Hydrogen Ion Equilibria of Conformational States of *Escherichia coli* Alkaline Phosphatase*

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ABSTRACT: Hydrogen ion equilibria of the enzymatically active dimer and the unfolded subunit of *Escherichia coli* alkaline phosphatase have been studied. The unfolded subunit has all ionizable groups exposed to the solvent and is a highly extended macromolecule at low pH. The native enzyme contains an average of 3 zinc ions which are bound to histidine residues; 16 of the 20 tyrosine residues/dimer are masked, but all other titratable groups are available for protonation at normal pK's.

Thus, the sites of interaction between two subunits apparently do not involve charged side chains. Above pH 9.0 the native dimer undergoes a small conformation change evidenced by an increase in viscosity and a decrease in the electrostatic interaction factor. This conformation change is not accompanied by ultraviolet spectral perturbations or an alteration of enzymatic activity.

A study of the formation of secondary, tertiary, and quaternary structure in the protein alkaline phosphatase has been reported recently (Reynolds and Schlesinger, 1967). Unfolded subunits of the protein were obtained by acidification of an aqueous solution of the native dimer. From their hydrodynamic properties these unfolded subunits were inferred to be random coil polypeptide chains. However, optical rotatory dispersion data for the monomer at pH 2 gave $b_0 = -89$, a value significantly different from that anticipated for a polypeptide chain in random coil conformation ($b_0 = 0$ for 100% coil; Schellman and Schellman, 1962). This species also has a specific rotation at 2330 Å equal to -5400 which is unexpectedly large for a structureless polypeptide (Schellman and Schellman, 1962). The magnitude of this residual specific rotation may be related to the fact that individual amino acid residues contribute significantly to optical rotation even when there is no secondary or tertiary structure present (Tanford, 1967).

Neutralization of a solution of these acidified monomer units leads first to refolding of the individual chains followed by dimerization to an enzymatically active protein. The reactivated enzyme is identical with the native enzyme which is a globular protein of molecular weight 86,000 containing an average of three zinc ions per dimer. The refolded subunit was found by difference spectroscopy to contain four masked tyrosine residues per monomer unit while the active dimer has eight masked tyrosines per monomer.

The following questions arise regarding the conformational states described above. (1) Is the conformational state of the unfolded subunit at pH 2 truly a random coil, or is there some persistent ordered structure such as might be suggested by the optical rotatory dispersion data? (2) What structural differences, if any, in addition to the number of masked tyrosines exist between the refolded monomer at neutral pH and the reassociated active dimer? (3) What specific residues are involved in the interaction between subunits when the native dimer is formed? (4) What specific residues are involved in metal–protein binding? To answer some of these questions studies of hydrogen ion equilibria of alkaline phosphatase have been carried out. In the native dimer all ionizable groups are accessible to protons with normal pK's with the exception of 16 tyrosines/dimer and 3 imidazoles/dimer. The latter are shown to be involved in zinc binding. Thus, the specific site interactions between two subunits do not appear to involve charged side chains. No masked ionizable groups are observed when the unfolded subunit is titrated under conditions where refolding and reassociation cannot take place, and the electrostatic interaction factor between protons and the charged subunit is zero at positive protein charges greater than twenty. These findings provide additional support for the view that the acidified monomer contains little or no residual tertiary or secondary structure despite the fact that two disulfide bonds per monomer are still intact (Schlesinger, 1965a,b).

Experimental Section

(1) Methods of protein preparation and assay for enzymatic activity have been described previously (Reynolds and Schlesinger, 1967). (2) Hydrogen ion titration curves were obtained by continuous titration with an International Instrument Company Di-Functional recording titrator and Instrument Laboratory combination electrode 14043. A jacketed titration cell was thermostated by circulating liquid from a constant temperature bath held to $\pm 0.1°$. All titrations were carried out at $\mu = 0.1$ M KCl, protein concentrations 0.4–0.9% by weight, and in the absence of CO_2 (flushed continuously

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TABLE I: Titration Data of Native Alkaline Phosphatase.

	n	pK_i	w	Amino Acid Anal. This work[a]	Amino Acid Anal. Rothman and Byrne[b]	Small Molecule pK's[c] 25°
Side-chain COO^-	86	4.075	0.037	174[d]	174[d]	4.6
α-COO^-						3.8
Histidine	13	7.10	0.037	16	16	6.3
Tyrosine	4	10.5	0.001	20	20	9.6
Lysine	50	10.7	0.001	53	50	10.4
α-Amino	0			0[e]		
$Zn(H_2O)_2^{2+}$	3	8.0[f]	0.037	3		
	3	>12.0				
Arginine	19[g]	>12		20	24	>12.0

[a] Amino acid analysis from author's laboratory. [b] Rothman and Byrne (1963). [c] Tanford (1962). [d] Acid and amide. [e] M. J. Schlesinger, unpublished work. [f] Tanford and Epstein (1954). [g] See text for explanation.

with nitrogen). Standard HCl and KOH (protected against CO_2) were used.

Protein solutions were dialyzed against glass-distilled water and blank titration curves determined. In one experiment a solution of enzyme was dialyzed against EDTA to zero activity and both the protein solutions and the dialysate were titrated at 6°. Analysis on a Perkin-Elmer atomic absorption spectrophotometer showed no Zn^{2+} in this protein sample. The average Zn^{2+} content in the preparation of native alkaline phosphatase which was used in these experiments was also determined by atomic absorption spectrophotometry and found to be three per dimer. Other preparations from the laboratory have been found to contain from two to six Zn^{2+} per dimer. (3) The criteria for the presence of secondary and tertiary structure in both subunits and dimer were those previously employed (Reynolds and Schlesinger, 1967), *i.e.*, the identity of the far-ultraviolet optical rotatory dispersion spectra with that of active dimer and ultraviolet difference spectra in the aromatic transition region.

Results

The circles in Figure 1 show the experimental data obtained for the titration at 6° of the native enzyme, alkaline phosphatase. The titration curve is reversible between pH 4.0 and 12.0, but outside this range, optical rotatory dispersion and difference spectra show loss of secondary and tertiary structure (Reynolds and Schlesinger, 1967). In addition, hysteresis is observed in the back-titration curve from pH 2.0 (see below and Schlesinger, 1965a,b).

The spectrophotometric titration of tyrosine is shown in Figure 2, and these data taken together with that of Figure 1 were used to calculate the number of ionizable groups and their respective dissociation constants. These values are listed in Table I. The method of data analysis was essentially the iterative process described by Tanford (1962). Group counting was based on the amino acid analysis as well as the titration curve itself. The Linderstrøm-Lang (1924) equation was applied to the reversible region of the titration curve.

$$\mathrm{pH} = \mathrm{p}K_i + \log \frac{x}{1 - x} - 0.868wZ \qquad (1)$$

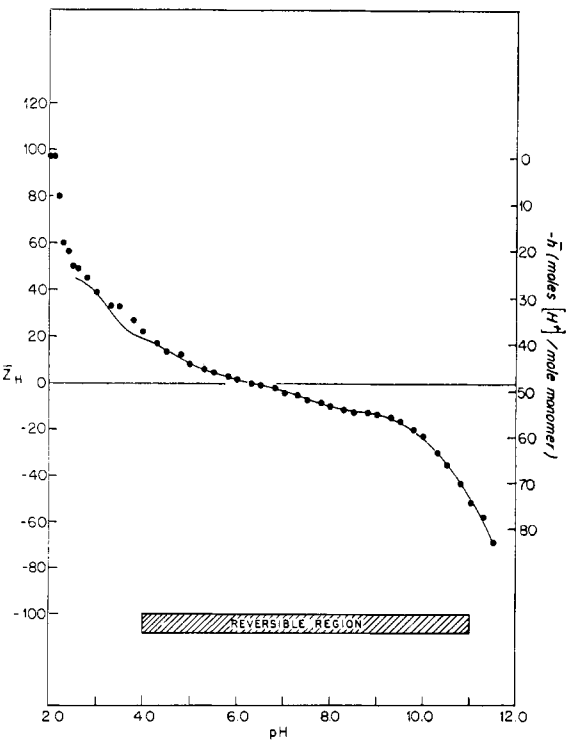

FIGURE 1: Titration of native enzyme. μ = 0.1 M KCl, temperature = 6°. (⊙) Experimental points; (——) theoretical curve.

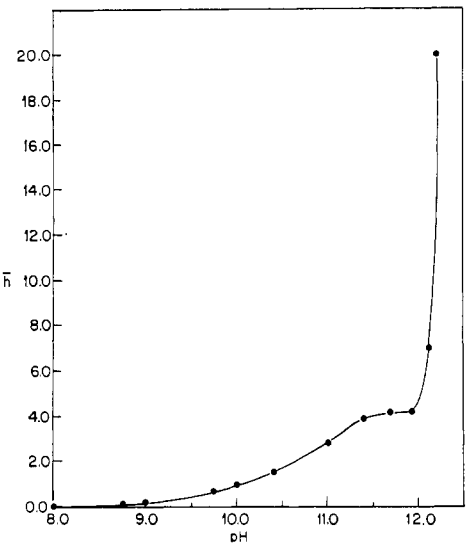

FIGURE 2: Spectrophotometric titration of tyrosine in native enzyme, μ = 0.1 M KCl, temperature = 6°.

where K_i = dissociation constant, x = fraction of protons dissociated from a particular set containing n groups of identical pK_i, w = electrostatic interaction between dissociating proton and charged macroion, and Z = charge on the protein. Z_h, which is the charge on the macroion based on the number of bound protons, was used instead of Z in this calculation since the binding of KCl to alkaline phosphatase has not been determined. Although the addition of 0.1 M KCl to protein at the isoionic point resulted in no pH change, binding of counterions could occur at higher protein charge and result in small differences between Z and Z_h. From eq 1 it can be seen that a plot of pH − log $(x/(1-x))$ *vs.* Z_h should be linear for each set of ionizable groups with identical pK_i's. pK_i equals pH − log $(x/(1-x))$ at $Z_h = 0$, and 0.868 w is the slope of the line. Plots of eq 1 were linear for carboxyl groups above pH 3.5 and for histidine groups between pH 6.0 and 8.0. Below pH 3.5 the slope of the line from the Linderstrøm-

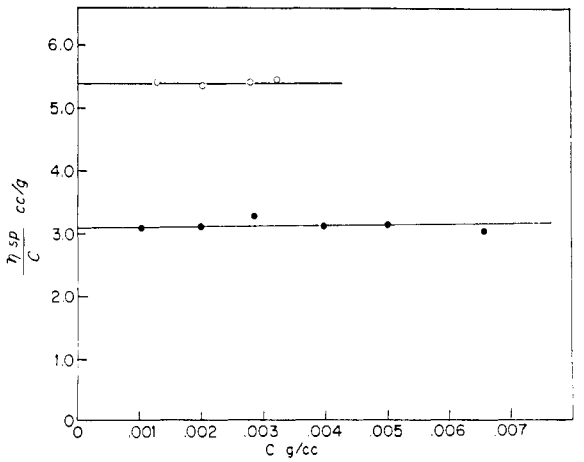

FIGURE 3: Viscosity of alkaline phosphatase dimer, temperature = 6°: (●) pH 6.3, μ = 0.1 M KCl, active enzyme; (○) pH 9.65, μ = 0.1 M Tris, active enzyme.

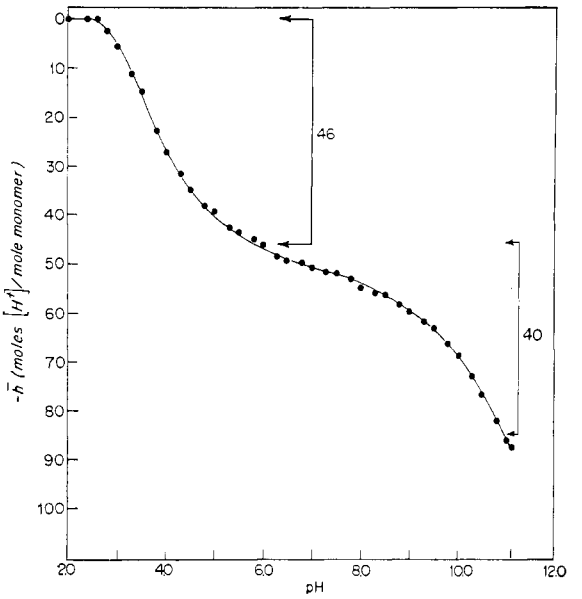

FIGURE 4: Titration of unfolded subunit. μ = 0.1 M KCl, temperature = 6°.

Lang plot was zero, a result consistent with the known dissociation and unfolding (Reynolds and Schlesinger, 1967) at high protein positive charge. Tyrosine and lysine groups gave a Linderstrøm-Lang plot with "w" approximately equal to 0.001, suggesting either a conformational change above pH 9.0 where these groups are ionized or the inapplicability of eq 1 due to the spacial arrangement of these groups in the protein. No change in optical rotatory dispersion is observed for a solution containing native alkaline phosphatase until the pH exceeds 12. However, an increase in viscosity does occur at pH 9.65 as shown in Figure 3 where η_{sp}/c for native dimer at pH 6.3 (isoionic) and 9.65 is plotted *vs.* protein concentration. The intrinsic viscosity, $[\eta]$, which is the value of η_{sp}/c at $c = 0$, must be corrected for the effect of protein density (Tanford, 1955) by addition of the term $(1 - \bar{v}_2\rho_0)/\rho_0$ to η_{sp}/c at $c = 0$.

Thus, $[\eta]$ for dimer at pH 6.3 = 3.4 cc/g and for active dimer at pH 9.65 = 5.65 cc/g.

The arginine content in Table I was calculated in the following manner. The number of carboxyl groups titrated below pH 6.3 in the native enzyme is 86/dimer (Table I). This is equal to the number of positively charged groups at the isoionic point of pH 6.3. There are 50 lysines and 11 histidines protonated at the isoionic point, and the difference between 86 and 61 is 25. Thus, the arginines plus acidic Zn^{2+} waters must equal 25. If we assume two metal–protein bonds per zinc ion, the total charge at pH 6.3 due to $3Zn(H_2O)^{2+}$ is +6, giving 19 arginines. If only one metal–protein bond exists, each Zn^{2+} has 3 acidic waters and the positive charge contribution due to metal is +9. In this case the arginine count is 16 which is incompatible with the amino acid analysis (Table I).

The values of n, pK_i, and w from Table I were used to calculate a theoretical titration curve which is shown as the solid line in Figure 1 and compared with experimental values in Table II. The agreement between theo-

TABLE II: Titration of Native Alkaline Phosphatase.

pH	Calcd Z	Exptl Z_h
2.5	+44.9	+50.2
3.0	+37.9	+39.1
3.5	+24.3	+32.9
3.8	+20.4	+28.0
4.0	+19.1	+21.9
4.3	+15.1	+17.5
4.5	+12.1	+14.7
4.8	+8.5	+12.0
5.0	+7.1	+7.8
5.3	+6.0	+6.2
5.5	+4.3	+4.7
5.8	+3.0	+3.9
6.0	+1.8	+1.6
6.3	0	0
6.5	−0.7	−1.0
6.8	−1.9	−2.7
7.0	−3.1	−4.1
7.3	−5.2	−5.4
7.5	−7.1	−6.8
7.8	−8.4	−8.4
8.0	−9.5	−9.5
8.3	−10.9	−11.9
8.5	−12.8	−12.3
8.8	−13.1	−12.5
9.0	−13.5	−13.1
9.3	−15.9	−14.2
9.5	−17.4	−15.8
9.8	−20.4	−20.0
10.0	−23.3	−22.1
10.3	−28.7	−29.6
10.5	−35.1	−34.8
10.8	−44.1	−43.0
11.0	−50.2	−51.6
11.3	−56.8	−58.0

retical and experimental values of Z_h is within ±1 group in the reversible portion of the titration curve.

Figure 4 presents the titration data for the unfolded subunit of alkaline phosphatase obtained by back-titration from pH 2, 6°, μ = 0.1 M KCl. It has been shown previously (Reynolds and Schlesinger, 1967) that under these experimental conditions no refolding or reassociation takes place. A group count shows 46 groups/monomer unit between pH 6.0 and 2.0 and an additional 40 groups/monomer between pH 6.0 and 11.0. This latter number agrees well with the analysis given in Table III (Tanford, 1962). Since approximately 3 histidines are expected to titrate below pH 6.0, only 43 of the 46 groups between pH 6.0 and 2.0 are carboxyls. It is apparent that all titratable groups are available in the unfolded monomer and that Zn^{2+} does not bind measurably to the unfolded protein throughout this pH range.

Linear polyampholytes do not in general obey the Linderstrøm-Lang equation since they expand and contract with changes in charge. Thus, w will vary with the

TABLE III: Analysis Based on Amino Acid Content and Small Molecule pK's.

	No. Titrated between pH 6 and 11
8 histidine—pK = 6.3	5.33
6 acidic H_2O groups—1.5 Zn/monomer with $2H_2O$ pK = 8.0 (Tanford and Epstein, 1954), $2H_2O$ pK = 12.0	3.30
10 tyrosines—pK = 9.6	10.00
25 lysines—pK = 10.4	20.8
	39.43

charge on the unfolded protein and straight-line plots will not result from eq 1. Figure 5 shows pH − log (α/(1 − α)) *vs.* α for the carboxyl groups in the unfolded monomer. At low pH (high positive charge) the slope is zero, indicating no electrostatic interaction. A change in slope occurs at pH 3.9 (α = 0.57) which agrees with previously published viscosity data (Reynolds and Schlesinger, 1967) showing a tenfold decrease in [η] for the unfolded monomer at pH 4 as compared with [η] at pH 2.

The sites for metal binding to the apoprotein were investigated by titration of the native enzyme and EDTA-treated dimer between pH 6.0 and 9.0. Figure 6 shows the number of protons dissociated as a function of pH for these species. The total number of protons dissociated in this range is 15–16. However, the active dimer contains three Zn^{2+} with an assumed pK_i (H_2O) = 8.0 (Tanford and Epstein, 1954), and therefore only 13 of the protons dissociated over this pH range are from imidazole groups. The apoprotein contains no Zn^{2+} and all 16 groups are considered to be imidazole. This statement depends on the observation (M. J. Schlesinger, unpublished work) that no α-amino groups can be detected in alkaline phosphatase.

Analysis of the apoprotein titration curve in Figure 6 shows that w = 0 for the imidazole groups (see Table IV for representative values of pH − log (x/(1 − x))).

Discussion

In the study of the formation of structure in a polypeptide chain it is important to define the amount of

TABLE IV: Titration of Apoprotein.

pH	n	pH − Log (x/(1 − x))
6.0	0	
6.5	4.25	6.939
7.0	7.63	7.038
7.4	11.2	7.031
8.0	14.6	6.968

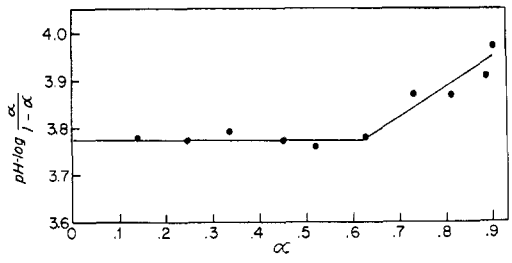

FIGURE 5: pH − log (α/(1 − α)) *vs.* α for carboxyl groups of unfolded subunit.

constraint initially present. Thus, although the disulfide bridges in the monomer units of alkaline phosphatase have not been broken, the unfolded subunit at pH 2 has been shown in the present work to have all ionizable groups exposed to solvent and to have zero electrostatic interaction between the dissociating proton and the charged central ion at high protein positive charges. These facts taken together with the previously published viscosity and difference spectra (Reynolds and Schlesinger, 1967) support the view that the acidified monomer contains little residual secondary or tertiary structure.

The present work also shows that histidine residues are involved in metal–protein interaction and are effectively masked by zinc ions. Tait and Vallee (1966) made a similar suggestion based on a study of the photooxidation of imidazole residues in the presence of rose bengal. When metal is removed from the native enzyme the electrostatic interaction factor decreases, suggesting some alteration in the shape or compactness of the macromolecule. A complete study of the apoprotein is currently under way and will be published elsewhere.

The electrostatic interaction factor, w, for the native enzyme decreases when the pH exceeds 9.0 and the intrinsic viscosity increases as the negative charge on the protein is increased. Neither optical rotatory dispersion nor ultraviolet difference spectra show any changes in secondary or tertiary structure as a function of pH between 6.0 and 12.0. In addition, the enzyme remains fully active over this range. A reduction in w may be expected from any one of the following factors: (1) a conformation change which may or may not include unmasking of ionizable groups inaccessible in the native state; (2) binding of counterions leading to an incorrect value of Z in eq 1; (3) inapplicability of the Debye–Hückel–Born model due to the particular location of the ϵ-amino and tyrosine residues on or above the protein "surface." The increase in $[\eta]$ between pH 6.3 and 9.65 suggests the first factor is at least partially involved in the change in w. However, the radius, R_e, of an equivalent hydrodynamic sphere is proportional to $[\eta]^{1/3}$. $\{[\eta] \sim 2.5N/M(4/3\pi R_e^3)\}$ so that R_e at pH 9.65 is only 1.42 times larger than R_e at pH 6.3. From Debye–Hückel theory

$$w = \frac{\epsilon^2}{2DRkT}\left(1 - \frac{KR}{1 + Ka}\right) \qquad (2)$$

where $\epsilon = 4.8 \times 10^{-10}$ esu (electronic charge), $D = 86$ (dielectric constant of the solvent), k = Boltzmann constant (1.38×10^{-16} erg/deg), $T = 279°$K, a = radius of protein plus H^+, R = radius of protein, and K = Debye reciprocal length. The experimental value of w (0.037) in Table I gives $R = 29 \times 10^{-8}$ cm at pH 6.3. Based on the hydrodynamic data, R at pH 9.65 may be expected to increase to 40.5×10^{-8} cm, and w then becomes 0.019. This value is approximately ten times larger than the experimental value for tyrosine and lysine. While there are obvious approximations inherent in both the hydrodynamic calculation of R_e and the application of this parameter to eq 2, it would appear that the increase in asymmetry and/or size of the protein at pH 9.0 is not large enough to account for the large decrease in w. The binding of counterions cannot be eliminated as a source of error, but a change in w of the magnitude observed requires extremely high mole ratios of bound electrolyte to protein.

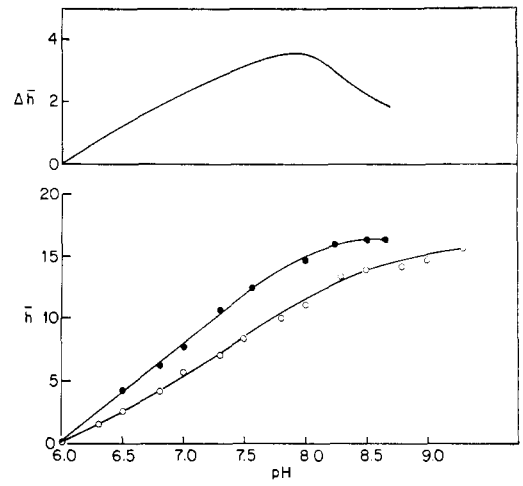

FIGURE 6: Titration of histidine residues. (●) Inactive dimer; (○) active dimer; temperature = 6°, μ = 0.1 M KCl.

The third factor, that of the inapplicability of the smeared charge model, has been treated theoretically by Tanford and Kirkwood (1957) and Tanford (1957). Location of the ionizable groups slightly above the surface of the macroion such as might occur if the surface is "frayed" by solvent penetration and swelling would tend to reduce the electrostatic interaction.

All three factors may thus contribute to a reduction in w, but the relative effects of each cannot be evaluated. It is, however, apparent from spectral studies and the hydrodynamic properties of the protein that no massive disorganization of structure takes place below pH 12.0.

All ionizable groups are exposed to solvent in the active enzyme except the 3 histidines to which zinc ions are bound and 16 tyrosines which are unmasked only above pH 12. The apparent hysteresis in the back-titration curve, which was reported previously (Schlesinger, 1965a,b) and is also observed in the present work between pH 2.0 and 6.0, is not the result of masked carboxyl groups but is due to a change in w with dissociation and unfolding plus the exposure of the three histidine residues when zinc is removed at low pH values. Since no anomalous ionizable groups are found in the native enzyme, with the exception of tyrosine, it is apparent, then, that the actual sites of contact between two

subunits do not involve charged groups. The interaction between subunits may be an entropy-driven process involving interaction between hydrophobic residues. It remains to be determined whether the four tyrosine groups per monomer which are exposed to water in the refolded monomer but masked in the native dimer are points of contact between the two subunits or are buried by a rearrangement which takes place during dimerization.

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Preparation of Leucine Aminopeptidase Free of Endopeptidase Activity*

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ABSTRACT: A new method is described for preparing leucine aminopeptidase from the supernatant fraction of hog kidney which provides this enzyme in 50% yield with a specific activity (C') greater than 100 min^{-1}/mg of nitrogen, a value higher than that previously reported on a preparative scale. The purified enzyme did not detectably hydrolyze cytochrome *c*, rabbit actin, or mercuripapain, proteins whose amino-terminal sequences are refractory to exopeptidase action. The present preparation is therefore believed to be suitable for protein sequence determination. Previously unrecognized factors affecting the reproducibility of the preparation, including a requirement that kidneys from inbred hogs be employed, are discussed. Multiple chromatographic forms of the enzyme of differing substrate specificity were partially resolved.

The leucine aminopeptidase of the supernatant fraction of swine kidney can hydrolyze most L-amino acids from the amino-terminal position of proteins and peptides (Smith *et al.*, 1952). Exploitation of this property for protein sequence determination, however, requires enzyme preparations free of contaminating endopeptidases, because such contamination leads to erroneous deductions of sequence (Frater *et al.*, 1965).

Since available methods for preparing the enzyme (Frater *et al.*, 1965; Hill *et al.*, 1958; Fasold *et al.*, 1962; Folk *et al.*, 1959) do not eliminate endopeptidases, we have investigated alternative means of purifying it and have established a relatively rapid and reliable procedure for obtaining highly purified leucine aminopeptidase free of measurable endopeptidase activity. The enzyme prepared by this method has a molecular weight of 300,000 as determined by Sephadex G-200 chromatography, in agreement with the value deduced by earlier workers from its sedimentation constant (Spackman *et al.*, 1955). Its substrate specificity, pH optimum, and activation by Mn^{2+} are also in agreement with previous reports (Smith and Spackman, 1955). However, multiple chromatographic forms of the enzyme have been detected.

The physical and enzymatic properties of swine leucine aminopeptidase reported in this study and observed originally by Smith and Hill (1960) closely resemble the properties of the crystalline leucine aminopeptidase isolated from beef lens by Hanson *et al.* (1965). The crystalline enzyme from beef lens was found to have approximately the same molecular weight, substrate specificity, specific activity, pH optimum, and metal dependence as the enzyme obtained from swine kidney supernatant fraction.

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