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Formamide-Induced Dissociation and Inactivation of *Escherichia coli* Alkaline Phosphatase. Metal-Dependent Reassociation and Restoration of Activity from Isolated Subunits†

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ABSTRACT: Alkaline phosphatase from *Escherichia coli* has been reversibly dissociated by treatment with low concentrations of formamide. The monomer retains the capacity to bind metals and to regenerate catalytically active dimer that is identical with the native dimeric enzyme. The rate and extent of dissociation of dimer to monomer depend upon pH, ionic strength, temperature, formamide concentration, and enzyme-bound metal. Under appropriate experimental conditions, reassociation can be greatly slowed, allowing the properties of the monomer to be examined in solution. The formamide-induced apo monomer has a conformation distinct from that of the dimer and zinc- or cobalt-containing monomers. The monomer tightly binds 1 mol of zinc or cobalt in a metal-binding site altered from those of the dimer but is catalytically inactive. pH, ionic strength, and formamide concentration all influence reassociation. Hydrophobic forces are implicated as important in subunit interactions. The effect of metal content on the dissociation–reassociation process underscores the essential role that metals play in maintaining enzyme tertiary structure and reveals a new role in stabilizing the quaternary structure.

Understanding the chemical basis for the effects of enzyme aggregation upon its biologic function is an important goal of enzymology. Studies of subunit interactions in oligomeric enzymes require elaboration of a system in which the monomer–oligomer reaction is completely reversible and which allows separate investigation of the properties of the monomer and oligomer. Recent studies on mitochondrial malate dehydrogenase (Wood et al., 1981; Jurgensen et al., 1981) utilizing pH changes to induce alterations in the state of aggregation have begun to reveal the importance of subunit interactions on thermal stability, fluorescence properties, and enzymatic activity. We have developed such a system to investigate the chemical basis of the effects of aggregation on the subunits of dimeric *Escherichia coli* alkaline phosphatase. This enzyme is of particular interest, exhibiting as it does complex metal-binding interactions and negative cooperativity.

E. coli alkaline phosphatase, molecular weight 89 000, is composed of two subunits of molecular weight 44 500 (Simpson & Vallee, 1968) and contains four tightly bound zinc atoms. Two zinc atoms are critical for the *catalytic* activity of the enzyme, the other two being thought necessary for *structural* integrity of the molecule. In addition, two magnesium atoms *regulate* enzyme activity (Simpson & Vallee, 1968; Anderson et al., 1975; Bosron et al., 1977). Recently, these metal-binding sites have been characterized more fully. The interrelationships of the metal-binding sites remain only partially defined, as is the distribution of metal-binding sites on the subunits (Otvos & Armitage, 1980a,b).

Alkaline phosphatase exhibits negative cooperativity in substrate hydrolysis (Simpson & Vallee, 1970), in the binding of inorganic phosphate (Lazdunski et al., 1969), and in conformational changes as revealed by NMR[1] (Chlebowski et al., 1977). Apparently, binding of a substrate or an inhibitor molecule to one subunit induces conformational changes in the other through as yet undefined subunit interactions. However, this negative cooperativity remains controversial; other investigators claim that under certain experimental conditions it can be eliminated completely (Bloch & Bickar, 1978; Otvos & Armitage, 1980a,b). Nevertheless, under some

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[1] Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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(if not all) conditions these subunit interactions are important. For delineation of such interactions as well as definition of the metal binding sites, it is necessary to compare the properties of the subunits and of the dimer under identical conditions.

In the past, dimeric alkaline phosphatase has been dissociated either by reduction in the presence of urea (Levinthal et al., 1962) or by treatment with acid (Schlesinger & Barrett, 1965). In each case the monomer unfolds completely and, hence, neither its catalytic activity nor its metal-binding properties can be examined. Structural mutants of *E. coli* alkaline phosphatase characterized by a defective, catalytically inactive subunit have been reported (Schlesinger, 1967). Studies of metal binding to the isolated subunit are precluded because the mutant subunit dimerizes readily in the presence of zinc; moreover, there is evidence to suggest that the mutation alters either the geometry or constituents of the metal-binding site (Applebury & Coleman, 1969). Stable subunits have been prepared (McCracken & Meighen, 1980, 1981) by immobilizing dimers via attachment to an inert support and subsequent dissociation of the dimer with high concentrations of guanidine hydrochloride. Some properties of the matrix-bound subunit were characterized, but it was difficult to reassociate the dimer and it was not possible with this system to quantitate and physicochemically characterize the subunit in solution.

We have reported a new method employing formamide to dissociate dimeric alkaline phosphatase under mild conditions (Falk, 1978). The present studies demonstrate this reaction to be fully reversible; thus, the monomeric unit retains both the capacity to bind metals and catalytic activity, and all other properties of the native dimers may be regenerated. Further, this system allows study of both monomeric and dimeric species under identical conditions. Such examination reveals that at least one pair of zinc atoms, which stabilizes the quaternary structure of alkaline phosphatase, can be localized. Moreover, the factors involved in subunit interactions can be differentiated, and their potential use to delineate their participation in the negative cooperativity is explored.

Materials and Methods

Preparation of Alkaline Phosphatase. Alkaline phosphatase was obtained from periplasmic extracts of *E. coli* strain C-90 by the method of Simpson et al. (1968). Highly purified enzyme obtained at pH 7.4 contained 4.0 ± 0.3 g-atoms of zinc, 1.2 ± 0.2 g-atoms of magnesium, and no other metals (Bosron et al., 1977). Apophosphatase was prepared as described (Simpson & Vallee, 1968). The apoenzyme contained no phosphate (Bosron & Vallee, 1975), less than 0.02 g-atom of zinc, and 0.04 g-atom of magnesium. The specific activity of the apoenzyme was 0.01 unit or 0.02% of that of the native enzyme.

Assays. Enzymatic assays were performed in 1 M Tris-HCl, pH 8.0 at 25 °C, with 4-nitrophenyl phosphate, 1 mM, as substrate. The sum of hydrolase plus transferase activities was measured routinely in 1 M Tris-HCl, pH 8, and hydrolase activity alone was measured in 20 mM Veronal–400 mM NaCl, pH 8 (Cohen & Wilson, 1966). Units of activity are expressed as micromoles of substrate hydrolyzed per minute per milligram of protein using a molar absorptivity of 1.68×10^4 at 400 nm to monitor changing concentration of the 4-nitrophenolate ion. Protein concentration was determined spectrophotometrically with $A^{1\%} = 7.2$ (Plocke et al., 1962), and all calculations involving molarity were based on a molecular weight of 89 000 (Simpson & Vallee, 1968). Protein concentrations of all samples containing formamide and of all monomeric phosphatase species were determined by the method of Lowry (Lowry et al., 1951) or by a modification of that method incorporating trichloroacetic acid precipitation (Dickinson et al., 1980). Purified apophosphatase served as the standard. Zinc and magnesium were determined by atomic absorption spectrometry.

The catalytic activity of the enzyme under various dissociation conditions was determined by withdrawing aliquots and diluting them 10-fold into ice-cold dilution–incubation buffer containing 50 mM Tris-HCl, pH 8, 1 M NaCl, and a 10-fold molar excess of Zn^{2+} (final concentrations). After 11 min of incubation at 5 °C, the diluted enzyme was assayed in 1 M Tris-HCl, pH 8 at 25 °C. Under these conditions, reassociation of the monomer is quite slow (Table IV) so that catalytic activity reflects reconstitution of the undissociated apo dimer. The zinc-reconstituted activity is expressed as a percentage of the control, i.e., zinc-reconstituted activity of the apo dimer treated similarly but omitting the formamide.

Preparation of Reagents. Analytical grade chemicals were used throughout. 4-Nitrophenyl phosphate (Sigma 104) and Tris-HCl (Trizma) were obtained from Sigma Corp., spectrographically pure zinc, cobalt, and magnesium sulfates were from Johnson-Matthey, Ltd., $^{65}ZnCl_2$ and $^{60}CoCl_2$ were from Amersham Corp., and formamide (Spectro grade) was from Eastman Organic Chemicals. All solutions were prepared from deionized, distilled water. Buffers were extracted with 0.001% dithizone in CCl_4 and stored over Chelex-100 (Anderson & Vallee, 1975). High pH buffers were passed over a Chelex-100 column. Formamide-containing solutions were prepared from metal-free stock buffer solutions and Chelex-treated formamide. Glassware and polyethylene containers were rendered metal free by treatment with nitric acid–sulfuric acid mixtures as described by Thiers (1957).

Ultracentrifugal Analysis. Sedimentation velocity experiments were carried out in a Spinco Model E analytical ultracentrifuge employing the Schlieren optical system. Sedimentation equilibrium studies employed the method of Yphantis (1964), using the interferometric optical system.

Light Scattering. Light scattering studies were performed with a Perkin-Elmer MPF-3 spectrofluorometer operated in the ratio mode. Both excitation and emission monochromators were set at 400 nm, and excitation and emission slits were set at 4 nm. All solutions were filtered through Millipore cellulose acetate–nitrate filters rendered metal free by treatment with 8-hydroxyquinoline-5-sulfonic acid, and deaerated. Quartz fluorescence cuvettes with a capacity of 4 mL and a 10-mm path length were employed, and the temperature was regulated by means of a thermostated cuvette holder. During operation at low temperature the instrument was flushed with N_2. Light scattering intensities are presented as percent transmittance with deionized, distilled H_2O as a standard, normalized to zero percent transmittance.

Succinylation Experiments. Succinylation of the holo dimer was performed according to Meighen & Yue (1975) with a Radiometer autotitrator in a jacketed cell with the temperature regulated at 5 °C. Alkaline phosphatase (6–7 mg/mL) in 50 mM Tris-HCl adjusted to pH 8 was treated with 2 mg of succinic anhydride/mL of solution. The pH was maintained at 8 by the addition of 2 M NaOH. The same procedure was employed for apo monomer and zinc monomer. The succinylation of apo dimer was not affected by the addition of 1 mM 8-hydroxyquinoline-5-sulfonic acid.

Electrophoresis. Disc gel electrophoresis was performed with 10% polyacrylamide separating and 3% stacking gels in Tris-HCl–glycine buffer, pH 9.4 (Gabriel, 1971). Protein was stained with Coomassie Brillant Blue (Smith, 1968), and phosphatase activity was visualized with α-naphthyl phosphate

Table I: Dissociation of Alkaline Phosphatase

sample	[formamide][a] (%)	enzyme	$s_{20,w}$[b]	M_r	relative light scattering
1	5	Zn_4Mg_2	6.06	89 000	1
2	5	apo	2.94		0.5
3	10	apo		44 850	
4[c]	5	apo monomer + Zn	2.06		0.58
5	22	Zn_4Mg_2	5.45		
6	22	apo	1.75		

[a] All buffers were 50 mM Tris-HCl, pH 9.5, and 250 mM NaCl except sample 3 which was 50 mM Tris-HCl, pH 9.5, and 1 M NaCl. [b] The first and last photographs of the sedimentation velocity experiment were taken 1 and 2 h, respectively, after mixing. $s_{20,w}$ values for 22% formamide were calculated on the basis of density = 1.047 g/mL and viscosity = 1.106 cP and for 5% formamide density = 1.014 g/mL and viscosity = 1.032 cP; experiments were performed at a speed of 56 100 rpm, a temperature of 22 °C, and an enzyme concentration of 6 mg/mL. A partial specific volume of 0.73 was assumed for all species. [c] Uncorrected $s_{t,b}$; this sample was run at 8 °C.

and Fast Red TR (Gabriel, 1971).

Visible and Ultraviolet Absorption and Ultraviolet–Circular Dichroic (UV–CD) Spectra. Ultraviolet and visible absorption spectra were recorded with a Cary 14 spectrophotometer and UV–CD spectra with a Cary 61 spectropolarimeter. Rectangular 1-cm quartz cuvettes were employed in jacketed cell holders that were flushed with N_2 at low temperatures. Each CD spectrum was corrected by subtraction of a reference spectrum recorded under identical conditions in the same cuvette, but omitting the protein. Circular dichorism is expressed as molar ellipticity θ, with units of degrees per square centimeter per decimole. The use of formamide-containing buffers precluded the measurement of absorption or CD spectra below 250 nm.

Metal-Binding Studies. Metal-binding studies were performed according to the method of Hummel & Dryer (1962). A 0.9 × 25 cm column of either Bio-Gel P4 or Sephadex G-25 was equilibrated at 5 °C with various buffers containing 5×10^{-7}–5×10^{-6} M $^{65}Zn^{2+}$. Apo monomer, 1×10^{-6}–1×10^{-5} M, was equilibrated with the same $^{65}Zn^{2+}$ buffer and passed through the column. The resultant fractions were counted in a γ counter, and the absolute amount of metal bound was calculated from the average of the metal peak and trough of the resulting chromatograph.

Results

Formation of Monomer. When metal-free alkaline phosphatase (apo dimer) at pH 9.5 and 23 °C is exposed to 5% formamide overnight or to 22% formamide for 1 h, the dimeric enzyme completely dissociates into monomers (samples 2 and 6, Table I). The resultant subunits sediment as a single symmetrical boundary with sedimentation coefficients of 2.94 S and 1.75 S in 5 and 22% formamide, respectively. Under the same conditions the Zn_4Mg_2 enzyme (holo dimer) sediments as a single, symmetrical boundary with sedimentation coefficients of 6.06 S and 5.45 S, respectively (samples 1 and 5, Table I). These values are similar to those observed previously for the native and dissociated enzyme where, in the absence of formamide, native apo- and holophosphatases both have sedimentation coefficients of 6.1 S at neutral and alkaline pH while that of the acid-dissociated monomer is pH dependent, with sedimentation coefficients of 2.3 S at pH 2 and 3.4 S at pH 4 (Reynolds & Schlesinger, 1967). The sedimentation coefficient of 2.94 S in 5% formamide at pH 9.5 (Table I) and the symmetrical boundary suggest that the protein is monomeric under these conditions, as verified by molecular weight determinations using equilibrium centrifugation (Table I, sample 3). The molecular weight, 44 900, confirms that only the monomeric species is present. Analysis was performed in 1 M NaCl to eliminate any charge effects and in 10% formamide to ensure complete dissociation at this high salt concentration (Table II, sample 7). Further, the light scattering intensity of the apo monomer in 5% formamide is exactly half that of the holo dimer. When all other parameters are held constant, the light scattering intensity will be directly proportional to the molecular weight, assuming that the differential refractive index increment is identical for both monomer and dimer (Tanford, 1967). These results confirm the ultracentrifuge data by a method that is independent of partial specific volume.

Table II: Effect of pH, NaCl Concentration, and Formamide Concentration on Rate of Loss of Zinc-Reconstituted Activity[a]

sample	pH	[NaCl] (M)	[formamide] (%)	$t_{1/2}$ (min)[b]
1	8.5	0.25	5	218
2	8.5	0.25	7.5	75
3	8.5	0.25	10	33
4	8.5	0.25	15	8.8
5	9.5	0.25	5	90
6	9.5	0.25	10	25
7	9.5	1.0	10	75
8	9.5	1.0	5	incomplete dissociation[c]
9	8.0	0.25	10	108
10	8.0	1.0	10	incomplete dissociation[c]
11[d]	8.0	0.25	10	168

[a] All solutions were buffered in 50 mM Tris-HCl. [b] $t_{1/2}$ of the loss of zinc-reconstitutable activity determined by incubating apo dimer at an enzyme concentration between 1 and 2 mg/mL in the buffers listed at 25 °C, as in Figure 1. The diluting buffer was the same as in Figure 1 except that it contained 1 M NaCl. [c] Experiments with $t_{1/2}$ up to 218 min proceed to greater than 95% dissociation, while those shown as incomplete are still partially dimeric after as long as 72 h. [d] 5 °C.

While the results of these techniques are conclusive, the techniques themselves are not ideal for the study of the factors that affect the formamide-induced dissociation. However, since the monomer is catalytically inactive (vide infra), catalytic activity proved a more convenient method to follow rates and extents of dissociation and was correlated with the physically measured dissociation. Since only the apoprotein can be dissociated while enzyme-bound metal is an absolute requirement for catalytic activity, Zn^{2+} (or Co^{2+}) ions must be added prior to assay to reconstitute the metalloproteins. Thus, for these experiments, apo dimer was incubated at 25 °C in 50 mM Tris-HCl, pH 9.5, 250 mM NaCl, and 5% formamide. In order to determine the extent of dissociation, we withdrew aliquots at various times and assayed them as described under Materials and Methods. In this assay, added metal reconstitutes catalytic activity under conditions in which reassociation is negligible. The factors affecting reassociation are discussed in greater detail below.

Table III: Effect of pH on Rate of Loss of Zinc-Reconstitutable Activity[a]

sample	pH	[formamide] (%)	$t_{1/2}$[b]
1	9.5	5	90
2	8.5	5	218
3	8.5	10	33
4	7.5	10	60

[a] 50 mM Tris-HCl-250 mM NaCl, 25 °C. [b] As in Table II.

Activity changes correlated well with the extent of dissociation as determined by physical methods. Thus, on incubation of the apo dimer in 5% formamide at 25 °C activity is lost in parallel both with changes in light scattering intensity and with the conversion of the 6.1 S apo dimer to the 2.9 S apo monomer. In contrast, under these conditions 5% formamide neither affects activity nor physical properties of the holo dimer. Changes in activity can therefore monitor the dissociation of formamide-treated alkaline phosphatase.

Utilizing this assay, we found both the rate and extent of dissociation of dimeric alkaline phosphatase to depend on a number of parameters; among these are formamide concentrations, pH, temperature, and ionic strength. Thus, increasing formamide concentration (samples 1–4, Table II), increasing temperature (samples 9 and 11, Table II), and decreasing salt concentrations (samples 6 and 7, Table II) all increase the *rate* of dissociation. On the other hand, lowering the pH while keeping the other parameters constant decreases the dissociation rate (samples 1–4, Table III). Increasing formamide concentration (samples 7 and 8, Table II) and decreasing salt concentration (samples 9 and 10, Table II), independent of protein concentration over the range 5×10^{-6}–1×10^{-4} M, all increase the *degree* of dissociation of the apo dimer. Concentrations of formamide below 5% do not induce dissociation.

Incubation of apo dimer with 2 or 4 equiv of zinc prior to the addition of formamide prevents dissociation. There is virtually no loss of activity of either the Zn_2 dimer or Zn_4 dimer after 72 h of incubation in concentrations of formamide up to 50%. However, at 60% formamide, losses of activity after 24 h are significant, and at 75% formamide activity is lost completely within 2 h. Again, loss of activity correlates with the appearance of the slower sedimenting monomeric species in sedimentation velocity experiments. Unlike zinc, magnesium does not affect the dissociation. The dissociation behavior of the dimer containing 2 g-atoms of magnesium is identical with that of the apo dimer.

Reassociation of Monomer to Dimer. At room temperature and at low formamide concentrations (5% or less, pH 9.5), addition of zinc or cobalt immediately initiates dimerization of the monomer. Conveniently, enzyme activity monitors this process also. Sedimentation velocity experiments show that the appearance of the faster sedimenting, dimeric species correlates directly with the extent of activity restored by zinc. A multiplicity of factors affects the rate and extent of recovery of activity, i.e., reassociation, but the dependence on metals is obligatory. Comparison of samples 1 and 2, Table IV, demonstrates the requirement of zinc for reassociation; this is also true for cobalt. Raising the temperature to 37 °C greatly increases the rate of reassociation. Magnesium alone (samples 1 and 7, Table IV) neither stimulates reassociation nor affects the zinc-promoted reassociation of the monomer (samples 2 and 3, Table IV). While at 4 °C 1 M NaCl retards the reassociation, at 37 °C no significant ionic strength effect can be demonstrated (samples 2 and 4, Table IV). Moreover, variation of pH affects reassociation only slightly (samples 5 and 6, Table IV). Addition of zinc to the dissociation mixture (i.e., at high formamide concentrations) does not promote reassociation, even at 37 °C (sample 8, Table IV).

Table IV: Metal Ion Dependent Reassociation of Formamide-Induced Monomers[a] to Dimers

sample	pH and molar metal excess	% act. after 3-h incubation 4 °C	37 °C
1	pH 8	<5	<5
2	pH 8, 10× Zn^{2+}	8	84
3	pH 8, 10× Zn^{2+}, 2.5× Mg^{2+}	8[b]	81[b]
4	pH 8, 10× Zn^{2+}, 1 M NaCl	<5	78
5	pH 7.5, 10× Zn^{2+}	<5	75
6	pH 9, 10× Zn^{2+}	<5	65
7	pH 8, 2.5× Mg^{2+}	<5[c]	<5[c]
8	pH 9.5, 10× Zn^{2+}, 275 mM NaCl, 22% formamide	<5[d]	<5[d]

[a] Apo dimer was dissociated in 55 mM Tris-HCl, pH 9.5, 275 mM NaCl, and 22% formamide for 1 h at 23 °C; aliquots were withdrawn and diluted 10× into the 50 mM Tris-HCl buffers listed and incubated as specified above. Activity was measured in 1 M Tris-HCl, pH 8, and compared to a control sample with Zn_4 holoenzyme treated similarly. [b] These values are corrected for previously reported magnesium activation of Zn_4 holoenzyme. [c] Aliquots of these samples were taken at the specified times and incubated for 10 min with a 10-fold molar excess of Zn^{2+} at 4 °C and then assayed as previously. [d] A 10-fold molar excess of Zn^{2+} was added directly to aliquots of the dissociation, and then they were treated as before.

The dissociation conditions themselves affect the extent of reassociation. Thus, long exposure to formamide, high formamide concentrations, or high temperatures during dissociation decrease the amount of activity that can ultimately be restored.

Properties of Monomers. Activity and Metal-Binding Capacity. The zinc content of the "apo dimer" is approximately 0.02 g-atom of zinc/mol (see Materials and Methods); as a consequence, a commensurate amount of activity remains even after extended incubation in formamide, under conditions where the Zn_2 dimer is stable. The residual activity, therefore, could result from a small concentration of undissociated dimer or could be intrinsic activity of the monomer.

To resolve this issue, we performed electrophoresis to separate residual, undissociated dimer from the monomer to identify the species responsible for the catalytic activity. To create a charge difference, we treated all samples first with succinic anhydride. While succinylation fails to affect the activity of the holo dimer, the succinylated monomer does not reassociate to form dimer; and the succinylated monomer and dimer can be separated easily be electrophoresis.

Therefore, phosphatase was dissociated in 50 mM Tris-HCl, pH 9.5, 250 mM NaCl, and 5% formamide overnight at room temperature. After addition of metal and subsequent succinylation, only 5% of the activity of the untreated holo dimer could be detected. It was not affected by dilution or heating at 37 °C. The succinylated enzyme was gel filtered through Sephadex G-25, previously equilibrated with 50 mM Tris-HCl, pH 8, and subjected to disc gel electrophoresis. Duplicate gels were stained for protein and activity. Only approximately 5% of the material migrated in the position of succinylated holo dimer, and all the catalytic activity is associated with this band. The bands due to succinylated monomer exhibited no catalytic activity. These results are consistent with the residual activity being due to undissociated dimer.

Under the conditions employed, the holoenzyme does not dissociate, and the residual zinc content of the apoenzyme represents the presence of holoenzyme, accounting for the

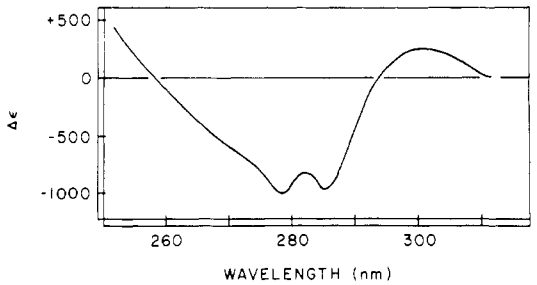

FIGURE 1: UV difference spectrum of apo monomers vs. apo dimers. Conditions: enzyme concentration 0.43 mg/mL in 50 Tris-HCl, pH 9.5, 250 nM NaCl, and 5% formamide. $\Delta\epsilon$ is expressed on the basis on the dimeric molecular weight, 89 000.

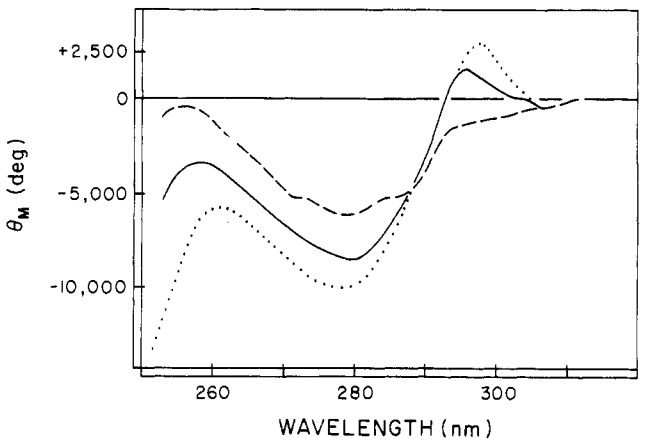

FIGURE 2: UV–CD of alkaline phosphatase. Conditions: 50 mM Tris-HCl, pH 9.5, 250 mM NaCl, and 5% formamide at 10 °C. (– –) apo monomers (4.2×10^{-5} M); (—) apo dimers (1.1×10^{-5} M); (···) Zn_4 dimer (1.1×10^{-5} M).

residual activity. Thus, minor metal contamination of the enzyme solution is a likely basis for the presence of the stable, dimeric phosphatase species. Therefore, under standard assay conditions monomeric alkaline phosphatase is catalytically inactive.

This could result from an inability to bind metals and/or a fundamental structural change that disrupts the active site. Examination of the metal-binding capacity of the monomer was performed by using the method of Hummel & Dryer (1962). These direct binding studies indicate that in 50 mM Tris-HCl, pH 8, and 1 M NaCl, at 5 °C, 1.0 ± 0.1 g-atom of Zn^{2+} binds/mol of monomer. The dissociation constant of the zinc monomer complex is $<10^{-6}$ M; limitations of the technique preclude precise determinations of a lower limit. The native dimer binds 4 mol of zinc, i.e., 2 g-atoms/subunit (Bosron et al., 1975), with dissociation constants of 2×10^{-11} M and 1×10^{-8} M (Cohen & Wilson, 1966). In contrast to the dimer, the presence of magnesium did not affect the binding of zinc to the monomer.

Spectral Properties. That dissociation induces the loss of one metal binding site coincident with the loss of catalytic activity suggests that the conformation of the monomer differs from that of the dimer.

Indeed, changes in the UV difference absorption and circular dichroic spectra subsequent to dissociation are consistent with conformational changes. The difference absorption spectrum of apo monomer vs. apo dimer (Figure 1) exhibits minima at 279 and 285 nm ($\Delta\epsilon$ = 1000) but only minor alterations above 290 nm. The CD spectrum of the apo monomer differs significantly from those of either the apo dimer or holo dimer (Figure 2). The positive extremum at 295 nm of the holo dimer decreases when the metal is removed to yield the apo dimer and becomes negative on dissociation to the apo monomer. Further, in the apo monomer, the intensity of the negative extremum of the holo dimer at 279 nm diminishes, and the band structure becomes more complex. Under non-dissociating conditions, formamide minimally affects the difference absorption and circular dichroic spectra of either the Zn_4 dimer or the apo dimer.

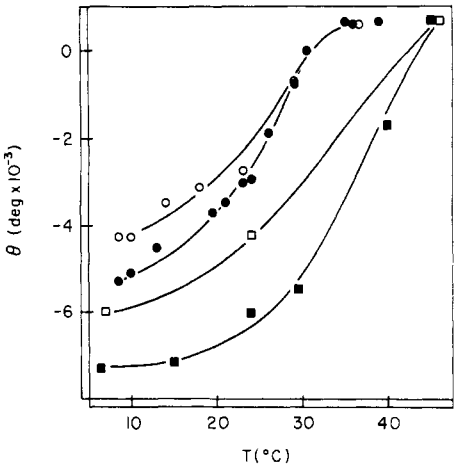

FIGURE 3: Temperature dependence at apophosphatase UV–CD. Conditions: (●) apo monomer, 2.09×10^{-5} M in 50 mM Tris-HCl, pH 9.5, 250 mM NaCl, and 5% formamide with heating; (○) apo monomer, same solution with recooling; (■) apo dimer, 1.05×10^{-5} M in 50 mM Tris, pH 9.5, and 250 mM NaCl with heating; (□) apo dimer, same solution with recooling.

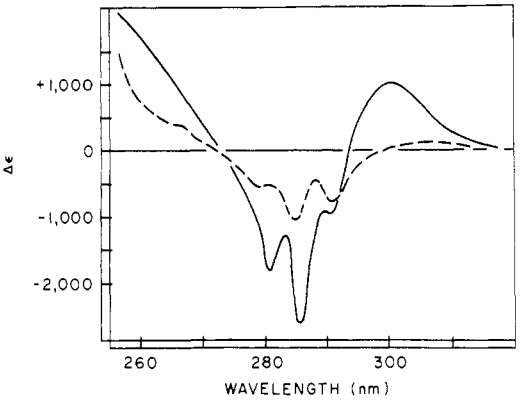

FIGURE 4: Temperature difference spectra. Conditions: (—) apo dimer, 1.05×10^{-5} M in 50 mM Tris-HCl, pH 9.5, and 250 mM NaCl; (– –) apo monomer, 2.09×10^{-5} M in 50 mM Tris-HCl, pH 9.5, 250 mM NaCl, and 5% formamide. The sample cell was maintained at 44 °C and the reference cell at 24 °C.

Importantly, both spectra and studies of thermal perturbations demonstrate that the monomer retains tertiary structure. The CD spectrum of the apo monomer at 279 nm is abolished by heating, with a transition midpoint temperature of 28 °C (Figure 3). The apo dimer has a higher transition midpoint temperature of 35 °C. The final CD spectra of the apo monomer and the apo dimer at 45 °C, however, resemble each other closely. Cooling largely restores the original CD spectra of both. Over this temperature range, the CD spectrum of the holo dimer undergoes only minor perturbations (not shown). Difference absorption spectra of enzyme at 44 °C vs. enzyme at 24 °C further characterize the conformational differences (Figure 4). Both apo dimer and apo monomer exhibit minima at 280, 286, and 291 nm, although for the apo monomer their amplitudes are much lower. Moreover, the maximum of the apo dimer absorption difference spectrum is at 300 nm, while that of the apo monomer is at 310 nm and of lower amplitude.

Addition of 1 g-atom of cobalt/apo monomer generates a visible absorption spectrum with a single broad maximum at

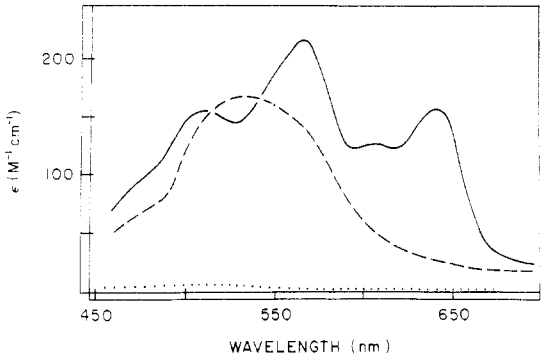

FIGURE 5: Visible absorption spectra of cobalt phosphatase and Co_2SO_4. (···) Co_2SO_4, (--) Co monomers, and (—) Co dimers. Conditions: 50 mM Hepes, pH 8, and 1 M NaCl, at 5 °C.

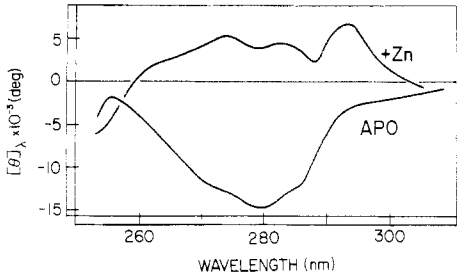

FIGURE 6: UV–CD of apo and Zn_1 monomers. Conditions: 2.09×10^{-5} M in 50 mM Tris-HCl, pH 9.5, 250 mM NaCl, and 5% formamide at 5 °C.

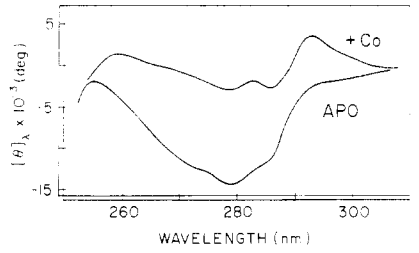

FIGURE 7: UV–CD of apo and Co_1 monomers. Conditions: 2.09×10^{-5} M in 50 mM Tris-HCl, pH 9.5, 250 mM NaCl, and 5% formamide at 5 °C.

530 nm (ϵ = 160 M^{-1} cm^{-1}). Addition of one more g-atom of cobalt to the Co_1 monomer rapidly regenerates the enzymatically active Co_4 dimer, which exhibits the normal spectrum of the Co_4 dimer (Anderson et al., 1975). Both differ from that of cobalt sulfate (Figure 5).

Addition of zinc to apo monomer (Figure 6) yields a distinctive CD spectrum for the Zn_1 monomer, which does not resemble that of either apo monomer, apo dimer, or holo dimer (Figure 2). The same is true of cobalt (Figure 7). CD titrations reveal that only 0.5 g-atom of zinc or cobalt/monomer is required to evoke the maximal spectral change. Both in the presence and absence of formamide, variation in pH between 8 and 9.5 does not affect this unexpected result (Table V). In the titration of apo monomer with cobalt, the presence of magnesium neither affects the stoichiometry nor the final spectrum.

The CD spectra of the Zn_1 and Co_1 monomers are stable for about 1 h at 5 °C, indicating minimal reassociation. Beyond that time, at 5 °C, these CD spectra slowly approach those of the Zn_2 or Co_2 dimer. The addition of one more g-atom of zinc/mol of monomer to the Zn_1 monomer brings about rapid (within 1 h) reassociation to the Zn_4 dimer.

Sedimentation velocity studies of the Zn_1 monomer at 8 °C in 50 mM Tris, pH 9.5, 250 mM NaCl, and 5% formamide reveal an uncorrected $s_{t,b}$ of 2.06 S (Table I) compared with 2.21 S for the apo monomer. Similarly, light scattering experiments in the same buffer at 5 °C show that treatment of the monomer with Zn^{2+} (1 g-atom/mol) does not immediately affect the state of aggregation of the monomer. However, a slow reassociation to the dimeric form is apparent under the experimental conditions employed.

Table V: Metal Stoichiometry by CD Titration

sample	metal	buffer[a]	stoichiometry
1	Zn^{2+}	pH 8, 1 M NaCl	0.5
2	Zn^{2+}	pH 8, 1 M NaCl	0.5
3	Zn^{2+}	pH 9.5, 1 M NaCl	0.56
4	Zn^{2+}	pH 9.5, 250 mM NaCl, 5% formamide	0.5
5	Zn^{2+}	pH 8.5, 250 mM NaCl, 5% formamide	0.55
6	Co^{2+}	pH 8.5, 250 mM NaCl, 5% formamide	0.6
7	Co^{2+}	pH 8.5, 250 mM NaCl, 5% formamide, Mg^{2+}	0.55
8	Zn^{2+}	pH 9.5, 1 M NaCl, 10% formamide	0.35
9	Zn^{2+}	pH 9.5, 0.1 M NaCl, 5% formamide	0.45

[a] All buffers were 50 mM Tris-HCl except sample 2, which was 50 mM Hepes; titrations were carried out at 5 °C.

Discussion

Since the equilibrium constant for dissociation of the apo dimeric form of *E. coli* alkaline phosphatase is approximately 2×10^{-6} M and much lower for the native, metal-liganded form (Applebury & Coleman, 1969), the soluble monomeric form of the wild-type enzyme has not been studied under physiological conditions. We have elaborated a system utilizing low concentrations of formamide in which wild-type alkaline phosphatase may be studied under a wide range of conditions in either its monomeric or dimeric forms. Measurements of the state of aggregation of apo alkaline phosphatase utilizing sedimentation velocity, sedimentation equilibrium, and light scattering demonstrate that formamide reversibly dissociates the dimer into monomers. Moreover, concomitant changes in catalytic activity, ultraviolet absorption, and circular dichroic spectra correlate directly with these changes in molecular weight.

The apo monomer retains an ordered tertiary structure, but one that which differs from that of the dimer. The $s_{20,w}$ value of 2.94 S in 5% formamide is intermediate between the value reported for the unfolded and partially refolded, acid-dissociated monomers at pH 2 and 3.4, respectively (Schlesinger & Barrett, 1965). The minima at 279 and 285 nm ($\Delta\epsilon$ = 1000) of the absorption difference spectrum of the apo monomer vs. the apo dimer (Figure 1) suggest that formation of the monomer places about half of the tyrosyl residues into a new environment, probably exposing them to the solvent (Scheraga, 1961). Heating the apo monomer to 45 °C exposes the remainder to solvent (Figure 4). The temperature difference spectra of the apo monomer and apo dimer both exhibit minima at 291 nm with similar $\Delta\epsilon$ (Figure 4), attributable perhaps to exposure of tryptophanyl residues to solvent (Scheraga, 1961).

The circular dichroic spectrum of the apo monomer differs from that of the apo dimer throughout the entire side-chain Cotton effect region, indicating that tyrosyl and tryptophyl residues of the two species exist in different environments (Figure 2). Heating of both species to 45 °C completely abolishes their near-ultraviolet–CD spectra, confirming the

disruption of tertiary structure.

Many characteristics of the formamide-induced monomer species differ from those of monomers reported previously but obtained under conditions different from those employed here. The circular dichroic and the absorption spectra of the mutant U47 alkaline phosphatase (Applebury & Coleman, 1969) and the absorption difference spectra of acid-dissociated alkaline phosphatase (Reynolds & Schlesinger, 1967) differ either in the positions of maxima and minima or in intensity from the corresponding spectra of the formamide-induced monomer. These differences in spectral properties and in metal-binding capacity, as well as the rate and extent of reassociation to dimeric alkaline phosphatase, suggest that the formamide-induced monomer is structurally distinct from monomers obtained by other methods. The spectral properties of the matrix-bound monomer were not reported, but its metal-binding stoichiometry and ability to reassociate suggest similarity to the formamide-induced monomer (McCracken & Meighen, 1980, 1981).

Direct binding studies indicate that the formamide-induced monomer tightly binds either one zinc or one cobalt atom (Table V). Sedimentation velocity experiments performed on the apo monomer in the presence and absence of zinc at 8 °C indicate that under both conditions the species remains monomeric. The small differences in sedimentation velocity between the apo monomer and zinc monomer may be due to differences in partial specific volume. Studies of light scattering intensity of the apo monomer with and without zinc at 5 °C confirm that monomer only is present. The absorption spectrum of the cobalt monomer has a single broad maximum at 530 nm (ϵ = 160 M^{-1} cm^{-1}), consistent with either tetra- or pentacoordinate geometry but inconsistent with octahedral geometry (Kaden et al., 1974). Substitution of Co^{2+} for Zn^{2+} in the octahedrally coordinated structural site of the dimer generates a visible absorption band at 510 nm of very low molar absorptivity (ϵ = 5 M^{-1} cm^{-1}). Complete substitution of Co^{2+} for Zn^{2+} in the dimer yields the more complex four-banded spectrum, thought to reflect distorted tetra- or pentacoordinate geometry (Simpson & Vallee, 1968). Since the spectrum of the Co monomer differs significantly from that of Co dimer, dissociation into monomer results not only in the loss of one Co^{2+} (Zn^{2+}) binding site but apparently alters the remaining Co^{2+} (Zn^{2+}) binding site. This is consistent with conclusions drawn with the matrix-bound monomer, which binds 0.9 mol of Zn^{2+} with a dissociation constant of 2×10^{-8} M or lower and is catalytically inactive (McCracken & Meighen, 1980).

Formamide thus induces a stable, monomeric apophosphatase that can tightly bind 1 g-atom of metal/mol and retain tertiary structure and the potential for restoration of catalytic activity. However, even when 1 g-atom of metal is bound, it remains catalytically inactive.

Utilizing this system, we have investigated the monomer in order to determine the effects of aggregation on the properties of the monomer. The apo dimer, holo dimer, apo monomer, and metallo monomer of phosphatase seemingly represent four distinct conformations each with characteristic properties.

In formamide, only the apo dimer dissociates to form the apo monomer, while the holo dimer does not. Conversely, only the metallo monomer will reassociate to form the holo dimer. Metal ions are integral features of the enzyme's quaternary structure, obligatory to reassociation, stabilizing the dimer, and preventing its dissociation. Moreover, when only 1 g-atom of zinc or cobalt is added to the monomer at 5 °C, reassociation occurs only slowly. At this temperature, addition of a second g-atom of metal greatly accelerates the rate of association. One possible explanation could be that the metal-binding site, which is lost on dissociation, is located sufficiently close to the areas of interactions between both subunits, thus accounting both for the stabilization of the quaternary structure and the metal effect on reassociation. Alternately, changes in the state of aggregation, which affect the conformation of the subunit, might thereby alter the metal-binding site.

Data on metal binding indicate that 1 g-atom of metal is bound per monomer, but metal titrations under a variety of conditions show that 0.5 g-atom of metal ion/monomer maximally perturbs the near-ultraviolet–CD spectra. The existence of two distinct populations of monomers could account for such observations, with the conformation of only one population reflected in a CD spectrum sensitive to metal binding at a single binding site. The other population of monomers would then bind metal to a single metal-binding site, but the occupation of this site by a metal would not evoke changes in the CD spectrum. The population of apo monomers could represent two conformations that are stable in the dimeric state, or the two subunits could differ in composition. Alternative explanations could be equally valid, but there are no unambiguous experimental data that could differentiate between any speculative hypotheses in this regard.

Dissociation induces new conformations of the monomers affecting the environment about the metal atoms and, hence, the catalytic activity. These conformational changes could be related to those postulated to account for the demonstrated negative cooperativity of this enzyme (Simpson & Vallee, 1970; Lazdunski et al., 1969; Chlebowski & Mabrey, 1977). The binding of substrate or inhibitor to one subunit may induce a conformational change, which in turn could induce a conformational change in the other subunit resulting in altered ligand geometry about its catalytic metal atom and rendering the dimer less active. Thus, two originally identical subunits could assume new nonidentical conformations, one more and one less active.

The present experiments emphasize the critical role that metal atoms play in each step of such a hypothetical process. Metal atoms stabilize quaternary structure; they are necessary for reassociation and prevent dissociation of active, dimeric alkaline phosphatase. Dissociation of apophosphatase induces at least one new conformation of the apo monomer in which metal-binding sites are lost and in which the geometry of the remaining metal-binding site is altered. Previous reports show the metal to be necessary for catalytic activity, which is also affected by conformational changes.

The state of aggregation of alkaline phosphatase also affects the thermal stability of the subunit. Recent advances in calorimetry have made possible the study of the thermal unfolding of dilute protein solutions. Differential scanning calorimetry and circular dichroism at 220 nm as a function of temperature at pH 9 have shown a transition for apophosphatase at 38 °C (Chlebowski & Mabrey, 1977) and apo monomer at 33.8 °C (Chlebowski et al., 1979). An extrapolation to pH 9.5 of the pH dependence of that transition temperature profile closely agrees with a transition temperature of 35 °C, as observed for the apo dimer by circular dichroic measurements at 278 nm (Figure 3). By this criterion, the monomers undergo a similar transition, but with a transition temperature of about 28 °C, reflecting a destabilization of the tertiary structure of the monomers relative to the dimer.

The conditions required for dissociation of the apo dimer form a basis for a tentative identification of the forces that

might play a role in subunit interactions. Among them, hydrophobic interactions are most probable, quite consistent with the known solvent ordering effects of formamide on tertiary and quaternary structure in proteins (Singer, 1962) and the effects of ionic strength. The exposure of tyrosyl residues to solvent in the course of dissociation to monomers is in accord with this conclusion (Figure 1). If these tyrosyl residues were to be located in the region of subunit interactions, they might thereby be shielded from the solvent in the apo dimer.

Involvement of hydrogen bonds and electrostatic forces would seem less likely. Formamide is a weakly protic solvent with a dielectric constant higher than water and capable of forming hydrogen bonds by acting as an acceptor. Since formamide forms hydrogen bonds equivalent in energy to those formed by water and peptide bonds (Pimental & McClellan, 1960), low concentrations of formamide would be expected to disrupt peptide hydrogen bondings minimally. Electrostatic interactions cannot be primary in the stabilization of subunit interactions that are disrupted by formamide, since increasing the concentration of supporting electrolyte, NaCl, from 0.25 to 1.0 M at pH 9.5 inhibits dissociation completely (samples 5 and 8, Table II). This inhibition is equally apparent on variation of salt concentration at a pH much closer to the isoelectric point of the enzyme (samples 10 and 11, Table II).

The present study suggests roles of metal atoms in *E. coli* alkaline phosphatase in addition to those known previously, i.e., stabilizing tertiary and quaternary structures. They represent novel indications of the forces involved in the interaction of subunits and suggestive evidence as to the locus of one pair of metal-binding sites.

References

Anderson, R. A., & Vallee, B. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 394–397.

Anderson, R. A., Bosron, W. F., Kennedy, F. S., & Vallee, B. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2989–2993.

Applebury, M. L., & Coleman, J. E. (1969) *J. Biol. Chem. 244*, 308–318.

Bloch, W., & Bickar, D. (1978) *J. Biol. Chem. 253*, 6211–6217.

Bosron, W. F., & Vallee, B. L. (1975) *Biochem. Biophys. Res. Commun. 66*, 809–813.

Bosron, W. F., Kennedy, F. S., & Vallee, B. L. (1975) *Biochemistry 14*, 2275–2282.

Bosron, W. F., Anderson, R. A., Falk, M. C., Kennedy, F. S., & Vallee, B. L. (1977) *Biochemistry 16*, 610–614.

Chlebowski, J. F., & Mabrey, S. (1977) *J. Biol. Chem. 252*, 7042–7052.

Chlebowski, J. F., Armitage, I. M., & Coleman, J. E. (1977) *J. Biol. Chem. 252*, 7053–7061.

Chlebowski, J. F., Mabrey, S., & Falk, M. C. (1979) *J. Biol. Chem. 254*, 5745–5753.

Cohen, S. R., & Wilson, I. B. (1966) *Biochemistry 5*, 904–909.

Dickinson, L. C., Rose, S. L., & Westhead, E. W. (1980) *J. Inorg. Biochem. 13*, 353–366.

Flak, M. C. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1287.

Gabriel, O. (1971) *Methods Enzymol. 22*, 565–604.

Hummel, J. P., & Dryer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.

Jurgensen, S. R., Wood, D. C., Mahler, J. C., & Harrison, J. H. (1981) *J. Biol. Chem. 256*, 2383–2388.

Kaden, T. A., Holmquist, B., & Vallee, B. L. (1974) *Inorg. Chem. 13*, 2585–2590.

Lazdunski, C., Petitclerc, C., Chappelet, D., & Lazdunski, M. (1969) *Biochem. Biophys. Res. Commun. 37*, 744–749.

Levinthal, C., Signer, E. R., & Fetherolf, K. (1962) *Proc. Natl. Acad. Sci. U.S.A. 48*, 1230–1237.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

McCracken, S., & Meighen, E. (1980) *J. Biol. Chem. 255*, 2396–2404.

McCracken, S., & Meighen, E. (1981) *J. Biol. Chem. 256*, 3945–3950.

Meighen, E., & Yue, R. (1975) *Biochim. Biophys. Acta 412*, 262–272.

Otvos, J. D., & Armitage, I. M. (1980a) *Biochemistry 19*, 4021–4030.

Otvos, J. D., & Armitage, I. M. (1980b) *Biochemistry 19*, 4031–4043.

Pimental, G. C., & McClellan, A. L. (1960) *The Hydrogen Bond*, p 234, Freeman, San Francisco, CA.

Plocke, D. J., Levinthal, C., & Vallee, B. L. (1962) *Biochemistry 1*, 373–378.

Reynolds, J. A., & Schlesinger, M. J. (1967) *Biochemistry 6*, 3552–3559.

Scheraga, H. A. (1961) *Protein Structure*, p 85, Academic Press, New York.

Schlesinger, M. J. (1967) *J. Biol. Chem. 242*, 1604–1611.

Schlesinger, M. J., & Barrett, K. (1965) *J. Biol. Chem. 240*, 4284–4292.

Simpson, R. T., & Vallee, B. L. (1968) *Biochemistry 7*, 4343–4349.

Simpson, R. T., & Vallee, B. L. (1970) *Biochemistry 9*, 953–958.

Simpson, R. T., Vallee, B. L., & Tait, G. H. (1968) *Biochemistry 7*, 4336–4342.

Singer, S. J. (1962) *Adv. Protein Chem. 17*, 1–69.

Smith, I. (1968) *Chromatographic and Electrophoretic Techniques*, Vol. 2, p 383, Interscience, New York.

Tanford, C. (1967) *Physical Chemistry of Macromolecules*, pp 275–315, Wiley, New York.

Thiers, R. E. (1957) *Methods Biochem. Anal. 5*, 273–335.

Wood, D. C., Jurgensen, S. R., Geesin, J. C., & Harrison, J. H. (1981) *J. Biol. Chem. 256*, 2377–2382.

Yphantis, D. E. (1964) *Biochemistry 3*, 297–317.