Purification and Properties of Rat Liver 6-Phosphogluconate Dehydrogenase. Activity at Normal *in Vivo* Concentration of Coenzyme†

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ABSTRACT: Rat liver 6-phosphogluconate dehydrogenase has been purified to homogeneity. The native enzyme has a molecular weight of 102,000 ± 3000 and is a dimer composed of subunits with identical molecular weights of 52,000 ± 300. Optimal conditions of ionic strength, pH, and ion composition for proper assay of the enzyme are reported. Under optimal conditions K_m's for 6-phosphogluconate and $NADP^+$ were 7.1×10^{-5} and 1.3×10^{-5} M, respectively. The complexing of either substrate to the enzyme is independent of the presence of the second substrate. NADPH inhibition is competitive with respect to $NADP^+$ ($K_i = 2 \times 10^{-5}$ M). Calculation of the activity expected for 6-phosphogluconate dehydrogenase at *in vivo* concentrations of $NADPH^+$ and NADPH suggest that this enzyme is almost completely inhibited *in vivo*. The great sensitivity of the enzyme to changes in the $NADP^+$:NADPH ratio suggests that 6-phosphogluconate dehydrogenase may contribute to the maintenance of the proper ratio of $NADP^+$:NADPH in the cell.

This laboratory is currently investigating mechanisms regulating the levels and activities of lipogenic enzymes *in vivo*. These studies have focused on the pentose phosphate pathway dehydrogenases and this paper reports the purification and properties of rat liver 6-phosphogluconate dehydrogenase [6-phospho-D-gluconate:$NADP^+$-oxidoreductase (decarboxylating), EC 1.1.1.44]. Veech *et al.* (1969) have shown that a number of NAD^+ or $NADP^+$ cytoplasmic dehydrogenases in rat liver are at equilibrium with their substrates and products and that the ratio of free $NADP^+$:NADPH changes somewhat under different nutritional conditions. We have examined the nature and extent of the inhibition of purified 6-phosphogluconate dehydrogenase by NADPH. The results show that this enzyme is greatly inhibited at concentrations of $NADP^+$ and NADPH found *in vivo*. The great sensitivity of the enzyme to changes in the $NADP^+$:NADPH ratio suggests that 6-phosphogluconate dehydrogenase may contribute toward maintenance of the proper $NADP^+$:NADPH ratio in the cell.

Materials and Methods

Enzyme Assays. The mixture used to assay for 6-phosphogluconate dehydrogenase activity contained 100 μmoles of Bicine[1] buffer (pH 8.3), 100 μmoles of sodium acetate (pH 8.3), 1 μmole of $NADP^+$, 2 μmoles of 6-phosphogluconate, and enzyme in a final volume of 1.0 ml. One unit of enzyme is that amount of enzyme which will form 1 μmole of NADPH in 1 min under the assay conditions described above. In the kinetic studies, initial reaction velocities were obtained using a Gilford 2000 spectrophotometer with the expanded scale and a constant temperature of 30°. Protein was determined by the method of Lowry *et al.* (1951).

Any enzyme preparation with a specific activity less than 32 which was used in kinetic experiments was checked for contaminating enzymes capable of removing 6-phosphogluconate, $NADP^+$, or NADPH. An excess of enzyme was incubated with each of these for 15 min at 30° and the incubation mixture was then used to measure the 6-phosphogluconate, $NADP^+$, or NADPH remaining. Only enzyme preparations free of such contaminating enzymes were used in these experiments. The concentration of solutions of $NADP^+$ or NADPH was determined spectrophotometrically using extinction coefficients of 18×10^3 M^{-1} cm^{-1} at 259 mμ or 6.22×10^3 M^{-1} cm^{-1} at 340 mμ, respectively. 6-Phosphogluconate concentrations were standardized by the method of Hohorst (1963).

Estimation of Molecular Weights. The molecular weight of the native protein was determined by the method of Hedrick and Smith (1968) using 5–10% polyacrylamide gels and double the usual concentration of asparagine in the buffer. A linear standard curve was obtained using ferritin, catalase, aldolase, pepsin, and ovalbumin as standards. 6-Phosphogluconate dehydrogenase activity was detected by putting gels in the usual assay mixture containing 0.24 mg of nitroblue tetrazolium and 42 μg of phenazine methosulfate per ml. Other proteins were detected by using Coomassie Brilliant Blue R-250 (Chrambach *et al.*, 1967). The molecular weight of the subunit was determined by the method of Weber and Osborn (1969) using two-thirds the usual amount of methylenebisacrylamide and 10% polyacrylamide. A linear standard curve was obtained using catalase, ovalbumin, aldolase, lactic dehydrogenase, pepsin, and trypsin as standards.

Purification of 6-Phosphogluconate Dehydrogenase. Young rats (about 200 g) were fasted for 2 days and then fed a 60% carbohydrate, non-fat diet for 4 days in order to increase the level of 6-phosphogluconate dehydrogenase (Rudack *et al.*, 1971). Livers from these animals were homogenized in four volumes of 0.15 M KCl and centrifuged at 66,000*g* for 60 min in order to obtain the crude rat liver supernatant fraction. All subsequent steps were carried out at 4°.

Ammonium Sulfate Fractionation. The crude rat liver supernatant fraction was treated with an equal volume of a solution of saturated ammonium sulfate (pH 7.0), allowed to stand for 30 min, and centrifuged. The 50% saturated super-

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[1] Abbreviation used is: Bicine, *N,N*-bis(2-hydroxyethyl)glycine.

TABLE I: Results from the Purification of 6-Phosphogluconate Dehydrogenase.[a]

Fraction	Vol (ml)	Total Protein (mg)	Total Act. (Units)	Sp Act. (Units/ mg of Protein)	Yield (%)
Crude soluble fraction	165	2970	510	0.17	100
Ammonium sulfate	150	645	405	0.63	79
DEAE-cellulose	193	83	403	4.81	79
CM-cellulose	310	6.3	216	34	42
Concentration	11.4	5.7	184	32	36

[a] The procedure for each step is described in the Methods section.

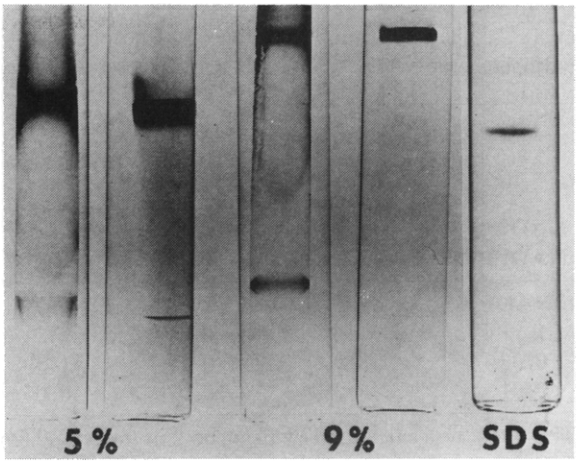

FIGURE 1: Disc gel electrophoresis of 6-phosphogluconate dehydrogenase. Five or nine percent polyacrylamide gels were stained for 6-phosphogluconate dehydrogenase activity (left-hand gels) or for protein (right-hand gels) as described in the Methods section. Gels stained for 6-phosphogluconate dehydrogenase activity were run using 0.016 unit of enzyme. Gels stained for protein were run using 20 μg of 6-phosphogluconate dehydrogenase. The procedure used for the sodium dodecyl sulfate (SDS) gel was described in the Methods section. The specific activity of the 6-phosphogluconate dehydrogenase was 32.

natant fraction was treated with 0.667 volume of saturated ammonium sulfate (pH 7.0), allowed to stand for 30 min, and centrifuged. The 50–70% precipitate was dissolved in 1 mM EDTA and dialyzed versus the same buffer at pH 7.0.

Column Chromatography. A DEAE-cellulose column (4.2 × 12 cm) was equilibrated with 5 mM potassium phosphate–1 mM EDTA (pH 7.0), prior to application of the dialyzed 50–70% ammonium sulfate fraction. The column was eluted using a 1-l. linear gradient from 5 to 40 mM potassium phosphate buffer (pH 7.0)–1 mM EDTA. Those fractions containing 6-phosphogluconate dehydrogenase activity were combined, dialyzed *vs.* 1 mM EDTA (pH 6.9), and applied to a CM-cellulose column (2.5 × 8 cm) previously equilibrated with 3 mM potassium phosphate buffer–1 mM EDTA (pH 6.9). The enzyme was eluted using a linear gradient between 3 and 12 mM (600 ml of each) potassium phosphate buffer–1 mM EDTA (pH 6.9). Fractions containing 6-phosphogluconate dehydrogenase were pooled and concentrated with a diaflo enzyme concentrator using a PM-30 ultrafiltration membrane.

Materials. Young, male Sprague-Dawley rats were obtained from local suppliers. Catalase, aldolase, lactic dehydrogenase (beef heart), trypsin, $NADP^+$, and NADPH were purchased from Boehringer Mannheim. Pepsin, ovalbumin, fructose 1,-6-diphosphate, DEAE-cellulose, and CM-cellulose were purchased from Sigma. Bicine and ferritin were obtained from Calbiochem.

Results

Table I summarizes the results from the purification procedure described in the Methods section. The final product had a specific activity of 32–34 and was obtained in 36% yield. The concentrated, purified, enzyme stored at 4° in 20% glycerol was stable for months. It was necessary to carry out the purification procedure as rapidly as possible (usually no more than 3 days) in order to obtain a satisfactory yield of pure enzyme.

The purity of the final product is illustrated in Figure 1. Excess amounts of purified enzyme were subjected to disc gel electrophoresis using 5 and 9% polyacrylamide gels. These were then stained for 6-phosphogluconate dehydrogenase activity or protein. In a separate experiment the purified enzyme was dissociated into subunits and subjected to disc gel electrophoresis by the procedure of Weber and Osborn (1969). The right-hand gel in Figure 1 illustrates the results from the latter experiment. Only one protein band was observed for the native protein and this protein stained for 6-phosphogluconate dehydrogenase activity. Likewise, only one protein band was observed when the purified enzyme was dissociated into subunits. These results demonstrate that the final product (specific activity 32) is a homogeneous preparation of rat liver 6-phosphogluconate dehydrogenase.

The molecular weight of native 6-phosphogluconate dehydrogenase was determined by the method of Hedrick and Smith (1968) and the molecular weight of the subunit by the method of Weber and Osborn (1969). Linear standard curves obtained using these methods gave molecular weights of 102,000 ± 3000 for the native enzyme and 52,000 ± 300 for the subunit. These data provide evidence that the native enzyme is composed of two subunits of identical molecular weight.

Initial characterization studies on the purified enzyme showed a pH optimum of 8.3 using Tris-Cl or Bicine-acetate buffers at a constant ionic strength of 0.07. The effect of ionic strength on enzyme activity is shown in Figure 2. There is a

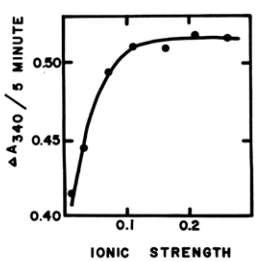

FIGURE 2: The effect of ionic strength on 6-phosphogluconate dehydrogenase activity. Each assay mixture was as described in the Methods section except that sodium acetate (pH 8.3) was varied to give the indicated ionic strength. The 6-phosphogluconate dehydrogenase specific activity equaled 18.

TABLE II: Calculation of the Inhibition of 6-Phosphogluconate Dehydrogenase at $NADP^+$:NADPH Ratios Observed *in Vivo*.[a]

$NADP^+$: NADPH	% V_m	μmoles of NADPH Produced/min per g of Liver: Control Rats	μmoles of NADPH Produced/min per g of Liver: Induced Rats
No NADPH	22	1.3	3.2
1	14	0.8	2.1
0.33	9	0.5	1.3
0.01	0.4	0.02	0.06

[a] The % V_m was calculated by using eq 2 in the text, 0.5 mM $NADP^+$ (A) + NADPH (Q), 30 μM 6-phosphogluconate (B), Michaelis constants of 1.3×10^{-5} and 7.1×10^{-5} M for $NADP^+$ and 6-phosphogluconate, respectively, and an inhibition constant (K_{iQ}) of 2×10^{-5} M for NADPH. Additional details are presented in the Results section.

substantial increase in enzyme activity until the ionic strength is 0.11 and enzyme activity is unchanged between ionic strength 0.11 and 0.25. Michaelis constants for the binding of $NADP^+$ and 6-phosphogluconate to the enzyme were estimated by the method of Florini and Vestling (1957). A double-reciprocal plot (Lineweaver and Burk, 1934) of $1/v$ *vs.* 1/6-phosphogluconate at five different constant concentrations of $NADP^+$ showed that all of the lines had a common point of intersection on the abscissa. Likewise, a plot of $1/v$ *vs.* $1/NADP^+$ at five different constant concentrations of 6-phosphogluconate showed that all of these lines had a common point of intersection on the abscissa. These results are consistent with a sequential mechanism for the reaction (Cleland, 1963) where the presence of one substrate has no effect on the complexing of the second substrate (Florini and Vestling, 1957). Michaelis constants calculated from these data were 1.3×10^{-5} and 7.1×10^{-5} M for $NADP^+$ and 6-phosphogluconate, respectively.

During experiments to determine the effect of ionic strength on 6-phosphogluconate dehydrogenase activity, several anions were found to inhibit this enzyme by competing for 6-phosphogluconate. Acetate does not inhibit and sodium acetate was used to maintain a constant ionic strength when determining the inhibition by other anions. Inhibition constants calculated from Lineweaver and Burk plots by the method of Dixon and Webb (1964) were 1.2×10^{-2}, 1.6×10^{-2}, 1.9×10^{-2}, and 9.7×10^{-2} M for SO_4^{2-}, PO_4^{3-}, HCO_3^-, and Cl^-, respectively. Neither Mg^{2+}, Mn^{2+}, K^+, Na^+, nor NH_4^+ (all 0.01 M) had any effect on 6-phosphogluconate dehydrogenase activity when these cations were added to the standard assay mixture.

Luzzatto and Afolayan (1971) have proposed that $NADP^+$ and NADPH levels may regulate glucose-6-P dehydrogenase activity in erythrocytes. Consequently, we investigated the effect of altered concentrations of $NADP^+$ and NADPH on the activity of 6-phosphogluconate dehydrogenase. The results from these experiments plotted by the method of Lineweaver and Burk (1934) demonstrated that NADPH was a competitive inhibitor with respect to $NADP^+$. The inhibition constant for NADPH was 2×10^{-5} M.

It is difficult to estimate the extent to which one would expect 6-phosphogluconate dehydrogenase to be inhibited by NADPH *in vivo*. Veech *et al.* (1969) proposed that the concentrations of free pyridine nucleotides determine the direction of reactions in which they participate. These authors have shown that the ratio of free $NADP^+$:NADPH is 0.01 in the liver of a normal rat. Although it was technically impossible for us to measure the activity of 6-phosphogluconate dehydrogenase at $NADP^+$:NADPH ratios of 0.01, it was possible to calculate the activity one might expect *in vivo*. Our kinetic experiments have shown that the complexing of one substrate to 6-phosphogluconate dehydrogenase is not affected by the presence of the second substrate. Alberty (1953) has shown that rate eq 1 applies to this special situation. Furthermore, Wratten and Cleland (1963) have derived a rate eq 2 for the effect of a competitive product inhibitor on the initial reaction velocity of eq 1. In this equation K_A and K_B are Michealis constants for substrates A ($NADP^+$) and B (6-phosphogluconate), respectively. K_{iQ} is the inhibition constant for the product of the reaction, Q (NADPH). Using eq 2 we have calculated the initial reaction velocity (v) expected at concentrations of $NADP^+$, NADPH, and 6-phosphogluconate found *in vivo*. In these calculations it was assumed that the total $NADP^+$ + NADPH concentration was 0.5 mM (Greengard *et al.*, 1968; Williamson *et al.*, 1969; Lagunas *et al.*, 1970) and that the 6-phosphogluconate concentration was 30 μM (Lagunas *et al.*, 1970). The validity of using eq 2 was checked by calculating the initial reaction velocity expected for the concentration of $NADP^+$, NADPH, and 6-phosphogluconate used in our kinetic studies. There was excellent agreement between the calculated and observed initial reaction velocities even at the highest $NADP^+$:NADPH ratio used (0.044).

$$v = \frac{V_m}{1 + \frac{K_A}{(A)} + \frac{K_B}{(B)} + \frac{K_A K_B}{(AB)}} \quad (1)$$

$$v = \frac{V_m}{1 + \frac{K_A}{(A)}\left[1 + \frac{(Q)}{K_{iQ}}\right] + \frac{K_B}{(B)} + \frac{K_A K_B}{(AB)}\left[1 + \frac{(Q)}{K_{iQ}}\right]} \quad (2)$$

Table II shows that at 0.5 mM $NADP^+$ and 30 μM 6-phosphogluconate the initial velocity is 22% of that observed when the enzyme is saturated with these substrates. When the NADPH concentration is increased at the expense of the $NADP^+$ concentration this inhibition increases so that, at a $NADP^+$:NADPH ratio of 0.01, the enzyme is almost completely inhibited (0.4% V_m). Thus, at the ratio of free $NADP^+$:NADPH observed *in vivo* (0.01), 6-phosphogluconate dehydrogenase is almost completely inhibited. In Table II we have also estimated the capacity of liver to oxidize 6-phosphogluconate. Measurement of the total amount of 6-phosphogluconate dehydrogenase present using optimal assay conditions shows that the maximal capacity of normal rat liver to oxidize 6-phosphogluconate is 5.8 μmoles/min per g of liver (Rudack *et al.*, 1971). If these rats are induced to produce higher levels of 6-phosphogluconate dehydrogenase, this capacity increases to 14.8 μmoles/min per g of liver. However, at the concentration of substrates and products expected *in vivo* this capacity decreases to 0.02 and 0.06 μmole per min per g of liver in the control and induced rats respectively. These estimates of the capacity of liver to oxidize 6-phosphogluconate are based upon the total activity of the enzyme measured at 30° and pH 8.0 (Rudack *et al.*, 1971). We have not attempted to convert these numbers to the more physio-

logical conditions of 37° and pH 7.4 since the small changes involved would not alter the conclusion that this enzyme is almost completely inhibited under the conditions expected *in vivo*.

Dyson and D'Orazio (1971) have reported that fructose 1,6-diphosphate is a competitive inhibitor (with respect to 6-phosphogluconate) of sheep liver 6-phosphogluconate dehydrogenase. We have not been able to demonstrate such an inhibition with the rat liver enzyme. In these studies the 6-phosphogluconate concentration was near the K_m value and $NADP^+$ concentrations were either limiting or saturating. Fructose 1,6-diphosphate was at 5×10^{-4} M. The experiments were run in the presence or absence of magnesium, at high or low constant ionic strength and at pH 8.3 or 7.7. We did not observe an inhibition by fructose 1,6-diphosphate under any of these conditions. The reasons for this apparent difference between the enzymes from sheep and rat liver are not clear at this time.

Discussion

The simple purification scheme outlined in Table I resulted from extensive modification of the procedure published by Villet and Dalziel (1969) for the enzyme from sheep liver. The purified enzyme was used for polyacrylamide disc gel electrophoresis by the procedures of Hedrick and Smith (1968) and Weber and Osborn (1969). Thus, the native enzyme was subjected to disc gel electrophoresis on several gels differing in percent polyacrylamide, or dissociated into subunits and then subjected to disc gel electrophoresis. In all of these experiments only one protein band was observed indicating that the enzyme had been purified to homogeniety. These procedures also revealed that the molecular weights of the subunits were identical and one-half of the molecular weight of the native protein. Thus, rat liver 6-phosphogluconate dehydrogenase is a dimer of two subunits of identical molecular weight. The molecular weight we have obtained for native rat liver 6-phosphogluconate dehydrogenase (102,000) agrees very well with the value of 104,000 reported by Dyson *et al.*[2] for the sheep liver enzyme but both of these estimates are considerably less than Villet and Dalziel's (1969) estimate of 129,000 for 6-phosphogluconate dehydrogenase from sheep liver.

The effect of ionic strength shown in Figure 2 necessitated running initial characterization studies at a constant ionic strength. Furthermore, the observation that many anions inhibited 6-phosphogluconate dehydrogenase required the use of a noninhibitory compound (sodium acetate) to adjust the ionic strength. Under these conditions, magnesium had no effect on the activity of rat liver 6-phosphogluconate dehydrogenase and the pH optimum for the enzyme was 8.3. Thus, differences in assay conditions probably account for the higher pH optimum and requirement for magnesium reported by Glock and McLean (1953).

The information contained in Table II is a crude attempt to calculate the activity of rat liver 6-phosphogluconate dehydrogenase expected *in vivo*. Obviously, such calculations involve a number of uncertainties about actual concentration of substrates and products present *in vivo*. In addition, more sophisticated hormonal or substrate level regulatory mechanisms yet to be discovered may be involved in the regulation of this or other enzymes involved in the oxidation of glucose-6-P in the pentose phosphate cycle. Nevertheless, some broad generalizations appear to be valid. Rat liver 6-phosphogluconate dehydrogenase activity would be almost completely inhibited at the ratio of free $NADP^+$:NADPH calculated by Veech *et al.* (1969) for the normal rat (0.01). The enzyme is very sensitive to changes in the ratio of $NADP^+$:NADPH. Changing this ratio from 0.01 to 0.33 would cause a 25-fold increase in the rate of 6-phosphogluconate oxidation. These calculations are in rough agreement with the observation that electron acceptors such as phenazine methosulfate can cause a 10-fold or greater increase in the rate of oxidation of glucose-6-P in the pentose phosphate pathway (Gumaa and McLean, 1969).

In mouse liver the turnover rate of 6-phosphogluconate is 0.1 μmole/min per g of liver (Reich *et al.*, 1968). Calculation of the estimated capacity for the oxidation of 6-phosphogluconate in rat liver when the ratio of free $NADP^+$:NADPH is 0.01 suggests that in the control rat the capacity for 6-phosphogluconate oxidation is 0.02 μmole/min per g of liver. In rats induced to higher levels of 6-phosphogluconate dehydrogenase, this capacity is increased to about 0.06 μmole/min per g of liver. In view of the uncertainties about actual concentrations of substrates and products present in liver, we regard this as fairly good agreement between our data and that of Reich *et al.* (1968) for the capacity of liver to oxidize 6-phosphogluconate *in vivo*. Our calculations also suggest that under conditions such as lipogenesis when NADPH is being rapidly oxidized in biosynthetic reactions, an increase in the $NADP^+$:NADPH ratio would decrease the inhibition by NADPH, increase the concentration of $NADP^+$, and rapidly increase the capacity of liver to oxidize 6-phosphogluconate and restore the $NADP^+$:NADPH ratio to the control value. Thus the $NADP^+$:NADPH ratio would be regulated in a manner functionally analogous to the regulation of the energy charge of a cell (Atkinson, 1968). Similar suggestions have been proposed for the regulation of NAD^+:NADH ratios by NAD-specific isocitrate dehydrogenase (Duggleby and Dennis, 1970) and by the pyruvate dehydrogenase complex (Shen and Atkinson, 1970).

[2] J. D. E. Dyson, R. E. D'Orazio, and W. H. Hanson, reported orally at the 62nd Annual Meeting of the American Society of Biological Chemists, San Francisco, Calif., June 1971.

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Role of Zinc(II) in the Refolding of Guanidine Hydrochloride Denatured Bovine Carbonic Anhydrase†

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ABSTRACT: Circular dichroism, ultraviolet difference spectroscopy, and activity measurements (*p*-nitrophenyl acetate as substrate) have been employed to study the denaturation and renaturation of bovine carbonic anhydrase B. This metalloenzyme is readily denatured by guanidine hydrochloride and refolds spontaneously upon removal of the denaturing condition, recovering essentially full (>95%) enzymatic activity. Denaturation in 3 M guanidine hydrochloride at 25° and pH 6, as judged by circular dichroism and ultraviolet spectroscopy, carries both the native and the apoenzyme from the same initial to the same final conformational state, exposing approximately six buried tryptophan side chains, disrupting interactions of aromatic side chains with dissymmetrical regions, and presumably destroying the specific zinc binding site of the enzyme. The transition between native and denatured conformational states appears thermodynamically reversible with or without Zn(II), although in the absence of the metal it occurs at a lower guanidine hydrochloride concentration (1.5 M *vs.* 1.0 M midpoint). Renaturation kinetics are complex and imply that intermediate species accumulate during the reaction. Under some conditions (dilution from 4.0 M to 1.0 M guanidine hydrochloride, pH 6, 25°) refolding occurs readily if Zn(II) is present during the initial stages of the reaction, whereas it occurs at an extremely low rate if Zn(II) is added later. This suggests that Zn(II) is bound during the initial steps of folding of the polypeptide chain and thus influences the pathway of the reaction although it does not affect the final conformational state.

Bovine carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is a metalloenzyme containing one atom of bound zinc(II) per molecule of 30,000 molecular weight. The zinc atom can be removed and replaced, as originally shown by Lindskog and Malmström (1962). Enzyme activity disappears on removal of the zinc, and returns when zinc is added to the apoenzyme (Lindskog, 1963). Thermodynamic parameters for the binding of zinc to the apoenzyme, obtained by combining the calorimetric results of Henkens *et al.* (1969) with equilibrium measurements of Lindskog and Malmström (1962), show that the zinc is very tightly bound and that an unfavorable enthalpy of binding is overwhelmed by a very large entropy increase. The binding of zinc(II) by the apoenzyme is relatively rapid while its dissociation is very slow (Henkens and Sturtevant, 1968; Henkens and Lochmüller, 1970). Zinc binding is accompanied by only very small changes in the ultraviolet spectrum (Henkens and Sturtevant, 1968) and almost no change in the ultraviolet circular dichroism. These observations, together with the observation that zinc binding has little effect on the rotational relaxation time or the sedimentation constant of the protein (Brewer *et al.*, 1968), indicate that the metal has little or no effect on the structure of the protein. The identity of the protein ligands coordinating the zinc is unknown. Presumably the zinc binding site, which in the human enzyme is located at the bottom of a crevice (Fridborg *et al.*, 1967), is destroyed on denaturation of the enzyme by guanidine hydrochloride.

Many enzymes denatured by guanidine hydrochloride have been shown to refold spontaneously to fully active enzymes, evidently native in all respects upon removal of the denaturing conditions (*cf.* review by Tanford, 1968). These experiments provide convincing evidence that the three-dimensional structure of an enzyme is determined by its primary structure, although they do not rule out the possibility that the presence or absence of specific ions or molecules may determine whether or not the native structure is achieved. In this regard, there has been no systematic study of the role of metal ions in the refolding of a metalloenzyme, although Teipel and Koshland (1971) reported some work on the metal-activated enzyme enolase and Reynolds and Schlesinger (1969) showed

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