Structural Role of Pyridoxal 5′-Phosphate, Pyridoxal 5′-Phosphate Analogs, and Other Agents in the Association of Subunits of *Bacillus alvei* Apotryptophanase†

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ABSTRACT: *Bacillus alvei* apotryptophanase readily dissociates at low protein concentration and sediments at 5.7 S (dimer) in 0.01 *M* potassium phosphate (pH 7.8) from 9 to 33°. With temperature held constant at 9°, increasing the potassium, sodium, or ammonium phosphate buffer concentration increases the sedimentation value to 8.0 S. Increasing the monovalent cation concentration alone does not have the effect. Imidazole and pyridoxal compete with phosphate, preventing the effect. Raising the temperature to 26° in the presence of high concentrations of potassium phosphate increases the sedimentation constant to 9.4 S. The addition of pyridoxal-P converts the dimer to a 9.4S tetramer. The conversion is dependent upon coenzyme concentration, temperature, and the nature of monovalent cation present. The K_m for pyridoxal-P for the sodium form of the enzyme is more than tenfold greater than the K_m for the potassium form of the enzyme. 2′-Methyl, 2′-hydroxyl, 6-methyl, and the *N*-oxide of pyridoxal-P are active in the association of dimer to tetramer but to differing extents. Analogs altered in the 4′-formyl position are also inactive structurally. Anthranilic acid, a competitive inhibitor of tryptophan, and 8-anilino-1-naphthalenesulfonic acid (ANS), a competitive inhibitor of pyridoxal-P binding, are both active in affecting the dimer to tetramer association but tryptophan is not. The dimer and tetramer are spectrally distinguishable through circular dichroic measurements, fluorescence quenching with pyridoxal-P or pyridoxal, and fluorescence enhancement with ANS. Pyridoxal-P causes the release of ANS from an ANS–apoenzyme complex.

Previous studies have shown that tryptophanase in crude extracts of *Escherichia coli* coexists as two species, one sedimenting between 6 S and 7 S (dimer) and one between 9 S and 10 S (tetramer). The dimer predominates (Newton et al., 1965; Feiss and DeMoss, 1965). Purification of *Bacillus alvei* tryptophanase carried out in the presence of excess pyridoxal-P[1] yields enzyme with a sedimentation coefficient of 9.7 S (Hoch and DeMoss, 1972) and it is this tetramer species which is responsible for both the α,β-elimination and β-replacement reactions whereas the dimer form is inactive (Whitt and DeMoss, 1973).

Purified tetramer holotryptophanase dissociates to dimers in the presence of Tris and absence of excess pyridoxal-P (Gopinathan and DeMoss, 1968). The apoenzyme at protein concentrations below 3 mg/ml also dissociates to molecular weight forms that are smaller than tetramer (Hoch and DeMoss, 1972).

In this report, we have investigated in vitro the role played by various chemical and physical conditions on the reversible shift between the dimer and tetramer forms. Purified apotryptophanase was exposed to ligands known to bind to the active site in order to assess their effects on the aggregational state. The effects of temperature, ionic strength, and a deforming buffer were also measured. Our goal was to determine more about the forces acting upon dimers to either inhibit or promote association to the higher molecular weight species.

Sucrose density gradient centrifugation was the technique chosen for these studies so that the sedimentation properties of the enzyme could be determined using protein concentrations approaching those used to study enzymatic activity. Enzyme concentrations used for sedimentation studies were also used in fluorescence analysis. In this manner, we could examine the spectral properties of forms of the enzyme with known sedimentation values.

Materials and Methods

Materials. Tryptophanase from *B. alvei* was purified by the procedure of Hoch et al. (1966). Apotryptophanase was prepared by cysteine treatment as described by Hoch and DeMoss (1972). Analogs of pyridoxal-P not commercially available were gifts from the following individuals: Dr. E. E. Snell (2-norpyridoxal-P and 2′-methylpyridoxal-P); Dr. S. Fukui (2′-hydroxypyridoxal-P and *N*-oxide of pyridoxal-P); Dr. A. Pocker (isopyridoxal-P); Dr. H. Dunathan and M. Karpeisky (6-methylpyridoxal-P). All other enzymes and chemicals were obtained commercially and used without further purification.

Sucrose Gradient Centrifugation. Sucrose density gradient centrifugation was according to the method of Martin and Ames (1961); 5-ml gradients were prepared from 5 and 20% sucrose solutions in the specified buffer, supplemented with compounds such as pyridoxal-P, pyridoxal-P analogs, substrates, or various salts. Gradients were prepared in Beckman cellulose nitrate tubes 3.5–5 hr before centrifugation and allowed to equilibrate at the temperature of the specific experiment. Centrifugation was for 16 hr in a

† From the Department of Microbiology, University of Illinois, Urbana, Illinois 61801. *Received March 13, 1975.* This work was supported by Research Grant AI 2971 from the National Institutes of Health, U.S. Public Health Service. H.C.I. was a predoctoral trainee supported by a Microbiology Training Grant (GM-510) from the National Institute of General Medical Sciences. This work is taken in part from a thesis submitted by H.C.I. to the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology.

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[1] Abbreviations used are: pyridoxal-P, pyridoxal 5′-phosphate; ANS, 8-anilino-1-naphthalenesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

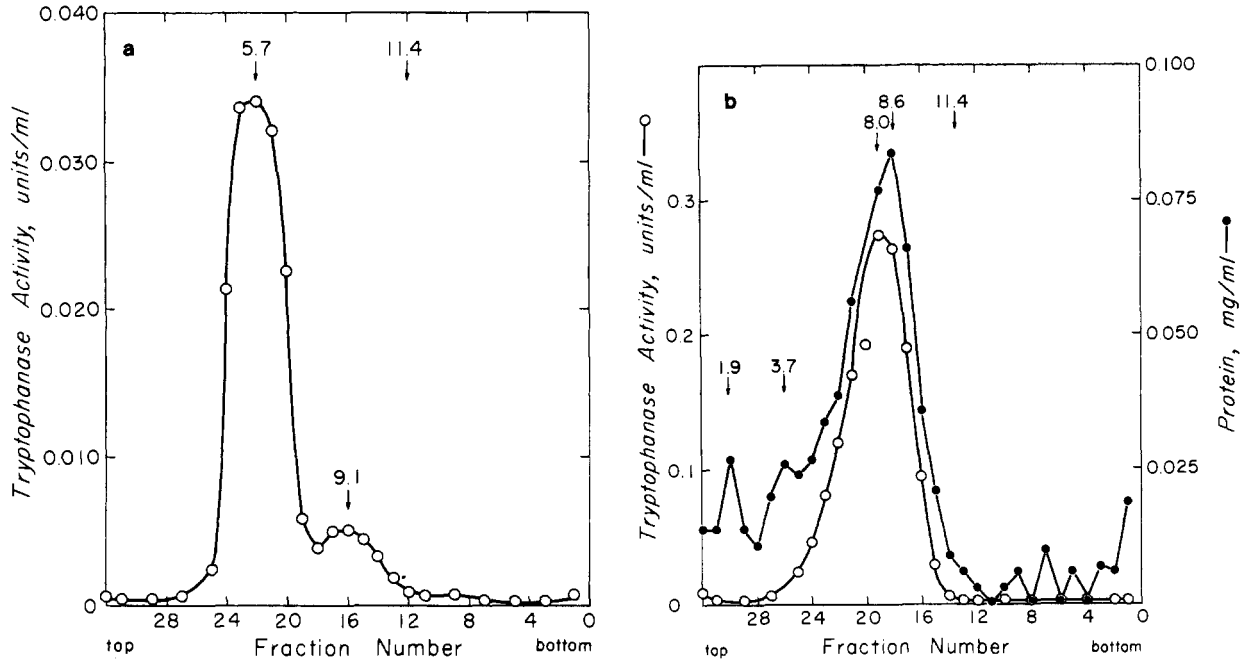

FIGURE 1: (a) Sucrose density gradient centrifugation of apotryptophanase from *B. alvei*. Linear sucrose density gradients were prepared from 5 and 20% sucrose solutions in 0.01 *M* potassium phosphate (pH 7.7). Sucrose density centrifugation was carried out as described in Materials and Methods. Centrifugation was for 16 hr at 32,000 rpm. Temperature inside the gradient after centrifugation was 9°. Samples were analyzed for tryptophanase and catalase. Catalase was used as the reference standard and a sedimentation coefficient of 11.4 S for catalase was assumed for calculations. (b) Effect of high potassium phosphate concentration on the sedimentation coefficient of apotryptophanase from *B. alvei* at low temperature. Sucrose solutions were prepared in 0.10 *M* potassium phosphate (pH 7.6). Temperature inside the gradient was 9°. (○) Tryptophanase activity distribution; (●) protein distribution which was determined colorimetrically by a modification of Lowry et al. (1951).

SW-39 rotor in a Spinco Model L ultracentrifuge. Temperatures varied from 3 to 33° inside the chamber and from 9 to 35° inside the centrifuge tube. Temperatures stated refer to the temperature of the gradient. A blank gradient was included in each centrifuge run for temperature measurement immediately after termination of centrifugation. Speed varied from 28,000 to 33,000 rpm depending upon temperature. Apoenzyme or holoenzyme (90–140 μg of protein in 25–60 μl volume) was layered on the gradient along with a marker enzyme. Fractions containing eight drops (approximately 160 μl) were collected and assayed for tryptophanase, catalase, or lactate dehydrogenase activity and protein. Samples (25–100 μl) were assayed for tryptophanase activity by a modification of the method of Pardee and Prestidge (1961). Fractions from gradients run with apoenzyme in the absence of pyridoxal-P were assayed for tryptophanase activity after preincubation in the presence of 2.6×10^{-4} *M* pyridoxal-P. In samples from gradients run in the presence of tryptophan but in the absence of pyridoxal-P, the tryptophanase reaction was initiated by the addition of pyridoxal-P; 100-μl samples were assayed for protein by the method of Lowry et al. (1951).

Marker Enzymes. Lactate dehydrogenase was included as the marker enzyme in all experiments in which 0.02 *M* Tris buffer (pH 7.8) (supplemented with 0.002 *M* EDTA and 0.005 *M* β-mercaptoethanol) was used. For all other gradients catalase was used as marker enzyme; 25–50 μg of marker enzyme was layered on each gradient.

Lactate dehydrogenase was used as a reference standard with a sedimentation coefficient of 7.2 S. Lactate dehydrogenase activity was measured by following the decrease in absorbance at 340 nm using 3 ml of a reaction mixture containing 6×10^{-4} *M* sodium pyruvate and 1.5×10^{-4} *M* NADH in 0.1 *M* KPO_4 (pH 7.0). The reaction was initiated with 20 μl of enzyme fraction. Activity was recorded as change in absorbance at 340 nm per min per 10 μl of enzyme fraction.

Catalase was used as a reference standard, and a sedimentation coefficient of 11.4 S assumed for calculations. Martin and Ames (1961) reported that the 11.4S value does not vary over the temperature range used in these experiments. Catalase activity was measured by the method of Martin and Ames (1961) and calculated as change in absorbance at 240 nm per min per 10 μl of enzyme fraction.

Fluorescence Quenching. Fluorescence quenching experiments were carried out as described by Eisen (1964). Data were corrected for dilution, fluorescence of the blank, and fluorescence attenuation. Attenuation was measured by adding appropriate concentrations of coenzyme to solutions of tryptophan or catalase.

Circular Dichroic (CD) Measurements. Circular dichroic measurements were made using a Jasco UV 5 ORD/CD recording spectropolarimeter. Apotryptophanase diluted to 0.5 mg/ml in 0.01 *M* and 0.06 *M* KPO_4 (pH 7.8) was scanned with emphasis on the region from 260 to 290 nm. Apotryptophanase diluted to 0.1 mg/ml in 0.01 *M* and 0.06 *M* KPO_4 (pH 7.8) was scanned with emphasis on the 205–240-nm region. All measurements were made at 25°. Data were reported in terms of change in absorbance.

Results

Sedimentation Value for Apotryptophanase at Low Protein Concentration. Sucrose density gradient centrifugation shows that apotryptophanase in 0.01 *M* potassium phosphate (pH 7.7) at 9° sediments primarily as a 5.7S dimer (Figure 1a). From 5 to 10% of the tryptophanase activity sediments as tetramer; the percent of residual tetramer was consistent for eight different preparations of apoenzyme. The protein profile parallels the activity profile except for two small peaks at 3.8 S and 1.6 S suggesting the presence

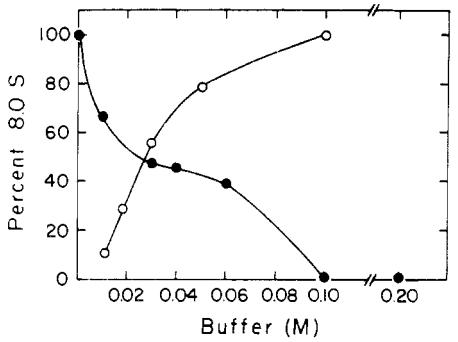

FIGURE 2: Competitive effects of potassium phosphate and imidazole on percent of tryptophanase activity sedimenting at 8.0 S. Conditions as in Figure 1. Temperature was 9-10°. (○) Values obtained for enzyme layered on gradients prepared in increasing concentrations of potassium phosphate, pH held constant at 7.7; (●) gradients prepared in 0.1 *M* potassium phosphate supplemented with increasing concentrations of imidazole, pH held constant at 7.7.

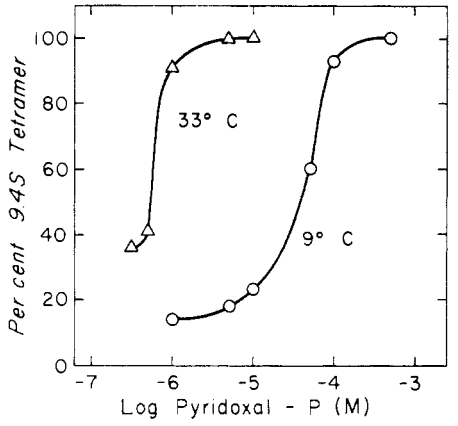

FIGURE 3: Effect of varying the concentration of pyridoxal-P on the association of the 5.7S dimer form of apotryptophanase to the 9.4S tetramer at 9° (○) and at 33° (△). Individual sucrose gradients were prepared in 0.01 *M* potassium phosphate (pH 7.7) supplemented with the appropriate concentrations of pyridoxal-P. At 9°, centrifugation was for 16 hr at 32,000-33,000 rpm. At 33°, centrifugation was for 16 hr at 28,000-29,000.

of species smaller than dimer which are not reactivated in the presence of excess pyridoxal-P. Raising the temperature from 9 to 25 or 33° did not affect the sedimentation profile.

Dependence of s Value on Potassium Phosphate Concentration. When *B. alvei* apotryptophanase, stored in 0.01 *M* potassium phosphate, was centrifuged 16 hr in gradients containing higher ionic strength buffer (0.1 *M* potassium phosphate (pH 7.6)), a single symmetrical peak of activity at 8.0 S was obtained. Protein distribution paralleled the activity profile with an additional shoulder at 5.7 S and two small peaks at 3.7 S and 1.9 S (Figure 1b). When apoenzyme was dialyzed for 72 hr at 4° against 0.1 *M* potassium phosphate (pH 7.7) prior to being layered on the gradient, the same results were obtained. Substitution of either ammonium or sodium phosphate for potassium phosphate also failed to modify the sedimentation value.

The increase in sedimentation coefficient varied as a function of potassium phosphate concentration with 50% of the enzyme sedimenting at 8.0 S at 27 m*M* potassium phosphate (Figure 2). Apoenzyme centrifuged in 0.01 *M* potassium phosphate gradients supplemented with either 0.1 *M* potassium or ammonium chloride sedimented at 5.9 S and 5.7 S, respectively, implying that the increase in sedimentation value was not due to high monovalent cation concentration. To further test the role of phosphate ions, imidazole phosphate buffer was used. To obtain a 0.05 *M* phosphate concentration at pH 7.4, the imidazole concentration had to be 0.25 *M*. Apoenzyme in imidazole phosphate sedimented at 5.9 S. Further investigation showed that imidazole not only competed with the potassium phosphate effect (Figure 2) but also dissociated holotryptophanase.

Table I: The Effect of Temperature on Sedimentation Value.

Temp (°C)[a]	Sedimentation Velocity (S)	Temp (°C)[a]	Sedimentation Velocity (S)
9.0	7.60	21.5	8.47
14.0	7.80	26.0	9.45
16.0	8.18	26.5	9.45
17.0	8.27	33.0	9.42

[a] Temperature was determined in the following manner. A blank gradient was run along with sample gradients. Immediately upon removal of the rotor from the centrifuge, a thermometer was inserted into the gradient, and the temperature recorded.

Table II: Effect of Monovalent Cations on Distribution of the Dimer and Tetramer Forms of Tryptophanase in Sucrose Density Gradients.

Sample Applied to Gradient	Sedimentation Velocity in 0.02 *M* Tris-HCl, 0.002 *M* EDTA, and 0.005 *M* β-mercaptoethanol at pH 8.0 (S)		
	No Additions	+0.1 *M* KCl	+0.1 *M* NaCl
B. alvei			
Apotryptophanase	5.2	5.1	5.5
Holotryptophanase[a]	5.3	5.4	5.5
Holotryptophanase[b] in 10^{-4} *M* Pyridoxal-P	9.0 (5.7)[c]	9.6 (6.0)	6.9
E. coli[d]			
Apotryptophanase	8.3	6.3	6.4
Holotryptophanase	8.3	9.8	6.5

[a] Gradients were not supplemented with pyridoxal-P. [b] Gradients were supplemented with 10^{-4} *M* pyridoxal-P. [c] The major component was tetramer but dimer was also present. A concentration of 10^{-3} *M* pyridoxal-P was needed to completely overcome the dissociating effect of Tris (Gopinathan and DeMoss, 1968. [d] Morino and Snell (1967).

Effect of Temperature on the Potassium Phosphate Effect. When the temperature at which high potassium phosphate (0.1 *M*, pH 7.8) gradients were centrifuged was varied, the *s* value for apoenzyme changed from 7.6 S at 9° to 9.4 S at 26°. Above 26°, a constant value of 9.4 S was obtained (Table I).

Conversion of Dimer to Tetramer as a Function of Pyridoxal-P Concentration. The extent of association to tetramer as a function of concentration of pyridoxal-P was measured at both 9 and 33° (Figure 3). The concentration of pyridoxal-P required for aggregation is temperature dependent. A 100-fold higher concentration of coenzyme was required at 9° (5×10^{-4} *M*) than was needed at 33° (5×10^{-6} *M*) for 50% conversion to the 9.4S species.

Effect of Sodium Ion on the Binding of Pyridoxal-P. When *B. alvei* holotryptophanase was centrifuged in Tris gradients, 10^{-4} *M* pyridoxal-P was not sufficient to overcome completely the dissociating action of Tris (Table II). In 0.1 *M* potassium chloride, 68% of the holotryptophanase activity sedimented as the tetramer. If, however, 0.1 *M* sodium chloride was used, no tetramer was observed. The relative effects of sodium and potassium on pyridoxal-P binding were investigated further (Figure 4). When apotrypto-

Table III: Structural Role of Kinetically Active Pyridoxal-P Analogs in the Conversion of 5.7S Dimer to 9.4S Tetramer.

Pyridoxal-P Analog (10^{-2} *M*)	Ability to Catalyze the 5.7S–9.4S Association[a]	
	Percent Dimer	Percent Tetramer
None	95	5
Pyridoxal-P	7	93
2'-Hydroxypyridoxal-P	32	68
2'-Methylpyridoxal-P	63	37
6-Methylpyridoxal-P	14	86
N-Oxide of pyridoxal-P	50	50

[a] The temperature inside the gradients was 9.5°.

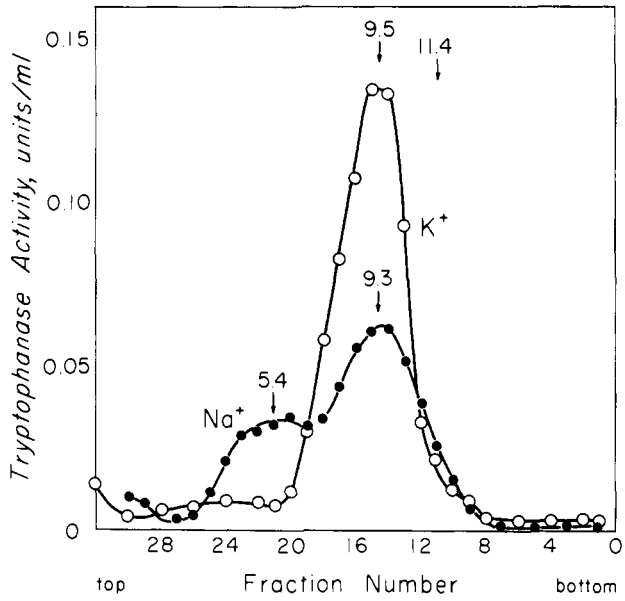

FIGURE 4: Effect of Na^+ as compared with K^+ on the ability of pyridoxal-P to induce the association of the 5.7S dimer form of apotryptophanase to 9.4S tetramer. Individual sucrose gradients were prepared in 0.01 *M* potassium phosphate (pH 7.7) supplemented with 10^{-4} *M* K^+ pyridoxal-P (○) and in 0.01 *M* sodium phosphate (pH 7.7) supplemented with 10^{-4} *M* Na^+ pyridoxal-P (●). Temperature was 9°. Curves were normalized to the catalase peak.

phanase was centrifuged in low potassium phosphate, supplemented with 10^{-4} *M* potassium salt of pyridoxal-P, association to tetramer was complete. In the presence of the sodium salt of pyridoxal-P, only 64% of the activity sedimented at 9.4 S. Decreased affinity of sodium apoenzyme for the coenzyme was also measured kinetically. The K_m for sodium apoenzyme is 17 μM which is tenfold higher than the K_m of potassium apoenzyme (Isom and DeMoss, 1975). The V_{max} values show that the sodium enzyme has only 35% the activity of the fully activated reconstituted potassium enzyme.

Structural Role of Catalytically Active Pyridoxal-P Analogs in Dimer–Tetramer Conversion. Apoenzyme was centrifuged in 0.01 *M* potassium phosphate supplemented with 10^{-4} *M* concentration of various pyridoxal-P analogs at 9.5°. As indicated in Table III, all of the catalytically active analogs tested (Isom and DeMoss, 1975) induced association to 9.4 S but to different extents. 6-Methylpyridoxal-P was almost as effective structurally as pyridoxal-P, while the *N*-oxide and 2'-methyl analogs were considerably less active.

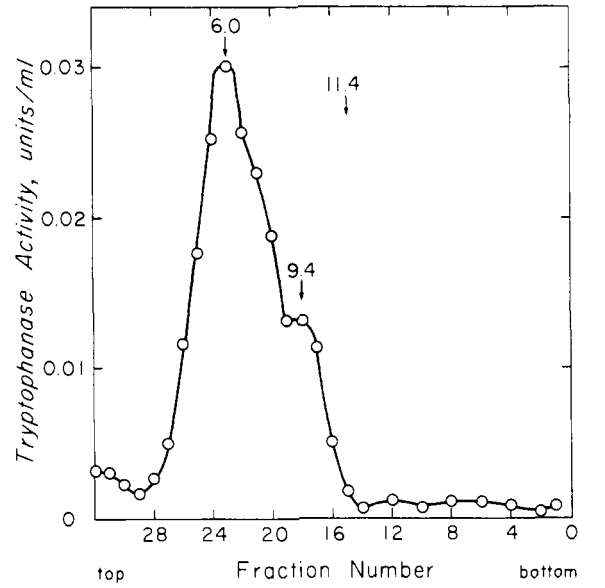

FIGURE 5: Dissociative effect of pyridoxal on the high potassium phosphate induced 8.0S tetramer form of apotryptophanase from *B. alvei*. Individual sucrose gradients were prepared in 0.1 *M* potassium phosphate (pH 7.8) supplemented with 10^{-2} *M* pyridoxal. Temperature was 9°.

Structural Role of Pyridoxal and Other Kinetically Inactive Pyridoxal-P Analogs. Since pyridoxal binds to apotryptophanase (Isom and DeMoss, 1975), its structural effect was tested over a range from 10^{-4} to 10^{-2} *M*. Supplementing low potassium phosphate (0.01 *M*, pH 7.8) gradients with pyridoxal had no aggregational effect; the enzyme sedimented at 5.7 S. Supplementing high potassium phosphate (0.1 *M*, pH 7.8) gradients with pyridoxal not only failed to increase the sedimentation value to 9.4 S, but rather, interfered with the phosphate effect such that 76% of the activity was found at 6.0 S and 24% as a shoulder in the 9.4S region (Figure 5). Centrifugation of apotryptophanase in high potassium phosphate gradients supplemented with the catalytically inactive analogs 4-deoxypyridoxine-P (10^{-2} *M*) and pyridoxamine-P (5×10^{-3} *M*) failed to show any interference by these two analogs with the high phosphate effect. Single symmetrical peaks were seen at 7.9 S and 8.1 S, respectively. These compounds also failed to alter the sedimentation profile in low potassium phosphate gradients.

Pyridoxine, pyridoxamine, 4-deoxypyridoxine, and isopyridoxal-4'-P were also unable to convert dimer to tetramer. Concentrations from 10^{-4} to 10^{-2} *M* analog were tested in low potassium phosphate.

Substrate and Competitive Inhibitor Induced Structural Changes. Since pyridoxal-P which is bound to the active site is capable of inducing the association of apoenzyme subunits to tetramer, other ligands which bind at the active site were tested for their ability to cause association.

5 m*M* L-tryptophan (17-fold the K_m value) had no structural effect from 9 to 25° and in gradients with either low or high potassium phosphate.

1 m*M* anthranilic acid, a competitive inhibitor of tryptophan in the tryptophanase reaction (K_i of 0.1 m*M*, Hoch et al., 1966) had no structural effect in either low or high potassium phosphate gradients at 9.5°. However, when the temperature was raised to 25° and the inhibitor concentration increased to 5 m*M*, anthranilic acid converted dimer to tetramer. When apotryptophanase was centrifuged at 25°

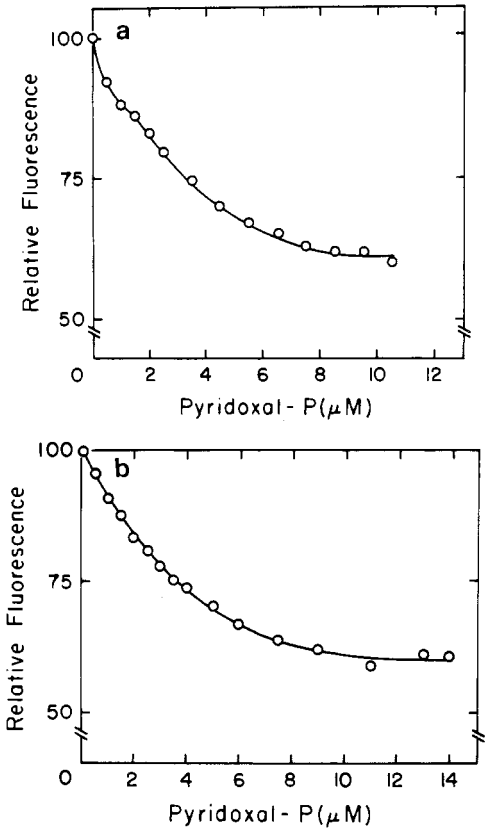

FIGURE 6: (a) Fluorescence quenching of 0.09 mg of *B. alvei* apotryptophanase/ml of 0.01 *M* potassium phosphate with pyridoxal-P. Excitation was at 280 nm; emission was measured at 350 nm 10 min after each addition. Temperature was 29°. Data were corrected for the fluorescence of the blank, for dilution, and for fluorescence attenuation, before plotting. (b) Fluorescence quenching of 0.09 mg of *B. alvei* apotryptophanase/ml of 0.10 *M* potassium phosphate with pyridoxal-P.

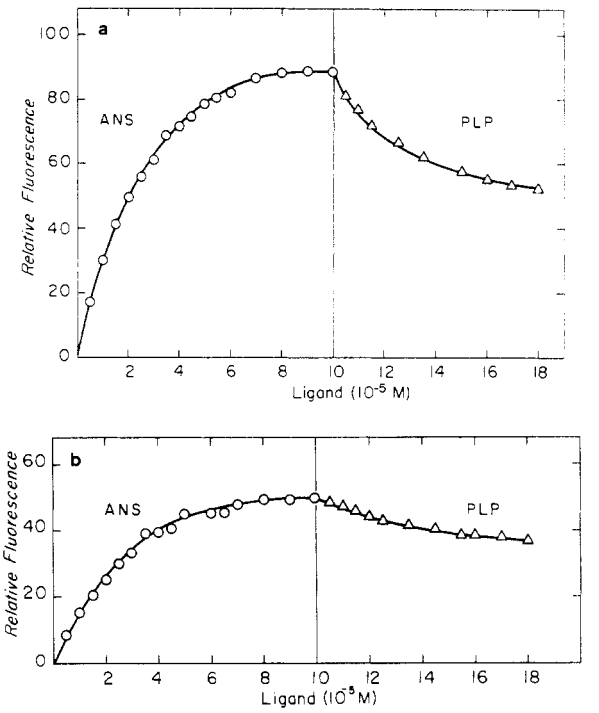

FIGURE 7: (a) Fluorimetric titration of 0.18 mg of apotryptophanase per ml of 0.10 *M* potassium phosphate (pH 7.8) with ANS followed by the effect of titration with pyridoxal-P on the apoenzyme–ANS fluorescence. Excitation was at 360 nm; emission was measured at 470 nm 10 min after each addition of ANS or pyridoxal-P. Temperature was 27°. At the final point, the concentration was 0.10 m*M* ANS and 0.08 m*M* pyridoxal-P. (○) ANS; (△) pyridoxal-P. (b) Fluorimetric titration of 0.18 mg of apotryptophanase per ml of 0.01 *M* potassium phosphate (pH 7.8) with ANS followed by the effect of titration with pyridoxal-P on the apoenzyme–ANS fluorescence. At the final point, the concentration was 0.10 m*M* ANS and 0.08 m*M* pyridoxal-P. (○) ANS; (△) pyridoxal-P.

in gradients supplemented with 5×10^{-3} *M* anthranilic acid and 10^{-7} *M* pyridoxal-P, association to tetramer was enhanced.

8-Anilino-1-naphthalenesulfonic acid (ANS), a competitive inhibitor of pyridoxal-P binding (Isom and DeMoss, 1975), is active in the association of dimer to 9.4S tetramer apoenzyme. When apotryptophanase was centrifuged at 9.5° in low potassium phosphate supplemented with 10^{-3} *M* ANS, 34% of the enzyme activity sedimented at 9.4 S while in high potassium phosphate with 10^{-3} *M* ANS, 52% of the activity was in the 9.4S band.

Spectral Differences in Apotryptophanase Samples. The *s* value of apoenzyme samples can be manipulated by adjusting temperature and potassium phosphate concentration. Difference spectroscopy and absolute fluorescence intensity values failed to distinguish between enzyme sedimenting at 5.7 S or at 8.0 S.

Circular dichroic measurements of apoenzyme (100 μg/ml) measured at 25° in both low (5.7S dimer predominates) and high potassium phosphate (9.4S tetramer) revealed differences in the optical activity of the two forms below 250 nm. Two bands with ellipticity minima at 216 and 207 nm were evident in the spectra of both species, but both bands were less intense when the 9.4S form predominated.

The fluorescent quenching patterns for the 5.7S and 9.4S forms also differ. In Figure 6a, the first four points of the titration curve for the 5.7S dimer constitute one curve which breaks at 1.6 μ*M* pyridoxal-P as the second curve begins. These results were reproducible for three preparations of apoenzyme. The curve for quenching of the 9.4S form is smooth and does not show this effect (Figure 6b).

Fluorescence Enhancement with ANS. ANS was also used to examine differences in the enzyme forms. After dilution into 0.1 *M* potassium phosphate (pH 7.8), apoenzyme in high potassium phosphate was titrated with increasing amounts of ANS until fluorescence enhancement plateaued (Figure 7a). The same experiment was carried out using apoenzyme diluted into low potassium phosphate (Figure 7b). A comparison of the data shows that the fluorescence enhancement when ANS was bound in low potassium phosphate was only 56% of that in high potassium phosphate; i.e., the fluorescence intensity of the ANS–dimer was one-half the fluorescence intensity of the ANS–tetramer complex. The binding constant, for ANS, determined fluorimetrically, varied from 1.1×10^{-5} to 3.0×10^{-5} *M* which was in agreement with the kinetically determined value of 3.1×10^{-5} *M*.

Under conditions of both low and high potassium phosphate, apoenzyme saturated with ANS was titrated with incremental amounts of pyridoxal-P. In high potassium phosphate, pyridoxal-P quenched the ANS fluorescence 41% (Figure 7a), while in low potassium phosphate, pyridoxal-P only quenched 25% (Figure 7b).

Discussion

Previously reported ultracentrifugation data showed that the sedimentation values for *B. alvei* apotryptophanase (0.01 *M* potassium phosphate (pH 7.0)) decreased with de-

creasing protein concentration, but the extent of dissociation was not determined (Hoch and DeMoss, 1972). In this paper we have shown that apoenzyme exists as a dimer; removal of pyridoxal-P is not sufficient to cause any significant dissociation to monomer.

The ease with which *B. alvei* apoenzyme dissociates and the failure of the tetramer form to aggregate to multiples of tetramer distinguish this enzyme from tryptophanases purified from other organisms. The *B. alvei* enzyme dissociates to dimer form under milder conditions than are required for the *E. coli* enzyme (Table II). Removal of pyridoxal-P is sufficient to cause dissociation without elevation of salt concentration and without reduction of temperature to 4°. The *B. alvei* enzyme, on freezing and thawing, does not aggregate to multiples of tetramers as was observed for purified tryptophanase from *Aeromonas liquefaciens* (Cowell and DeMoss, 1973). Preparations of *B. alvei* tryptophanase used in gradients were thawed 5–7 times and neither active (activity profile) nor inactive (protein profile) aggregates were ever observed.

We have shown in this paper that the active center, defined as the site where pyridoxal-P binds, is involved in the association process from dimer to tetramer. The types of associations reported can be subdivided into two classes: (1) those in which tetramer species produced are inactive and induced by high phosphate at 25°, or by millimolar anthranilic acid at 25°, and (2) those in which tetramer is catalytically active and induced by micromolar concentrations of pyridoxal or analogs of pyridoxal-P.

We have also reported that apoenzyme sediments as a catalytically inactive peak at 8.0 S in high potassium phosphate at low temperature. Enzyme at 8.0 S may represent a conformationally distinct form assumed by the enzyme during a step in the catalytic process or it may simply be an average peak representing the equilibrium position of a dissociating and reassociating dimer–tetramer system.

The interconversions of dimer, tetramer, and sedimentation of enzyme at 8.0 S are all influenced by small molecular weight compounds which bind at the active center. For example, a high concentration of phosphate ions favors association of dimers. It is known from previous work in this laboratory that the phosphate ester at the C_5 position of the coenzyme is important in the orientation of pyridoxal-P in tryptophanase (Isom and DeMoss, 1975) and with other enzymes the phosphate ester has been implicated in having a vital role in coenzyme binding (Furbish et al., 1969). If a site on the apoprotein interacts ionically with the phosphate group from the coenzyme, phosphate ions may also bind at this position. The mechanism may be to neutralize ionic groups so that dimer subunits previously repulsed by charge can readily associate. The ability of pyridoxal to compete with the phosphate association and to hold apoenzyme as dimers suggests that pyridoxal is unable to neutralize the charges repulsing dimers and is oriented at the active site so that it blocks the action of phosphate ions.

A second example of a small molecule acting at the active center in the association of dimer to tetramer is the sodium ion. We have found that although sodium has only a slightly different hydrated radius from potassium, sodium alters by an order of magnitude the ability of pyridoxal-P to bind to apoenzyme and reduces the V_{max} to 30–35% of fully activated enzyme. Recent studies using circular dichroism indicate differences in the spectral behavior of the sodium form of *B. alvei* holotryptophanase as compared to the potassium form (Fenske and DeMoss, manuscript in preparation). The ability of anthranilic acid and ANS, which compete for substrate and coenzyme binding, to cause association is further support for the involvement of the active center in subunit association.

Even a slight modification of the coenzyme molecule, as seen in the analog experiments, can cause a major difference in its effectiveness on dimer association. For example, we have found that association of dimers has an absolute requirement for an intact 4′-formyl group. *B. alvei* dimers do not form tetramers in the presence of pyridoxamine-P or pyridoxine-P (10^{-2} *M*) which is in contrast to the data reported for the *E. coli* apotryptophanase for which both pyridoxamine-P and pyridoxine-P function as associative agents (Morino and Snell, 1967).

Examination of the structurally active analogs reveals that the degree of effectiveness of each is not simply a function of the affinity of the apoprotein for the analog. Each analog was used at concentrations greater than tenfold the K_m. No correlation exists between the K_m value and the relative structural ability. The *N*-oxide of pyridoxal-P has the highest K_m, but the 2′-methyl analog is the least effective structurally.

The differences in CD spectra and fluorescence quenching are small but indicate that the 5.7S and 9.4S forms can be distinguished spectrally. A more striking distinction between the properties of dimer and tetramer was observed with ANS. The dye, ANS, which is only weakly fluorescent in a hydrophilic environment, becomes highly fluorescent with a blue shift in its emission maximum in nonpolar solvents or upon binding to hydrophobic regions of proteins. Excitation of ANS free in solution at 360 nm resulted in a weak fluorescence emission peak at 520 nm. When ANS bound to apoenzyme, the fluorescence intensity was enhanced and the emission band shifted to 480–485 nm. From the spectral shift and enhancement we know that ANS binds to hydrophobic regions in both the 5.7S and 9.4S forms. However, the maximum intensity for ANS–tetramer was twofold that of ANS–dimer suggesting that either (1) the environment surrounding ANS binding sites in the tetramer is more hydrophobic than in the dimer, or (2) additional hydrophobic sites to which ANS can bind become available in the tetramer.

When pyridoxal-P was added to ANS–tetramer, the fluorescence was quenched and the spectral emission maximum shifted to a 500-nm band which is characteristic of the apoprotein–pyridoxal-P complex. Pyridoxal-P has a greater affinity and causes ANS to be released resulting in a decrease in fluorescence enhancement. The fluorescence data confirm the kinetics which showed that ANS is a competitive inhibitor of pyridoxal-P binding with a 20-fold higher K_i than the K_m for pyridoxal-P.

Competitive kinetics, ability to induce the 5.7S to 9.4S conversion, and fluorescence competition all indicate that ANS and pyridoxal-P compete for the same sites. Since ANS binds at the active center and fluoresces, we can conclude that within the active center of *B. alvei* tryptophanase, a hydrophobic region exists.

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Tryptophan and Cysteine Residues of the Acetylcholine Receptors of *Torpedo* Species. Relationship to Binding of Cholinergic Ligands†

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ABSTRACT: Several methods were used to analyze for tryptophan in the acetylcholine (ACh) receptors purified from the electric organs of the electric rays, *Torpedo californica* and *Torpedo marmorata*. The best value of tryptophan was 2.4 mol %. When excited at 290 nm, both receptors fluoresced with a maximum at 336, but there was no change in the fluorescence emission spectra upon binding of carbamylcholine, *d*-tubocurarine, ACh, or decamethonium. The free SH content of the *Torpedo* receptors varied in different preparations, and was highest in that purified from fresh *T. californica* using deaerated solutions and dialysis under nitrogen, and lowest in that prepared from the aged lyophilized membranes of *T. marmorata*. The maximum free SH content was 20 nmol/mg of protein or 0.22 mol %, equal to at most 18% of the total cysteic acid residues. Reaction of either 33% or of all the SH residues with *p*-chloromercuribenzoate reduced maximum ACh binding to the pure receptor prepared from fresh *T. californica* by only 23%.

Tryptophan and cysteine residues are of particular importance in studies of the relationship between the structure and function of proteins. This is both because they are present in relatively small amounts in most proteins and because reagents have been developed which react specifically with each of these amino acids. In addition, tryptophan has an intense fluorescence, which is very sensitive to its local environment, so that changes in conformation often result in changes in the tryptophan fluorescence.

Acetylcholine (ACh)[1] receptors have been purified from the electric organs of four fish species (Karlsson et al., 1972; Olsen et al., 1972; Klett et al., 1973; Schmidt and Raftery, 1973; Eldefrawi and Eldefrawi, 1973a; Biesecker, 1973; Karlin and Cowburn, 1973; Chang, 1974; Meunier et al., 1974; Lindstrom and Patrick, 1974; Michaelson et al., 1974; Ong and Brady, 1974) and their amino acid compositions determined in those of the electric ray, *Torpedo marmorata* (Eldefrawi and Eldefrawi, 1973a; Heilbronn and Mattson, 1974), *Torpedo californica* (Michaelson et al., 1974), and *Torpedo nobiliana* (Moore et al., 1974), as well as the electric eel, *Electrophorus electricus* (Klett et al., 1973; Meunier et al., 1974). Because of the difficulties involved in the analysis of tryptophan and the interference caused by detergents that are present in all these receptor preparations, only a few analyses of tryptophan were made. Its presence was detected in three of these studies (Eldefrawi and Eldefrawi, 1973a; Meunier et al., 1974; Moore et al., 1974), and not in a fourth (Klett et al., 1973).

In this paper, we use the ACh receptor purified from *T. californica* and compare it with that from *T. marmorata*. This *T. californica* receptor has been characterized immunologically. Rabbits and rats, immunized with this protein, developed skeletal neuromuscular defects, which seem to result from an impairment of their own ACh receptor function (Sanders et al., 1975). Similar observations were previously made on the ACh receptor purified from *Electrophorus electricus* (Patrick and Lindstrom, 1973; Sugiyama et al., 1973). This receptor has been reconstituted into black lipid membranes and carbamylcholine shown to induce monovalent cation selective conductance (Eldefrawi et al., 1975). The ACh receptor purified from the same *californica* species had also been reconstituted into lipid vesicles, and, in some preparations, activators were shown to accelerate ^{22}Na efflux (Michaelson et al., 1974). We report on the cysteic acid residues and free SH groups of the *californica* receptor and the effect on ACh binding of modifying them. Because of the very low content of detergent in our receptor

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[1] Abbreviations used are: ACh, acetylcholine; SH, sulfhydryl; PCMB, *p*-chloromercuribenzoate; NbS_2, 5,5′-dithiobis(2-nitrobenzoic acid).