Conformational States of the Subunit of *Escherichia coli* Alkaline Phosphatase*

Jacqueline A. Reynolds and Milton J. Schlesinger

ABSTRACT: The refolding and reassociation of subunits of alkaline phosphatase from *Escherichia coli* have been studied as a function of extent of protonation. The subunit exists as an extended coil in aqueous solution at pH 2, as a solvent-permeable, globular species at pH 4, and as a refolded, compact macromolecule at pH 6–8. This last conformational state has the same α-helix content as the native dimer and appears to differ from the active enzyme only in the exposure of four additional tyrosine residues per monomer to the aqueous solvent. Dimerization of the refolded subunit returns these tyrosine residues to a hydrophobic region suggesting either a conformational change accompanying the protein–protein interaction or the tyrosines as a point of contact between two monomer units. Zn^{2+} binding, which is essential for dimerization and enzymatic activity, is unnecessary for refolding of the subunit.

Investigations of interactions between protein subunits in the formation of quarternary structure have been severely limited because of the difficulty in observing the formation of secondary and tertiary structure within the subunits as an independent process (for recent reviews, see Reithel, 1963; Scheraga, 1961; for a specific, intensive investigation, see Stellwagen and Schachman, 1962).

Alkaline phosphatase from *Escherichia coli* is a system which lends itself to the study of the relationships between secondary, tertiary, and quaternary structure. It is a globular dimer containing two identical subunits (Rothman and Byrne, 1963), and recent studies have shown that this protein can be reversibly dissociated into subunits by extensive protonation (Schlesinger and Barrett, 1965; Schlesinger, 1965).

In the present investigation several distinct conformational states of the subunit have been characterized as a function of extent of protonation during the refolding and reassociation process. Ultraviolet difference spectra and optical rotatory dispersion studies have been used to characterize the short-range properties of the protein while hydrodynamic measurements are used to define the size and shape. The results show that refolding takes place in the subunit prior to reassociation and together with kinetic studies of reactivation of the acid-treated subunit suggest the protein states and pathway from highly protonated monomer to active dimer given in reaction 1.

The active dimer is a zinc–metalloprotein and is shown in the present investigation to contain three Zn^{2+} per dimer. The metal is not required to form the refolded monomer but is essential for the dimerization process. Note that in the proposed pathway between various conformational states steps 2 and 4 are not reversible under the experimental conditions employed and that no postulate is made regarding the number of Zn^{2+} or role of this ion in the mechanism of reassociation.

Experimental Section

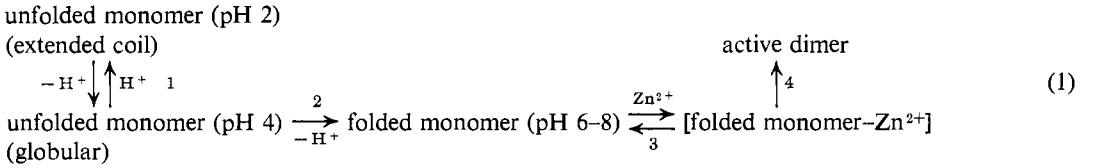
Materials. Growth conditions of *E. coli*, strain CW-3747, and purification of the alkaline phosphatase produced by the bacterial cells have been described previously (Schlesinger and Barrett, 1965). The procedure was identical with that in the reference cited with the following exceptions. (a) $ZnSO_4$ (5×10^{-6} M) was added to the growth media in order to ensure formation of predominantly one isozyme (Schlesinger and Andersen, 1967). (b) After lysozyme treatment the dialyzed supernatant solution was not subjected to a 67° treatment but was pumped directly onto the DEAE-cellulose column. (c) The lyophilized, purified protein was redissolved in deionized water and dialyzed extensively against glass-distilled water at 4°. (d) Purity and homogeneity were established by acrylamide gel and starch gel electrophoresis in 0.04 M Tris-Cl buffer at pH 8.0. The protein was judged >94% pure by these criteria.

Guanidine hydrochloride was recrystallized three times from methanol and a 6 M solution had a resultant optical density <0.1 above 2300 A.

Methods. The enzyme assay procedure has been previously described (Schlesinger and Barrett, 1965).

Difference spectra were obtained using a Cary 14 double-beam spectrophotometer with a thermostatted cell compartment. In all experiments four matched cells of identical path length were used, and reagents added to the protein solution in the sample beam were compensated in the reference beam. The native enzyme

* From the Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri. *Received July 18, 1967.* Supported by Research Grant GB 5973 from the National Science Foundation to one of us (M. J. S.).



(1)

is always present in the reference beam in a concentration identical with the reagent-treated protein in the sample beam.

Spectrophotometric titrations were carried out with the Cary 14 and a Radiometer pH meter. $\Delta\epsilon_{2950}$ was measured by means of difference spectra as a function of pH and the value $\Delta\epsilon^{1\,cm}_{2950}$ (1 tyrosine) of 2450 was used to calculate the number of tyrosine residues titrated. The value $\Delta\epsilon$ 2450 was obtained by dividing the total $\Delta\epsilon^{1\,cm}_{2950}$ for unfolded protein at pH 12.5 by the number of tyrosines present by amino acid analysis.

Optical rotatory dispersion measurements were made on a Cary 60 spectropolarimeter using a 1-cm path-length thermostatted cell. Protein concentrations were adjusted over the accessible wavelength range to keep the dynode voltage below 0.6 kv. The slit was programmed to give a constant intensity over the wavelength studied.

Viscosity measurements were carried out in Cannon microviscometers in a constant-temperature bath controlled to $\pm 0.005°$. Flow times in all experiments exceeded 300 sec and were reproducible to ± 0.2 sec.

Protein concentrations were determined from the optical density at 2800 using $\epsilon^{1\,cm}_{0.1\%}$ 0.77 (Rothman and Byrne, 1963).

Zn^{2+} assays were performed on a Perkin-Elmer Model 303 atomic absorption spectrophotometer.

Results

Protein Conformational States. The following protein conformations were observed as a result of alterations in the number of protons bound to the native macromolecule. The experimental verification of these states is described in the following sections.

(1) Titration of the native dimer to pH 2 at 0° and $0.2 \geq \mu \geq 0$ results in the formation of a subunit in a highly extended configuration which is referred to herein as an extended coil. (2) Decreasing the number of protonated residues by titration back to pH 4 from state 1 produces a single polypeptide chain species which occupies a reduced hydrodynamic volume and is referred to as unfolded subunit, globular. (3) Further titration to pH 6–8 and elevation of the solution temperature to $\geq 20°$ for approximately 1 min produces a monomer unit containing secondary and tertiary structure which is referred to as a refolded subunit. To prevent reassociation of this species to the native, enzymatically active dimer the temperature of the protein solution was lowered to less than 15° immediately after refolding was observed. (4) Incubation of the refolded subunit at 20° or above leads to reassociation to a dimeric species which is experimentally indistinguishable from the native protein. In all experiments described herein unless otherwise noted the Zn^{2+} concentration was that initially present on the native enzyme, *i.e.*, three times the molar concentration of the active dimer.

Viscosity. Previous publication from this laboratory (Schlesinger, 1965) presented sedimentation data for alkaline phosphatase at a single protein concentration as a function of extent of protonation. In the current work the intrinsic viscosity has been measured at various solution hydrogen ion concentrations. These values provide a quantitative definition of the macroscopic state of the protein and are presented in Table I together with the previously published sedimentation data and molecular weights.

It is obvious that increasing H^+ to 10^{-2} M results in dissociation of the enzyme into two inactive subunits and the expansion of the monomer as evidenced by the molecular weight, the high value of $[\eta]$, and the small value of $s_{20,w}$. As the H^+ concentration is lowered to 10^{-4} M the monomer assumes the hydrodynamic shape of a spherical protein ($[\eta] = 3.4$ cc/g). It will be shown, however, that this protein structure is still permeable to solvent while the native form with an identical $[\eta]$ is not. Furthermore, the intrinsic viscosity of the species at $H^+ = 10^{-4}$ M increases with temperature, a phenomenon typical of random coil polymers in poor solvents (Fox and Flory, 1949).

Tanford *et al.* (1967) have published a study of the intrinsic viscosity of a number of proteins in 6 M guanidine hydrochloride and 0.1 M mercaptoethanol. These authors have shown approximate random coil behavior in this solvent system through the linear relationship $\log [\eta] = \log 0.716 + 0.66 \log n$, where n is the number of amino acid residues per chain. The viscosity of alkaline phosphatase in aqueous solution at pH 2 may be compared to the Tanford data by extrapolation of $[\eta]$ to infinite ionic strength in order to compensate for the contributions to the viscosity from electroviscous effects. Plotting $[\eta]$ *vs.* $(2/(\mu)^{1/2})$ gives a linear relationship and $[\eta]_{\mu \to \infty} = 23.5$ g/cc. Log $[\eta] = 1.372$ and log $n = 2.58$ (from amino acid analysis, Rothman and Byrne, 1963). These values do not agree exactly with the Tanford data which predict a value of $\log [\eta] = 1.56$ in guanidine hydrochloride for this protein. However, the solvent–segment interactions in aqueous solution may be expected to differ somewhat from those in guanidine hydrochloride and indeed a different straight-line function between log $[\eta]$ and log n may exist in aqueous solvents. The extremely high value of $[\eta]$ is an indication that the subunit at pH 2 exists in a highly extended state despite the fact that the two disulfide bonds (Schlesinger, 1965) are still intact.

The data in Table I for alkaline phosphatase in 6 M guanidine hydrochloride give log $[\eta] = 1.52$, a value very close to that predicted by the Tanford data even

TABLE I: Hydrodynamic Properties of Alkaline Phosphatase (T = 6°).

State of Protein	pH	μ	[η] (cc/g)	Mol Wt[a]	$s_{20,w}$ (S)[a]	Enzymatic Act. (units/mg)
Native	7.4	0.1 (Tris-Cl)		86,000	6.1	1,100
	5.6–6.5	0.1, 0.01 (NaCl)	3.4			1,650
Unfolded monomer (extended coil)	2.0	0.2 (NaCl)	29.0			0
	2.0	0.1 (KCl and NaCl)	31.7	43,000	2.3	5
	2.0	0.01 (NaCl)	41.5			0
Unfolded monomer (globular)	4.0	0.01, 0.1 (NaCl)	3.4 7.4[b]			5
	4.2	0.1 (NaAc)		43,000	3.4	5
Native in 6 M Guanidine hydrochloride	6.0		33.0[c]			0

[a] Schlesinger (1965). [b] T = 20°. [c] T = 25°.

though the alkaline phosphatase monomers contain two disulfide bridges. It is therefore evident that these disulfide bonds in alkaline phosphatase restrict only a small portion of the subunit in this solvent.

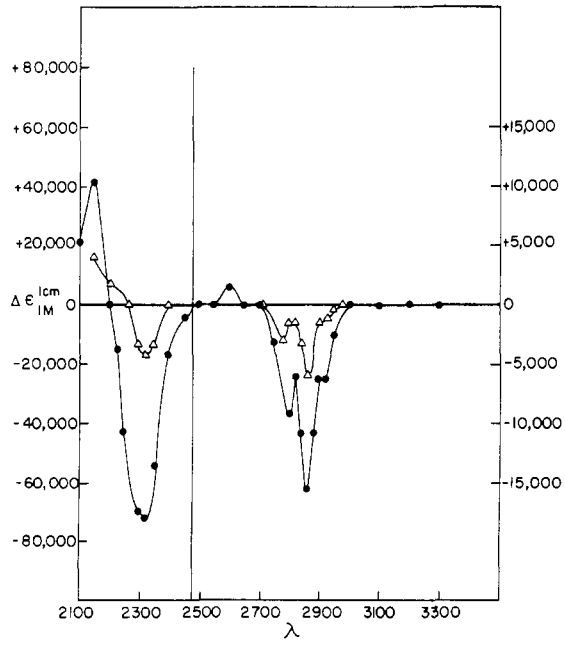


FIGURE 1: Difference spectra of unfolded and refolded subunits *vs.* native dimer; μ = 0.1 NaCl, temperature = 15°, protein concentrations = 0.1–2.0 g/l. Note the change in scale for $\Delta\epsilon$ at λ 2450. (●) pH 2 and 4 and (△) pH 6–8.

Difference Spectra. At pH 2 native alkaline phosphatase is dissociated into subunits with hydrodynamic properties closely approximating extended coils. The difference spectra in Figure 1 of these unfolded subunits *vs.* native protein show a tyrosine blue shift (negative peaks at 2860 and 2800 A) and a second blue shift at 2330 A which is associated with both peptide and aromatic absorption. It is interesting that the six tryptophans present by amino acid analysis (Rothman and Byrne, 1963) do not appear to contribute measurably to the long-wavelength difference spectrum. This may indicate that these residues are on the outside surface of the native enzyme. The observed blue shift in Figure 1 is characteristic of the removal of tyrosines and peptide bonds from a region of higher to one of lower refractive index.

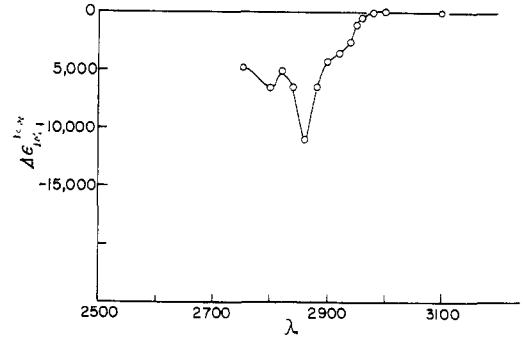


FIGURE 2: Difference spectra of subunits in 6 M guanidine hydrochloride *vs.* native dimer. Temperature = 15°, protein concentrations = 0.1–2.0 g/l.

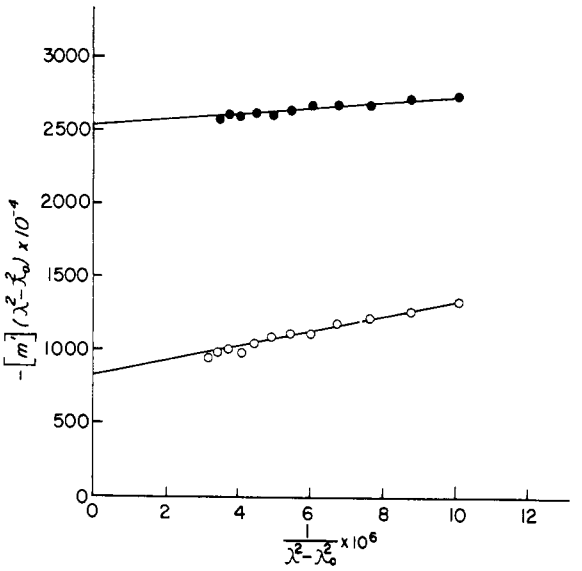


FIGURE 3: Moffitt–Yang plot of optical rotatory dispersion, native dimer, and unfolded subunit. Temperature = 15°, μ = 0.01 and 0.1 NaCl, protein concentrations = 2–5 g/l. (●) pH 2 and 4 and (⊙) native dimer.

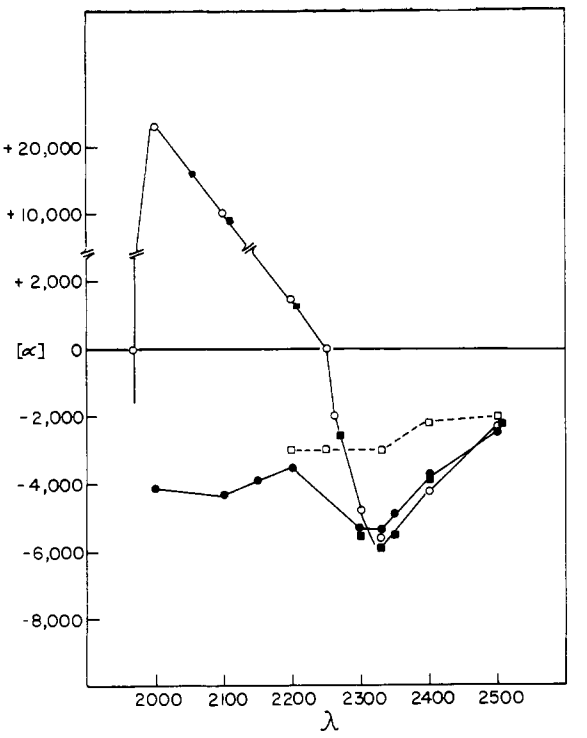


FIGURE 4: Optical rotatory dispersion spectra of native dimer, and unfolded and refolded subunits. Protein concentrations = 2–4 × 10^{-2} g/l. (⊙) Native dimer, μ = 0. (■) Native dimer, μ = 0.1; refolded subunit in presence or absence of EDTA, pH 6–8; EDTA-inactivated native dimer. (●) Unfolded subunit at pH 2 and 4, μ = 0.1. (⊡) Unfolded subunit in 6 M guanidine hydrochloride, pH 6.

A decrease in H^+ from 10^{-2} to 10^{-4} M results in no apparent refolding of the subunit by the criterion of difference spectra despite the fact that $[\eta]$ decreases to 3.4 g/cc, a value characteristic of the native globular protein. When the H^+ is further decreased to 10^{-6}–10^{-8} M under conditions where $\mu \leq 0.1$ and the protein concentration is $\leq$0.3 mg/ml, a large reduction in the difference spectrum amounting to 60% at 2860 A and 75% at 2320 A is observed (Figure 1) with no concomitant return of enzymatic activity. The protein in this state is referred to as a refolded monomer. The refolding observed in Figure 1 takes place within one minute at temperatures $\geq 20°$.

Figure 2 shows the difference spectrum which results when native enzyme is placed in 6 M guanidine hydrochloride. The results are qualitatively similar to dissociation and unfolding as the result of protonation although the absolute magnitude of the effect is somewhat smaller in guanidine. Again, the predominant contribution to the blue shift in the long-wavelength region appears to be made by tyrosine residues (see, for example, Wetlaufer, 1962).

Optical rotatory dispersion curves for the native enzyme and the acidified subunit were analyzed between 3600 and 6000 A using the Moffitt–Yang relationship

$$[m'](\lambda^2 - \lambda_0^2) = a_0\lambda_0^2 + \frac{b_0\lambda_0^4}{\lambda^2 - \lambda_0^2} \qquad (1)$$

where $[m']$ = effective residue rotation, the residue molecular weight = 123, λ_0 = constant (arbitrarily taken here as 2120 A), a_0 = constant dependent on solvent, and b_0 = constant empirically related to α-helix content. A plot of the left-hand side of eq 1 *vs.* $1/(\lambda^2 - \lambda_0^2)$ is shown in Figure 3. Calculation of b_0 from these curves gives −225 for native enzyme and −89 for the subunit. Based on values of b_0 = −630 for 100% α helix and b_0 = 0 for random coil, an estimated helix content for the native protein is 40% and for the unfolded monomer at pH 2 is 14%. Optical rotatory dispersion data for a solution of the unfolded protein in which the pH has been raised to 4 are superimposable on the curve for unfolded monomer at pH 2 in Figure 3 implying that no alteration in helix content has occurred as the result of the change in proton binding.

Refolded monomer could not be examined in the visible region since high concentrations of protein are required for accurate determination of the rotation in this region. At concentrations >0.3 mg/ml rapid regeneration of activity (reassociation to dimer) takes place.

Unfolded subunits can be regenerated to the dimer with >90% of the original enzymatic activity by incubating monomers in a low ionic strength solution ($\mu \leq 0.1$) at 37° and pH 6–8. The optical rotatory dispersion of this solution throughout the wavelength

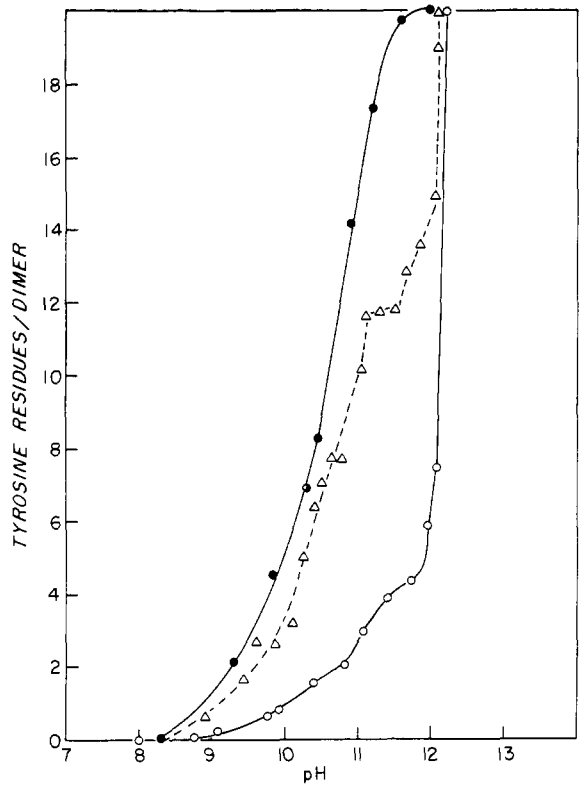


FIGURE 5: Spectrophotometric titration of tyrosine residues. Each conformational species is thermodynamically distinct. λ 2950, μ = 0.1 KCl, temperature = 12°. (○) Native dimer, protein concentrations = 0.2–2 g/l. (●) Unfolded monomer, protein concentrations = 0.2–2 g/l. (△) Refolded monomer, protein concentration = 0.2 g/l.

range 3600–6000 A is closely similar to that of the native enzyme (Figure 3).

Figure 4 shows the optical rotatory dispersion spectra from 2000 to 2600 A of native dimer, unfolded subunits at pH 2 and 4, and refolded subunits at pH 6–8. The significant features are the following: (1) the extremely small decrease (~10%) in the Cotton effect trough at 2330 A when the native enzyme is dissociated and unfolded at pH 2; (2) the loss of the 2000-A Cotton effect peak on unfolding and the accompanying formation of a new minimum at 2100 A; and (3) the large reduction in the 2330-A trough in the presence of 6 M guanidine hydrochloride.

An estimate of helix content from the magnitude of the levorotation at 2330 A by the method of Simmons *et al.* (1961) can be made through the use of the following equation

$$f_h = \frac{m'_{2330}\,(\text{native}) - m'_{2330}\,(\text{unfolded})}{-11{,}000} \qquad (2)$$

assuming m'_{2330} (unfolded protein) = 2000. For the native enzyme f_h = 36% and for the unfolded subunit

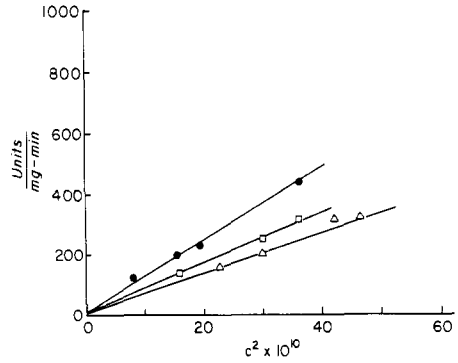


FIGURE 6: Initial rates of reactivation of refolded subunit. Each point is obtained by extrapolating to zero time the change in enzymatic activities measured at frequent intervals during the first 5 min. Under the conditions of assay (protein concentration <1.0 μg) no reactivation occurs in the assay solution itself. Temperature = 37°, μ = 0.05 KCl, pH 7.5. (●) 10^{-5} M excess Zn^{2+}. (□) 5×10^{-6} M excess Zn^{2+}. (△) 2.5×10^{-6} M excess Zn^{2+}.

at pH 2 and 4, f_h = 31%. The latter value does not agree well with the per cent helix approximated from b_0 calculations. It is evident that a significant contribution is made to the magnitude of the 2330-A trough by some structure other than α helix.

Optical rotatory dispersion of a solution containing refolded monomer is superimposable on the curve for native enzyme in this short-wavelength region. Thus, all secondary structure is regained in the refolding process and no major contribution is made to the optical rotation by the interactions associated with dimerization.

Spectrophotometric titrations of tyrosine residues in the native alkaline phosphatase, unfolded subunit, and refolded subunit are shown in Figure 5. Amino acid analysis of the protein gives 19 tyrosine residues/dimer (Rothman and Byrne, 1963). However, if the change in absorbance at 2950 A/tyrosine titrated is assumed to be 2450, a value very close to that reported for other proteins (Tanford, 1962), spectrophotometric titration indicates a total of 20 tyrosines/dimer. The estimated error in the ordinate of Figure 5 is ±0.5 tyrosine/dimer.

In the native protein five tyrosines per dimer are available to the solvent at 12°. The other 15 residues are titrated above pH 12 where alkaline phosphatase becomes rapidly inactive. While alkaline inactivation has not been thoroughly investigated, it would appear that an extensive conformation change takes place in this region.

The unfolded subunit has all tyrosines available in the normal titration range. Refolding was prevented during the course of the titration by carrying out the experiment at 12°.

Analysis of the titration curve of the refolded monomer shows 12 tyrosines/dimer available to the solvent.

TABLE II: Zinc:Protein Ratios.

Protein (moles/l.)	Zn^{2+} (moles/l.)	Zn:Protein	Act. (units/mg)	Dialysis Treatment
6.6×10^{-6}	1.97×10^{-5}	2.99	2050	H_2O
7.55×10^{-6}	2.31×10^{-5}	3.06	2050	H_2O
5.42×10^{-6}	1.54×10^{-8}	0.00284	2.7	10^{-2} M EDTA

The optical rotatory dispersion of this material was measured in the 2000–2600-A range to determine that refolding by this criterion had actually occurred, and the difference spectrum from 2500 to 3200 A at pH 8 was identical with that shown in Figure 1 for refolded monomer.

A check on the number of accessible tyrosine residues is provided by the difference spectrum at 2860 A. In the unfolded subunit $\Delta\epsilon_{1\ M}^{1\ cm}$ is 15,000 ± 1000 corresponding to the 15 residues that are masked in the native enzyme. Each tyrosine thus contributes $\Delta\epsilon_{1\ M}^{1\ cm}$ 1000 to the difference spectrum. In the refolded subunit $\Delta\epsilon_{1\ M}^{1\ cm}$ is 6000 ± 1000 suggesting that 6 ± 1 residues per dimer remain in the aqueous environment. Since 5 ± 0.5 tyrosines are already exposed in the native protein, a total of 11 ± 1 residues may be expected to titrate in the normal pH range in the refolded subunit. This corresponds to the 12 residues observed in the spectrophotometric titration of the refolded subunit.

Of major interest is the fact that reassociation of the two refolded subunits leads to a further conformational change involving removal of between six and eight tyrosines per dimer from the aqueous environment. Since optical rotatory dispersion data show the helix is already formed in the refolded species, these tyrosines must be a part of the tertiary or quaternary structure. Thus, reassociation may involve not only the collision of two subunits, but a structural rearrangement as well.

The Refolded State as a Subunit. The present study has demonstrated the existence of a refolded protein species when highly extended monomer coils in aqueous solution at pH 2 are neutralized to pH 6–8. It was necessary to determine whether this state was an inactive dimer which was reactivated by a further conformational change or a subunit which could be dimerized to the active state. Direct molecular weight measurements are difficult in this case due to the rapidity with which the refolded macromolecule regains enzymatic activity at high protein concentrations. Therefore, the following indirect method was used to determine the state of aggregation of the refolded protein species. Acidified protein solutions at pH 2, μ 0.05, and concentrations from 1 to 6 $\times$ 10^{-5} g/cc were neutralized to pH 7.5 and excess Zn^{2+} was added. These solutions were warmed to room temperature to allow refolding (as evidenced by optical rotatory dispersion and spectral properties) and immediately placed in a water bath at 37°. Initial rates of reactivation were measured by sampling frequently in the first 5 min and assaying for enzymatic activity by the standard procedures. Figure 6 demonstrates that the initial rates are second order in protein concentration and increase with the amount of Zn^{2+} present. These results indicate that the reacting species, in this case the refolded protein, is a subunit undergoing dimerization during the reactivation process and further suggest that a rapid equilibrium between the subunit and Zn^{2+} precedes the dimerization step.

Additional support for the view that the refolded species is a subunit and not an inactive dimer is provided by the fact that in zone sedimentation in a sucrose gradient the refolded protein (as determined by reassociation followed by assay for enzymatic activity) sediments more slowly than the active dimer.

The Role of Zn^{2+} in the Refolding and Reassociation of Alkaline Phosphate. E. coli alkaline phosphatase is a zinc metalloprotein in which the metal is essential for both enzymatic activity and reassociation of subunits. Preparations of the purified native protein used in the present studies were analyzed by means of an atomic absorption spectrophotometer for zinc content and a value of three Zn^{2+} per dimer obtained (see Table II). This value is higher than one previously reported for this enzyme (Plocke *et al.*, 1962) and other samples of enzyme prepared in this laboratory have been found to have a Zn:protein ratio close to 2.0. These latter preparations have lower enzymatic activities and preliminary studies indicate that the relationship between zinc content and activity is complex.

A sample of this preparation was dialyzed against EDTA to the point of 0% activity. Zn^{2+} analysis showed no metal bound to the protein under these conditions. In addition, it has been shown (Schlesinger, 1965) that the above treatment does not lead to subunit dissociation. The ultraviolet difference spectrum between 2500 and 3200 A of the EDTA-inactivated dimer *vs.* native dimer was zero indicating no conformational change involving tyrosine or tryptophan residues upon the removal of metal from the enzymatically active protein. Small, nonreproducible changes occurred at short wavelengths (λ <2350) which are attributed to small protein concentration changes that result from the addition of reagents to the solutions in the absorption cells. Furthermore, the optical rotatory dispersion spectrum of the EDTA-inactivated alkaline phosphatase is identical with that of native dimer (Figure 4) indicating that no helix disruption occurs when Zn^{2+} is removed.

The addition of EDTA to the unfolded subunit at

TABLE III: Summary of Physical Parameters of Alkaline Phosphatase Conformational States.

State of Protein	Act. (units/mg)	[η] (cc/g)	b_0	% Helix	$[\alpha]_{2330}$	$[\alpha]_{2100}$	No. of Tyrosines Exposed	
							Titration Data	$\Delta\epsilon_{2870}$
Native	1,650	3.4	−255	40.5	−6,000	+10,000	5 ± 1	5 ± 1
pH 2	(0)	μ = 0.01, 41.5	−89	14.1	−5,400	−5,400	20 ± 1	20 ± 1
		μ = 0.1, 32.0						
		μ = ∞, 23.5						
pH 4	(0)	3.4	−89	14.1	−5,400	−5,400	20 ± 1	20 ± 1
pH 7.5	(18)	3.4			−6,000	+10,000	12 ± 1	11 ± 1
(refolded)								
6 M guanidine hydrochloride	(0)	33.0			−3,000		20 ± 1	20 ± 1

pH 2 results in no alteration in the difference spectrum shown in Figure 1 (unfolded subunit *vs.* native dimer). Refolding in the presence of this reagent gives the same difference spectrum between 2500 and 3200 A as that shown in Figure 1 (refolded subunit *vs.* native dimer). In addition, regeneration of the helical content of the protein occurs even in the presence of EDTA as evidence by the identity of the optical rotatory dispersion spectra shown in Figure 4.

Further investigation of the role of Zn^{2+} in enzymatic activity and conformational states is underway. From these studies, however, it is evident that refolding of the monomer but not reassociation can occur in the absence of Zn^{2+}, and that no apparent disruption of secondary or tertiary structure takes place when the metal is removed from the native enzyme. While Zn^{2+} is necessary for reassociation of the subunits and the recovery of enzymatic activity, its exact role is not obvious from the present investigation.

Discussion and Summary

Table III summarizes the physical parameters applicable to each protein conformational state occurring during the process of reactivation. High degrees of protonation (pH 2) of native alkaline phosphatase lead to dissociation of the protein into two disorganized subunits. The hydrodynamic properties of this macromolecular state are a close approximation to the random coil. All tyrosines are exposed to the solvent and b_0 values from optical rotatory dispersion give an α-helix content of 14%. When the pH is raised to 4 the unfolded subunit assumes a smaller hydrodynamic volume but there are no alterations in optical properties. These two results are not inconsistent since a common phenomenon in synthetic polymer chemistry is the lowered viscosity shown by random coil macromolecules when introduced into a poor solvent (*e.g.*, polyisobutylene; Fox and Flory, 1949). As some of the positive charge on the protein is neutralized by increasing the pH, the aqueous solvent becomes a less favorable environment and the protein assumes a smaller hydrodynamic volume to minimize its solvent–segment interaction. The net charge on the molecule is apparently still too large to allow assumption of the native secondary and tertiary structure. This state is defined as a solvent-permeable coil in a restricted volume element. When the solution containing the biopolymer at pH 4 is titrated to neutrality and the temperature raised to 20°, the α-helix structure is regained and approximately four tyrosine residues per monomer are placed in a hydrophobic environment. This step proceeds in the presence or absence of Zn^{2+}. The reassociation step and restoration of enzymatic activity occurs with bimolecular kinetics and only in the presence of Zn^{2+}. This reactivation involves no alteration in optical rotatory properties of the refolded species but does remove approximately four additional tyrosines per monomer from the aqueous environment. The extent of reorganization of tertiary structure in this final step is currently being examined by comparison of hydrogen

ion titration curves and will be reported in a subsequent paper.

References

Fox, T. G., and Flory, P. J. (1949), *J. Phys. Colloid Chem. 53*, 197.
Plocke, D. J., Levinthal, C., and Vallee, B. L. (1962), *Biochemistry 1*, 373.
Reithel, F. J. (1963), *Advan. Protein Chem. 18*, 123.
Rothman, F., and Byrne, R. (1963), *J. Mol. Biol. 6*, 330.
Scheraga, H. (1961), Protein Structure, New York, N. Y., Academic.
Schlesinger, M. J. (1965), *J. Biol. Chem. 240*, 4293.
Schlesinger, M. J., and Andersen, L. (1967), *Proc. N. Y. Acad. Sci.* (in press).
Schlesinger, M. J., and Barrett, K. (1965), *J. Biol. Chem. 240*, 4284.
Simmons, N. S., Cohen, C., Szent-Gyorgyi, A. G., Wetlaufer, D. B., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 4766.
Stellwagen, E., and Schachman, H. K. (1962), *Biochemistry 1*, 1056.
Tanford, C. (1962), *Advan. Protein Chem. 17*, 69.
Tanford, C., Kawahara, D., and Lapanje, S. (1967). *J. Am. Chem. Soc. 89*, 729.
Wetlaufer, D. (1962), *Advan. Protein Chem. 14*, 303.

The Synthesis of Triaminoacyl-insulins and the Use of the *t*-Butyloxycarbonyl Group for the Reversible Blocking of the Amino Groups of Insulin*

Daniel Levy† and Frederick H. Carpenter

ABSTRACT: *t*-Butyloxycarbonyl azide reacts with insulin in dimethylformamide to give a derivative in which all three amino groups of insulin are covered and which has a diminished biological activity. Upon treatment of the *t*-butyloxycarbonyl-insulin with anhydrous trifluoroacetic acid, the *t*-butyloxycarbonyl groups are removed to yield a crystalline insulin with high biological activity. Reaction of the *p*-nitrophenyl esters of *t*-butyloxycarbonylamino acids with insulin results in the introduction of three amino acid residues; one on each of the amino terminals of the A and B chain and one on the ε-amino group of lysine 29 of the B chain. Upon treatment of the derivative with anhydrous trifluoroacetic acid, the *t*-butyloxycarbonyl groups are removed to yield triaminoacyl-insulins. Derivatives prepared in this manner included trialanyl-, triasparaginyl-, trilysyl-, trimethionyl-, and by a slight modification, triglutamyl-insulins.

The products were characterized by amino acid composition, DEAE Sephadex chromatography, chemical and enzymatic degradations, and biological activity. Despite the introduction of groups of different size, charge, and hydrophylic–hydrophobic properties, the resulting triaminoacyl-insulins all possessed about the same biological activity amounting to 40–50% of that of bovine insulin in the mouse convulsion assay with slightly higher values in the immunochemical assays.

A continuing study in this laboratory is concerned with the relationship of structure to biological activity of insulin. A recent review (Carpenter, 1966) summarized a number of findings that were obtained by subjecting insulin to various *enzymatic* degradations The present report is concerned with *chemical* modifications involving the free amino groups of insulin.

Reactions at the free amino groups of insulin have been the subject of several previous investigations. Fraenkel-Conrat and Fraenkel-Conrat (1950) covered the amino groups by acetylation with acetic anhydride to yield products which showed little loss of biological activity. On the other hand, Mills (1953) reacted the amino groups with 2,4,5-trinitrotoluene to give products with no biological activity. Since the acetyl and dinitrotolyl residues cannot be removed under mild conditions, they can be considered as irreversible blocking groups. Although several reagents have been proposed for use in the reversible blocking of the amino groups

* From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received July 28, 1967.* This study was supported in part by a grant (AM 00608) of the National Institutes of Health and a grant (GB-5931) of the National Science Foundation. A preliminary account of this work has been presented at the Pacific Slope Biochemical Conference, Davis, Calif., June 15–17, 1967.

† Postdoctoral fellow of the National Institutes of Health, 1965–1967.