were "hidden" (dead time) changes in ANS fluorescence in some cases, which suggested that metal ion or salts produce conformational changes in the protein which precede subunit association or dissociation. The fluorescence changes in ANS *observed* using the stopped flow were found to be entirely a function of subunit dissociation, and the kinetics of the ANS fluorescence changes tended to be simple first and second order. This is in contrast to the association of the α and β subunits of human chorionic gonadotropin,[43] which was influenced by a subsequent first-order ("annealing"?) step.

The second-order data could be converted to subunit association rate constants using estimates of subunit dissociation equilibrium constants obtained from activity measurements (below). The values calculated were in the same range ($10^4 - 10^6$ $M^{-1}sec^{-1}$) as those obtained in studies of association of other proteins.[46–48] The different rates obtained were interpreted as a result of different conformations of the protein. Another possibility, however, is described in Section II.E.4. It was also noted that the "B" form of enolase dissociated more extensively than the "A" form.

Enzyme activity data indicated that associated enzyme became active on addition of

magnesium ion within the dead time of the stopped flow, whether or not magnesium ion was originally present.[41,44] So the important factor appeared to be subunit association, not metal ion per se. That is, the "annealing" reaction depends on subunit association.

2. Relation between Subunit Structure and Enzymatic Activity

The relation between subunit structure and activity has been the subject of some controversy. Although Brewer and Weber[25] and Gawronski and Westhead[40] found that subunits dissociated in the absence of magnesium ion are inactive,* Keresztes-Nagy and Orman[28] and later Holleman[31] showed that at 40°, in the presence of magnesium (II) and substrate, yeast enolase would dissociate into monomers which retained all the activity of the dimeric enzyme (Figure 2). Thus, the data of Brewer and Weber[25] and Gawronski and Westhead[40] were attributed to effects of the salts used for dissociation, although Brewer and Weber[25] obtained similar results in the absence of salts.

If the subunits dissociated in the absence of magnesium ion retain some potential residual activity, the ratio of initial activities of partially dissociated to fully associated enzyme should approach a constant value as the protein concentration drops and the extent of dissociation becomes more complete. Accordingly, such experiments were carried out over two or three orders of magnitude of protein concentration (and over several years).[26] The experiment involved adding substrate to enzyme incubated with excess magnesium ion (associated enzyme) and magnesium ion and substrate to enzyme incubated with excess EDTA (partially dissociated enzyme). The relative initial activities, A+ and A− respectively, can be used to obtain a calculated subunit dissociation constant, K_D:

$$K_D = \frac{4A_o\left(\frac{A+}{A-} - 1\right)^2}{\frac{A+}{A-}} \qquad (3)$$

whose constancy is a test of the assumption that the monomers are inactive. A_o in the equation is the molar concentration of dimeric enzyme, assuming complete association. While the calculated values of K_D were extremely sensitive to errors in the activities, there was no observed effect of concentration. This indicates the monomers produced in the absence of metal ion, that is, with excess EDTA present, are indeed inactive, and this could not be an effect of the salt, since the same result was obtained at low (approximately 0.05) ionic strength.[26]

To see why the subunits are inactive,the kinetics of the 295 nm absorbance change produced in a chromophoric competitive inhibitor, AEP, when it binds to the magnesium-enzyme (see Section III.E.4)[51] was measured. Spectrophotometric titrations have shown that a significant part of the absorbance change occurs when only enough metal ion to produce subunit association is present and it has been shown that the subunits bind and react to added magnesium ion very quickly.[41] These results are shown in Figure 3. It is clear that similar processes are rate limiting when magnesium ion or magnesium ion plus AEP are added to dissociated enzyme. Either the AEP cannot bind to the magnesium subunits until after they have associated (and "annealed") or, since it has been suggested that a direct interaction between the bound metal ion and the amine

* Gawronski and Westhead[40] also observed a loss of activity in KBr and KCl solutions even in the presence of 0.5 to 2 m*M* magnesium which had characteristics of the effect of subunit dissociation. Since high concentrations of salts are known to sharply reduce, by several orders of magnitude, the affinity of magnesium and other metals for the enzyme,[49,50] it seems likely that the drop in initial activity was not due to monomers with a reduced activity but to an insufficient metal concentration.

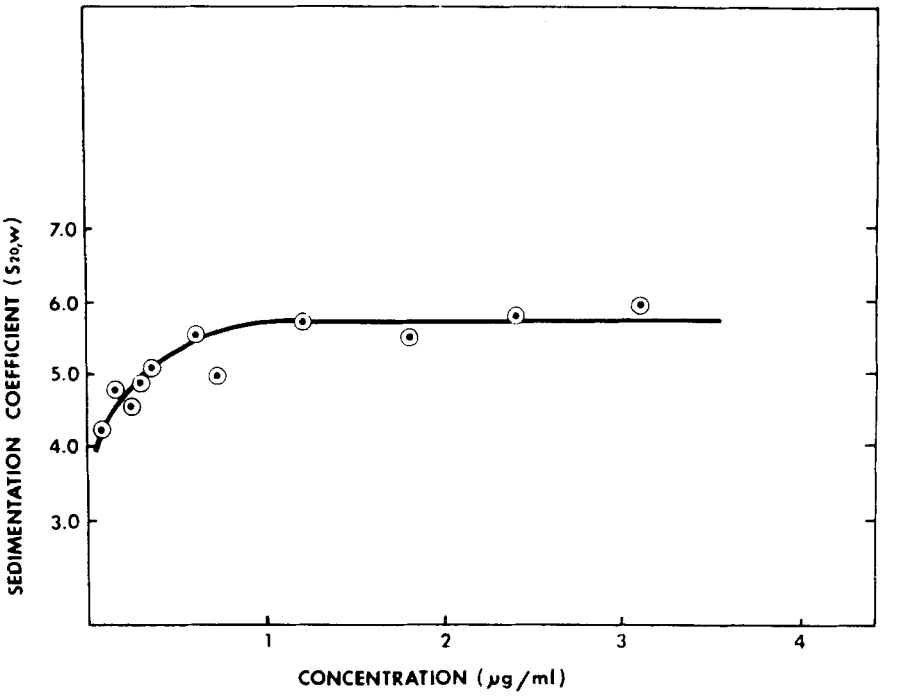


FIGURE 2. Sedimentation constant of yeast enolase as 40°. Data from "active enzyme centrifugation" experiments, carried out in the presence of 1 m*M* $MgCl_2$ and 2.4 m*M* 2-phosphoglyceric acid at 60,000 rpm. The concentration at which 50% dissociation has occurred is estimated to be 0.2 μg/mℓ (approximately $2 \cdot 10^{-9}$ *M*). In the presence of metal only, the concentration at 50% dissociation is about 40 μg/mℓ, or about $4 \cdot 10^{-7}$ *M*. (Data from Holleman, W. H., *Biochim. Biophys. Acta*, 327, 176, 1973. With permission.)

group on the AEP occurs (see Section III.E.3), this interaction cannot take place until the subunits associate. In any event, the substrate binding site is altered in some way upon subunit association or dissociation in the absence of metal ion.[26] Note, however, that Keresztes-Nagy and Orman's[28] and Holleman's[31] data show that the active site is at least potentially complete on each subunit — no parts of the active sites are "shared" between subunits.

3. Characteristics of the Subunit Association Reaction

Since the subunits of yeast enolase dissociated in the absence of magnesium ion are inactive, it is theoretically possible to use enzymatic activity to measure the extent of subunit association, as has been done in the case of formyltetrahydrofolate synthetase.[52] However, certain other conditions must hold before enzymatic activity can be used to quantitate the extent of subunit association. For one thing, whether or not the rate of subunit association is affected by ligands added which change the position of equilibrium, the rate must remain slow enough to allow initial activities to be measured. For another, all dissociated enzyme must be inactive and all associated enzyme fully active. In the case of yeast enolase, the rates of subunit association were measured and found to be sufficiently slow.[41,44] There was evidence for active monomers of the enzyme at 20° in the presence of magnesium ion and 1 *M* KCl.[26] Still, enzyme at the concentration normally used was nearly completely associated in 1 *M* KCl with excess magnesium ion present.

The existence of an annealing reaction subsequent to subunit association can make

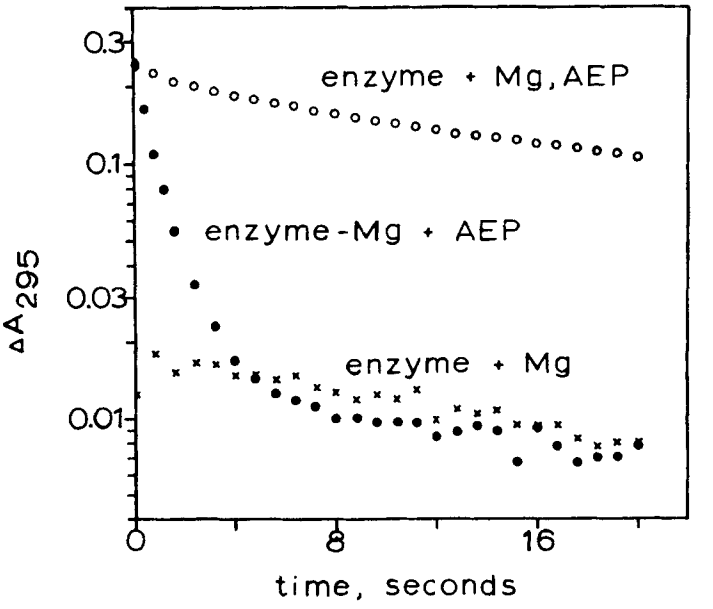


FIGURE 3. Kinetics of reaction of associated and dissociated enzyme with 3-aminoenolpyruvate-2-phosphate ("AEP").[26] $6 \cdot 10^{-6}$ *M* enzyme and 5 m*M* $MgCl_2$ and 1 m*M* EDTA (●) or 1 m*M* EDTA alone (○ and x) was mixed in a stopped-flow spectrophotometer with 10^{-4} *M* AEP and 1 m*M* EDTA (●), 10 m*M* $MgCl_2$ (x), or 10 m*M* $MgCl_2$ and 10^{-4} *M* AEP (○). 1 *M* KCl and 0.10 ionic strength tris-HCl, pH 7.9 was present in all solutions. (From Brewer, J. M., in *Biophysical Discussions: Fast Biochemical Reactions in Solutions, Membranes and Cells,* Parsegian, V. A., Ed., The Rockefeller University Press, 1978, 53. With permission.)

enzymatic activity measurements nonquantitative indicators of subunit association if the annealing reaction does not go to completion:[26,44]

$$2EL \rightleftharpoons E_2L_2 \rightleftharpoons E_2^* L_2 \qquad (4)$$

This is because E_2L_2, which is inactive, would be counted as monomeric enzyme. The only way to see whether this occurs is to measure subunit dissociation by some physical method and compare the values obtained with those calculated from activity measurements.*

Osmotic pressure data indicated that the activity measurements in tris buffers give a value of the dissociation constant that was consistently too high by about a factor of 4. This was explained[26] as an "annealing" effect — subunits which are associated in 1 *M* KCl, are not all "annealed"; the equilibrium of the annealing reaction in tris buffers is of the order of 2 to 3:

$$\begin{array}{l} \text{annealed dimer (active upon + Mg [II])} \\ 2E \rightleftharpoons E_2 \rightleftharpoons E_2^* \\ \text{unannealed dimer (not active upon + Mg [II])} \end{array} \qquad (5)$$

* Because of the cooperative nature of protein structure, a perturbation of one part of a protein will tend to affect other parts, and the resulting conformational change will be attained in a series of first-order reactions.[53] Consequently, there is in principle always an annealing reaction; whether it can be detected depends on whether it, or some other measurable reaction (such as subunit association) preceding it, is rate limiting.

Even so, the enzymatic activity measurements appear to be qualitatively reliable. The enzyme is more dissociated at more alkaline pHs according to isopycnic density gradient centrifugation data.[54] Measurement of the subunit dissociation constant using enzymatic activity and osmotic pressure confirmed this.[26] There appeared to be no groups titrating over the pH range 5 to 9 which affect subunit dissociation. The dependence of subunit dissociation on pH was low: the results obtained using both techniques suggested 0.3 to 0.5 mol of protons are released on dissociation per mole of protein. Since this represents the difference in proton binding to monomer and dimer, the nonintegral value is not especially significant.

The activity data also indicated that different buffers produce different apparent dissociation constants. This was not completely unexpected, considering that tris base is known to interact with apoenolase (this is the origin of much of the difference spectral change produced by magnesium) and other buffers such as HEPES compete effectively with tris.[26] Different buffers presumably act by shifting the "annealing" reaction.

Unfortunately, the osmometer used was not sufficiently precise to permit measurement of the dissociation constant in the absence of salt.

Subunit association in yeast enolase appeared to be pressure dependent, as the sedimentation constant of partially dissociated enzyme (1 *M* KCl) was a function of rotor speed.[26] This suggests that ionic or hydrophobic interactions predominate in producing subunit association.[55]

The pressure dependence of subunit dissociation was examined in detail by Paladini and Weber.[56] They used a pressure cell which enabled measurement of the polarization of the fluorescence of the tryptophans in the native protein and of DNS-conjugates of the enzyme. They were able to get results which were reversible and of high precision. Their measurements were made at low ionic strengths, with 0.1 *M* magnesium ion present. In the interpretation of their data, they assumed that two factors would be involved in the volume change occurring upon subunit dissociation. As dissociation occurs, the protein surface exposed enables solvent molecules to pack more efficiently about the newly exposed residues. And any movement of the newly exposed residues which results in a smaller overall volume will occur at higher pressures. They expressed these factors in the following equation:

$$\frac{d \ln Kd}{dP} = \Delta V - \frac{dVs}{Vs\, dP} \cdot Vs \cdot P \qquad (6)$$

ΔV is the standard volume change at one atmosphere and Vs is the effective volume of the residues that becomes subject to compression on dissociation. The term $\frac{1}{Vs} \cdot \frac{dVs}{dP}$ is their average compressibility. The values obtained from their measurements were 18 mℓ/mol for ΔV and 36 mℓ/mol/kbar for $\frac{dVs}{dP}$. The value of $\frac{1}{Vs} \cdot \frac{dVs}{dP}$ was calculated as 0.029/kbar, lower than the values for water or other solvents. Evidently this protein at least has a rather low compressibility.

4. Origin of Subunit Structure

There is considerable evidence, much of it indirect, that hydrophobic interactions predominate in producing subunit association in yeast enolase.[26] Brewer and Weber[25] presented evidence that 1 *M* potassium chloride or removal of magnesium ion with EDTA facilitated dissociation while 1 *M* postassium acetate did not. This suggested that electrostatic or hydrogen bond interactions were not involved in maintenance of dimeric structure.

Gawronski and Westhead[40] studied the dissociation of the enzyme produced by

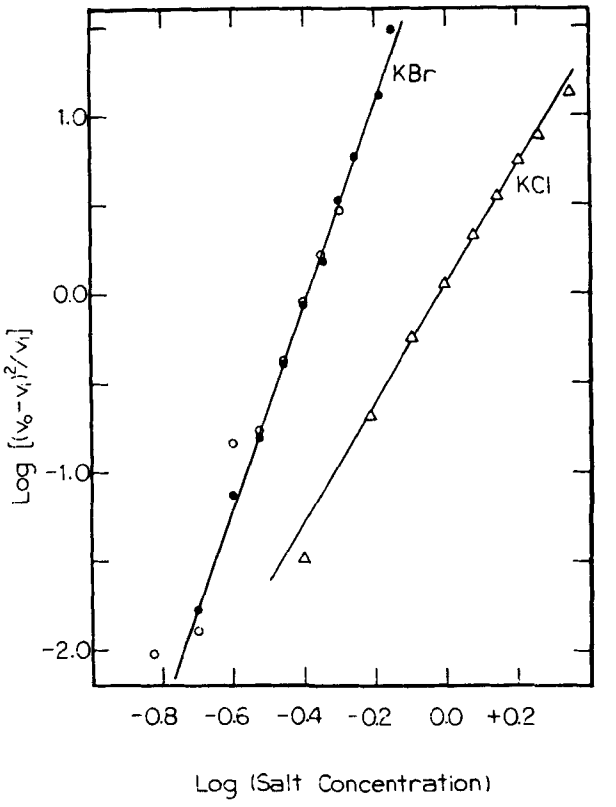


FIGURE 4. Effects of salt concentration on activity. The ordinate is essentially the logarithm of (inactive enzyme)^2/(active enzyme), that is, (activity lost)^2/(activity at low ionic strength), and those quantities were measured by incubating enzyme in buffer and magnesium plus the concentrations of salts shown and then adding substrate. The slopes of the lines indicate 6 mol of bromide and 3 of chloride are involved in the loss of activity, which was related to subunit dissociation.[40] (Reprinted with permission from Gawronski, T. and Westhead, E. W., *Biochemistry*, 8, 4261, 1969. Copyright 1969, American Chemical Society.)

potassium bromide (Figure 4). On the basis of enzymatic activity measurements, they found that relatively few moles of bromide were required for dissociation (or conversely, were bound after subunit dissociation) and that the heat capacity of the enzyme changed sharply upon dissociation, indicating exposure of hydrophobic regions when the subunits separated. This latter observation was consistent with that of Brewer[44] who found that the fluorescence of ANS increased when enolase was dissociated.

Brewer[54] found by isopycnic density gradient centrifugation in cesium chloride or sulfate that subunit dissociation was not accompanied by a significant change in water of hydration. This also suggested that subunit dissociation involved largely exposure of hydrophobic surfaces, which would not be heavily hydrated.[32] In addition, titration of isoionic (deionized) enzyme with chloride and sulfate salts indicated that relatively little difference in net anion binding occurred under associating (excess sulfate or magnesium ion present) or dissociating conditions. Of course, conformational changes subsequent to dissociation could complicate the interpretation of these data.

Enzymatic activity measurements showed that D_2O appeared to reduce subunit dissociation, consistent with its tendency to strengthen hydrophobic interactions.[57]

More direct evidence as to the nature of the factors producing subunit interaction in yeast enolase has come from measurements of the temperature dependence of the subunit dissociation constant, as measured by enzymatic activity under dissociating (excess EDTA before mixing with excess magnesium ion and substrate) and associating conditions[26] (Figure 5). The trend is clear — the enthalpy of subunit association at 20 to 25° is zero, indicating that hydrophobic interactions predominate. Johnson and Faller[58] independently obtained similar results examining absorbance difference spectral changes as a function of temperature. The convex profile reinforces Gawronski and Westhead's[40] finding that the heat capacity of the protein changes on dissociation. A concave profile was obtained by Aune et al. in their study of the effect of temperature and pH on α-chymotrypsin dimerization.[59]

Note that the pH and temperature effects on subunit dissociation are similar in 1 *M* KCl and at 0.05 ionic strength, suggesting that similar ligands are involved in intersubunit interactions in both solvents.

The evidence strongly suggests that subunit association in yeast enolase involves hydrophobic interactions almost exclusively. Little or no change in number of anion binding groups occurs upon dissociation.[40,54] In addition the data suggest that the anion chloride reduces the favorable entropy of subunit interaction.

The mechanisms by which magnesium cation and anions such as chloride affect hydrophobic interactions are not known, though there is some evidence as to the origin of the effects of so-called chaotropic anions. Brewer[60] showed that potassium acetate in fact tended to reverse the effects of potassium chloride on fluorescence emission and subunit dissociation and that the chaotropic anions thiocyanate and sulfate facilitated exposure and burial, respectively, of tryptophans in the protein. That is, the effects of the anions were competitive and were consistent with their positions in the "Hofmeister series". This was interpreted as a result of differing solubilities of anion-amide complexes.[61]

The stopped-flow kinetic data of Brewer[55] were interpreted in terms of changes in protein conformation resulting from addition or removal of metal ion or salts which preceded subunit association or dissociation. An examination of the effect of 1 *M* potassium chloride upon the laser Raman spectrum of yeast enolase has however shown no effect of metal ion (or substrate) or 1 *M* potassium chloride on the Raman spectrum of the enzyme.[26] This indicates that the salt or magnesium ion induced conformational change does not involve extensive changes in hydrophobic or amide groups. And it does not support the suggestion by Robinson and Jencks[61] that chaotropic salts function by direct interactions with amide groups. No changes in intensity in the amide regions of the spectrum were found.*

On the other hand, the salt did change the spectrum of the solvent (water). The results were similar to those obtained earlier by Walrafen,[62] who examined the effect of a number of electrolytes on the Raman spectrum of water. Anions such as chloride and thiocyanate disrupt water structure. This should in turn reduce the strength of hydrophobic interactions, since these depend on the strength of water-water interactions.[63] Anions such as fluoride and possibly sulfate have the opposite effect.[62] A similar suggestion has been advanced by Luck.[64]

* Note that these spectra were obtained using protein concentrations above 1 m*M* (90 mg/mℓ); at these concentrations, little subunit dissociation can occur so the spectra would not show the effect of dissociation, just that of chloride binding.

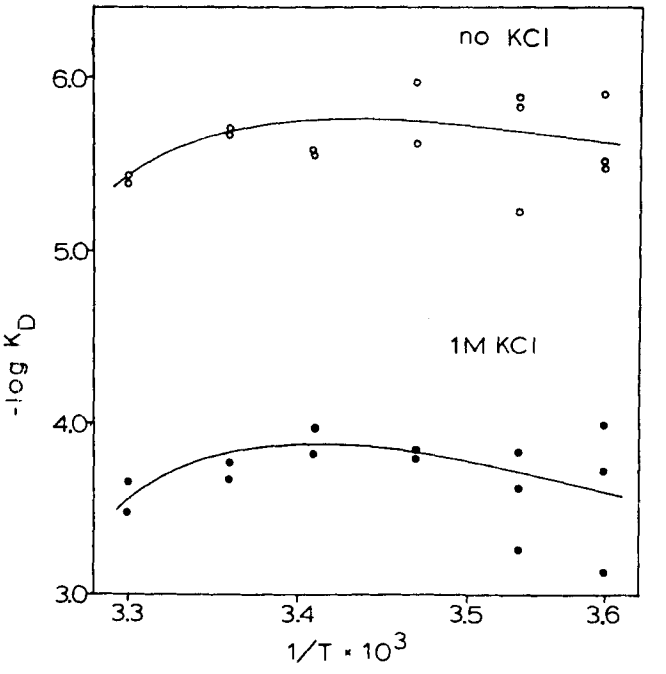


FIGURE 5. Effect of temperature on subunit dissociation constant ("K_D") as measured using enzymatic activity.[26] The enzyme concentrations used were 15 μM in the presence of 1 M KCl and 1 μM at 0.05 ionic strength. The data are corrected to pH 7.8. (From Brewer, J. M., Faini, G. J., Wu, C. A., Goss, L. P., Carreira, L. A., and Wojcik, R., *Physical Aspects of Protein Interactions*, Catsimpoolas, N., Ed., Elsevier-North Holland, New York, 1978, 57. With permission.)

III. INTERACTION OF METALS WITH APOENOLASE

A. Preparation and Assay of Apoenolase

Yeast enolase as prepared by the method of Westhead and McLain[6] contains firmly bound magnesium ion.[30] Two main procedures have been used to prepare apoenolase: treatment with EDTA (1 to 5 mM is required) and its removal by dialysis,[65] chromatography on Sephadex® G-25[30] or adsorption of the enzyme onto phosphorylated cellulose and washing[6]; or chromatography on a chelating or mixed bed ion exchange resin.[66] Dialysis is slow (1 week for complete EDTA removal). There is evidence that EDTA binds to the enzyme.[26] Removal of EDTA is best monitored using ^{14}C-EDTA. Chromatography on ion exchange resins is fast and reasonably complete provided the usual precautions are taken — plastic is used wherever possible. The apoenolase is somewhat less stable than the magnesium enzyme. This is partly because the latter is more resistent to proteolytic attack, and partly because the apoenzyme dissociates to some extent and is less stable when dissociated.[15,20] Consequently the apoenzyme is best stored concentrated.[20]

Residual metal ion content has been assayed using enzymatic activity,[30] atomic absorption spectroscopy,[30,66] and from the 296 nm absorbance change in the enzyme produced on adding excess EDTA, then excess magnesium.[26,30] Use of enzymatic activity for assays of residual metal ions is a poor method, both because many divalent metals do not activate this enzyme and because up to two moles of magnesium ion can bind without

producing much activity (see Section V.D). Atomic absorption is reliable for those metals whose absorbance is measured, but this does not tell about metals not measured. The 296 nm absorbance change seems to be a reasonable index of total metal ion content, since most divalent metal ions appear to produce it. However, the change itself is small, and the difference spectrum is buffer dependent,[26] depending on interaction of the apoenzyme with tris base. Despite these limitations, measurement of 296 nm absorbance seems best for routine use.

B. Specificity of Binding

An absorbance change in the enzyme, usually monitored at 296 nm, is produced by most metal ions whether these produce enzymatic activity ("activating" metals) or not. The magnitude of the change varies somewhat with the metal, but again does not appear related to the level of activity produced.[67] Nor is there specificity for divalent cations: terbium (III) binds very strongly[39] and apparently produces the absorbance change.

C. Stoichiometry and Strength of Metal Ion Binding

1. Magnesium (II)

Brewer and Weber[30] presented evidence from fluorimetric titrations that the enzyme bound a single equivalent of magnesium ion per 67,000 g of enzyme. Later, Hanlon and Westhead[49] performed equilibrium dialysis measurements of divalent calcium, magnesium, and manganese binding to the enzyme in 0.5 *M* KCl.* They found that 1.6 mol of magnesium ion per 67,000 g of enzyme bound at a free metal ion concentration of 0.8 m*M* in the absence of substrate. Unfortunately, their work, and that of Brewer and Weber,[30] suffered from ignorance of the correct molecular weight.

More recently, Faller and Johnson[66] and Brewer[65] independently measured the enthalpy of magnesium ion binding to enolase. The agreement in results was remarkable: 2 mol of magnesium ion bound strongly to the enzyme, apparently with different enthalpies (see below). The more extensive data of Faller and Johnson are shown in Figure 6.[66] Faller and Johnson confirmed this stoichiometry, measuring the proton release upon addition of magnesium ion to apoenzyme.[68] Faller et al.[67] were also able to show that the enzyme binds up to 2 mol of magnesium ion in the absence of substrate, using an ion-specific electrode to measure free metal ion concentrations. The same stoichiometry has been obtained using spectrophotometric titrations[67] and ultrafiltration.[39] Clearly, 2 mol of magnesium ion bind to the enzyme in the absence of substrate. This is, presumably, one per subunit.

The fluorimetric titration data of Brewer and Weber[30] were consistent with a dissociation constant of $3 \times 10^{-6} M$ (0.05 ionic strength). Hanlon and Westhead's[49] equilibrium dialysis data were fit to two dissociation constants differing by a factor of 50: $10^{-5} M$ and $5 \cdot 10^{-4}$ *M* (0.5 *M* KCl). It was later found, however, that magnesium ion binds much more strongly to the enzyme at low ionic strengths. Brewer and Weber[30] prepared apoenzyme by adding excess EDTA and chromatography on Sephadex®. It seems likely that the dissociation constant Brewer and Weber[30] obtained was influenced by unremoved EDTA in the protein preparation, and possibly by a difference in the nature of the metal ion binding sites (see below).

Faller and Johnson[66] obtained a good fit of their calorimetric data to theoretical independent sites with dissociation constants of $2 \cdot 10^{-9}$ *M* and 10^{-7} *M*. The pH titration data of Faller and Johnson[68] were fit assuming dissociation constants of $2 \cdot 10^{-8}$ *M* and

* The salt was used in this and other studies because it appeared to eliminate substrate inhibition[7] and also to reduce "nonspecific" manganese binding (discussed in a review by Malmstrom).[10] The author believes it is not very helpful.

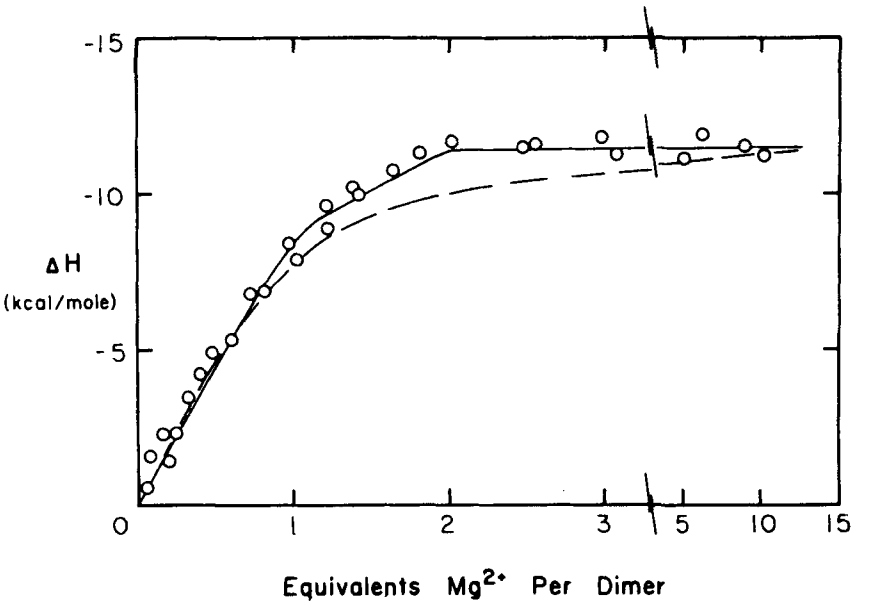


FIGURE 6. Enthalpy of reaction of magnesium with yeast enolase.[66] The titration was performed in 0.05 ionic strength tris-acetate, pH 7.5, and 25°. The solid line is a theoretical binding profile, assuming anticooperative binding and a 50-fold difference in dissociation constant. The dashed line assumes binding at a single site. See text. (From Faller, L. D. and Johnson, A. M., *Proc. Natl. Acad. Sci. U.S.A.*, 71, 1083, 1974. With permission.)

10^{-6} *M* and independent sites. The ion specific electrode measurements yielded estimates of $4 \cdot 10^{-9}$ *M* and $2 \cdot 10^{-7}$ *M*.[69] Because the protein concentration was much greater than the estimated values of dissociation constants in all these measurements, the absolute values of dissociation constants calculated are not very precise. It is clear, however, that the metal ion binds strongly, probably strongly enough for the enzyme to conform to Vallee's definition of a metalloenzyme.[70]

Since the subunits are identical in sequence, the apparent difference in the binding sites is of interest because of its possible relevance to problems of protein structure and ligand binding site interactions. If one assumes a 50-fold difference in dissociation constant for the two metal binding sites, the calorimetric titrations suggest that the difference in dissociation constant is due to a difference in enthalpy of metal ion binding to the two sites.[65,66] Either the binding sites are inherently nonidentical in the dimer, or there is an anticooperative effect generated by metal ion binding.

Faller and Johnson[68] found the sites were identical as far as proton release was concerned: a maximum of slightly over 1 mol of protons was released per mole of magnesium ion bound at 0.05 ionic strength. However, spectrophotometric titrations of apoenzyme with magnesium ion show the first mole of metal ion appears to produce more than half the total absorbance change.[67] This is consistent with the calorimetric data[65,66] and Hanlon and Westhead's equilibrium dialysis data,[49] which suggest or are consistent with a difference in the magnesium ion binding sites.

However, two points should be stressed: the first is that the unequivocal demonstrations of binding site differences that have been published have all been obtained in tris buffers. The effect of tris on the absorption difference spectral change in the enzyme[26] suggests the difference in binding sites may be a buffer effect. The second point is that, from an operational point of view, the sites appear to be equivalent:

substrate analogue binding seems identical to the two sites, though there is no published evidence as yet on turnover numbers.

2. Other Metal Ions

Hanlon and Westhead[49] reported that 4 to 6 mol of calcium ion per 67,000 g of enzyme bound to enolase in the absence of substrate. They measured calcium binding by gel filtration methods. However, spectrophotometric titrations of apoenzyme in tris buffers with divalent calcium, magnesium, and nickel show that only 2 mol of strongly bound metal ion perturb the enzyme.[67] Titrations at the same ionic strength (0.05 to 0.1), but at a more alkaline pH (i.e., higher tris base concentration),[26] show the absorbance changes produced by the metal ions are greater. This indicates that the nature of the spectrophotometric change produced by calcium ion is the same as that produced by magnesium ion, that is, the binding sites are the same. And some recent binding studies using ultrafiltration suggest only 2 mol of calcium ion bind to enolase.[39]

Terbium (III) also binds to the enzyme, and enzymatic activity measurements (the metal inhibits), 296 nm absorbance measurements, fluorescence titrations (see below), and binding measurements show 2 mol of the metal ion bind per mole of enzyme.[39,71] The binding of trivalent terbium is very strong so that it can displace bound divalent magnesium, but even high concentrations (1 m*M* or more) of magnesium ion alone do not displace terbium ion.

While these data show that only 2 mol of magnesium (II), terbium (III), and possibly calcium (II) bind to the enzyme, at least to the 10^{-3} *M* level of free metal ion concentration, other studies indicate that more than 2 mol of some other metal ions bind in the absence of substrate.

Chien and Westhead[72] examined the EPR spectra of manganese (II) free, bound to various chelating agents, and bound to yeast enolase. The work was again done in 0.5 *M* KCl. They did find two strong manganese ion binding sites and obtained evidence at 77°C for additional manganese (II) binding at ratios of manganese ion to enzyme of above two (corrected for a molecular weight of 93,000). No conclusions could be drawn as to the ligands coordinating the metal ions. More recently, a study of copper (II) binding to the enzyme in HEPES buffer (0.05 ionic strength) also showed more than 2 mol of copper (II) bind.[73] Manganese (II) is an "activator", that is, it produces enzymatic activity, while copper (II) does not. Westhead and Rose have shown that 4 mol of cobalt (II) bind in the absence of substrate.[74] Cobalt (II) is also an activator. Ultrafiltration measurements showed that 4 mol of zinc (II) bind to the enzyme in tetrapropylammonium borate buffer (see Section VI).[39] There is little information about dissociation constants of these metal ions.

The author finds it convenient to divide these metals into two categories, irrespective of whether they are "activators" or not and irrespective of the level of activity produced: those of which only 2 mol/mol of enzyme (presumably one per subunit) bind and those of which 4 mol/mol bind. This again is in the absence of substrate and at a free metal concentration of approximately 1 m*M*. The former category includes magnesium (II), terbium (III), and possibly calcium (II); the latter group includes the transition metal ions. The basis for this division seems to be that the dissociation constants for additional, "inhibitory" metal ions (see Section VI) are much smaller for the transition metals, so the additional binding is more readily detected. We will defer discussion of "inhibitory" sites until later; our discussion of binding sites will until then refer to those which potentially strongly bind magnesium ion in the absence of substrate.

D. Chemical Nature of the Binding Sites

The pH titration studies of Faller and Johnson[68] showed that the pH profile of proton

release on binding magnesium ion was consistent with a group in the protein with a pK of 7.3. The implication that imidazole groups were involved in metal ion binding is consistent with interpretations of obtaining similar pKs by other workers.[4,75] However, it should be noted that these pKs are also consistent with binding of metal to carboxyl groups which are in a hydrophobic environment (see below).

Earlier, Westhead[76] presented the results of photooxidation studies, using rose bengal, that implicated a single histidine (per 67,000 mol wt) whose loss could be correlated with loss of activity (see below). The effect of addition of EDTA showed that the strongly bound magnesium ion reduced the rate of activity loss, though addition of substrate as well provided additional protection. In addition, George and Borders[38] showed that magnesium ion offered a significant protection against inactivation by ethoxyformic anhydride.

These data suggest that histidine is involved in metal ion binding. However, any such involvement may be indirect, through a conformational change. EPR studies of copper (II) binding, apparently to the same sites, show no nitrogenous ligands coordinate with copper ion.[73] Copper (II) is not an activator but nickel (II) is and a recent abstract states that the nickel (II) enolase absorption spectra also indicate no nitrogenous ligands coordinating with the metal ion.[77] In tris buffer, there is evidence for nitrogenous ligand(s), almost certainly the tris.[20] Terbium (III) apparently binds at the same sites, and the increase in the fluorescence of the metal ion is similar to that produced by EDTA and carbonate.[71]

Three moles of water remain coordinated to enzyme-bound terbium ion.[71] Nowak et al.[78] found that two rapidly exchanging water molecules remained coordinated to enzyme-bound manganese (II) in 0.05 *M* tris buffer and 0.5 *M* KCl in the absence of substrate (see Section III.E.3). So these metal ion binding sites include carboxyl groups, though one or more coordination sites on the bound metal ion is accessible to the solvent at least in the absence of substrate.

The lack of energy transfer from enzyme to bound terbium (III) noted by Brewer et al.[71] was interpreted as evidence that the coordinating ligands for the terbium ion do not include tyrosines or tryptophans.

Preliminary measurements of the visible CD spectra of cobalt (II) and nickel (II) enolases show the bound metal ions are optically active.[79]

E. Effects of Metal Ion Binding

1. On the Enzyme

The metal ion produces a conformational change in the enzyme, as measured by absorption, fluorescence, and thermal stability.[30,71] However, absorption and fluorescence are influenced by the "tris effect"[26] (which can be considered another indicator of the conformational change), and by the effect of the metal ion on subunit association. Still, even after discounting these effects there appears to be a metal ion-induced conformational change, observable by ultraviolet difference spectroscopy in HEPES, etc. buffers.

The effect of magnesium ion on the subunit dissociation constant is well documented but here again the reason for the effect is unknown. While anions such as acetate and sulfate also promote subunit association, this is through strengthening hydrophobic interactions (see above), and the mechanism of action of magnesium ion may well be different. There is little evidence concerning the specificity of subunit association for different divalent metals. Presumably, most divalent metals would produce subunit association, but this is speculation.

The strongly bound metal ion which produces the conformational change has been called "conformational" or "structural" metal, to distinguish it from additional metal

which may be bound, depending on the circumstances. This nomenclature can be criticized as implying these cations have no other function. As long as the reader understands the designation "conformational" refers to only one function of the metal ion, the author feels the terminology is on the whole useful.

2. On Binding of Other Ligands

Another effect of the metal ion that is well documented is to enable the enzyme to bind the substrate and several competitive inhibitors. Calorimetric studies show no substrate or inhibitor binding in the absence of metal ion.[20,66] The metal ion probably does not do this entirely by interacting directly with these compounds while bound to the enzyme. If the metal ion interacts directly with substrate, it seems most likely it is interacting with the hydroxyl on carbon-three (see below); yet, glycolic acid phosphate binding to rabbit muscle enolase also requires magnesium ion,[80] as does phosphate binding to the yeast manganese (II)-enzyme (see below). Again, the "conformational change" mentioned above must be invoked.

There is no specificity for an activating conformational metal ion: calcium ion permits 2-phosphoglycerate binding[67] and calorimetric experiments indicate the substrate binds to the terbium (III)-enzyme.[71] Substrate also binds to the copper (II)-enzyme.[73]

3. Effects of Substrate and Substrate Analogue Binding on the Environment of Conformational Metal Ions

While conformational metal ions control the binding of substrate and substrate analogues, the question arises as to whether there is a reciprocal effect of substrate binding on conformational metal ions. In particular, one may ask whether there is a direct coordination between conformational metal ions and any part of the substrate.

There is considerable evidence that substrate or competitive inhibitors change the environment of conformational metal ions. The hyperfine structure of the EPR signal of enzyme-bound manganese (II) (again, an activating metal) is reduced by addition of a strongly bound substrate analogue (the 3-amino analogue of phosphoenolpyruvate), indicating a distortion of the metal ion environment.[81] EPR studies of a nonactivator, copper (II), show that substrate produces a "rhombic and tetragonal distortion of an octahedral geometry".[73]

There is a large effect of substrate on the enzyme-bound cobalt (II) absorption spectrum: substrate or a substrate analogue reduces the intensity of the visible absorption band of the metal ion.[20] Substrate also affects the spectrum of nickel (II) bound to the enzyme, producing a red shift in the near ultraviolet absorption maximum and an increase in integrated intensity of the transition.[20] The effect of substrate on enolase-bound nickel ion is similar to the effect of a competitive inhibitor on the spectrum of nickel-carboxypeptidase A,[82] and to the effects of competitive inhibitors and substrates on the spectrum of nickel-phosphoglucomutase.[83]

There is also evidence for a slight alteration in the environment of conformational terbium (III) by substrate.[71] So while environmental effects can be found in plenty, there is no direct evidence as yet for direct coordination between any part of the substrate and conformational metal ion. Note that in all these studies on enolase the assumption is that metal ion in the conformational sites largely remains in the conformational sites when substrate or analogues are added. The validity of this assumption has not been rigorously tested as yet.

There is some indirect evidence supporting the suggestion that the conformational metal ion interacts directly with the hydroxyl of 2-phosphoglycerate, i.e., the hydroxyl replaces a water molecule of the inner coordination sphere of the metal ion (in the reverse reaction, the metal-bound water would add to carbon-three of phosphoenolpyruvate).

This hypothesis of Nowak et al.[78] is based on calculated manganese (II)-phosphorous and manganese (II)-proton distances, obtained using ^{1}H- and ^{31}P-NMR and a weak substrate, α-(dihydroxyphosphinylmethyl)-acrylate (see Section IV.A). Less than 2 mol of manganese ion per mole of enzyme was present, so Nowak et al.[78] were presumably dealing with conformational metal ions. The calculated distances of approximately 6 Å from the phosphorous and 7.0 ± 0.5 Å from the carbon-bound protons of the substrate were too great for the metal ion to coordinate directly with the phosphate or carboxyl group, but were about right for coordination with the hydroxyl of 2-phosphoglycerate. These experiments were carried out in 0.5 *M* KCl.

These authors also obtained evidence suggesting that the substrates and substrate analogues they used allowed 0.3 to 1.0 mol of rapidly exchanging water molecules to remain on the manganese ion. They suggested the analogues "immobilized" 1 mol of metal-bound water so that it exchanged very slowly.[78] This assumes, of course, that the coordination number of the manganese ion remains the same as in the absence of substrate.

The importance of binding of added ligands to conformational metal ions is illustrated by the recent findings of Maurer and Nowak[84] that fluoride binds directly though weakly to conformational manganese (II). This then strongly decreases (by about two orders of magnitude) the dissociation constant for phosphate, a competitive inhibitor, which binds 6-7Å away. Phosphate binding correspondingly decreases the dissociation constant for fluoride. These observations suggest that the unoccupied coordination sites on conformational metal ion are indeed important for substrate binding and hint at its importance for catalysis (see Section V.E).

4. Relevance to Catalysis

The binding of magnesium ion at conformational sites is not sufficient for catalysis, as Faller et al. demonstrated.[69] However, while it does not produce the complete reaction, it does seem more important to catalysis than merely enabling the substrate to bind.

Nowak et al.'s[78] data suggested direct participation of conformational metal ions in the catalytic mechanism. There is more recent evidence as well. Spring and Wold showed that the aldehyde analogue of the substrate ("TSP") and the amino analogue of the product ("AEP") were competitive inhibitors which bound much more strongly than substrate or product.[51] Consequently, the suggestion was made that these might be "transition state analogues".[51] Of more interest was the finding that both compounds exhibited large ($\Delta\epsilon_{1\ M,1cm} \sim$ 10-20,000) ultraviolet (285 or 295 nm) absorbance changes on binding to enolase in 1 m*M* magnesium ion. Spectrophotometric titrations of apoenzyme-AEP solutions with magnesium ion showed that two equivalents of magnesium ion (one/subunit), which produces 10% of full activity, gives 67% of the AEP absorbance change.[67] This indicates the magnesium ion added initially affects the AEP absorbance more than enzymatic activity, and everything we know about the mechanism of action of this enzyme suggests the magnesium ion added is added initially to the conformational sites. So conformational magnesium ion is more important to producing the absorbance change in AEP than in producing enzymatic activity with 2-phosphoglycerate.

The possible structural basis for the absorbance changes in these compounds is discussed in Section V.D.2. The current working hypothesis of the authors is that the absorbance changes represent "partial reactions". These reactions involve (in the case of TSP) enolization[51] and (both analogues) twisting the molecules so the double bond between carbons two and three is aplanar.[67]

Spectrophotometric titrations of apoenzyme-AEP solutions with various divalent metals (Table 3) show that a 295 nm absorbance change in AEP occurs only with

Table 3
SPECTROPHOTOMETRIC TITRATIONS OF ENOLASE WITH VARIOUS METALS IN THE PRESENCE OF AEP

Metal	Activating?	Relative ΔA_{295} (%) moles metal/mole enzyme 1	2	100
Mg (II)	Yes	35	69	100
Mn (II)	Yes	39	75	105
Ni (II)	Yes	34	65	102
Fe (II)	Yes	25	50	—
Zn (II)	Yes	—	—	91[a]
Cd (II)	Yes	—	—	57[a]
Ca (II)	No	5	6	2
Ba (II)	No	3	6	3
Sr (II)	No	4	6	2
Cu (II)	No	2	5	—
Hg (II)	No	—	—	0
Pb (II)	?	—	14	—
Be (II)	No	3	4	—

Note: 10^{-5} *M* enzyme in 0.05 ionic strength tris-HCl, pH 7.8, or 0.05 M HEPES-NaOH, pH 7.7, was titrated. $3\text{-}4 \cdot 10^{-5}$ *M* AEP was also present.

[a] At pH 8.7 after 10 min (the absorbance was still increasing).[67]

From Brewer, J. M. and Collins, K. M., *J. Inorg. Biochem.*, 13, 151, 1980. With permission.

activating metal ions and apparently to the same extent* with all activating metal ions. Zinc (II) and cadmium (II), both activators, also produce the absorbance change but much more slowly, over many minutes under the conditions of measurement. In other words, the reaction seems to be "all or none".[67] And addition of only 1 mol per subunit of several activating metal ions produces the bulk of the absorbance change in the AEP. So all activating metal ions are essentially equivalent as conformational metal ions in producing the "partial reaction" in the AEP. It has been suggested[67] that this is a reflection of a uniformity of effect of activating conformational metal ions on the substrate (see Section IV.D.2).

Nonactivating metal ions produce the same change in absorbance of the enzyme as they do in the absence of AEP (see Section III.E.1). This is not because AEP does not bind to the calcium-enzyme; e.g., both AEP and substrate reduce the apparent rate of magnesium (II) or calcium (II) loss on reaction of the enzyme-conformational metal ion-AEP complex with excess EDTA.[67]

The absolute specificity of production of the AEP absorbance change for an activating metal ion suggests two things about the role of metal ion(s) in the enolase reaction. The first is that the conformational metal ion must bind the substrate in a particular way for the reaction to proceed at all. The second is that the rate of catalysis is controlled by

* Assuming that the absorption maximum is at 295 nm irrespective of the metal. Actually, it is at 300 nm for the nickel-complex, but this does not change those results significantly.[67]

something else, since the nickel (II) and magnesium (II) enzymes produce the absorbance change in TSP at the same rate (see below),[67] although the maximum velocity of the nickel (II)-catalyzed enzymatic reaction is much lower than that catalyzed with magnesium (II).[4]

A corollary of the first point is that the reason some metal ions can activate and others cannot must involve some property of these metal ions that differs absolutely from metal to metal. At present, the only property the author can suggest is the ability to form complexes with enzyme and substrate in which the conformational metal ion can assume a certain coordination geometry.[67]

The idea that the geometry of the ligands coordinating the metal ion in a metal-activated enzyme is important if not new. This was discussed, for example, in a review by Malmstrom and Rosenberg published in 1959.[85] The more recent "entatic state" hypothesis of Vallee and Williams[86] also suggested that a special metal ion geometry was involved in catalysis. We do not yet know the geometry of the metal ion coordination in the conformational sites. The absorbance data mentioned require additional information before conclusions as to the geometry can be drawn. We are of course particularly interested in the geometry of the complex with substrate, and a few speculations only can be offered that may be pertinent.

It seems reasonable that a metal ion enzyme complex must change in conformation on converting substrate to product (if the metal ion is involved in the reaction), since the conformation of the substrate itself changes on becoming product. It follows that the geometry of the ligands about the metal ion must be able to change also, and without kinetically retarding the reaction. So the metal ion in an enzyme-substrate complex must be readily convertible to metal ion in an enzyme-product complex.

It has been suggested that conformational nickel (II) in enolase is converted to five-coordinate geometry on binding substrate.[77] The basis for this suggestion is that the visible absorption change in the nickel ion resembles that in nickel (II) carboxypeptidase A[82] when a competitive inhibitor binds (see above). Five-coordinate geometry has the unusual property of encompassing a variety of forms which typically readily interconvert.[87]

The known activating metals are magnesium (II), which is known to form five-coordinate complexes, and the divalent transition metals from manganese to zinc, including cadmium but with the exception of copper. (Chromium II was recently found to activate under anaerobic conditions.[88]) So far as is known, all, including copper (II), can form five-coordinate complexes, and while calcium (II) and terbium (II) rarely if ever form such complexes, this particular theory fails to explain why copper (II) cannot activate enolase.

IV. INTERACTION OF SUBSTRATES AND COMPETITIVE INHIBITORS WITH ENOLASE

A. Structure and Specificity

A number of compounds exist which can serve as competitive inhibitors, and a much smaller number which serve as substrates. Wold[5] examined the kinetic properties of a number of compounds and concluded that the binding site for these compounds consisted of a relatively large or flexible binding site for a carboxyl group, and relatively rigidly defined sites, space, and geometrical relationships for a phosphate, a hydrogen on the second carbon, and a hydroxyl group on the third carbon. Since his review[5] was written, six new substrates have been synthesized, as well as six inhibitors (Figure 7).

Two of the six latest substrates are phosphoenolpyruvate analogues, involving substitution of fluorine for a hydrogen on carbon-three (phosphoenol-3-fluoropyruvate)

or methylene for the oxygen between carbon-two and phosphorous (α-(dihydroxyphosphinylmethyl)-acrylic acid).[89] One new substrate, 2-phospho-3-butenoic acid, is an analogue of 2-phosphoglycerate in which the methanolic carbon-three is replaced by ethylene.[90] The enzyme catalyzes a shift in position of the double bond from between carbons three and four to between carbons three and two, a β, γ-α, β isomerization, to form phosphoenol-α-ketobutyrate.[90]

The value of V_{max} obtained for the two phosphoenolpyruvate analogues using the rabbit muscle enzyme is about 1% of that for phosphoenolpyruvate itself;[89] their dissociation constants with the yeast enzyme are an order of magnitude or more smaller.[91] The 2-phospho-3-butenoic acid reacted with a maximum velocity which was 0.1% of that of 2-phosphoglycerate using the yeast enzyme, but the Michaelis constants were similar.[90] The mechanistic implications of these findings are discussed in Section V.D.3).

Three β-halo analogues of 2-phosphoglycerate have been synthesized and their properties reported. The β-iodolactic acid phosphate reacts with loss of HI but no other data are reported.[92] Loss of HCl and HF from the β-chloro- and β-fluorolactic acid phosphate compounds is catalyzed by the yeast and rabbit muscle enzymes.[93] These two compounds react more than two orders of magnitude more slowly than 2-phosphoglycerate and have Michaelis constants which are of the order of 50-fold greater.

The suggestion that the analogues AEP and TSP underwent "partial reactions" has been mentioned. Another competitive inhibitor, 2-phosphoglycolate, has also been found to undergo exchange of an α-hydrogen in the presence of the enzyme (see Section V.D.2).[93]

The new competitive inhibitors include a phosphoenolpyruvate analogue, substituted on carbon-3 with methyl (phosphoenol-α-ketobutyrate).[89] The dissociation constant for phosphoenol-α-ketobutyrate is an order of magnitude smaller than the Michaelis constant for phosphoenolpyruvate.[91]

An analogue of 2-phosphoglycerate with carboxyl replacing the methanolic carbon-3, 2-phosphotartronate, has an inhibition constant that is nearly 50-fold larger than the Michaelis constant for the substrate.[94] Some implications of these findings are also discussed below.

Three other phosphoglycerate analogues — 2-phosphobutyric acid, 2-phospho-3-butynoate, and 2-phospho-3-chloro-3-butenoate — are also shown (Figure 7). The latter two were synthesized in the hope they would be "suicide substrates", but this was not found to be the case.[93]

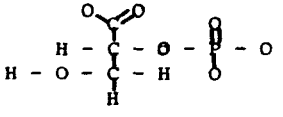
Another analogue of the substrate, with a nitro group replacing the carboxyl, methylene replacing the oxygen between carbon-2, and phosphorous and hydrogen replacing the methanolic carbon-3 (2-nitroethylphosphonic acid), has been found to be a very potent competitive inhibitor at alkaline pH.[95] The carbanionic form is believed to have a dissociation constant of less than 10^{-9} M (see below).[96] Several carbanionic nitro analogues of substrates with carboxyl groups have been found to be potent competitive inhibitors of fumarase and aspartase.[97] The latter enzyme, which catalyzes the elimination of ammonia from aspartate, requires magnesium ion for activity.

B. Stoichiometry and Strength of Substrate and Inhibitor Binding

Cardenas and Wold,[80] using the Hummel-Dryer technique, found that rabbit muscle enolase bound up to 2 mol of glycolic acid phosphate in the presence of excess magnesium ion. The yeast enzyme was shown to bind up to 2 mol of TSP and AEP.[51]

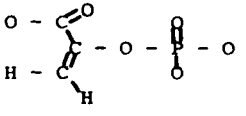
"Substrate" (an equilibrium mixture of 2-phosphoglycerate and phosphoenolpyruvate) and competitive inhibitors produce changes in the enzyme absorption and fluorescence if excess magnesium ion (or some other activating metal ions are present).[50]

1. Substrates



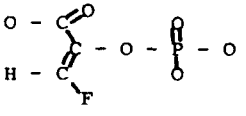
2-phospho-D-glycerate

(0.2 mM)



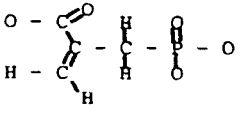
phosphoenolpyruvate

(0.2 mM)



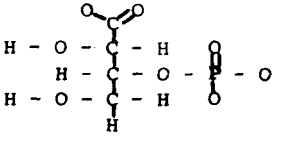
(Z)-phosphoenol-3-fluoropyruvate

(0.02 mM)



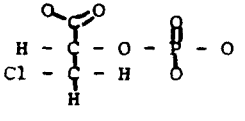
α-(dihydroxyphosphinyl-methyl)-acrylate

(0.008 mM)



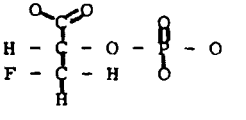
D-erythronate-3-phosphate

(0.3 mM)



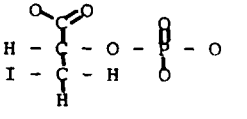
β-chlorolactic acid phosphate

(6.2 mM)



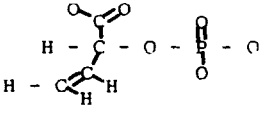
β-fluorolactic acid phosphate

(5 mM)



β-iodolactic acid phosphate

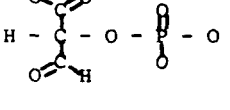
(?)



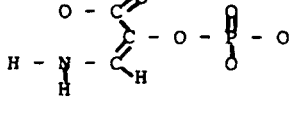
2-phospho-3-butenoic acid

(0.35 mM)

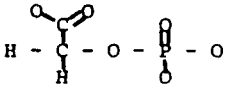
2. Compounds Which Undergo "Partial Reactions"



D-tartronic



3-aminoenolpyruvate-



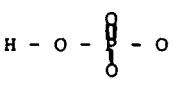
glycolic acid phosphate

semialdehyde 2-phosphate (AEP) (0.4 mM)

phosphate (TSP) (0.0005 mM)

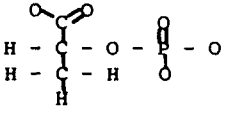
(0.004 mM)

3. Competitive Inhibitors



phosphate

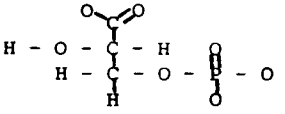
(4 mM)



lactic acid

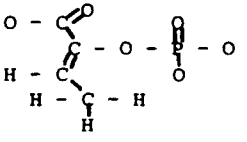
phosphate

(0.4 mM)



3-phospho-D-glycerate

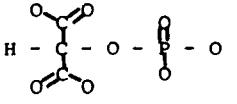
(0.5 mM)



(Z)-phosphoenol-

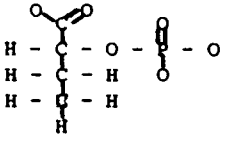
α-ketobutyrate

(0.012 mM)



2-phosphotartronate

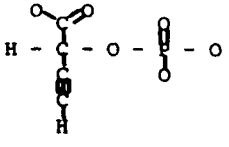
(5.6 mM)



2-phosphobutyric

acid

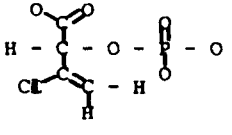
(0.34 mM)



2-phospho-3-

butynoate

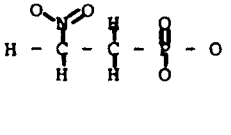
(3.3 mM)



2-phospho-3-chloro-

butenoate

(11 mM)



2-nitroethyl

phosphonic acid

(depends on pH)

FIGURE 7. Substrates and competitive inhibitors of yeast enolase. Previously known substrates are shown.[5] Also shown are those compounds reported since Wold's review[5] or those mentioned in this one. Carboxyl and phosphoryl groups are drawn as anionic forms. The values in parenthesis are Michaelis constants in the case of the substrates and the inhibition or dissociation constants in the case of the substrate analogues.[37,89-96] These were determined in the presence of 1-5 m*M* magnesium, in 0-0.4 *M* KCl. The values do not appear to be reliable to within more than about a factor of 2. See text.

Fluorimetric titrations showed that the substrate and inhibitors dissociation constants tended to decrease with increasing magnesium ion concentration, but were otherwise in good agreement with their Michaelis constants.

The calorimetric titrations of Faller and Johnson[66] showed that the binding sites for the competitive inhibitor 3-phosphoglyceric acid were independent and identical. This is in agreement with the results from fluorimetric[50] and spectrophotometric titrations.[51]

C. Chemical Nature of the Substrate Binding Sites

Reaction of the enzyme with 2,3-butanedione modifies a single arginine per subunit which is correlated with loss of activity.[36] Substrate or AEP protect the enzyme against loss of activity and loss of arginine. The tryptic arginine-containing peptide has been isolated and the critical arginine identified as arg-414.[17] The enzyme still binds AEP after inactivation by butanedione, but with a two- to fivefold greater dissociation constant ($K_D = 5.10^{-7}$ *M)* and with a lower extinction coefficient: $\Delta\epsilon_{295} = 14{,}000\ M^{-1}cm^{-1}$. Some of these findings were confirmed by Borders et al.,[37] though those authors obtained two arginines per subunit modified with butanedione, one of which was protected by substrate. Phosphate (a competitive inhibitor) also protected, suggesting that phosphate binds to the arginine.[37] The AEP data cited[36] would suggest the modified arginine can still allow approach of the phosphate group.

It should be noted that these experiments were carried out in the presence of excess (1 m*M)* magnesium ion as well as substrate. Since additional divalent metal binds in the presence of substrate and perhaps substrate analogues, the possibility exists that the protecting species is the additional metal ion bound, rather than the substrate or phosphate (assuming the additional metal ion binds if phosphate is present); see Section V.

D. Effects of Substrate or Analogue Binding

In considering the effects of substrate binding to the enzyme-conformational metal ion complex, it must be reiterated that the extent to which conformational metal ion shifts to other (i.e., catalytic) sites is not known. For magnesium ion, the evidence indicates the extent of the shift is small, since perhaps two or more orders or magnitude separate the dissociation constants of the conformational sites from the next strongest.[69]

Another point to keep in mind is that few studies have as yet been done using levels of metal ion sufficient for saturation of the conformational sites only.

1. On the Substrate or Analogue Itself

Kinetic studies have shown that the enzymatically important species is the trianionic (fully charged) form of the substrate.[4] However, there is some evidence that the bound species is the dianion. Calorimetry in the presence of excess (1 m*M)* magnesium ion has demonstrated proton uptake on binding the competitive inhibitor 3-phosphoglycerate.[66] Some unpublished observations show proton uptake upon "substrate" binding to the enzyme-conformational magnesium ion complex,[20] and ^{31}P-NMR measurements suggest the dianionic "substrate" is bound under these conditions.[98] Anderson and Cleland[95] have arrived at the same conclusion on the basis of detailed kinetic studies.

The effects of binding of AEP to the magnesium-enzyme have been discussed. There is some evidence as to its overall nature, measured in the presence of excess magnesium ion, and this is described in Section V.D.2.

2. On the Enzyme

There are few demonstrated effects of substrate binding alone on the enzyme; the fluorescence and absorption changes[50] referred to above are almost certainly due to

additional metal ion binding (see below). The most important effect, from a functional point of view, of substrate binding is that it allows the enzyme-conformational metal ion-substrate complex to bind more metal ion.

V. INTERACTION OF METAL IONS WITH THE ENZYME-CONFORMATIONAL METAL ION-SUBSTRATE COMPLEX

Measurements of enzymatic activity as functions of metal ion and substrate concentrations have demonstrated that the metal ion-substrate complex is not the actual substrate, but is in fact kinetically inert.[4,75] The data are discussed by Malmstrom.[10]

A. Stoichiometry and Dissociation Constants

The first direct demonstration that additional divalent metal was bound by enolase when substrate is present was by Hanlon and Westhead, using equilibrium dialysis.[49] Approximately two more moles of manganese (II) or magnesium (II), using a molecular weight of 93,000,[1] were bound by the E-M-S complex. Their work was done in 0.5 *M* KCl.

Brewer also demonstrated additional binding of magnesium by fluorimetric titrations, both at approximately 0.05 ionic strength and in 0.5 *M* KCl.[50] No information about stoichiometry could be obtained. In addition, calorimetric titrations of the E-M-S complex — more precisely the $E_2M_2S_2$ complex — with extra magnesium have demonstrated binding of additional metal.[20]

Faller et al.[69] carried out measurements of magnesium ion binding by enolase using an ion-specific electrode. Two additional moles of magnesium ion bound in the presence of substrate, up to a free metal ion concentration of approximately 3 m*M*, at 0.05 to 0.1 ionic strengths.

Ultrafiltration measurements have also demonstrated that 2 mol of additional zinc (II) binds in the presence of substrate.[39] So again a stoichiometry of 2 mol of metal ion has been demonstrated, and again we assume that one binding site is present on each subunit. This seems reasonable in view of the data of Keresztes-Nagy and Orman[28] and of Holleman.[31]

Some measurements of dissociation constants of the additional metal ion bound in the presence of substrate have been made. In general, binding of the additional metal ion is weaker than that of conformational metal ion. Hanlon and Westhead's[49] equilibrium dialysis data gave dissociation constants of ca. 1 m*M*, values in good agreement with those obtained in 0.5 *M* KCl by Brewer.[50] On the other hand, dissociation constants obtained at low ionic strengths were also about 0.5 m*M*.[50] Faller et al.[69] obtained a dissociation constant at 0.05 to 0.1 ionic strength of 50 μM, so there is a serious discrepancy between the values as measured by fluorimetric titrations[50] and using an ion-specific electrode.[69] This may be related to the fact that enzyme activity drops at higher metal ion concentrations and is discussed further in Section VI.

No information on dissociation constants of the extra zinc (II) bound was obtained, but under the conditions of measurement the dissociation constants could be as high as approximately 1 μM.[39]

In contrast to conformational metal ion binding, substrate-dependent metal ion binding to the two sites seems to be independent and identical.[69]

B. Specificity

Based on the studies of Warburg and Christian,[2] Malmstrom,[3] and Wold and Ballou,[4] metal ions can be divided into activators and nonactivators, and the activating metal ions show varying maximum velocities.[2-4] The only information on the effect of substrate on binding of nonactivating and weakly activating metal ions by the enzyme comes from

Sephadex® filtration measurements on copper (II) and cobalt (II).[73,74] Westhead and coworkers[73,74] have evidence that substrate strengthens binding of approximately 2 mol of each metal ion to produce a total binding of 4 mol of divalent metal per mole of enzyme. The very limited data on copper (II) are especially interesting, since copper (II) is not an activator. There is a question, however, about the possible relevance of Westhead and coworkers' observations[73,74] to "inhibitory" metal ion binding, which will be discussed in Section VI.

There is little other evidence, and that indirect, as to whether the substrate-dependent metal ion binding sites can be filled by any metal ion if the conformational sites are filled by a nonactivator. The little evidence that exists comes from measurements of the AEP absorbance on mixing enzyme-metal$_1$ -AEP with excess metal$_2$, metals 1 and 2 being calcium (II) or magnesium (II). The kinetics suggest it is much easier to replace conformational calcium ion than magnesium ion. Since the reactions show very different kinetics from the EDTA reactions mentioned before, the data suggest initial formation of an enzyme-metal$_1$ - AEP-metal$_2$ complex.[67]

The question of specificity can be extended to the substrate analogue. Is all of it required for additional metal ion binding? The only evidence on this point comes from the equilibrium dialysis data of Hanlon and Westhead,[49] who found up to 4 mol of manganese (II) bound at high (300 μM) concentrations of manganese ion using 2-phospholactic acid and inorganic phosphate; 3-phosphoglycerate produced binding of less than 3 mol of metal ion. This may, however, be from inhibitory plus conformational metal ion (see Section VI).

C. Chemical Nature of the Binding Sites

It has been suggested that because of the apparent interdependence of metal ion and substrate dissociation constants and concentrations that part of the substrate-dependent binding site consists of the substrate.[50] There is calorimetric evidence for proton release on additional metal ion binding.[20] The origin of the proton(s) is unknown.

George and Borders recently demonstrated that carbodiimide inactivation of the enzyme was prevented by magnesium ion (1 mM) and substrate but the effect of different concentrations of metal ion and substrate were not examined.[99] The pH dependence of the inactivations suggested the reactive carboxyl group had a pK of approximately 6.7, suggestive of a hydrophobic environment (see below).[100]

Recently, the photooxidation experiments of Westhead, using rose bengal as photoactive agent, were repeated and extended.[101] In contrast to Westhead's[76] findings, conformational magnesium ion had less effect in protecting against loss of activity. Addition of substrate as well as conformational metal ion had no effect at all. But addition of higher concentrations of metal ion did protect strongly. Four histidines were lost with conformational metal ion only present, and substrate and additional metal ion reduced this loss by one residue per subunit. Evidently three histidines uncorrelated with activity are photooxidized under Elliott and Brewer's conditions.[101] On digestion of enzyme which had been photooxidized with and without substrate and additional magnesium ion and electrophoresis of the digests, the loss of activity was correlated with loss of one histidine-containing tryptic peptide. This had the composition NH_2-his-(asx, leu)-lys-COOH. The critical histidine was then assigned the position his-191 in the amino acid sequence.[17]

However, the concentration of additional metal ion required for protection was rather high; the concentration for 50% of the protective effect (against loss of enzymatic activity) was 2.5 mM. This value was from steady-state rates. Under the conditions of these measurements, 2.5 mM was about eight times the concentration needed for half saturation of the substrate-dependent metal ion binding sites, using Faller et al.'s[69] value of

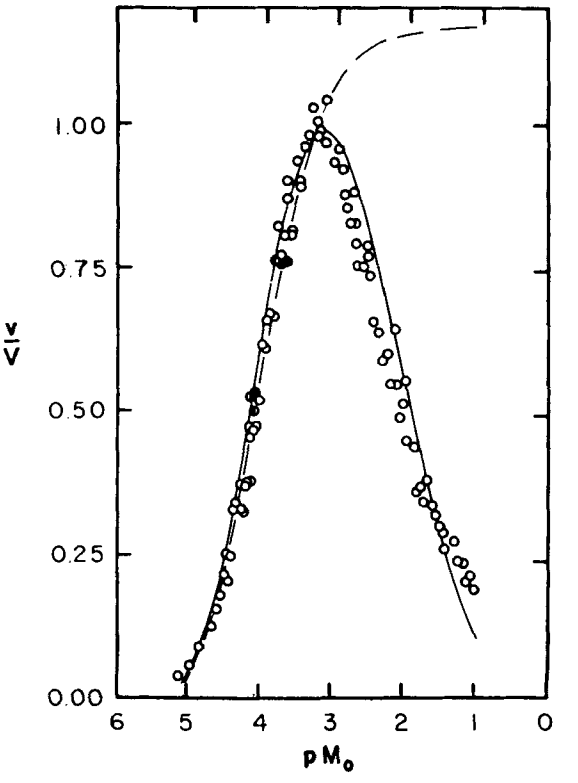


FIGURE 8. Dependence of enzyme activity on magnesium concentration. Stopped-flow measurements were made at pH 7.5, 0.05 ionic strength in tris-HCl (○), PIPES (●), and mixing enzyme and magnesium with substrate or enzyme with magnesium and substrate (◐) in those buffers. The dashed line assumes just 4 mol of magnesium bind; the solid line assumes two additional, inhibitory equivalents of metal bind. (Reprinted with permission from Faller, L. D., Baroudy, B. M., Johnson, A. M., and Ewall, R. X., *Biochemistry,* 16, 3864, 1977. Copyright 1977, American Chemical Society.)

50 μM for the dissociation constant. On the other hand, the midpoint value was a factor of 16 below the concentration required for 50% inhibition of enolase activity (40 mM, from steady-state rates).[101] Since protection by the metal ion is in a sense competing with rose bengal binding, we conclude the substrate-dependent metal ion bound is protecting the histidine. Whether it does this by direct coordination to the imidazole ring or whether its effect is mediated through a conformational change is not known.

Interestingly, titration of the photoinactivated enzyme with AEP showed the AEP again bound with the same affinity as to native enzyme, but with a lower difference extinction at 295 nm ($\Delta\epsilon$ = 10,700 $M^{-1}cm^{-1}$).[101] Since the conformational metal ion binding sites were left intact, this is reasonable (see Section III.E.4).

D. Effect of Additional Metal Ion Binding

1. On Substrates

It has long been known that magnesium ion which is more weakly bound than conformational metal ion was necessary for enzymatic activity.[30] Faller et al.[69] recently

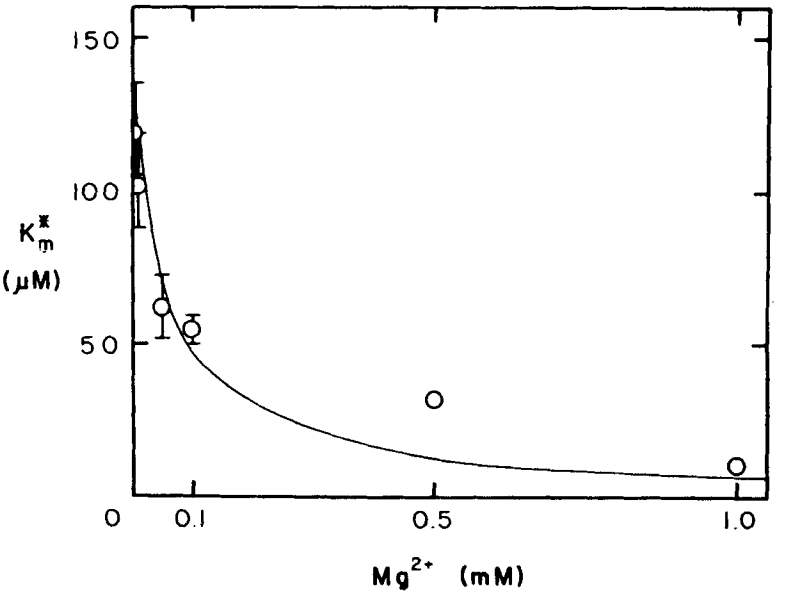


FIGURE 9. Dependence of substrate Michaelis constant (K_m^*) on magnesium concentration. (Reprinted with permission from Faller, L. D., Baroudy, B. M., Johnson, A. M., and Ewall, R. X., *Biochemistry*, 16, 3864, 1977. Copyright 1977, American Chemical Society.)

measured enzymatic activities using a stopped-flow apparatus and obtained a precise correlation between additional metal ion binding and appearance of enzymatic activity (Figure 8). Conformational metal ion alone was not sufficient for enzymatic activity. The authors could not say whether 1 mol of two bound produced all the activity or whether each independently did, but the latter possibility seems far more likely.

The major effect of additional metal ion binding in the presence of substrate is catalysis. The data of Faller et al. are strong evidence that enzyme with conformational metal ion only is inactive.[69] These findings predict a strictly ordered sequential mechanism of action for yeast enolase. Originally, Hanlon and Westhead[102] found no evidence for an ordered sequence, i.e., the Michaelis constants for metal ion and substrate showed no dependence on the concentration of the other ligand. However, they were using relatively high magnesium (II) and manganese (II) concentrations. Nowak et al.[78] used lower metal ion and substrate concentrations, and later Faller et al.[69] using a stopped-flow apparatus and lower metal ion concentrations obtained the expected variations in K_M for magnesium ion with substrate concentration (and vice versa), confirming that yeast enolase shows the kinetics expected for an ordered sequence (Figure 9). In Vallee's terminology, yeast enolase would be a metal ion-activated metalloenzyme.[70]

The metal ion specificity of the production of the AEP absorbance change suggested[67] the "catalytic" metal ion controls the rate of a step aside from substrate "activation", which is controlled by conformational metal ion. This was supported by results from experiments in which enzyme with two equivalents of magnesium (II), nickel (II) (nickel II is the weakest activator), or calcium (II) was mixed in the stopped flow with two equivalents of the same or another metal ion and 1 to 2 m*M* substrate[67] (Table 4). The enzymatic activities demonstrate that exchange between catalytic and conformational sites is slow enough to show that the presence of an activating metal ion as conformational metal ion is important, and that the rate of the reaction seems to depend

Table 4
EFFECT OF MIXING ENZYME-CONFORMATIONAL METAL COMPLEX WITH SUBSTRATE AND VARIOUS CATALYTIC METALS

Present initially	Added with substrate	Relative activities (%)
0	0	0
2 Mg (II)	0	10
2 Mg (II)	2 Mg (II)	73
2 Mg (II)	2 Ca (II)	23
2 Mg (II)	2 Ni (II)	22
2 Ca (II)	2 Ni (II)	1
2 Ca (II)	2 Ca (II)	0
2 Ca (II)	2 Mg (II)	18
2 Ni (II)	2 Mg (II)	50
2 Ni (II)	2 Ca (II)	10
2 Ni (II)	2 Ni (II)	10

Note: $3 \cdot 10^{-5}$ enzyme with metals as shown was mixed with 2 m*M* 2-phosphoglycerate with metals as shown. All reactions were in 0.05 ionic strength tris-HCl, pH 7.8 at 26°. Data are expressed relative to a control experiment in which $5 \cdot 10^{-4}$ *M* magnesium was present.

From Brewer, J. M. and Collins, K. M., *J. Inorg. Biochem.*, 13, 151, 1980. With permission.

more on the metal ions added with the substrate. This is provided that an "activating" metal ion (one that would produce the absorbance change in AEP) was added with the enzyme.

The relative maximum rates are believed to be in the order: Mg(II) > Zn (II) > Mn(II) > Fe (II) > Cd(II) > Co(II), Ni(II) (the position of Cr II is not available).[2-4] However, this reflects not only the effect of the metal ion in promoting the rate of the limiting step, but probably also the effect of binding metal ion at "inhibitory" sites (see Section VI).* It is possible there is some as yet undemonstrated kinetically limiting effect of conformational metal ion as well; this is suggested by the slowness of zinc (II) and cadmium (II) in producing the absorbance change in AEP (above).[67]

A related point is illustrated in Table 4. It is possible that a nonactivating metal ion might function as a "catalytic" metal ion if an activating one were in the conformational sites.

There does not appear, however, to be a single process that completely determines the overall rate of the enolase reaction. Shen and Westhead have carried out steady-state kinetic studies of the effect of substitution of deuterium for hydrogen at carbon-2 of the substrate.[103] With magnesium ion, they obtained a primary isotope effect of 3 which disappeared at pH 8.5 and above (Figure 10). The isotope effect for manganese (II) was smaller, but showed the same pH-dependence. No isotope effect was obtained using cobalt (II). An isotope effect of 3 is about one third of the expected effect if proton

* Though this work[2-4] was done before it was discovered that the enzyme contained strongly bound magnesium ion,[30] the author believes it is still largely valid. In the presence of an excess of another metal ion, the conformational magnesium ion would be replaced, so steady-state kinetics would be determined by the metal ion in great excess. This is particularly true in the presence of 0.4 to 0.5 *M* KCl. On the other hand, measurements of metal ion binding stoichiometries and dissociation constants should probably be regarded with suspicion.

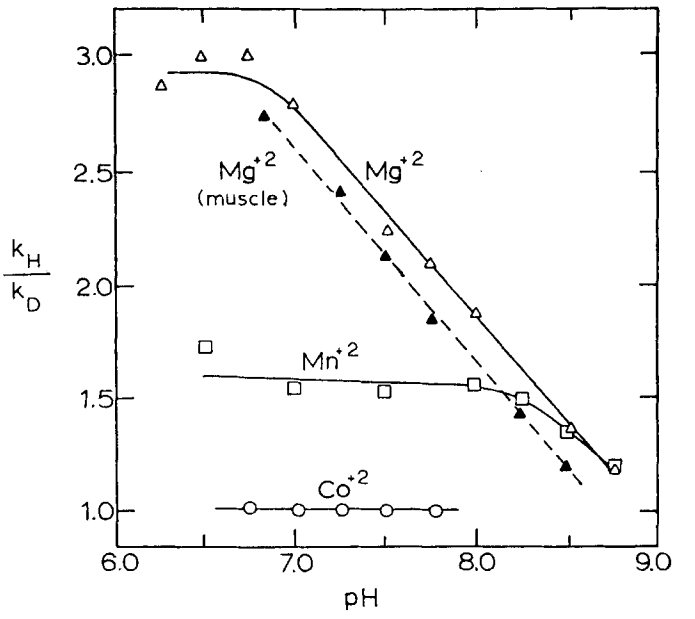


FIGURE 10. Effect of the pH on isotope effect (k_H / k_D) in dehydration of 2-deutero-2-phosphoglycerate. The solid lines represent data obtained using the yeast enzyme; the dashed line gives data obtained using rabbit muscle enolase. The triangles were obtained using magnesium, the squares, manganese, and the filled circles, cobalt. (Reprinted with permission from Shen, T. Y. S. and Westhead, E. W., *Biochemistry*, 12, 3333, 1973. Copyright 1973, American Chemical Society.)

removal from carbon-2 were completely limiting. Shen and Westhead concluded that at least three steps were important: proton abstraction, hydroxyl removal, and product release, apparently in that order.[103] So with magnesium (II) or manganese (II), proton removal from carbon-2 is somewhat limiting, except at alkaline pHs. At alkaline pHs, or at all pHs with the cobalt-enzyme, hydroxyl removal or possibly product release was limiting. Shen and Westhead presented evidence that an unprotonated base with a pK of 6.7 is involved in removal of the carbon-2 proton and that at least one protonated group is involved in some other step.[103] The unprotonated base may be the active-site carboxyl group demonstrated by George and Borders.[99]

In a recent abstract, Anderson and Cleland[95] reported results of measurements of ^{18}O and deuterium isotope effects and correlation of these with kinetic parameters. Only magnesium (II) was used. They confirmed and extended the findings of Shen and Westhead.[103] Their measurements indicated that C—O bond breakage occurs 13 times faster than C—H bond breakage, at lower pHs. At more alkaline pHs, release of 2-phosphoglycerate (rather than hydroxyl removal) is rate limiting. Their results were interpreted in terms of a carbanion mechanism involving loss of a proton from carbon-2 independently of loss of the hydroxyl on carbon-3.

Lane and Hurst[91] attempted to verify whether a carbanion intermediate might be involved in the enolase reaction. (This question was discussed in some detail in the review by Wold,[5] for example.) Lane and Hurst used carbanion-trapping reagents, but found no evidence of carbanions.[91] If such an intermediate exists, it is not readily accessible to the solvent.

Lane and Hurst,[91] using stopped-flow, temperature-jump methods, also found a signal of moderate amplitude which involved binding of phosphoenolpyruvate to the enzyme.

No proton equilibria were involved, and no firm conclusions could be drawn as to the origin of the signal. It was attributed to phosphoenolpyruvate distortion by enzyme-metal (see below). Two substrate analogues — (Z)-phosphoenol-3-fluoropyruvate and (Z)-α(dihydroxyphosphinylmethyl)-acrylate — produced a similar signal, and perhaps significantly, the half-times of their signals were 10- to 30-fold longer than that produced by phosphoenolpyruvate.

2. On TSP and AEP

These compounds show marked changes in absorbance[51] on binding to enzyme in the presence of activating metal ions.[67] Both AEP and TSP are unstable,[104] and the structures given are only inferred. The TSP absorbance change is probably due partly to an enolization of the aldehyde,[104] a suggestion supported by recent NMR data.[93] If the structures are correct, then as Spring and Wold pointed out[51] one must consider the cis-trans isomerization possibilities in the case of AEP, and they suggested that the absorbance increase obtained on binding to the magnesium-enzyme might involve a shift in cis-trans equilibria. However, the only work on AEP binding kinetics does not support this suggestion.

Measurements of AEP binding by stopped-flow techniques confirmed Spring and Wold's[51] observation that the association of the inhibitor was slow — a value for k_{assoc} of $5 \times 10^4/M/\text{sec}$ was obtained.[91] Lane and Hurst also found that the binding was accounted for by a simple binding scheme:[91]

$$E + AEP \rightleftharpoons E.AEP \qquad (7)$$

This was in the presence of excess magnesium ion. However, the kinetics observed were not consistent with one form of the two cis-trans isomers being bound preferentially; the AEP does bind slowly but there is no suggestion of two forms in solution which interact differently.[91] These observations suggest that AEP in solution has a great deal of single bond character in the bond between carbons two and three. The low association (rate) constant might occur as the result of a positive charge on the nitrogen. TSP on the other hand bound in a two-step reaction, with the initial bimolecular step being too fast to be measured in the stopped flow:

$$E + TSP \rightleftharpoons E.TSP \rightleftharpoons E.TSP^* \qquad (8)$$

The second step probably involves enolization of the TSP.

However, the red shifts and increases in integrated absorbance seen in both compounds could be produced by a twisting of a double bond between the second and third carbons of the bound species.[105] Some CD data support this suggestion;[67] TSP bound to magnesium-enolase shows a negative CD difference spectrum with a positive component at longer wavelengths. AEP, which should have no asymetric carbon, shows a strong negative CD difference spectrum. These spectra are also observed when these compounds are bound to nickel-enolase but not to calcium-enolase.[67] Evidently both compounds become optically active when bound to enzyme in the presence of an activating divalent metal, and a twisted carbon-carbon double bond would be expected to have such properties. Brewer and Collins[67] pointed out that such a structure would appear to be a likely intermediate or "transition state".

It should be noted that while conformational metal ion appears to produce the bulk (and perhaps all) of the absorbance change in AEP, there is a strong possibility that the additional metal ion enhances the effect. Very limited CD data are consistent with this possibility.[67]

Stubbe and Abeles[93] found that the yeast and rabbit muscle enzymes catalyze an ex-

change of the α-hydrogen of TSP (see below). Spring and Wold[51] were unable to demonstrate this reaction. Possibly the different experimental conditions account for this discrepancy: Stubbe and Abeles used 0.4 *M* KCl and relatively high concentrations of magnesium ion (5 m*M*).[93]

3. On Other Analogues

The failure of Lane and Hurst[91] to detect carbanion formation has been mentioned. On the other hand the extremely strong binding to the yeast enzyme of 2-nitroethylphosphonic acid which has lost a proton from the α carbon strongly supports the carbanion mechanism.[96] These observations can be reconciled if the carbanion produced is not accessible to the solvent.

More recently, Stubbe and Abeles[93] reported that yeast enolase and manganese (II) produced a slow (net) exchange of the α-hydrogens of phosphoglycolic acid. There was no deuterium isotope effect on the reaction, an observation which is also consistent with the above suggestion that the abstracted proton is "stored" internally and does not exchange readily with the solvent. The rate-limiting process would be the exchange with the solvent, not the actual proton abstraction and concomitant carbanion formation.

Additional evidence[93] supporting a carbanion mechanism was the finding of a small secondary isotope effect produced by deuterium substitution on carbon-3 of β-chlorolactic acid phosphate. A concerted mechanism — loss of both hydroxyl and proton at the same time — would involve a larger secondary isotope effect. The fact that HF was eliminated faster than HCl from the halo analogues was also consistent with a carbanion mechanism, since fluoride would be eliminated more slowly if carbon-halogen bond cleavage were to occur in the transition state. The primary isotope effect on hydrogen abstraction from the β-chloro analogue was large, 7 with the yeast enzyme, suggesting a completely rate-limiting step.[93]

It has also been noted that the β, γ-α, β isomerization catalyzed by the enzyme is consistent with a carbanion mechanism,[90] in which abstraction of the α-hydrogen would be the key step in producing the isomerization.

Stubbe and Abeles[93] measured the rate of exchange of the α-hydrogen of a number of substrate analogues catalyzed by the yeast and rabbit muscle enzymes. Some of their findings have been mentioned. They found that replacement of the β-hydroxyl of 2-phosphoglycerate or its methanolic group with various substituents drastically reduced the rate of abstraction of the α-hydrogen. These authors concluded that the β-hydroxyl had a large effect on the rate of breakage of the α-hydrogen bond. They suggested the β-hydroxyl interacted specifically with the active site, in agreement with Nowak et al.'s[78] hypothesis. The finding that the fluoro analogue reacted faster than the chloro despite the fact that chloride is a better "leaving group", should be considered in light of Maurer and Nowak's observations concerning the mechanism of inhibition of yeast enolase by fluoride.[84] The suggestion of Brewer and Collins[67] that TSP and AEP are bound in a particular configuration when activating metals are present is also pertinent.

Overall, these data are in agreement that the elimination of water from the substrate occurs with an initial abstraction of the α-hydrogen, forming a carbanion, then loss of hydroxyl in a nonconcerted step.[93]

4. On the Enzyme

The effect of substrate and excess metal ion on the absorption and fluoresence of the enzyme has been mentioned,[50] as has the conclusion that the effect is almost certainly due to additional, possibly "catalytic" metal ion binding. Activating metal ions produced the effect, and only with substrates or competitive inhibitors. The magnitude of the effect varied with the metal ion roughly according to the catalytic efficiency of the metal ion,

and with the phosphate ester according to no recognizable pattern.[50] While the "substrate" binding constants were close to the average Michaelis constant for substrate and product, the apparent magnesium ion binding constants in 0.05 ionic strength solutions tended to be an order of magnitude greater (approximately 0.5 m*M*) than the metal ion Michaelis or dissociation constant (approximately 50 μM).[69] On the other hand, they were also smaller than the apparent binding constants for "inhibitory" magnesium ion by an order of magnitude (see below). This discrepancy has never been resolved.

Holleman[31] showed the enzyme will dissociate reversibly at 40° in the presence of magnesium ion alone (1 m*M*). In the presence of magnesium ion and substrate, the apparent dissociation constant is reduced by a factor of 200, so there may well be an effect of catalytic metal ion on the conformation of the protein. Of course, this last effect could be due to substrate binding alone.

It has also been suggested[101] that substrate with magnesium ion at the 1 m*M* level has protective effects on more than one histidine through a conformational change.

The suggestions that catalytic metal ion binding affects the conformation of the enzyme has possible relevance to the mechanism of its participation in catalysis.

E. The Role of Metal Ions in Catalysis

There is no evidence proving that either metal ion interacts directly with any part of the substrate. Nowak et al.'s[78] data were interpreted in terms of interaction between conformational metal ion and the hydroxyl on the third carbon because the metal ion was too far from the phosphate and the hydrogens on carbon-two to be consistent with interaction between the carboxyl, the phosphate, or the hydrogens. Still, their hypothesis dominates thinking about the role of conformational metal ion.

In light of Maurer and Nowak's data on the interaction of fluoride and phosphate with the manganese-enzyme,[84] one can postulate that the phosphoryl and hydroxyl group of the substrate interact with the magnesium-enzyme in the same synergistic fashion. This interaction changes the geometry of the conformational metal ion in some as yet undefined way. The AEP and TSP data[67] suggest the hydroxyl of the substrate is bound in this complex at a particular angle to the rest of the molecule, an angle optimal for the reaction to take place.

There are two pieces of evidence which seemingly contradict the postulation of a direct interaction. The first is the relatively weak binding of the 3-carboxyl analogue.[94] However, all the evidence[5] on substrate specificity indicates the spatial requirements of the 3-hydroxyl region are strict, and the 3-carboxyl may simply not "fit". The second is the observation that AEP binds more strongly to the magnesium-enzyme than TSP.[51,91] The strength of binding of these two compounds certainly suggests a direct interaction between the electron-rich enolate oxygen or amine nitrogen and something, but if the something were conformational magnesium ion, we would expect a stronger interaction with TSP.[67] However, Stubbe and Abeles have pointed out that TSP exists in solution predominantly as the hydrated aldehyde, and that means its actual affinity for the enzyme must be at least 20-fold greater, possibly greater than that of AEP.[93]

The location and function of catalytic metal ion must be entirely speculative at present. Since the nearly rate-limiting step at acid pHs concerns proton abstraction from carbon-two[103] and since catalytic metal ion is thought to control the rate of the reaction,[67] it seems simplest to suggest the catalytic metal ion binds at a site which consists partly of the carboxyl group of the substrate. This would tend to make the α-hydrogens more acidic and hence facilitate formation of a carbanion. Binding to the phosphate would not seem to be so effective as the metal ion would be one atom farther away from the α-hydrogen. On the other hand, Stubbe and Abeles[93] have noted that the exchange of the α-hydrogen

of two substrate analogues as well as the natural substrate is increased by manganese (II), relative to magnesium (II). This tends to contradict the idea of direct interaction with the carboxyl.

The level of activity, the value of V_{max}, does not appear to differ more than an order of magnitude, comparing the values obtained using nickel (II) or cobalt (II) and magnesium (II).[39,103] The level of activity is roughly inversely proportional to the electronegativity of the metal ion.[87] It is also proportional to the readiness of the metal ion to form complexes which are other than octahedral.[106,107] Again, there is a suggestion that a particular geometry of complex may be involved.

VI. INHIBITORY METAL ION BINDING TO YEAST ENOLASE

It has long been known also that a sufficient excess of any activating metal ion inhibits the enzyme.[10] This can be through simple chelation of the substrate by the metal ion, but kinetic studies have shown that metal ions may inhibit by binding to the enzyme, either at a separate site or in a different way from catalytic metal ion. See, for example, the article by Wold and Ballou.[4]

A. Stoichiometry and Dissociation Constants

There are currently two theories about how the above-mentioned inhibition occurs. One theory, based on the work of Westhead and co-workers,[73,74] is that metal ion binds to the catalytic sites in the absence of substrate, and the binding of substrate strengthens the metal ion binding. The 2 mol of metal ion at the catalytic sites then assume different functions: the more strongly bound one produces catalysis while the more weakly bound one inhibits. This theory is based on the observations that only 4 mol of cobalt (II) or copper (II) bind per mole of enzyme whether or not substrate is present. Substrate strengthens the binding of 2 of the 4 mol of cobalt (II). The activity of the enzyme rises with increasing cobalt ion concentration, with an activation constant of 3.5 μM, then falls, with an inhibition constant of 18.5 μM. Binding of metal ion was measured using chromatography on Sephadex®.[74]

The second theory is based on the work of Elliott and Brewer.[39] Those authors, using ultrafiltration to measure metal ion binding, found a total of 6 mol of zinc (II) bound under conditions of near-total inhibition of enzymatic activity. Addition of 0.05 M imidazole relieved the inhibition and removed 2 mol of zinc (II), leaving four bound in the presence of substrate.

The reason for the discrepancy between these two theories, which are based on different methods of measurement of metal binding, is not known. It seems odd that the catalytic metal ions should assume different roles and dissociation constants, but it is certainly not impossible. There are some data in the literature which suggest binding of metal ion to the enzyme beyond 4 mol in the presence of substrate. Hanlon and Westhead[49] used equilibrium dialysis to measure manganese (II) binding to the enzyme in 0.5 M KCl. If their data are corrected for the true molecular weight, 2.8 mol of manganese are bound at 500 μM free manganese (II) in the absence of substrate and 5.3 mol in its presence. Two and 4 mol of magnesium (II) were bound under the same respective conditions. It seems more likely that inhibition is produced through binding of metal ions to an entirely separate class of sites, but it is by no means certain.

Faller et al.[69] showed that inhibition of the enzyme at high concentrations of magnesium ion occurred at a midpoint metal concentration of 9 mM, measuring enzyme activity using stopped-flow techniques. In a recent abstract[95] Anderson and Cleland noted an inhibitory effect of magnesium ion with an inhibition constant of 12 mM.

Measurements of enzyme activity have also been used to obtain relative inhibition

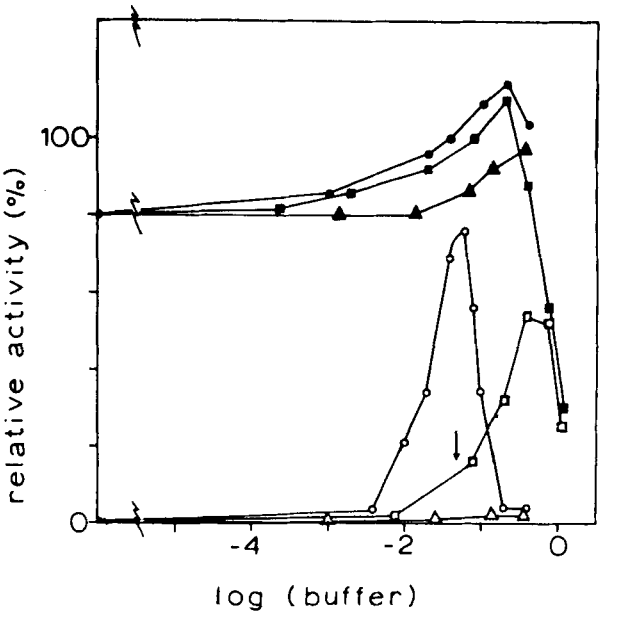


FIGURE 11. Effect of concentration of various buffers on activity. 2.5 m of 10^{-8} *M* yeast apoenolase with no buffer (far left points), (adjusted to pH 7.8 with tetrapropylammonium hydroxide) or with pH 7.8 buffers at the concentrations shown was assayed for enzymatic activity by addition of 0.1 mℓ of 25 m*M* 2-phosphoglycerate (tricyclohexylammonium salt). The solid points (upper three curves) were obtained using 1 m*M* $MgCl_2$; the open points with 1 m*M* $ZnCl_2$. The circles were obtained using imidazole-HCl, the squares with tris-HCl, and the triangles with tetrapropylammonium-HCl. (From Elliott, J. I. and Brewer, J. M., *J. Inorg. Biochem.*, 12, 323, 1980. With permission.)

constants of other metal ions. The measurements were made in the presence of 1 m*M* magnesium ion so the results appear to be valid as relative measures of affinity. Apparent dissociation constants for inhibition decreased according to the series: Cu(II) > Zn(II) > Cd(II) > Mn(II), Co(II) > Ni(II).[39] Since complete or nearly complete inhibition was produced by all the metal ions, irrespective of the level of enzymatic activity they could produce in other circumstances, it was concluded that the mechanism of inhibition was the same (see below).[39] Calcium (II) and terbium (III) also inhibit, but this is believed by replacement of conformational magnesium ion.[39]

B. Chemical Nature of the Binding Sites

The fact that inhibition is produced so readily — even in the presence of 1 m*M* magnesium ion — by transition metal ions, argues that the ligands are quite different from those involved in conformational metal ion binding. The inhibition by the transition metal ions was relieved by 0.05 to 0.1 *M* imidazole buffer, and this appeared to be a simple chelation phenomenon.[39] Tris was less effective; tetrapropylammonium borate was used for producing optimal inhibition (Figure 11). This lead to the suggestion that nitrogenous ligands in the enzyme such as imidazole (from histidine) may be involved in binding inhibitory metal ions. From the relative effectiveness of the transition metal ions in the series given above, it can be argued that tetrahedral geometry is involved.[106]

C. Relation to Catalysis

The major effect of inhibitory metal ion binding is nearly complete inhibition of enzymatic activity.[39] The mechanism by which this is achieved is not certain. Elliott and Brewer[39] suggested inhibition occurs because the metal ion coordinates to a residue which is necessary for enzymatic activity, perhaps his-191. Another possibility is through a conformational change in the enzyme. This is discussed further below. Anderson and Cleland[95,96] suggested that inhibitory magnesium ion coordinated to the phosphate of the substrate and acted by slowing release of substrate and product.

D. Effect of Binding on the Enzyme

Addition of high concentrations of transition metal ions causes the appearance of some denatured, insoluble enzyme. This may be an effect that is confined to a small fraction of soluble but already denatured protein present. The discrepancies between catalytic metal ion binding constants[69] and those measured using protection from photooxidation[101] and from fluorimetric titrations[50] have been mentioned. The possibility of a contribution from inhibitory metal ion binding to these phenomena cannot be dismissed.

There are also effects of inhibitory metal ion on the kinetics of the enzyme. The effect of the inhibitory magnesium ion occurs at a midpoint metal ion concentration of about 9 m*M* if the initial reaction is measured using stopped-flow techniques.[69] If steady-state rates are measured, the midpoint for the inhibition shifts to about 40 m*M*, and an acceleration in the reaction at high metal ion concentrations becomes noticeable, one extending over perhaps 10 sec.[101] Faller et al.[69] also noted the appearance of nonlinearity at high magnesium ion concentrations. The author hypothesizes that the binding of the inhibitory metal ion produces a conformational change in the complex which leads to less inhibited enzyme:

$$E_2M_2S_2M_2 + nM \overset{\text{fast}}{\rightleftharpoons} \underset{\text{(inhibited)}}{E_2M_2S_2M_2 \cdot M_n} \qquad (9)$$

$$E_2M_2S_2M_2 \cdot M_n \overset{\text{slow}}{\rightleftharpoons} \underset{\text{(less inhibited)}}{{}^*E_2M_2S_2M_2 \cdot M_n} \qquad (10)$$

There appears to be some dynamic relationship between the inhibitory metal ion and the catalytic complex which is as yet undetermined.

VII. RELATION TO OTHER ENZYMES

A number of other enzymes appear to use a "two metal ion" mechanism, or at least require 2 mol of metal ion per mole of active site. Some, like yeast enolase, can be considered metal ion-activated metalloenzymes and some are apparently metalloenzymes that utilize metal ion complexes as substrates. In two cases, inhibitory metal ion binding sites are also present.

A. Enzymes with ATP as Substrate or Product

These all use metal ion-nucleotide phosphate complexes as the substrates.

Pyruvate kinase — Pyruvate kinase (rabbit muscle) is a very well-studied enzyme. It has been shown that divalent metal and monovalent cations can produce conformational changes in the enzyme.[108] More recently it has also been shown that additional divalent cations, bound to the ATP, are also required for activity.[109] For these experiments, a "partial reaction", the enolization of pyruvate, was used as the assay along with an inert chromium-ATP complex. The "conformational" metal ion binds to the enzyme but not

apparently to the pyruvate — the monovalent cation interacts with the pyruvate. The conformational metal ion has little effect on the enolization reaction, but the metal ion bound to the ATP determines the rate of that reaction. The rate is a function of the pKa or electrophilicity of metal-bound water.

Glutamine synthetase — The *E. coli* enzyme binds 2 mol of divalent metal per subunit, one of which can be bound as the metal-ADP complex.[110] This more weakly bound metal ion is involved with ADP binding, the more strongly bound near the glutamate binding site. It has been suggested that the more strongly bound metal ion interacts with the γ-carboxyl of glutamate while the more weakly bound one activates the ADP or ATP.[111]

Carbamoyl-phosphate synthetase — This enzyme, also from *E. coli,* has one strong divalent metal binding site, and uses divalent metal-nucleotide complexes as substrates.[112] There also appears to be an inhibitory site/subunit which prefers divalent zinc, manganese, or cobalt.

Adenosine triphosphatase — Adolfsen and Moudrianakis[113] presented kinetic evidence that showed that the true substrate for heat-activated ATPase from the 13S coupling factor of oxidative phosphorylation from *A. faecalis* is Mg (II)·ATP. Magnesium ion also inhibited by binding at a separate site on the enzyme. It appears possible that a strong binding site on the enzyme also exists, which is involved in enzyme activation.

Phosphoribosylpyrophosphate synthetase — Li et al. used a cobalt-ATP coordination complex to examine the mechanism of metal activation of the enzyme from *S. typhimurium.*[114] Using a combination of EPR and kinetic studies, they were able to demonstrate that enzyme-bound metal ion was also necessary for the reaction.

B. Non ATP-Utilizing Enzymes

Most of these can be classified as metal ion-activated metalloenzymes.

Liver alcohol dehydrogenase — Liver alcohol dehydrogenase, a dimeric enzyme, has recently been found to have two classes of metal ions, a "catalytic" and a "structural", one of each per subunit. Sytkowski and Vallee[115] were able to substitute cobalt (II) for the normal zinc (II) for each category. They found, as in the case of enolase, that the catalytic metal ion determined the rate of reaction. (The exchange of metal ions is so much slower with the dehydrogenase than with enolase they could do these experiments by static methods.)

Alkaline phosphatase — Alkaline phosphatase from *E. coli* binds 2 mol of divalent zinc per monomer, one structural and one catalytic;[116] this enzyme is also a dimer.[117] The enzyme also binds additional magnesium ion which appears to enhance activity and thermal stability. A recent examination of this enzyme by ^{31}P and ^{113}Cd NMR has been published.[118] This enzyme binds only a single phosphate — there is a strong anticooperative interaction between the phosphate binding sites. The environments of the two strongly bound metal ions, originally identical, change when the phosphate binds to nonidentical ones. Additional metal ion further changes the ^{31}P NMR spectrum. However, the exact relation between activity and metal ion content is not as clear as in the case of enolase or some other enzymes.

Pyrophosphatase — Pyrophosphatase from yeast has been shown to have two classes of bound zinc ion per monomer which differ in binding affinity. The more weakly bound appears more involved with activity.[119]

Fructose-1,6-bisphosphatase — The enzyme from rabbit liver is a tetramer and has recently been shown using gel filtration and equilibrium dialysis techniques to bind 4 mol of divalent zinc.[120] The dissociation constants range from below 10^{-7} *M* to 10^{-6} *M* suggesting negative cooperativity. In the presence of a substrate analogue under conditions where 2 mol of analogue bind, 4 more moles of zinc ion bind to the enzyme.

These additional sites have been suggested as "catalytic" sites because with manganese ion as activator, the Michaelis constant and dissociation constant were somewhat higher (1.5×10^{-5} *M*), and because of similar saturating levels for activity and "catalytic" zinc ion binding. Additional zinc (II) can bind under some circumstances, but is associated with denaturation of the enzyme.

Orotate phosphoribosyltransferase — This enzyme, from yeast, is a dimer. Kinetic and EPR studies suggest that a metal-substrate complex is the actual substrate and that a metal-enzyme-substrate-metal complex is the predominant species under optimal conditions. Victor et al.[121] also felt that the metal ion-free enzyme was active with the metal-substrate complex, however.

C. Summary

The significance of the "conformational-catalytic" mechanism of metal ion activation is not known. It is still at the state of being established for some enzymes. Right now, only speculations can be offered. All the enzymes mentioned have subunits and all have strongly negatively charged phosphate-containing substrates.

As a preliminary hypothesis, it may be suggested that one of the 2 mol of metal ion involved functions to "lock" one of the substrates into a configuration so that when the second one attacks that or another substrate, electrons are withdrawn from crucial bonds and catalysis occurs. Either metal ion or both or neither may do this by interacting directly with a substrate.

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