Formation and Properties of a Tetrameric Form of *Escherichia coli* Alkaline Phosphatase*

Jacqueline A. Reynolds and Milton J. Schlesinger

ABSTRACT: Alkaline phosphatase from *Escherichia coli* has been studied extensively in a dimeric state containing four bound Zn^{2+} per dimer. It has now been shown that at free Zn^{2+} concentrations $>10^{-5}$ M rapid and reversible self-association occurs between pH 7.0 and 8.0. At pH 8.0 and an equilibrium concentration of $Zn^{2+} = 10^{-4}$ M all of the protein is present as tetramer containing 16 Zn^{2+}/tetramer. Hydrodynamic studies show no significant alteration in shape of the tetramer compared with dimer, and optical measurements indicate that one tryptophan residue per chain is removed from contact with solvent as the result of association. There is an increase in phosphate binding with tetramerization.

It has been well established that alkaline phosphatase from *Escherichia coli* is isolated from the cell as a dimer (mol wt 86,000) containing two identical subunits (Rothman and Byrne, 1963) and four Zn^{2+} per dimer.

Previous studies (Reynolds and Schlesinger, 1967–1969) of the *in vitro* formation of structure in alkaline phosphatase from *E. coli* have led to the following observations. (1) The dimer is reversibly dissociated into linear structureless polypeptide chains in 6 M guanidine hydrochloride or 10^{-2} M HCl. (2) These polypeptide chains can be refolded to a structured monomer which has an optical rotatory dispersion spectrum identical with that of the enzymatically active dimer but which has four to five of the ten tyrosine residues exposed to solvent as compared with two in the native state. (3) Association of the folded monomers can take place in the absence of bound metal. (4) Reversible binding of four Zn^{2+} to the apo dimer results in the removal of two to three tyrosine residues per chain from the aqueous environment and the appearance of enzymatic activity. These four Zn^{2+} ions are tightly bound to the enzyme and are not removed by exhaustive dialysis against glass-distilled water containing 10^{-7} M Zn^{2+}.

It has been observed previously (Reynolds and Schlesinger, 1969) that in the presence of excess Zn^{2+} ($>10^{-5}$ M) self-association of the alkaline phosphatase dimer occurs. The present work deals with the structural alterations in the enzyme accompanying reversible aggregation to the tetrameric state, the effect of proton and metal binding on the association reaction, and the functional results of tetramerization.

Experimental Section

Materials. The preparation and purification of *E. coli* alkaline phosphatase, strain CW 3747, have been described previously (Reynolds and Schlesinger, 1967; Schlesinger and Barrett, 1965). Protein concentrations were determined from the optical density at 2800 using an $\epsilon_{1\text{ cm}}^{0.1\%}$ of 0.77.

Reagent grade anhydrous $ZnCl_2$ was obtained from Fisher Chemical Co.

^{32}P-Labeled phosphoric acid was obtained carrier free from Mallinckrodt Chemical Co. and diluted with standard solutions of KH_2PO_4 in glass-distilled water.

Methods. Ultraviolet difference spectra were obtained using a Cary 14 spectrophotometer equipped with a thermostatted cell compartment and tandem cells of 1-cm path length. In all experiments reagents added to the sample cell were compensated in the reference beam. The validity of Beer's law was established by determining the difference spectra at a number of protein concentrations.

Circular dichroism was measured with a Cary 60 recording spectropolarimeter using 1- and 0.1-cm cells. A constant band width of 15 Å was used over the entire wavelength range.

Equilibrium dialysis of alkaline phosphatase with Zn^{2+} was carried out at 20° in 0.05 M KCl and the pH was adjusted with KOH. Protein concentrations between 5 and 10 mg per ml were used. Analyses of Zn^{2+} concentrations were obtained by both atomic absorption spectrophotometry and the spectral method was described below. The pH of the protein and dialysate was measured at the end of each dialysis experiment.

Equilibrium dialysis of alkaline phosphatase with inorganic phosphate was carried out at 20° under a variety of buffer and salt conditions. Concentrations of phosphate were determined for solutions on both sides of the membrane using 0.1-ml samples in 10 ml of Bray's scintillation solution (Bray, 1960) and a Packard scintillation counter. Differences in counts per unit of volume on the two sides of the membrane were converted into concentration differences and to the average mole ratio of phosphate/86,000 molecular weight.

Sedimentation velocity was determined in a Spinco Model E ultracentrifuge at a rotor speed of 52,640 rpm. The ultracentrifuge was equipped with a schlieren optical system and photographs were taken at 4-min intervals after reaching constant speed.

Number-average molecular weights were determined with a Mechrolab high-speed membrane osmometer 503 equipped with a variable temperature controller and B-19 membranes. Equilibrium pressure readings were always reached within 20 min. Protein concentrations between 1 and 15 mg per ml were used.

* From the Department of Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110. *Received May 29, 1969.*

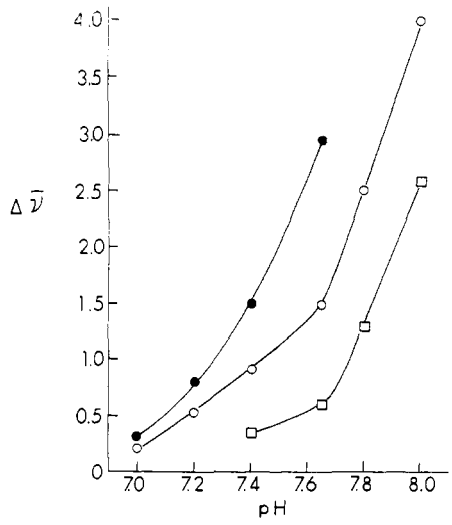

FIGURE 1: Zn^{2+} binding per dimer in excess of four Zn^{2+} per dimer, protein concentrations = 5–10 mg/ml. (●) 1.58×10^{-4} M Zn^{2+}, (○) 10^{-4} M Zn^{2+}, and (□) 6.9×10^{-5} M Zn^{2+}.

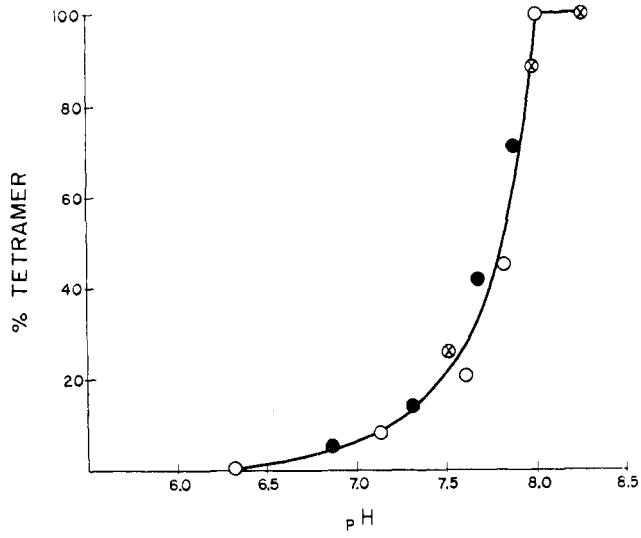

FIGURE 3: Mole per cent tetramer *vs.* pH. C_{eq} $[Zn^{2+}] = 1.2 \times 10^{-4}$ M–0.05 M KCl. (○) $T = 20°$, osmometry; (⊗) $T = 20°$, spectrophotometric; and (●) $T = 13°$, osmometry.

A spectrophotometric assay for Zn^{2+} using ammonium purpurate (Eastman) was developed. Fresh solutions of 10^{-4} M purpurate in 10^{-2} M Tris-Cl (pH 8.0) were prepared daily. The addition of aliquots of Zn^{2+} solutions to this reagent leads to a difference spectra (blue shift) with a trough at 5250 Å and a peak at 4575 Å. The magnitude of this difference spectra is a linear function of the metal ion concentration between 10^{-6} and 10^{-5} M. ΔOD_{4575}/mole of Zn^{2+} per l. = 3.87×10^4. Analyses using this technique agreed within 1% with atomic absorption determinations.

Results

Zn^{2+} Binding to Alkaline Phosphatase and Molecular Weight Changes as a Function of Metal and Proton Binding. The pH dependence of Zn^{2+} binding in excess of 4 Zn^{2+}/86,000 molecular weight is shown in Figure 1 for three different equilibrium concentrations of the metal. Partial binding isotherms at pH values between pH 7.2 and 8.0 are given in Figure 2 as a function of Zn^{2+} concentration.

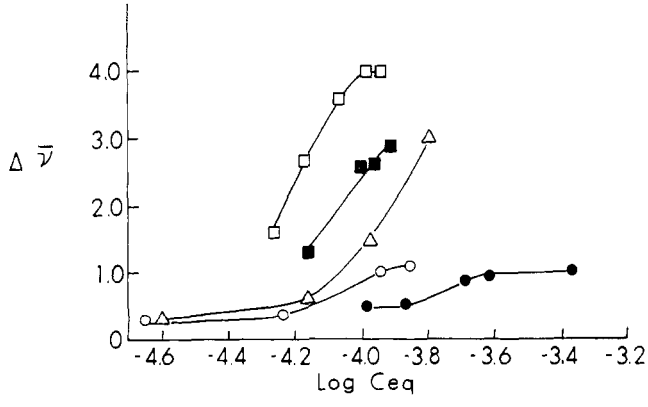

FIGURE 2: Zn^{2+} binding per dimer in excess of four Zn^{2+} per dimer, protein concentrations = 5–10 mg/ml. (□) pH 8.0, (■) pH 7.8, (△) pH 7.65, (○) pH 7.4, and (●) pH 7.2.

At $[Zn^{2+}] = 1.2 \times 10^{-4}$ M alkaline phosphates undergoes an increase in number-average molecular weight as a function of pH to a maximum at pH 8.0 of 172,000. No higher aggregates are observed in the ultracentrifuge, and mixtures of dimer and tetramer at lower pH values sediment with a single boundary-moving intermediate to that of pure dimer and pure tetramer. The data from Figures 1 and 2 were used to calculate bound and total metal ion concentrations for a series of protein solutions, $C_{eq}[Zn^{2+}] = 1.2 \times 10^{-4}$ M (where $C_{eq}[Zn^{2+}]$ represents the concentration of Zn^{2+} in solution not bound to the protein), and the molecular weights of the protein were determined as a function of pH at this constant Zn^{2+} concentration. Assuming only dimer and tetramer are present in any given solution, the mole per cent tetramer can be calculated from the apparent number-average molecular weight. These results are shown in Figure 3.

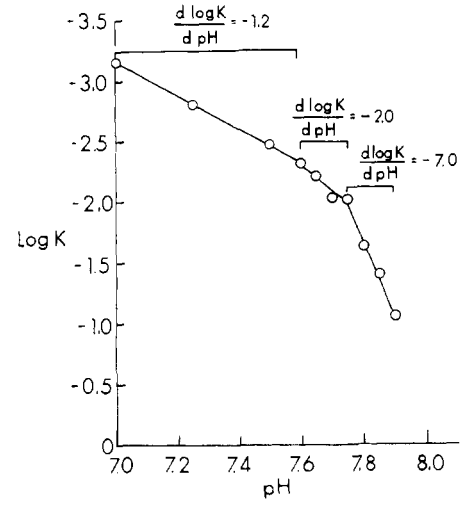

FIGURE 4: Log *K vs.* pH for tetramer formation.

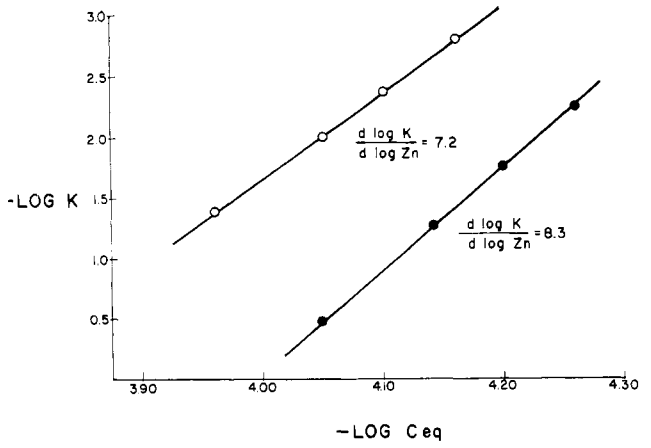

FIGURE 5: Log K *vs.* log $C_{eq}[Zn^{2+}]$ for tetramer formation. (○) pH 7.8 and (●) pH 8.0.

The equilibrium constant at any given pH for the reaction

$$2D \overset{K}{\rightleftharpoons} D_2$$

can be calculated from the data in Figure 3 and the first derivative of log K with respect to log hydrogen ion activity is the difference between protons bound to the tetramer and to two dimer molecules (Wyman, 1964). Figure 4 shows log K (where D and D_2 are expressed as mole per cent) *vs.* pH, and the slope of the curve is seen to vary from −1.2 to −7.0 between pH 7.0 and 7.9.

The mole per cent tetramer formed at constant pH as a function of the Zn^{2+} concentration was determined from the magnitude of the difference spectra at 2910 Å (see below) and the equilibrium Zn^{2+} concentration was obtained from the data in Figures 1 and 2. Log K for the association reaction at pH 8.0 and 7.8 is plotted *vs.* log C in Figure 5. The slope of these lines is $\Delta\bar{\nu}[Zn^{2+}]$ between 2 dimer molecules and a tetramer molecule, where $\bar{\nu}$ is the mole ratio of bound Zn^{2+} to protein. At pH 7.8 this value is 7.2/2 dimers and at pH 8.0,

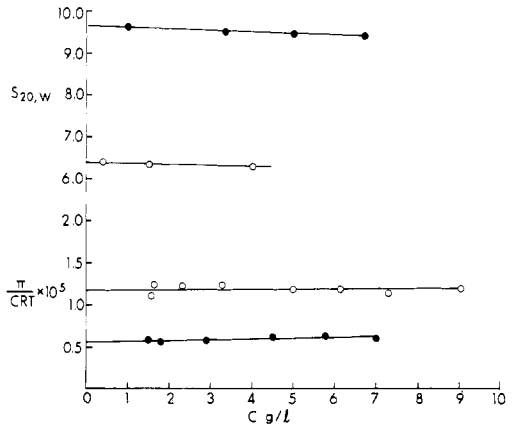

FIGURE 6: Osmotic pressure and sedimentation coefficients, 20°. (○) Dimer, pH 6.3, 0.05 N KCl. (●) Tetramer, pH 8.0, 0.05 N KCl, $C_{eq}[Zn^{2+}] = 1.2 \times 10^{-4}$ M.

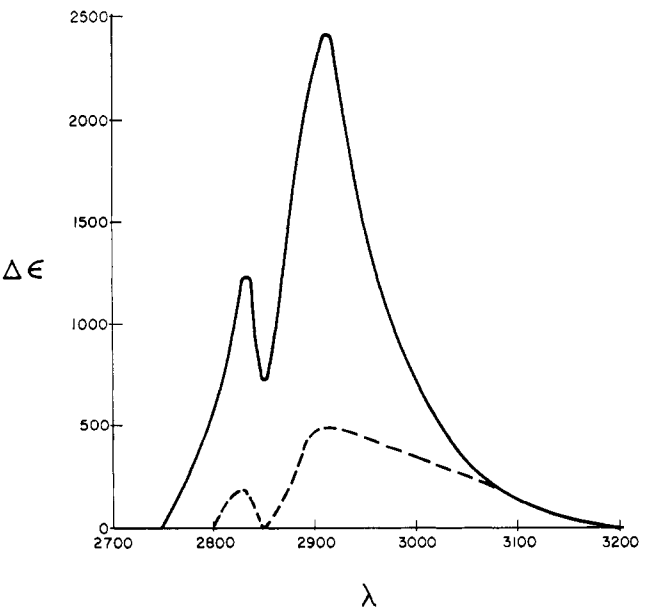

FIGURE 7: Difference spectra in 0.05 M KCl, protein concentration = 1–2 mg/ml. (—) Tetramer, pH 8.0, $[Zn^{2+}] = 1.2 \times 10^{-4}$ M; (- - -) 18% tetramer + 82% dimer, pH 7.4, $[Zn^{2+}] = 1.2 \times 10^{-4}$ M.

8.3/2 dimers. These numbers agree well with the maximum binding for these two pH values shown in Figure 2 where at pH 7.8, $\Delta\bar{\nu}[Zn^{2+}]$ = 3.5/dimer or 7.0/2 dimers, and at pH 8.0, $\Delta\bar{\nu}[Zn^{2+}]$ = 4.0/dimer or 8.0/2 dimers.

The osmotic pressure and sedimentation coefficients for dimer and tetramer are shown as a function of protein concentration in Figure 6. The reciprocal of the intercepts of π/RTc (where π is the osmotic pressure, c is the protein concentration in grams per liter, R is the gas constant, and T is the temperature) is the number-average molecular weight and is equal to 85,000 and 174,000 for the dimer and tetramer, respectively. $s^0_{20,w}$ is 6.4 and 9.65 S for these two species, and the ratio $s^0/M^{2/3}$ is 3.32×10^{-3} and 3.18×10^{-3} for dimer and tetramer.

Difference Spectra. The tetramerization and the binding of 4 additional Zn^{2+}/86,000 molecular weight lead to the difference spectra shown in Figure 7. The association reaction results in a red shift in the absorption band due to tryptophan residues, and the peak height at 2910 Å is a linear function of the mole per cent tetramer as determined independently by osmometry. The maximum $\Delta\epsilon^{1\,cm,\,1\,M}_{2910}$ is 2400 which can be attributed to 1 tryptophan residue/chain removed from contact with the aqueous media (Donovan, 1969).

Circular Dichroism. The circular dichroic spectra of alkaline phosphatase are shown in Figure 8. Between 2500 and 2000 Å in the wavelength region frequently ascribed to peptide absorption (Beychok, 1967), the spectra of tetramer and dimer are experimentally indistinguishable. However, the extrinsic Cotton effect band centered at 2790 Å increases in magnitude as Zn^{2+} is bound to the apodimer and also as the dimer associates and binds additional metal. This band is usually associated with tyrosine residues (Beychok, 1967); however, no experimentally observable perturbation of tyrosine was noted in the difference spectra discussed above.

Binding of P_i. P_i is a competitive inhibitor for alkaline phosphatase. A previous publication (Reynolds and Schles-

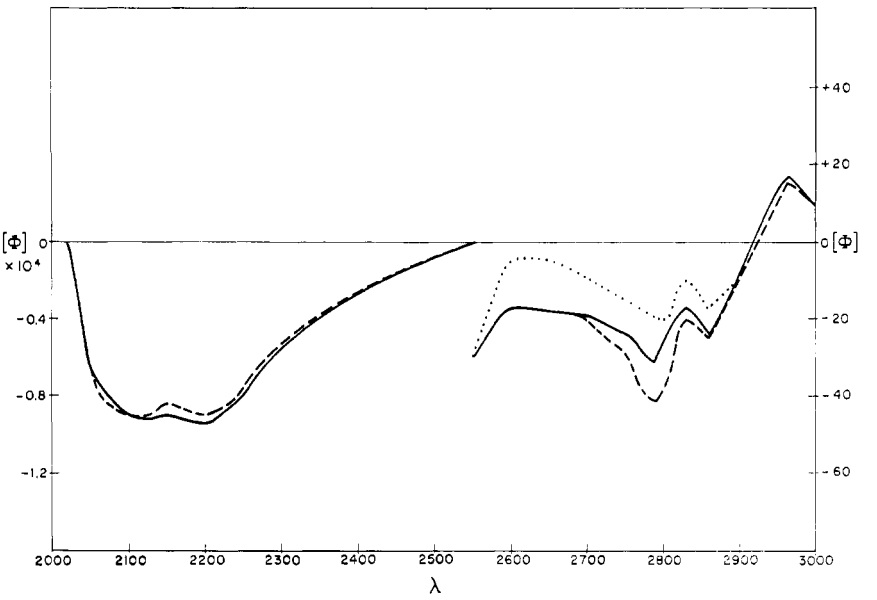

FIGURE 8: Circular dichroic spectra, [ϕ] = molar ellipticity = (deg − cm³)/dmole. (· · · ·) Apodimer, pH 6.3, 0.05 N KCl (0 Zn^{2+}/dimer); (—) dimer, pH 6.3, 0.05 N KCl (4 Zn^{2+}/dimer); (- - -) tetramer, pH 8.0, 0.05 N KCl, $C_{eq}[Zn^{2+}] = 1.2 \times 10^{-4}$ M (8 Zn^{2+})/dimer).

inger, 1969) reported 1 binding site for phosphate/86,000 molecular weight at pH 9.0, 0.1 M KCl, 0.01 M Tris-Cl, and an association constant of 4.2×10^5 M^{-1}. Table I contains additional data on phosphate binding obtained under other solvent conditions. The number of phosphate binding sites on the active dimer is not altered by changes in pH, buffer conditions, or Zn^{2+} binding up to 4 Zn^{2+}/dimer. However, at pH 7.6 in 0.05 M KCl, $C_{eq}[Zn^{2+}] = 1.3 \times 10^{-4}$ M, 6.2 Zn^{2+}/86,000 molecular weight, the number of phosphate binding sites increases to 3.6/86,000 molecular weight. Molecular weight measurements of the protein under these solvent conditions gave 33 mole % tetramer. Figure 9 shows the reciprocal plot of $1/\bar{\nu}$ *vs.* $1/C_{eq}$ for the binding of P_i to this mixture of dimer and tetramer. It is apparent that tetramerization and concomitant Zn^{2+} binding lead to an increase in the number of phosphate sites on alkaline phosphatase.

TABLE I: Phosphate Binding to Alkaline Phosphatase.

pH	Mole % Tetramer	n	K	Solvent	Zn^{2+}/Dimer
7.6	33	3.6	1.6×10^5	0.05 N KCl–2×10^{-4} M Zn^{2+}	6.2
6.85	0	1.1	2.0×10^5	0.05 N KCl–2×10^{-4} M Zn^{2+}	4.0
9.0	0	1.1	4.2×10^5	0.1 N KCl–0.01 M Tris	4.0
9.0	0	1.0	3.6×10^5	1.0 M Tris	4.0
5.6[a]	0	0.9	4.2×10^5	0.1 M acetate	2.7

[a] Ko and Kezdy (1967).

Discussion

The data presented here show that alkaline phosphatase associates reversibly and rapidly to a tetramer, M_n = 174,000 and $s^0_{20,w}$ = 9.65 S, in the presence of $Zn^{2+} > 10^{-5}$ M at pH values between 7.0 and 8.0. No major changes in hydrodynamic shape accompany the association as evidenced by the constancy of the ratio $s^0_{20,w}/M^{2/3}$ for dimer and tetramer. However, spectral changes suggest that one tryptophan per chain is removed from contact with the solvent for each molecule of tetramer formed, and a perturbation of the extrinsic Cotton effect trough at 2790 Å is observed in the circular dichroic spectra. It is not possible to distinguish between Zn^{2+} binding and protein–protein interaction effects on the optical properties examined since these two parameters are altered simultaneously.

The difference in the number of protons bound to the tetramer and the dimer was shown in Figure 4 to vary from −0.3 to −1.8 per polypeptide chain over the pH range 7.0–7.9. Simultaneously, the number of metal ions bound per chain varies from 0.1 to 1.6. While it is tempting to associate the binding of each Zn^{2+} with the release of one proton, it

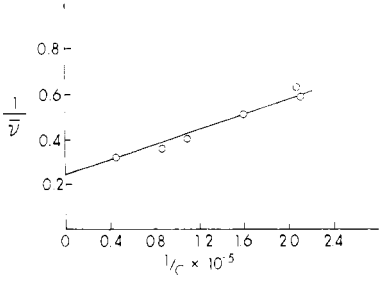

FIGURE 9: Reciprocal plot of P_i binding to alkaline phosphatase 0.05 M KCl, pH 7.6, C_{IJ} $[Zn^{2+}] = 1.2 \times 10^{-4}$ M.

must be kept in mind that the titration curve of tetramer would be expected to shift relative to that of the dimer due to a reduction in the electrostatic interaction between protons and the macromolecular species with increasing molecular weight. Thus, the difference in protons bound to the two species is not only a function of metal ion binding but also of the altered titration curve of the protein itself.

The number of binding sites on alkaline phosphatase for the competitive inhibitor, P_i, is 1/dimer under a variety of experimental conditions but increases to 3.6/dimer when 33 mole % of the protein is present as tetramer. Assuming there are only two species present, 0.67 (±0.07) mole of dimer and 0.33 (±0.03) mole of tetramer and, further, that each dimer contains only one site, the simple calculation belows shows that 17 ± 1 moles of P_i are bound to the tetrameric form of the protein. It appears that approximately

$$0.33\left(\frac{x}{2}\right) + 0.67(1) = 3.6$$

$$x = 17.8$$

1 mole of P_i is bound for each mole of Zn^{2+} on the protein in the tetrameric state.

Stopped-flow kinetic experiments comparing the tetramer and dimer as to number of catalytic sites and velocity of reaction will be reported in a subsequent communication.

Although alkaline phosphatase is isolated from *E. coli* cells as a highly stable dimer, it is possible that the ionic conditions in the periplasmic space where the enzyme is localized favor the tetramer form. Under derepressed conditions of growth, there are approximately 10^4 enzyme molecules/cell confined to an estimated space of 10^{-18} cm^3, or about 10 g/l. Experiments are in progress to determine the state of alkaline phosphatase *in situ*.

References

Beychok, S. (1967), *in* Poly-α-amino Acids, Fasman, G. D., Ed., Marcel Dekker, p 293.

Bray, G. A. (1960), *J. Anal. Biochem. USSR 1*, 279.

Donovan, J. W. (1969), *J. Biol. Chem. 244*, 1961.

Ko, S. H. D., and Kezdy, F. J. (1967), *J. Am. Chem. Soc. 89*, 7139.

Reynolds, J. A., and Schlesinger, M. J. (1967), *Biochemistry 6*, 3552.

Reynolds, J. A., and Schlesinger, M. J. (1968), *Biochemistry 7*, 2080.

Reynolds, J. A., and Schlesinger, M. J. (1969), *Biochemistry 8*, 588.

Rothman, F., and Byrne, R. (1963), *J. Mol. Biol. 6*, 330.

Schlesinger, M. J., and Barrett, K. (1965), *J. Biol. Chem. 240*, 4248.

Wyman, J. (1964), *Advan. Protein Chem. 19*, 224.

Secondary Modification of Cytochrome *c* by *Neurospora crassa**

W. A. Scott† and H. K. Mitchell

ABSTRACT: Two molecular species of cytochrome *c*, C_I and C_{II}, were detected in *Neurospora crassa*. The two cytochrome *c*'s differ in structure at residue 72. This position is occupied by lysine in C_{II}. The equivalent residue in C_I, a lysine derivative, has been identified as ε-trimethyllysine (DeLange, J. R., Glazer, A. N., and Smith, E. L. (1969), *J. Biol. Chem. 244*, 1385). A sequential synthesis of the two cytochrome *c*'s occurs in *Neurospora* cultures. Pulse-chase experiments with [^{14}C]lysine indicate that C_{II}, the early cytochrome *c*, is converted into C_I presumably by a specific methylation of lysine-72 in C_{II} to give ε-trimethyllysine in C_I. The kinetics of C_I and C_{II} synthesis in the *poky* mutant also suggest that the conversion of C_{II} into C_I reflects the binding of the cytochrome *c* to the mitochondrion.

Cytochrome *c* does not normally occur free in the cytoplasm of a cell. Apparently there is coordination between the rate of cytochrome *c* synthesis and its binding to the mitochondrial matrix. However, an exceptional situation exists in the *poky* strain of *Neurospora crassa*. This mutant, which exhibits cytoplasmic inheritance (Mitchell and Mitchell, 1952), accumulates up to 16 times the normal amount of cytochrome *c* found in the *wild-type* strain (Haskins *et al.*, 1953), over half of which is not bound (Hardesty, 1961). This extreme condition exists only in very young cultures. The cytochrome *c* accumulation is diluted out as the mold grows and ages. Using very young cultures and alkaline extraction, Hardesty (1961) obtained evidence that the *poky* mutant contains more than one molecular species of cytochrome *c*.

With this background, the *poky* mutant clearly presented a favorable situation in which to examine the biosynthesis of cytochrome *c* and to investigate further the question of the existence of more than one species of molecule. As reported here, there are indeed two cytochrome *c*'s produced in the mold, one being derived from the other.

* From the Division of Biology, California Institute of Technology, Pasadena, California 91109. *Received June 27, 1969.*

† Present address: The Rockefeller University, New York, N. Y. 10021.