L-Rhamnulose 1-Phosphate Aldolase of *Escherichia coli*. The Role of Metal in Enzyme Structure†

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ABSTRACT: L-Rhamnulose 1-phosphate aldolase (L-rhamnulose 1-phosphate L-lactaldehyde-lyase, EC 4.1.2.b) is a metalloenzyme which contains 2 g-atoms of zinc per mole of enzyme (mol wt 135,000) (Schwartz, N. B., and Feingold, D. S. (1972), *Bioinorg. Chem. 1*, 233). All the half-cystine groups of the aldolase could be titrated with 5,5′-dithiobis(2-nitrobenzoic acid) or with *p*-mercuribenzoic acid to yield an inactive enzyme. Sodium dodecyl sulfate was an inhibitor strictly competitive with L-rhamnulose 1-phosphate and dihydroxyacetone phosphate and noncompetitive with L-lactaldehyde. The combined action of sodium dodecyl sulfate and 2-mercaptoethanol converted the enzyme to inactive, metal-free subunits of molecular weight 35,000. Dialysis of the latter against 2-mercaptoethanol and Mn(II), Co(II), or Zn(II) could restore respectively at least 47, 25, and 22% of the initial activity; no activity was restored in the absence of 2-mercaptoethanol or when EDTA was present during the dialysis. Reactivated enzyme preparations contained material with the gel electrophoretic mobility and immunological properties of native enzyme. Electron micrographs of the crystalline aldolase showed that the enzyme contains four subunits arranged in a square.

A metalloenzyme which contains 2 g-atoms of Zn(II) per mole of enzyme (Schwartz and Feingold, 1972a) is L-rhamnulose 1-phosphate (L-rhamnulose-1-P) aldolase from *Escherichia coli*. The metal participates in enzyme activity at least to the extent of binding ketose phosphate substrate (Schwartz and Feingold, 1972b), and probably also aids in the deprotonation of the substrate by polarizing the carbonyl group. Such an effect of metal has been demonstrated by nuclear magnetic resonance with D-fructose 1,6-diphosphate (FDP) aldolase from yeast (Mildvan *et al.*, 1971), which contains 2 g-atoms of Zn(II) per two subunits of molecular weight 40,000 (Harris *et al.*, 1969).

The metal ion of FDP aldolase from yeast is essential for enzyme activity; although the metal can be removed without causing dissociation (Harris *et al.*, 1969), it is not known whether it is required for reassociation of the subunits. The results presented in this paper show that in L-rhamnulose-1-P aldolase the metal is required for the subunits to form active tetramer, in addition to being essential for enzyme activity.

Experimental Section

Enzyme. The purification and assay procedures for L-rhamnulose-1-P aldolase have been described previously (Chiu and Feingold, 1969).

Reagents. Sources and preparation of reagents used have been described previously (Schwartz and Feingold, 1972a). Salts of divalent metals used were spectrometrically standardized materials obtained from Johnson Matthey, London, England.

Disc Gel Electrophoresis. Polyacrylamide gel electrophoresis was performed as described by Ornstein (1964), and Davis (1964). The anionic gel system provided final acrylamide concentration of 7.5% and a running pH of 9.3; protein was located on the gels by staining with Coomassie Brilliant Blue. In addition, protein bands were located and their relative quantity was determined after electrophoresis by direct absorbance measurements at 245 nm with a Model 2410-S Linear Transport system attached to a Gilford 2400 spectrophotometer (Gilford Instruments, Yellow Springs, Ohio). The molecular weight of polypeptide chains was determined by electrophoresis in 5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate according to the procedure of Shapiro *et al.* (1967).

Immunochemical Methods. Antiserum to L-rhamnulose-1-P aldolase was prepared in rabbits as described previously (Chiu and Feingold, 1969). Immunodiffusion was performed by the method of Ouchterlony (1958) is preformed discs obtained from Miles Laboratories, Inc. (Kankakee, Ill.), or on Noble agar (Difco) plates on microscope slides. The center well of the discs contained 0.2 ml of antiserum to native enzyme and the peripheral wells contained 75 μl of solutions (2 mg of protein/ml) of the antigens.

Kinetic Studies. Experimental details of kinetic studies are given under Results. All determinations were performed at least twice. The slopes and intercepts which best fit the experimental points by the criterion of least squares (Reichmann, 1961) were used in all calculations.

Sulfhydryl Titrations. 5,5′-Dithiobis(2-nitrobenzoic acid) (Nbs_2)[1] purchased from Aldrich Chemical Co. (Milwaukee, Wisc.) was used for sulfhydryl determinations according to the colorimetric method of Ellman (1957). Reaction mixtures contained enzyme (0.02 mM) and Nbs_2 (1.0 mM) in 50 mM phosphate (pH 8.0). Readings were made at 412 nm. The sulfhydryl groups of L-rhamnulose-1-P aldolase were also titrated spectrophotometrically with PMB (Boyer, 1954). Reaction mixtures contained enzyme (0.02 mM) and PMB (0.5 mM) in 50 mM sodium phosphate (pH 7.0). Readings were made at 250 nm.

Electron Microscopy. Preparations were negatively stained

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[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); PMB, *p*-mercuribenzoic acid; DHAP, dihydroxyacetone phosphate; HSEtOH, 2-mercaptoethanol.

with 0.5% aqueous solutions of ammonium tungstate (meta) (pH 6.2) or potassium silicotungstate (pH 7.0). The specimens were prepared from active, crystalline-, or sodium dodecyl sulfate dissociated enzyme at a concentration of 0.5–1.0 mg/ml in 0.1 mM sodium phosphate (pH 7.0). The material was diluted directly on the grid support membrane with the staining solution. Specimens were examined in an AEI EM6B electron microscope at an accelerating voltage of 80 kV and were recorded at instrument magnifications of × 80,000 or × 100,000.

Results

Effect of Sodium Dodecyl Sulfate on L-Rhamnulose-1-P Aldolase. L-Rhamnulose-1-P aldolase (2.75 mg in 1 ml of 0.1 M potassium phosphate buffer (pH 7.1)) was diluted with an equal volume of 1% sodium dodecyl sulfate in the same buffer. At the times indicated below, 10-μl samples were removed and assayed for aldolase activity, using the coupled assay described previously (Chiu and Feingold, 1969). The following percentages of initial enzyme activity were recovered at 1, 2.5, 5, 15, 60, and 300 min respectively 65, 38, 30, 30, 10, and 0.

Kinetic studies of the inhibition of L-rhamnulose-1-P aldolase by sodium dodecyl sulfate were performed as described previously in studies of the inhibition of the enzyme by 1,10-phenanthroline (Schwartz and Feingold, 1972b). The effect of sodium dodecyl sulfate on the rate of cleavage of L-rhamnulose-1-P is presented in Figure 1. At low concentrations, sodium dodecyl sulfate was an inhibitor strictly competitive with L-rhamnulose-1-P. Under the described experimental conditions, higher concentrations of inhibitor than those indicated in Figure 1 caused the kinetic pattern to deviate from the strictly competitive type, *i.e.*, the effect of inhibitor could not be overcome by increasing concentrations of substrate, probably due to slow irreversible inactivation of the aldolase. When the kinetic data of Figure 1 are replotted as slope *vs.* inhibitor concentration (Cleland, 1963), a plot linear with respect to slope is obtained (insert), emphasizing the strictly competitive nature of the inhibition. The effect of sodium dodecyl sulfate on the rate of condensation of DHAP and L-lactaldehyde, when the concentration of the latter was held constant and DHAP concentration was varied, was examined as follows. Reaction mixtures contained (μmoles): glycylglycine (pH 7.5), 10; $(NH_4)_2SO_4$, 40; L-lactaldehyde, 3.9; L-rhamnulose-1-P aldolase, 13 μg; DHAP over the concentration range 0.125–0.5 mM, and sodium dodecyl sulfate as indicated in a total volume of 0.5 ml. All reagents were adjusted to pH 7.5 before mixing. At appropriate intervals, samples were removed and their L-rhamnulose-1-P content was determined as described previously (Chiu and Feingold, 1969).

In the absence of sodium dodecyl sulfate, V_{max} was 0.25 μmol/min, and a plot of $1/V$ *vs.* $1/S$ had a slope of 0.029 min. No change in V_{max} occurred in the presence of sodium dodecyl sulfate; however, the slopes of the reciprocal plots varied as follows for sodium dodecyl sulfate concentrations of (mM) 0.175, 0.35, and 0.70 respectively 0.03, 0.069, and 0.022. These results are consistent with strictly competitive inhibition.

The effect of sodium dodecyl sulfate when DHAP concentration was held constant and L-lactaldehyde concentration was varied was shown as follows. Reaction mixtures contained (μmoles) glycylglycine (pH 7.5), 10; $(NH_4)_2SO_4$, 40; DHAP, 3.5; L-rhamnulose-1-P aldolase, 13 μg, and L-lactaldehyde over the concentration range 0.3–1.25 mM in a total volume of 0.5 ml. All reagents were adjusted to pH 7.5 before mixing. Determination of L-rhamnulose-1-P was performed as described in the previous experiment. In the absence of sodium dodecyl sulfate, V_{max} was 0.30 μmol/min, and a plot of $1/V$ *vs.* $1/S$ had a slope of 0.62 min. In the presence of sodium dodecyl sulfate, V_{max} decreased while there was a slight increase in the slopes of the reciprocal plots. The V_{max} and slopes varied as follows for sodium dodecyl sulfate concentrations of (mM) 0.175, 0.35, and 0.70: 0.35, 0.84; 0.45, 0.96; 0.70, 1.10. These results are consistent with noncompetitive inhibition.

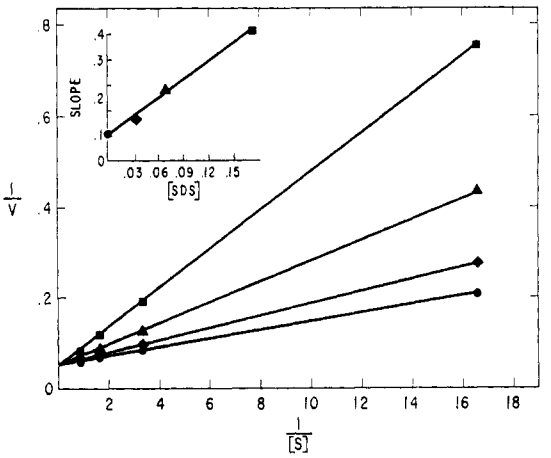

FIGURE 1: Effect of L-rhamnulose-1-P concentration on reaction velocity in the presence of sodium dodecyl sulfate. Reaction mixtures contained (μmoles) KCl, 50; Tris-HCl buffer (pH 7.5), 45; L-rhamnulose-1-P concentration was varied and sodium dodecyl sulfate included as indicated below in a total volume of 1 ml; 2 μl of solution containing 6.2 μg of L-rhamnulose-1-P aldolase (2 units/mg) was added and the reaction mixture was incubated at 30° for 5 min. It then was held at 100° for 2 min to terminate the reaction, cooled, and NADH (0.2 μmol) and α-glycerol phosphate dehydrogenase (50 μg) were added. The total change in absorbance was measured at 340 nm with a Gilford Model 2000 recording spectrophotometer. One unit of enzyme activity is the amount required to release 1 μmol of DHAP/min at 30°. Sodium dodecyl sulfate concentrations: (●) 0.0 mM; (■) 0.035 mM; (▲) 0.07 mM; (□) 0.127 mM. V = μmoles L-rhamnulose-1-P cleaved/min; S = mM L-rhamnulose-1-P.

Subunit Molecular Weight. Preliminary results by Chiu *et al.* (1968) suggested that L-rhamnulose-1-P was constituted of four identical subunits of molecular weight approximately 35,000. This value was confirmed by disrupting the enzyme in sodium dodecyl sulfate and HSEtOH and examining the product by electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate (Shapiro *et al.*, 1967). A single protein band of mobility corresponding to a molecular weight of 35,000 was obtained.

Preparation of Aldolase Subunits. Subunits were prepared from native enzyme by treatment with sodium dodecyl sulfate and HSEtOH, followed by dialysis against 0.02 M phosphate buffer (pH 7.0), as described previously. These preparations were enzymically inactive, contained no metal as shown by atomic absorption spectroscopy (Schwartz and Feingold, 1972b), and as will be demonstrated in the following, differed from native enzyme in their electrophoretic and immunologic properties.

Regeneration of Active Enzyme from Subunits. When the subunit preparation was dialyzed against a solution containing certain divalent cations and HSEtOH, enzyme activity was again detectable. The rate and extent of recovery of enzyme activity were greatest at pH 9.0. More than 65% of the recovery occurred during the first 20–24 hr and more slowly thereafter, tending toward a maximum at 6 days.

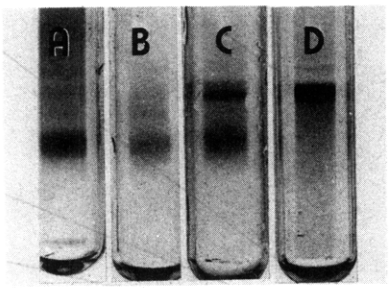

FIGURE 2: Regeneration of native aldolase structure demonstrated by disc gel electrophoresis. Polyacrylamide gel electrophoresis was performed on each of the following. (A) Aldolase subunits (50 μg). (B) Aldolase subunits dialyzed against HSEtOH and EDTA (20 μg). Aldolase subunits (2 mg/ml) in 0.02 M potassium phosphate (pH 7.4), 0.05 M in HSEtOH, were dialyzed for 20 hr at 4° against 100 volumes of 0.02 M potassium phosphate (brought to pH 9.0 with NaOH), 0.05 M in HSEtOH and 0.125 M in EDTA. The dialysis vessel was flushed with nitrogen and kept tightly covered during the process. (When the dialysis solution contained neither HSEtOH nor EDTA, but was 0.01 M in $ZnCl_2$, the identical pattern was obtained.) (C) Aldolase subunits dialyzed against HSEtOH and $ZnCl_2$ (50 μg). Subunits were dialyzed as in B; the dialysis solution contained no EDTA and was 0.05 M in HSEtOH and 0.01 M in $ZnCl_2$. (D) Native enzyme.

Therefore, most regeneration experiments were performed over a 20-hr period. (Further details are presented in the legend to Figure 2.) A protein band which had the characteristic mobility of native enzyme when examined by disc gel electrophoresis was present in the subunit preparations which had been dialyzed against a solution containing an appropriate divalent cation and HSEtOH. Furthermore, when examined by the Ouchterlony (1958) double diffusion method, the reactivated enzyme preparations yielded an additional precipitin band identical with that obtained with native enzyme.

In Figure 2 are presented polyacrylamide gel electrophoretic patterns obtained from variously treated aldolase subunit preparations. In the presence of EDTA or in the absence of HSEtOH in the dialysis solution, the patterns in columns A or B were obtained showing a requirement for thiol as well as metal to produce an electrophoretic band with the mobility of native enzyme. When 2-mercaptoethanol alone was present in the reactivation mixture, approximately 2% of the activity of the native enzyme was recovered, accompanied by the appearance of a faint band with the mobility of native enzyme. Since no attempt was made to remove divalent cations from the dialysis buffer, these effects were probably due to traces of metal in the reagents used. Mn(II), Co(II), and Zn(II) were equally effective in restoring "native structure" (defined as the protein band corresponding in electrophoretic mobility to untreated aldolase) and yielded the electrophoretic pattern shown in column C. As shown by absorbance scans of the gels, in each case, approximately 50% of the total protein was present in the band corresponding in mobility to native enzyme. The specific activities of the enzyme solutions obtained with the above metals were respectively 47, 25, and 22% of that of native enzyme. K_m values for L-rhamnulose-1-P, on the other hand, determined as described previously (Schwartz and Feingold, 1972b), did not differ significantly from the value for native enzyme.

Double diffusion patterns in agar gel obtained when the enzyme preparations described in Figure 2 were reacted with antiserum to native enzyme by the Ouchterlony technique (Ouchterlony, 1958) gave the following results. Native enzyme yielded two clearly separated precipitin bands; the innermost of these corresponded to the precipitin band obtained with subunits alone. Previously, Chiu and Feingold (1969) found only one band with crystalline enzyme. The two bands detected in this study could be due either to increased sensitivity of the rabbits used in the antiserum preparation or to a greater proportion of subunits in the enzyme immunogen, or to a combination of both factors. Subunits dialyzed in the presence of EDTA or in the absence of HSEtOH yielded only one precipitin band, corresponding to that obtained with untreated subunits. On the other hand, when subunits were dialyzed againt the same concentration of an appropriate metal salt (either $MnCl_2$, $CoCl_2$, or $ZnCl_2$) and 2-mercaptoethanol, they yielded a pattern indistinguishable from that given by native enzyme.

TABLE I: Reactivation of PMB-Treated Subunits.[a]

Expt	Additions	Per cent[b] Initial Specific Activity
1. Control (no prior treatment with PMB)	Zn(II), HSEtOH	20
2.	Zn(II), HSEtOH	60
3.	EDTA, HSEtOH	0
4.	Zn(II)	0

[a] A solution of aldolase subunits (2.5 μM in 0.02 M potassium phosphate (pH 7.0)) was dialyzed against 100 volumes of 5 mM PMB in the same buffer for 6 hr, during which time nitrogen gas was slowly bubbled through the solution. Aliquots of the retentate were then dialyzed for 20 hr against 100 volumes of 0.02 M potassium phosphate adjusted with 1 M NaOH to pH 9.0. The dialysis solutions were in addition: in experiments 1 and 2 0.01 mM $ZnCl_2$ and 0.05 M in HSEtOH, in experiment 3 1 mM in EDTA and 0.05 M in HSEtOH, and in experiment 4 0.01 mM in $ZnCl_2$. [b] Expressed as per cent of the specific activity (5.0) of the enzyme preparation from which the subunits were prepared.

Free Sulfhydryl Groups. All the potentially available sulfhydryl groups of L-rhamnulose-1-P aldolase could be titrated with Nbs_2. When titrated with a 50-fold molar excess of Nbs_2, eight sulfhydryls reacted within 30 sec; four more residues reacted in an additional 8–10 min, after which there was no further reaction. The 5′-thionitrobenzoate-substituted enzyme had no activity. Titration of aldolase subunits with Nbs_2 yielded a value of three sulfhydryl groups per subunit.

PMB also was used to titrate the sulfhydryl groups of native enzyme. With a 25-fold molar excess of the reagent, approximately eight groups reacted within 30 sec; four more groups per mole of enzyme reacted during an additional 30 min, after which there was no more reaction. The PMB substituted enzyme was inactive.

Reassociation of Sulfhydryl-Substituted Subunits. Since enzyme activity seemed to be dependent on the presence of free sulfhydryl groups, the effect on the reassociation of subunits of blocking these groups with PMB was examined. As can be noted from Table I, PMB-treated subunits did not yield active enzyme when incubated with Zn(II) alone. However, activity was restored when both Zn(II) and HSEtOH were present in the reaction mixture. Remarkably, much

more activity was recovered from PMB-treated subunits than from untreated subunits used as control.

Electron microscopy showed that crystalline L-rhamnulose-1-P aldolase consisted of a homogeneous population of enzyme molecules (Figure 3a). A large proportion of the structures was well defined and contained four subunits arranged in a square (Figure 3b). The distance measured along a side of the molecule was approximately 70 Å and the diameter of an individual subunit was approximately 35 Å. In some specimens oriented aggregates (microcrystals) could be detected (Figure 3c). These structures likewise display the square profile of the tetrameric enzyme molecule. Identical patterns were obtained with active, succinylated enzyme (Schwartz and Feingold, 1972b). Only amorphous material, probably representing unstructured aggregates of subunits, was present in electron micrographs of sodium dodecyl sulfate dissociated enzyme. Both stains used in this work gave essentially identical results.

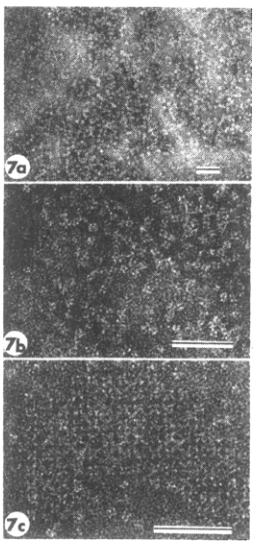

FIGURE 3: Crystalline L-rhamnulose 1-phosphate aldolase showing a homogeneous population of enzyme molecules (a), which at higher magnification appear as squares with subunits at the corners (b). The arrangement of the subunits is also apparent in crystalloid aggregates of the enzyme (c). Preparations a and b were negatively stained with potassium silicotungstate and (c) with ammonium tungstate (meta). The bar indicates 50 nm.

Discussion

Within a range of concentration and experimental conditions, the anionic detergent sodium dodecyl sulfate behaves almost exactly like the metal chelator 1,10-phenanthroline (Schwartz and Feingold, 1972b). Sodium dodecyl sulfate is an inhibitor competitive with L-rhamnulose-1-P and DHAP and essentially noncompetitive with L-lactaldehyde. At higher sodium dodecyl sulfate concentrations, the enzyme undergoes irreversible, time-dependent denaturation, and upon prolonged treatment with sodium dodecyl sulfate and HSEtOH is converted to subunits.

Such effects of protein denaturants have been observed previously in studies of other enzymes. Inagaki (1959) showed that urea (0–2 M) inhibited glutamate dehydrogenase competitively with L-glutamate or ammonium ions and uncompetitively with α-ketoglutarate, whereas at higher concentrations of urea (3.3 M), complete enzyme inactivation with protein denaturation occurred. Much higher concentrations of urea (8 M) resulted in subunit formation. These results are very similar to those presented in this work, except that urea rather than sodium dodecyl sulfate was used. A more recent study of the effect of sodium dodecyl sulfate on glutamate dehydrogenase (Rogers and Yusko, 1969) has shown that the anionic detergent inactivates the enzyme by two different mechanisms: reversible uncompetitive inhibition at low sodium dodecyl sulfate concentration and irreversible denaturation at higher levels of inhibitor. The authors conclude that inhibition at low sodium dodecyl sulfate concentration reflects a specific combination of inhibitor only with enzyme–substrate–coenzyme complex, and that inhibition at higher sodium dodecyl sulfate concentration reflects more general hydrophobic interactions leading to eventual disruption of the protein. Our findings can be interpreted similarly. Initially sodium dodecyl sulfate binds to the enzyme at or near the bound metal, possibly because of the high positive charge density in the vicinity, effectively competing with ketose phosphate for the site and, in addition, possibly causing slight, although still reversible, structural perturbation of the enzyme. This initial relatively site-specific interaction of sodium dodecyl sulfate with the enzyme is probably followed by progressively more extensive hydrophobic interactions between the protein and the hydrocarbon chains of the inhibitor. The latter interaction, although so slow as to be insignificant at low inhibitor concentrations, is much more extensive at higher sodium dodecyl sulfate levels. The dual action of sodium dodecyl sulfate suggests that the aldolase structure is maintained by two types of forces, those specifically associated with the ketose phosphate binding site and those characterized by hydrophobic interactions.

Prolonged treatment of L-rhamnulose-1-P aldolase with sodium dodecyl sulfate in the presence of a reducing agent (HSEtOH) causes complete denaturation of the enzyme accompanied by release of metal and formation of polypeptides of molecular weight 35,000. Since the molecular weight of active L-rhamnulose-1-P aldolase is in the order of 140,000 (Chiu and Feingold, 1969), these results confirm preliminary evidence for the tetrameric nature of the enzyme (Chiu *et al.*, 1968). Further confirmation for this structure is afforded by electron micrographs of native enzyme to be discussed in detail later.

The reactivation experiments show that metal plays a role in the structure as well as in the function of L-rhamnulose-1-P aldolase. When inactive, metal-free subunits are dialyzed against divalent cations and HSEtOH, there is reappearance of enzyme activity and of material with the gel-electrophoretic and immunological properties of native enzyme. If the divalent cation is made unavailable by EDTA, no activity is recovered, nor can protein with the characteristic properties of native enzyme be detected in the reactivation mixture. Since under the conditions employed, each of the metals tested effected the conversion of approximately 50% of the total protein present to material with the gel-electrophoretic mobility of the native aldolase, the particular metal ion used does not seem to determine the extent to which the monomers reassociate. However, probably not all of this reassociated material, although it was electrophoretically identical with native enzyme, was enzymically active.

The specific activity of Zn(II)-reactivated subunits (calculated as: (specific activity of the mixture)/(fraction of protein with the gel-electrophoretic mobility of native enzyme)) was 44%, and the specific activity of the Co(II)-reactivated subunits was 50% of that of native enzyme. Co(II)-containing native enzyme is not available; however, at least 75% of the

Zn(II) of native L-rhamnulose-1-P aldolase can be replaced by Co(II) to yield an enzyme with 400% of the specific activity of the starting material (Schwartz and Feingold, 1972b). The greater specific activity of this Co(II)-substituted enzyme probably is due to the effect of the metal on V_m; K_m (L-rhamnulose-1-P) was not significantly different for Zn(II)- and Co(II)-reactivated subunits. On the basis of the increased activity observed with the Co(II)-substituted enzyme, one would expect the Co(II)-reactivated subunits to have approximately 200% the specific activity of native enzyme. The lower than expected specific activity of the Co(II)-reactivated subunits may be due to a relatively small proportion of active tetramer among the total tetramer population.

The difference in specific activities of reconstituted enzyme obtained with different metals can be ascribed to: (1) the effect of the metal on structure, evidenced by the ability of the particular divalent cation to align subunits to yield enzymically active tetramers, and (2) the effect of the metal on function, evidenced by the dependence of V_m on the metal used.

In general, all class II aldolases have an exaggerated requirement for sulfhydryl-containing reagents such as HSEtOH (Rutter, 1964) probably due to a need to keep thiol groups reduced. In this respect, L-rhamnulose-1-P aldolase is no exception. Not only is 2-mercaptoethanol required to maintain enzyme activity during storage, upon reaction with Nbs_2 or PMB, enzyme activity is progressively lost. The number of sulfhydryl groups which react with these reagents is equal to the total half-cystine residues present in the molecule (Chiu and Feingold, 1969), showing that the active enzyme contains no disulfide linkages. It is therefore not surprising that unless HSEtOH is present in reactivation mixtures, active tetrameric enzyme is not formed. Electrophoretic examination of reactivation mixtures without HSEtOH showed the presence of a large number of bands (at least 12) with mobilities both greater and less than that of native enzyme. These bands probably represented oligomers in which the subunits were randomly linked by disulfide bonds. The question remains concerning the function of the sulfhydryl groups. When the PMB-substituted sulfhydryl groups of the subunit were freed by treatment with HSEtOH during reactivation, approximately three times more activity was recovered than when the sulfhydryl groups were initially free (Table I). These results suggest that native conformation is influenced by hydrogen bonding in which the sulfhydryl groups participate. If the formation of random bonds by sulfhydryl groups is prevented, intersubunit interactions characteristic of the native enzyme are more likely to occur and a greater proportion of active conformers results.

Electron microscopy shows that L-rhamnulose-1-P aldolase is a tetramer and attests to the purity and homogeneity of the enzyme preparations. Furthermore, the diameter of the subunits estimated from the micrograph is consistent with a subunit molecular weight of approximately 35,000. As expected, individual subunits obtained by disruption of the enzyme with sodium dodecyl sulfate could not be resolved by electron microscopy. The subunits of tetrameric L-rhamnulose-1-P aldolase are almost without exception arranged in the form of a square. From the profiles of single enzyme molecules and the perpendicular orientation visualized in the microcrystals, it may be concluded that the aldolase possesses cyclic symmetry similar to that exhibited by the tetrameric proteins, tryptophanase (Morino and Snell, 1967) or pyruvate carboxylase (Valentine *et al.*, 1966). It is instructive to compare the electron micrographs of L-rhamnulose-1-P aldolase with those of class I FDP aldolase from mammalian tissues, a tetramer of molecular weight 160,000 (Penhoet *et al.*, 1967). Electron micrographs of the latter enzyme show both squares and triangles, which led Penhoet *et al.* (1967) to propose a tetrahedral arrangement of subunits (dihedral symmetry (Klotz *et al.*, 1970)). This structure has been substantiated by low-resolution X-ray diffraction studies (Eagles *et al.*, 1969). Thus, although both aldolases are tetramers of similar molecular weight, they appear to differ markedly in their quaternary structure.

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