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Hydrogen–Tritium Exchange of Partially and Fully Reconstituted Zinc and Cobalt Alkaline Phosphatase of *Escherichia coli*†

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ABSTRACT: The influence of metals upon the rate of hydrogen–tritium exchange of *Escherichia coli* alkaline phosphatase has been studied by the gel filtration technique. At pH 8.0, 20°, nearly 90% of the 1200 potentially exchangeable hydrogens of the metal-free apoenzyme exchange within a few minutes; an additional 8% exchange more slowly over the ensuing 12 hr. In contrast, at all times of exchange enzymes *fully* reconstituted with 4 g-atoms/mol of Co or Zn retain 60 or 70 more hydrogens than the apoenzyme indicating that the metal atoms markedly stabilize the dynamic protein structure. The greater conformational stability of the metalloenzymes is also apparent from examination of the temperature dependence (4–35°) of the exchange reaction. The rates of exchange of phosphatases only partially reconstituted with metals, *i.e.,* 2 g-atoms/mol of either Zn or Co, differ both from apo- and holometalloenzymes and from each other. Thus, addition of only two Zn atoms appears to stabilize a discrete set of equilibrium conformers which differ from those generated by two Co atoms, suggesting differing modes of metal binding; both, in turn, are less stable than the equilibrium structures of their corresponding holometalloenzymes. However, in the presence of phosphate, the proton exchange rates of the partially reconstituted metallophosphatases coincide with those observed for their corresponding holometalloenzymes. Hence, phosphate appears to act in concert with metals to promote conformational stabilization. Phosphate also protects the partially reconstituted Zn enzyme against a slow loss of catalytic activity evident under these experimental conditions.

The metal atoms in metalloenzymes may function in two important ways: metals may participate in the catalytic process directly as components of active enzymatic sites; alternatively, they may affect protein tertiary or quaternary structure and may thereby influence catalysis indirectly. In some enzymes, different atoms of the same metal or atoms of different metals appear to have catalytic and structural roles, respectively (Vallee and Wacker, 1970). Delineation of the precise manner in which metals participate in enzyme action has provided important approaches to understanding the mechanisms of action of metalloenzymes. As would be anticipated, investigations of polymeric systems have proven considerably more complex than have comparable studies of monomeric proteins (Ulmer and Vallee, 1971).

Escherichia coli alkaline phosphatase, a dimeric metalloenzyme, mol wt 89,000, exemplifies these problems since its zinc atoms appear to have *both* functional and structural roles (Simpson and Vallee, 1968). Preparations of the enzyme isolated in our laboratory consistently contain 3.8–4.2 g-atoms of zinc/mol of enzyme (Simpson *et al.,* 1968) which may be removed and replaced completely with corresponding loss and restoration of catalytic activity. Moreover, enzymatic function can be restored also by substitution of cobalt for zinc (Plocke and Vallee, 1962; Simpson and Vallee, 1968). On the basis of kinetic and physicochemical studies employing both zinc and cobalt, the metal atoms in alkaline phosphatase appear to fall into at least

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two distinct classes. Only two of the four metal atoms are thought to be involved directly in catalysis at the active enzymic sites of the two subunits (Simpson and Vallee, 1968). However, all four metal atoms appear to influence either tertiary (Reynolds and Schlesinger, 1969) or quaternary (Simpson and Vallee, 1969; Applebury and Coleman, 1969) structure. The relative contribution of active *vs.* nonactive site metals to conformation remains uncertain, however.

In order to further explore potential, metal-controlled, structural changes in alkaline phosphatase, we have now employed hydrogen isotope exchange, thought to be among the most sensitive of techniques for the delineation of small alterations in protein conformation. This experimental procedure has permitted a comparison of the dynamic structural properties of partially reconstituted enzymes and those bearing a full complement of four metal atoms.

The results indicate that both "active site" and "nonactive site" metal atoms affect the conformational stability of alkaline phosphatase. Moreover, the hydrogen isotope exchange technique provides a novel approach to the delineation of new relationships between the structural and catalytic effects of metals in this enzyme.

Methods

E. coli alkaline phosphatase was prepared as described previously and fractions having the highest specific activity were pooled (Simpson *et al.*, 1968). The zinc content of such preparations ranged from 3.9 to 4.1 g-atoms/mol, as measured by atomic absorption spectroscopy.

Apoalkaline phosphatase, (P'ase)[1] in concentrations of 20–30 mg/ml, was prepared by dialysis at room temperature, against 0.05 M 8-hydroxyquinoline-5-sulfonic acid (pH 7.5) followed by dialysis against metal-free Tris-Cl, 0.01 M (pH 8.0), 4°. However, similar experimental results were obtained with enzyme rendered metal-free by (1) dialysis against 0.01 M EDTA (pH 7.5), 4°, followed by exhaustive dialysis against Tris-Cl, 0.01 M (pH 8.0), to remove all traces of EDTA (Ulmer and Vallee, 1971), or (2) by continuous stirring of the enzyme with 50% (by volume) Chelex-100 (Bio-Rad), equilibrated with Tris-Cl, 1 M (pH 8.0), for 48 hr followed by centrifugation at 10,000*g* to remove the resin. All three procedures for removing metal result in apoenzyme containing less than 2.6% residual zinc.

All solutions were rendered metal-free either by extraction with dithizone (Thiers, 1957) or by passage over a column of Chelex-100 (Bio-Rad) resin (Himmelhoch *et al.*, 1966). All glassware was acid-cleaned and rinsed with deionized water as described previously (Vallee and Hoch, 1955). Polyethylene containers were employed whenever possible. Cuvets were cleaned by soaking in dilute EDTA or 8-hydroxyquinoline-5-sulfonic acid and were rinsed exhaustively with metal-free distilled water immediately prior to use.

Enzymatic (hydrolase) activity was determined employing 1×10^{-3} M 4-nitrophenyl phosphate (Sigma) as substrate, in 1 M NaCl–0.01 M Tris-Cl (pH 8.0). Initial rates were estimated by the change in absorbance at 400 nm employing a Beckman spectrophotometer with a Gilford external recorder; the cell compartment was thermostated at 25°. Under these conditions, the ϵ_{278} of the product is 1.68 $\times 10^4$ M^{-1} cm^{-1}. Enzyme concentrations were determined spectrophotometrically employing the value $\epsilon_{78}(0.1\%)$ 0.72. A molecular weight of the protein of 89,000 was used for all calculations (Simpson *et al.*, 1968).

Johnson-Matthey "spec pure," sulfate salts of zinc and cobalt were dissolved in metal-free distilled water prior to their use for reconstituting the metalloenzyme from apoalkaline phosphatase.

The rates of hydrogen-tritium (H-T) exchange of metallo- and apophosphatases were determined by the "exchange out" technique of Englander (1963). The apoenzyme (20–30 mg/ml) was tritiated to equilibrium in the presence of THO (New England Nuclear), 1 mCi/ml, either for 2 days at 20°, pH 8.0, or for 1 week at 4°, pH 8.0.[2] Exchange was initiated by passing the catalytically inactive, tritiated apoprotein quickly (1–2 min) over a large, 1.5 × 30 cm, water-jacketed column (Ace glass) packed with coarse Sephadex G-25 (Pharmacia Inc.), previously rendered metal-free and preequilibrated with buffer at the desired pH, salt concentration, and temperature. Hydrogen exchange of the metalloprotein was determined on aliquots of the same tritiated apoprotein to which suitable amounts of solutions of zinc sulfate or cobalt sulfate were added 15 min prior to application of protein to the column. To avoid extrinsic metal contamination, the apoenzyme was always the *first* sample applied to the metal-free large Sephadex column; the reconstituted metalloenzyme was then applied immediately after washing with several column volumes of buffer to remove the unbound tritium.

After passage over the metal-free, large gel filtration column, the tritiated apoalkaline phosphatase stock solution contains less than 0.1 g-atoms of zinc/mol of protein. After similar recovery from the column, metalloenzyme stock solutions, reconstituted by addition of appropriate excess of metals to the protein prior to gel filtration, exhibit the enzymatic activities characteristic of native zinc alkaline phosphatase or of the cobalt enzyme described previously (Simpson and Vallee, 1968).

Enzymes eluted from the large column, having been separated from bulk tritium, provide stock solutions for the exchange of apo- or metalloproteins which are incubated at the desired temperature and pH until further measurements are required. Aliquots of 0.8 ml of this stock are withdrawn at intervals and filtered rapidly through small, 0.9 × 30 cm, columns packed with coarse Sephadex G-25, permitting removal of tritium lost from the protein during each interval. The small columns were jacketed to maintain a temperature of 0° in order to minimize the degree of exchange taking place during passage over the column.

The protein peak from the small columns was collected in three or more tubes and, after appropriate dilutions, the concentration of protein in each was determined spectrophotometrically to result in an absorptivity at 278 nm varying from 0.2 to 0.7. From each of the peak tubes 0.5 ml was then added to 19.5 ml of scintillation fluid (Bray, 1960) and, after cooling, was then counted in a Packard "Tri-Carb" scintillation counter for from 10 to 20 min to result in a minimum of 5000 total counts. Samples were arranged such that counting was accomplished between 2 and 4 hr after the protein was dissolved in the scintillation fluid and cooled in the dark to 4°. Equivalent time points for enzymes

[1] The abbreviations used are (P'ase), apoalkaline phosphatase; [(P'ase)Zn_4] and [(P'ase)Co_4], the enzyme fully reconstituted with 4 g-atoms of zinc or cobalt, respectively; [(P'ase)Zn_2] and [(P'ase)Co_2], the enzyme partially reconstituted with 2 g-atoms of zinc or cobalt.

[2] Due to the protective effect of the metal atoms, tritiation of the metalloprotein requires a much longer time period to achieve equilibrium.

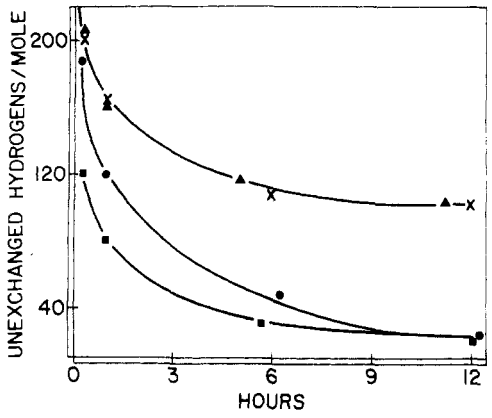

FIGURE 1: Comparison of H–T exchange of (P'ase) with that of [(P'ase)Zn_4] and [(P'ase)Zn_2] ± phosphate. All exchanges were performed in 0.16 M Tris-Cl (pH 8.0), 20°, except [(P'ase)Zn_2] + P_i (0.1 M Tris-Cl and 0.01 M phosphate), so as to offset the ionic strength of the added phosphate. Protein concentrations were approximately 30 mg/ml prior to passage over the large column and during subsequent incubations. The columns were washed and equilibrated as described in the Experimental Section. (■) (P'ase); (●) [(P'ase)Zn_2]; (▲) [(P'ase)Zn_2] + P_i; (X) [(P'ase)Zn_4].

with varying metal contents were counted alternately. For the three or more peak tubes, the precision of counts per mg of protein was ±3%. For successive experiments, the repeatability of the entire exchange procedure was 3–5%.

Results

The time course of H–T exchange of apoalkaline phosphatase, (P'ase), in 0.16 M Tris-Cl (pH 8.0), 20°, is shown in Figure 1; 15 min after separation of bulk tritium, (P'ase) retains approximately 120 tritium atoms per molecule. Hence, under these conditions, of its total of 1200 potentially exchangeable hydrogens almost 90% of the apoenzyme had already exchanged out so rapidly as to indicate that these groups are almost freely exposed to solvent water. During the ensuing 6 hr, an additional 80 hydrogens are exchanged, while still another 10 are lost from the protein even more slowly over the subsequent 6 hr. The sluggish exchange of these 90 hydrogens is attributed ordinarily to constraints imposed by conformational features of the protein (see Discussion). The remaining bound tritium, approximately 30–40 atoms, constitutes an inert "core" which, under these experimental conditions, exchanges only very slowly to a limited extent, over many days. Therefore, this group of hydrogens is presumed to be shielded nearly completely from solvent.

In contrast to apophosphatase, the fully reconstituted zinc enzyme [(P'ase)Zn_4] retains about 200 tritium atoms per molecule after initial gel filtration (Figure 1); hence, compared to the apoenzyme, the holometalloenzyme shelters an additional 80 tritium atoms per mole of protein, and this difference is maintained throughout the entire time course of its exchange (Figure 1). Thus, the presence of a full complement of four zinc atoms protects nearly 7% of the total hydrogens of the molecule from exchange.[3] Under the same experimental conditions, apophosphatase and the fully reconstituted cobalt enzyme, [(P'ase)Co_4], differ by the exchange of only 50–60 hydrogens (Figure 3), a highly significant but less marked difference than that observed for [(P'ase)Zn_4] (Figure 1).

TABLE 1: Temperature Dependence of Hydrogen–Tritium Exchange of (P'ase) and [(P'ase)Zn_4].[a]

Temp (°C)	Time (min)	Unexchanged Hydrogens/mole	
		(P'ase)	[(P'ase)Zn_4]
4	15	313	379
	130	253	329
	240	210	287
25	15	244	312
	120	164	235
	210	146	211
35	20	190	240
	105	117	178
	160	100	158

[a] 0.01 M Tris-Cl, pH 7.5.

The rates of hydrogen isotope exchange are strongly dependent upon the temperature and pH at which the exchange reaction is performed. Thus, variations in either solvent, temperature, or pH may be employed to investigate exchange mechanisms (Hvidt, 1964; Hvidt and Nielsen, 1966). On this basis, hydrogen–tritium exchange rates of (P'ase) and [(P'ase)Zn_4] were determined over a range of temperature from 4 to 35° (Table I). The apo- and metalloenzymes differ consistently by approximately 70 hydrogens per mole of protein when stored at 4 and 25°, respectively. As the temperature is increased to 35°, the difference becomes slightly smaller.

According to Hvidt and Nielsen (1966) (see Discussion), most proteins exchange by an "EX_2" mechanism, in which the temperature-controlled exchange of exposed peptide groups functions as the rate-limiting step, as in model compounds. If exchange occurs by this mechanism, the process of the exchange reaction should describe a composite first-order decay function of the product of time and temperature (as well as H^+ or OH^- concentrations) (Hvidt and Nielsen, 1966). Figure 2 shows the exchange data for zinc and apophosphatase at temperatures ranging from 4 to 35°; the proportion of unexchanged hydrogens is plotted *vs.* the logarithm of the products of temperature and duration of the exchange reaction as suggested by Willumsen (1966). The data for both forms of phosphatase can be fitted to a regularly decreasing function, precisely as would be anticipated if, as temperature is raised, exchange accelerates solely due to a temperature-dependent catalysis of the rate-limiting step. Hence, it appears likely that, over the range examined, the hydrogen exchange of exposed peptide hydrogens in both the alkaline and zinc apophosphatases depends directly upon temperature and is, therefore, in accord with an "EX_2 mechanism" (Hvidt and Nielsen, 1966).

Previous kinetic and spectral observations of metallophosphatases have suggested that the four metal binding sites of this enzyme are nonequivalent, and may comprise two discrete types, resulting in metal complexes of different geometry and symmetry, as can be discerned particularly when cobalt is substituted for zinc to serve as a spectral probe (Simpson and Vallee, 1968). Therefore, the structural role of each metal was examined further by comparing

[3] The difference in hydrogen exchange properties of (P'ase) and [(P'ase)Zn_4] is independent of the chelating agent employed to prepare the starting apoenzyme, *i.e.*, Chelex-100 resin, EDTA, or 8-hydroxyquinoline-5-sulfonic acid.

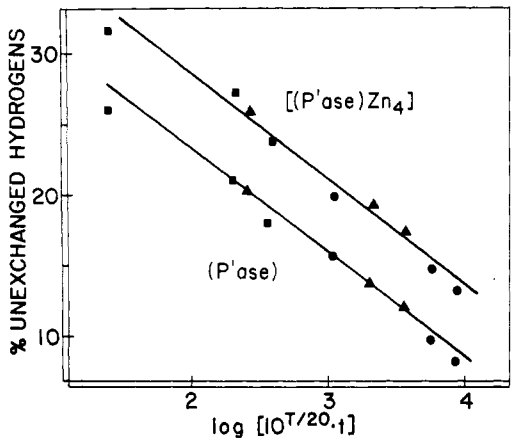

FIGURE 2: Temperature dependence of H-T exchange of apo- and fully reconstituted zinc alkaline phosphatase. Exchanges were carried out at 4, 25, and 35° in 0.01 M Tris-Cl (pH 7.5). All experiments were performed on aliquots of a single, large, tritiated sample. After passage over the large column, protein concentrations were 2.0 ± 0.3 mg/ml. The data were plotted according to Willumsen (1966), using a simplified form of the complex Willumsen equation, since the pH was held constant. (■) 4°; (▲) 25°; (●) 35°.

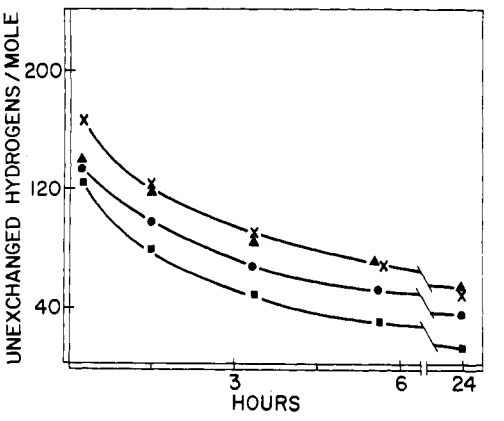

FIGURE 3: Relative rates of H-T exchange of (P'ase), [(P'ase)Co_4], and [(P'ase)Co_2] ± P_i. Exchanges were carried out in 0.16 M Tris-Cl (pH 8.0), 20°, except [(P'ase)Co_2] + P_i, (0.1 M Tris-Cl-0.01 M phosphate). Protein concentrations were 2.0 ± 0.3 mg/ml after passage over the large column. The columns were washed and equilibrated as described in the Experimental Section. (■) (P'ase); (●) [(P'ase)Co_2]; (▲) [(P'ase)Co_2] + P_i; (X) [(P'ase)Co_4].

the hydrogen exchange characteristics of the partially reconstituted enzymes, [(P'ase)Zn_2] and [(P'ase)Co_2], with those of fully reconstituted holometalloenzymes, [(P'ase)Zn_4] and [(P'ase)Co_4], and that of the apoenzyme.

The kinetic H-T exchange rate of [(P'ase)Zn_2] in 0.16 M Tris-Cl (pH 8.0), 20°, compared to that of the apoenzyme and [(P'ase)Zn_4] is shown in Figure 1. Significantly, the presence of only 2 g-atoms of zinc/mol appears to have a marked influence on exchange. At the earliest measurement, less than 15 min after removing bulk tritium and subsequent to addition of zinc, [(P'ase)Zn_2] retains almost as much tritium per molecule as does the native protein—186 *vs.* 200 unexchanged hydrogen atoms. Of itself, this would suggest that only two zinc atoms confer an initial degree of conformational stabilization nearly equivalent to that characteristic of the completely reconstituted holoenzyme. However, the subsequent loss of tritium from [(P'ase)Zn_2] is significantly more rapid than that observed for [(P'ase)Zn_4] (Figure 1): a marked difference in the degree of exchange between the zinc enzyme containing two and four zinc atoms, respectively, is evident within 1 hr and, after 6 hr, the course of [(P'ase)Zn_2] exchange approaches that of the apoenzyme, remaining similar thereafter. This indicates that the two zinc atoms bind to phosphatase such as to retard exchange of a portion, but not all of the tritiums which are protected in the holoenzyme, [(P'ase)Zn_4].

Phosphate, the product of substrate hydrolysis by alkaline phosphatase, has been shown to influence the spectral properties of the enzyme through interactions affecting the metal-binding sites. Moreover, phosphate retards the otherwise rapid inactivation of the enzyme through removal of the more labile zinc atoms upon exposure to 8-hydroxyquinoline-5-sulfonic acid (Simpson and Vallee, 1968). Hence, it was of interest to determine the effects of phosphate upon the rates of hydrogen-tritium exchange of the apo-, partially and fully reconstituted zinc phosphatases. Allowing for alterations owing to changes in ionic strength, phosphate does not influence exchange of (P'ase) or [(P'ase)Zn_4]. However, phosphate markedly affects exchange of [(P'ase)Zn_2]. In the presence of 0.01 M phosphate the rate of exchange of this partially reconstituted protein is identical with that of the holoenzyme (Figure 1). Thus, the presence of phosphate together with the first two zinc atoms maintains a hydrogen exchange pattern equivalent to that observed when all four zinc atoms are present.

The exchange of the partially reconstituted cobalt enzyme, [(P'ase)Co_2], differs from that of [(P'ase)Zn_2] (Figures 1 and 3). While the addition of two zinc atoms appears to shield groups of early, *i.e.*, rapidly exchanging hydrogens, the kinetic exchange curve of [(P'ase)Co_2] is similar to that of (P'ase) at the beginning of the experimental period (Figure 3). Subsequently, the exchange of [(P'ase)Co_2] progresses slightly less rapidly than that of the apoenzyme; after 8 hr, nearly 20 fewer hydrogens of [(P'ase)Co_2] have exchanged. In the presence of phosphate the kinetic hydrogen exchange curve of [(P'ase)Co_2] resembles that of [(P'ase)Co_4], a phosphate effect analogous to that observed with the partially reconstituted enzyme.

The hydrogen exchange properties of apophosphatase and those of the completely reconstituted holometalloenzymes appear to obey exchange mechanisms analogous to those observed in model systems (Figure 2). However, the time-dependent variations in rates of tritium loss from the partially reconstituted zinc and cobalt proteins (Figures 1 and 3) indicate alternate exchange mechanisms, and, hence, suggest potential differences in structure between the partially and fully reconstituted metalloenzymes.

The possibility that corresponding time-dependent changes in enzymatic activity might occur was explored with partially and fully reconstituted enzymes prepared in the same manner as those utilized for the hydrogen exchange measurements. Preliminary experiments have realized a time-dependent loss of activity of [(P'ase)Zn_2], when studied at a concentration of 2.2 mg/ml in 0.1 M Tris-Cl (pH 8.0), 20° (Figure 4). This loss of activity is fully reversible by the addition of a second increment of two zinc atoms and is largely obviated by incubation in the presence of phosphate.[4] The exact circumstances leading to this anomalous and previously unreported behavior and their relationships to the pronounced effect of phosphate on the hydrogen-tritium exchange of [(P'ase)Zn_2] are currently under active investigation.

[4] Phosphate is not present in the assay, accounting for the activities of [(P'ase)Zn_2] ± P_i which are nearly equal.

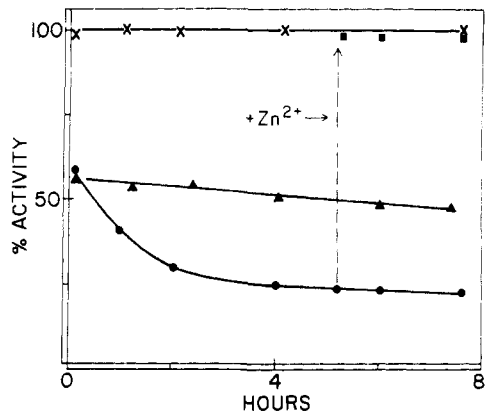

FIGURE 4: Effect of phosphate and zinc on the activity of [(P'ase)Zn_2]. Protein (2.2 mg/ml) was incubated in 0.1 M Tris-Cl ± 0.01 M phosphate (pH 8.0), 20°, followed by addition of 2 g-atoms/mol of zinc at the time indicated. Samples were reconstituted and assayed as described in the Experimental Section. Activities are expressed as the per cent of the activity of [(P'ase)Zn_4]. (●) [(P'ase)Zn_2]; (X) [(P'ase)Zn_4]; (▲) [(P'ase)Zn_2] + P_i; (■) [(P'ase)Zn_2] + Zn^{2+}.

Discussion

The principles of hydrogen exchange in proteins as well as the advantages and shortcomings of various methods of exchange have been reviewed (Schellman and Schellman, 1964; Hvidt and Nielsen, 1966; Englander and Staley, 1969; Willumsen, 1971; Ottesen, 1971; Englander *et al.*, 1972). In determining the hydrogen-exchange properties of apo- and metallophosphatases, we have employed the tritium gel filtration "exchange out" technique of Englander (1963). It is rapid, convenient, relatively sparing of enzyme, and capable of high precision. Concentrated protein samples of considerable volume may be tritiated to equilibrium and, thereafter, identical aliquots are withdrawn for exchange measurements under a variety of experimental conditions. Moreover, as in the present investigations, it is possible to compare the exchange of a protein in the presence and absence of smaller molecules such as substrates, or metal atoms under otherwise identical circumstances. In this manner, the structural consequences of the interaction of proteins with biologically important smaller molecules can be assessed, while cancelling out factors such as equilibrium isotope effects and other variables which can influence absolute exchange rates.

In practice, the gel filtration technique quantitates the exchange of only a fraction of the total, labile hydrogens of a protein, *e.g.*, at a pH near neutrality, a large number of hydrogens of most globular proteins are exchanged during removal of bulk tritium, or within a few minutes thereafter, at rates too rapid to determine with this experimental procedure. Thus, exchange of more than 9/10th of the total, potentially exchangeable hydrogens of alkaline phosphatase occurs during passage over the large column (Figure 1). The remaining hydrogens, most of which exchange over a period of hours to days, are designated as slowly exchanging, and it is their composite rate constants which are reflected in the exchange curves.

Most if not all such slowly exchanging groups in proteins have been found to be peptide amide hydrogens, as observed by Lenormant and Blout (1953). Hypotheses as to the nature of the constraints which retard their exchange have generated much of the interest in this experimental approach. Early attempts to correlate the number of slowly exchanging protons with the helical content of proteins have proven unsuccessful (Elliott and Hanby, 1958; Blout *et al.*, 1961; Hvidt, 1964; Hvidt and Nielsen, 1966; Nakanishi *et al.*, 1972), and more recently correlations with hydrogen bonded structures in general have been favored (Englander and Staley, 1969). However, the potential role of steric factors and local environmental effects have also been emphasized (Klotz and Mueller, 1969; Liechtling and Klotz, 1966). Hydrogen bonded amides do, indeed, exchange slowly relative to intrinsic rates of non-hydrogen bonded, free amides. This circumstance led to the suggestion that, in proteins, some "opening" of hydrogen-bonded structures might be required to permit exchange. This general concept was first advanced by Linderstrøm-Lang (1955) and formal mechanisms of exchange were proposed by his students (Hvidt, 1964; Hvidt and Nielsen, 1966). According to their motility model, local conformational changes alternately completely exclude hydrogens of a polypeptide from exchange with solvent, a "closed" conformation, or render them freely exchangeable, an "open" conformation. While the nature of the closed conformation is not specified, its abundance is related to the compactness of the protein, as defined by its secondary and tertiary structure; in contrast, open conformations, such as those which predominate in denatured proteins, exchange rapidly with solvent, at rates characteristic of model amides or random coil polypeptides. According to this model, proteins in aqueous solution are considered to undergo continuous fluctuations among differing conformational states—some "open" and some "closed"—which are in rapid equilibrium with one another. The relative prevalence of each conformational state is a function of its conformational energy. The interaction of the protein with small molecules or metal atoms through ligand groups potentially distant from each other in the primary structure might be expected to impose limitations on the number of possible conformations (Ulmer, 1970a,b). Metal atoms consequent to binding to proteins could shift the conformational equilibrium toward a time-average conformation significantly different from that predominant in their absence (Ulmer, 1969). Such a shift in the prevailing time-average conformation could alter the exchangeability of a sizeable number of hydrogen atoms, even in the absence of discernible alterations in gross hydrodynamic or optical properties, provided the static features of the metal-stabilized form remain very similar to those which are prevalent in the absence of the metal and, hence, account for the sensitivity of hydrogen exchange methodology.

The pronounced influence of metals upon the H–T exchange of alkaline phosphatase may be interpreted in this context. Initially, and throughout a prolonged period of observation, zinc and cobalt retard the exchange of 70–80 and 50–60 hydrogens, respectively, over and above those which exchange slowly in the apoenzyme (Figures 1 and 3). For a metalloprotein, these constant differences are among the largest observed thus far (Ulmer, 1969; Ulmer and Vallee, 1971), indicating a marked stabilizing effect of metal atoms upon dynamic protein structures.

The greater conformational stability of the metalloprotein is also apparent from examination of the temperature dependence of exchange. When analyzed in terms of the mechanistic scheme proposed by Hvidt and Nielsen (1966) and plotted in the manner suggested by Willumsen (1966), the data can be fitted to a simple, monotonically decreasing function (Figure 2). This suggests that the temperature-dependent biomolecular reaction between exposed peptide protons and catalytic hydroxyl ions is the rate-limiting step

of exchange. According to this formulation, a displacement of the entire decay curve to the left, along the abscissa, signifies that apophosphatase has a less stable macromolecular conformation than does the metalloenzyme (Ulmer, 1969; Ulmer and Vallee, 1971).

The hydrogen–tritium exchange of partially reconstituted metallophosphatases is of special interest (Figures 1 and 3). Thus the time course of exchange of [(P'ase)Zn_2] differs from both that of (P'ase) and of [(P'ase)Zn_4]. During the early period of exchange [(P'ase)Zn_2] retains hydrogens almost to the same extent as does the completely reconstituted holometalloenzyme. However, [(P'ase)Zn_2] loses hydrogens more rapidly than does [(P'ase)Zn_4] and, after several hours, the decay curve approaches and then becomes coincident with that of the apoenzyme. In contrast, while the time course of exchange of [(P'ase)Co_2] also differs from that of the apoenzyme, a group of later exchanging hydrogens are found to account for this difference (Figure 3). These observations suggest that zinc and cobalt partially reconstitute phosphatases such as to stabilize discrete but different conformers which are less stable than the corresponding holometalloenzymes. Such an interpretation is consistent with previous spectral and kinetic data which indicate that zinc and cobalt bind differently when only 2 g-atoms of either metal are added to the apoenzyme (Simpson and Vallee, 1968). The present hydrogen exchange data, however, cannot distinguish between possibilities such as, *e.g.*, small differences in binding of zinc and cobalt at the same protein sites, the binding to quite different sites, or reequilibration of metals between alternate sites.

Phosphate ions interact with alkaline phosphatase to form a phosphoseryl intermediate (Schwartz and Lipmann, 1961), to exchange labeled oxygen with water in the presence of the enzyme (Stein and Koshland, 1952), and phosphate can serve as a substrate to form phosphoesters with suitable alcohol receptors (Myerhoff and Green, 1949). It has been shown, moreover, that both the removal of the two most labile zinc atoms and the corresponding loss of catalytic activity, upon exposure of phosphatase to chelating agents, are greatly retarded by phosphate (Simpson and Vallee, 1968). This suggested that phosphate might further stabilize metallophosphatases with respect to hydrogen exchangeability. Figures 1 and 3 demonstrate that, indeed, the presence of phosphate further alters the time courses of hydrogen exchange of phosphatases partially reconstituted with either zinc or cobalt to resemble those of the completely reconstituted holometalloenzymes. Phosphate does not appreciably affect the exchange of either apo- or holometalloenzymes. Hence, the anion would seem to act in concert with the two metal atoms of the partially reconstituted protein to induce alterations of conformational equilibria which may be equivalent, but not necessarily identical with those of the enzyme bearing a full complement of metal atoms. Despite the apparent equivalence of conformational stabilization, phosphate can clearly not "replace" the missing set of metal atoms in a physical sense. Hence, it seems possible that among the large number of potential conformers of alkaline phosphatase only certain equilibrium sets are strongly favored energetically, *e.g.*, those represented by apophosphatase, the holometalloenzymes, and those characteristic of [(P'ase)Zn_2] and [(P'ase)Co_2]. In this sense, phosphate appears to promote various intermediate states, *i.e.*, the partially reconstituted zinc or cobalt enzymes, to more stable states characteristic of the holometalloenzyme. The second increment of metal atoms accomplishes the same result. In the case of phosphate such a promotion in stability might be mediated, for example, through bidentate chelation of the phosphorus oxydianion with the metal at its binding site; on the other hand, the second increment of two cobalt or two zinc atoms could act by interacting at quite different sites. The specificity of phosphate in altering stability of the partially reconstituted proteins has not yet been examined experimentally.

In addition to influencing the hydrogen exchange properties of [(P'ase)Zn_2], phosphate stabilizes the catalytic activity of the partially reconstituted enzyme. The mechanism which accounts for the loss of activity of [(P'ase)Zn_2] is obscure at present. We have reported that, under specified conditions, only two zinc atoms are required to restore the hydrolase activity of apophosphatase toward nitrophenyl phosphate (Simpson and Vallee, 1968), although alternate observations have also been reported (Petitclerc *et al.*, 1970). The reasons for such variations appear to stem both from differences in methods of preparation of apoenzyme (for a discussion see Ulmer and Vallee, 1971) and other technical details affecting the modes of metal restoration. The reversible loss of activity noted here and its prevention by phosphate addition may stem from as yet unidentified factors or might relate to possible intermediate conformations suggested by these hydrogen exchange measurements.

The multiple avenues by which zinc and cobalt influence the structure of alkaline phosphatase are likely to offer insight into the diverse means by which metals may importantly affect biologic events. Metals may participate directly in catalysis as components of the active sites of enzymes. However, metals may also control the number of active enzyme species through modulation of conformational equilibria, and thereby they may determine rates of enzyme degradation, influence transport processes, alter membrane functions, and play yet other roles in biochemical events.

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Two Distinct Pathways of the Streptokinase-Mediated Activation of Highly Purified Human Plasminogen†

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ABSTRACT: Two human plasminogens (Pg I and Pg II) were isolated in high yield and in pure form from pooled human plasma fraction $III_{2,3}$ by affinity column adsorption and elution on lysine-agarose beads followed by carboxymethylcellulose ion exchange chromatography, 4°, pH 5.0. These plasminogens both have molecular weights of about 85,000 and glutamic acid as their amino acid N-terminals. Following activation to plasmins, by streptokinase, active site equivalent weights equal to determined molecular weights were obtained. Proteolytic activities of both plasmins were about 40 CTA units/mg of protein. Plasminogen activation by catalytic streptokinase (SK) or urokinase (UK) as well as with stoichiometric quantities of streptokinase was investigated by stopping the reactions at the desired times with the active site titrant *p*-nitrophenyl-*p'*-guanidinobenzoate (NphOGdnBz). The reaction mixtures were reduced in sodium dodecyl sulfate (SDS) and the reaction products separated and quantitatively analyzed by SDS acrylamide gel electrophoresis. Native plasminogens Pg I and Pg II are single polypeptide chains with molecular weights of about 85,000. Conversion to plasmin (P) using either catalytic streptokinase or urokinase results in the cleavage of two characteristic peptide bonds yielding plasmin molecules containing three peptide chains ($P_{60,20,5}$), the order of cleavage being exclusively $Pg_{85} \rightarrow Pg_{80,5} \rightarrow P^a_{60,20,5}$ (where superscript a denotes the presence of a *p*-nitrophenyl-*p'*-guanidinobenzoate titratable active site and the subscripts indicate the approximate molecular weight in thousands of peptide chains involved). Interaction of Pg with stoichiometric amounts of streptokinase (SK) leads to the plasminogen activator complex $SK_{37,6,4} \cdot P^a_{60,20,5}$. Bond cleavages of Pg within this complex occur entirely by the reactions $Pg^a_{85} \rightarrow Pg^a_{65,20} \rightarrow P^a_{60,20,5}$. This order is the reverse of that found in catalytic activations. The SK peptide bond splits within the complex occur in the order $SK_{47} \rightarrow SK_{43,4} \rightarrow SK_{37,6,4}$. End group studies of isolated peptides from the NphOGdnBz treated $SK_{37,6,4} \cdot P^a_{60,20,5}$ complex showed the peptide orders (N)-SK_6-SK_{37}-SK_4-(C) for SK_{47} and (N)-P_5-P_{60}-P_{20}-(C) for Pg_{85} (the (N) and (C) designating the NH_2 and COOH termini, respectively).

Plasminogen (Pg)[1] activation by streptokinase (SK)[2] because of its practical importance in medicine and its unique nature has been the subject of many investigations. It is generally accepted that SK combines directly with either plasmin (P) or Pg to form a stoichiometric complex (Pg activator) capable of rapid conversion of Pg to P. A summary of data leading to these concepts can be found in a recent review by Amery and Claeys (1970). However, the direct role played by SK in the conversion of the active center of P, with little or no Pg activator activity, into an efficient enzyme for this action is not well understood.

In previous communications (McClintock and Bell, 1971a,b) we demonstrated that human plasminogen is able to combine directly with streptokinase in a 1:1 molar ratio to yield a complex. This complex then undergoes a time and temperature dependent unimolecular reaction which generates an active site. The results showed clearly that the for-

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[1] Unless otherwise stated, the terms Pg and P refer to plasminogens and plasmins of human origin.

[2] Abbreviations used are: ACTH, β-corticotropin; CM, carboxymethyl; CTA, Committee on Thrombolytic Agents, National Heart Institute; Dip-F, diisopropyl phosphorofluoridate; Dns-Cl, 1-dimethylaminonaphthalene-5-sulfonyl chloride; EACA, ε-aminocaproic acid; HSEtOH, 2-mercaptoethanol; Nph, *p*-nitrophenol; NphOGdnBz, *p*-nitrophenyl-*p'*-guanidinobenzoate; SDS, sodium dodecyl sulfate; SBTI, soybean trypsin inhibitor; SK, streptokinase; UK, urokinase.