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Studies on the Association of β-Chain Monomers of *Escherichia coli* Tryptophan Synthetase*

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ABSTRACT: Using the technique of sedimentation–diffusion equilibrium we have found measurable quantities of β-chain monomers, dimers, and possibly higher multimers in solutions of the B protein of *Escherichia coli* tryptophan synthetase. The multimers are in equilibrium; association constants for the monomer–dimer (K_2) and monomer–dimer–trimer (K_3) are estimated at 10.4 ± 0.5 and 1.4 ± 0.5 l./g, respectively, in 0.1 M potassium phosphate at pH 7.3. The association constant K_2 is markedly pH dependent over the range 6.5–9.5, and this change is reversible. K_2 is independent of temperature and ionic strength in the range 6–37.5° and up to $\Gamma/2 = 0.5$. We determined the distribution at sedimentation equilibrium of macromolecular species absorbing at 280, 335, and 415 nm. At 335 and 415 nm (absorption maxima due to the enzyme–cofactor complex) with low protein concentrations the solution appeared monodisperse at the dimer molecular weight of 89,000 daltons, suggesting that the monomer does not bind the cofactor. The monomer–dimer equilibrium is strongly affected by the presence or absence of cofactor, however.

Tryptophan synthetase from *Escherichia coli* is a heteropolymer separable into two components. Each of the separated components (termed A and B) will catalyze a partial reaction in the production of L-tryptophan from indoleglycerol phosphate and L-serine. It is well known that physical interaction between the A and B subunits takes place and that concomitant with this interaction a 10–100× increase is seen in the rate of each half-reaction (Crawford and Yanofsky, 1958). The A protein requires no cofactor, and contributes that portion of the active site of tryptophan synthetase involved in removal of the glycerol phosphate side chain from indoleglycerol phosphate. Primary amino acid sequence and molecular weight determinations have shown it to be a single polypeptide (α) of 28,700 daltons (Yanofsky *et al.*, 1967).

The B protein binds 2 moles of pyridoxal phosphate, and is involved in that part of the reaction attaching the alanyl moiety of L-serine to the indole portion of indoleglycerol phosphate. It is a homologous dimer (β_2) and although its primary sequence is not yet known, its molecular weight has been estimated to be 90,000 daltons (Hathaway *et al.*, 1969).

Previous investigations of the B protein in our laboratory showed it to be an associating system, with an association constant (K_2) for the monomer–dimer equilibrium not too large to preclude its measurement by presently available experimental techniques. This paper is concerned with the measurement of this type of interaction under various experimental conditions. Equations used in this work can be found in the Appendix.

Materials

Tris(hydroxymethyl)aminomethane, pyridoxal 5′-phosphate, and β-mercaptoethanol were purchased from the Sigma Chemical Co. Guanidine hydrochloride "ultra-pure" was obtained from Mann Research Laboratories. Doubly deionized or glass distilled, deionized water was used in all experiments. All chemicals were reagent grade and were used without further purification.

Methods

Analytical Gel Electrophoresis. Sodium dodecyl sulfate–acrylamide disc electrophoresis was performed using 7% gels essentially following the procedure of Shapiro *et al.*

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(1967) with the following modifications. (1) Reservoir buffer was 0.1 M sodium phosphate, pH 8.2, containing 1% sodium dodecyl sulfate. (2) Iodoacetamide was not used but dithioerythritol was added to the protein samples (1–2 mg of protein/ml, 0.01 *M* dithioerythritol).

Bovine serum albumin, myoglobin, lactic dehydrogenase, ovalbumin, and pepsin were used as standards of known molecular weight.

Preparation of Buffers. Potassium phosphate buffers were prepared as 1 M stock solutions by mixing 1 M monobasic and 1 M dibasic potassium phosphate until the desired pH was attained. Tris buffers were prepared by dissolving 12.1 g of the base in 900 ml of water, adjusting to pH with concentrated acid, and then bringing to 1 l. final volume. Tris–glycine buffer was prepared in the same way except that solid glycine was added to obtain the final pH.

Purification of Enzyme. The procedure for purifying B protein from *E. coli* strain A2/F'A2 has been described elsewhere (Wilson and Crawford, 1965). A protein was prepared from *E. coli* B8 following the procedure of Henning *et al.* (1962). All preparations showing residual contamination on analytical disc gels were further purified by preparative gel electrophoresis employing a Shandon apparatus and a Tris–glycine discontinuous pH system (Sulitzeanu *et al.*, 1967). Crystalline A protein was found to contain 5–10% of a contaminating protein which was removed by preparative electrophoresis but not by chromatography on Sephadex G-150. B apoenzyme was prepared according to Miles *et al.* (1968).

Tryptophan synthetase AB complex was prepared by mixing stoichiometric quantities of ammonium sulfate suspensions of the purified proteins. The mixture was centrifuged for 20 min at 39,000*g* and resuspended in 0.1 M potassium phosphate buffer, pH 7.2, containing 1.0 mM β-mercaptoethanol and 5 μg/ml of pyridoxal phosphate. The complex was separated from an excess of either component by upward-flow chromatography on a 2.5 × 30 cm, G-150 Sephadex column equilibrated with 0.1 M potassium phosphate buffer, pH 7.2, supplemented only with 1.0 mM β-mercaptoethanol. Elution was accomplished with this buffer. Fractions from the most rapidly eluting peak were pooled, precipitated with $(NH_4)_2SO_4$, and dialyzed overnight against pyridoxal phosphate supplemented elution buffer. The complex has also been prepared by including 0.1 M NaCl and 0.15 mM L-serine in the mixing step and 0.1 M NaCl in the elution buffer.

Protein Determination. Protein concentrations were determined both by absorption at 280 nm and colorometrically (Lowry *et al.*, 1951). The specific absorbancy of the B apoenzyme at 280 nm ($\epsilon_{1\text{ cm}}^{1\%}$) was redetermined and found to be 5.8 ± 0.2, in good agreement with that obtained earlier (Wilson and Crawford, 1965). The specific absorbancy of holoenzyme in 0.05 M potassium phosphate buffer, pH 7.3, containing 1 μg/ml of pyridoxal phosphate and 10^{-3} M β-mercaptoethanol was also determined, as well as the ratio of absorbance at 280 nm to that at 300, 335, and 415 nm (see Results). Volumetric dilutions and transferrals were made using gravimetrically calibrated micropipets or a Gilmont S-1300 ultra precision micrometer syringe. Dry weight comparisons between protein solution and dialysate were made using micro weighing vessels. The solutions were heated over phosphorus pentoxide in an atmosphere of dry N_2 at 80° for 12 hr. The oven was then evacuated and heated at 110° until constant weight was obtained. Color development of anhydrous B apoenzyme relative to standard bovine serum albumin solutions was also evaluated in this way.

Conformity to Beer's law was checked at 280 and 414 nm. In all cases Beer's law was obeyed.

Analytical Ultracentrifugation. Protein solutions to be analyzed by equilibrium centrifugation were always dialyzed 18–36 hr, with agitation. Buffer was generally changed once (500–1000 volumes) during the first 6 hr. Following dialysis, the solution was centrifuged for 30 min at 39,000*g*. The clear supernatant was diluted to the desired absorbance using the equilibrated dialysate buffer.

The Spinco Model E analytical ultracentrifuge was equipped with a scanner, ultraviolet optics, and multiplexer (Lamers *et al.*, 1963). Three samples were centrifuged simultaneously using charcoal filled epon, double sector centerpieces. Sapphire windows were used routinely. Radial velocities were selected to give distributions with σ_w values about one-half those suggested by Yphantis (1964) for high speed centrifugation. We felt this minimized convection, provided a wide concentration range, and facilitated meniscus clearing at the end of each run.

The time required to approach equilibrium was greatly reduced by using the overspeed technique of Richards *et al.* (1968). Protein distributions often appeared near equilibrium after only 8 hr and never gave $c_b:c_a$ ratios predicted for a monodisperse solution. At the end of a run, rotor speed was increased to 60,000 rpm and held for 1–2 hr. A final scan was then made after deceleration to the experimental velocity in order to make slight base-line corrections. Sucrose was often included in the buffers to prevent convection. Disconnecting the heater circuit also helped in this regard. A low rate of cooling could then be balanced against the heat conduction from the drive unit. In this way, temperature variations were reduced to ±0.1° over a 12-hr period.

The optical path length of the assembled cell was measured with a micrometer, and found to be 1.18 cm. Absorbances were measured using the scanner's calibration stairsteps as suggested by Chervenka (1969). Protein concentration at equilibrium was then determined by the equation:

$$c = \frac{\text{absorbance}}{\epsilon_{1\text{cm}}^{1\%} \times 0.12\text{ cm}}$$

We used 0.748 ml/g for the partial specific volume of all associating species except as noted in Table I (Wilson, 1965). Tangents were determined by a least-squares fit to closely spaced data and integration was performed using Simpson's one-third rule. All calculations were carried out using a programmable Hewlett–Packard 9100 A calculator.

Reversibility of pH Effect. Demonstration of the reversibility of pH effects was accomplished by dialyzing apoenzyme 18 hr against Tris-Cl buffers, pH 9.45 and pH 7.74. A portion was then removed from the dialysis bag at pH 9.45 and dialyzed another 18 hr against the pH 7.74 buffer. All three samples were brought to equilibrium under identical centrifuge conditions in a single experiment using a four-place rotor.

Results

Evaluation of the Monomer Molecular Weight. A rough estimate of the monomer molecular weight (M_1) can be

TABLE I: Determination of the Monomer Molecular Weight (M_1).

Experimental Method	Procedure	Pauci-dispersity	M_1 Obsd
Sedimentation equilibrium	lim M_w^a $c \to 0$	+	44,000 ± 3000
Sedimentation equilibrium	Eq 6	+	43,800 ± 2100[a]
Sedimentation equilibrium	5 M Gu·HCl	−	44,057 ± 1200[b]
Sedimentation equilibrium	5–8 M urea	−	44,500 ± 1640[b,d]
Sedimentation equilibrium	Tris-Cl, pH 9.3	−	43,675 ± 1340[c]
Sedimentation equilibrium	Tris-glycine, pH 8.9	Slight	45,800 ± 2500
Sedimentation equilibrium	Performate	Slight	45,000 ± 4500[d]
Polyacrylamide electrophoresis	Urea	+	45,000 ± 1350[d]
Polyacrylamide electrophoresis	Sodium dodecyl sulfate	+	42,000 ● 2500
Average			44,203 ± 1076[e]

[a] Average of two experiments. [b] Using $\bar{v}$ = 0.737 from amino acid analysis (Wilson, 1965). [c] Average of four experiments. Standard deviation shown. [d] Data from Hathaway *et al.* (1969). [e] Standard deviation shown.

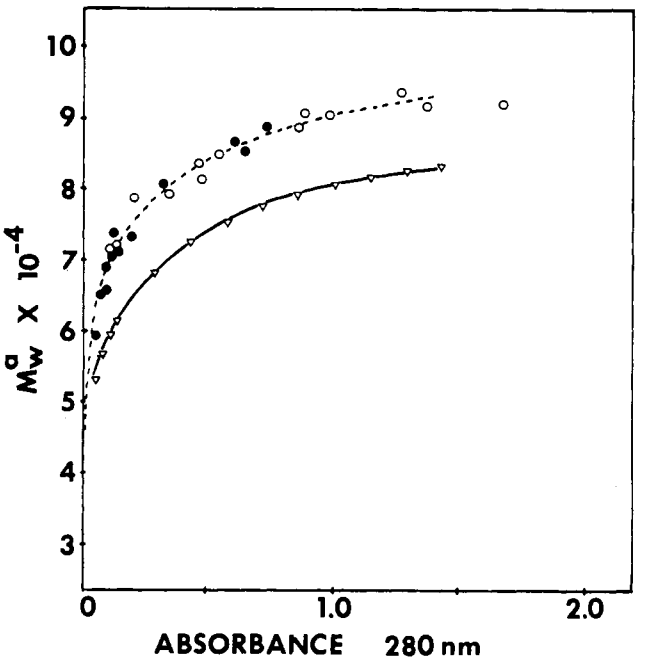

FIGURE 1: Equilibrium centrifugation of B protein in 0.1 M potassium phosphate buffer, pH 7.3, supplemented with 10^{-3} M β-mercaptoethanol and 5 μg/ml of pyridoxal phosphate. M_w^a recorded at 280 nm; initial absorbance 0.21 (—●—); recorded at 280 and 290 nm with initial absorbance 1.1 (—○—). Absorbance values at 290 nm were converted into 280 nm using the 280:290 ratio 2.03; number-average molecular weight (M_n^a) (—▽—). The dashed line represents the best fit of the data using K_2 = 10.4 l./g and K_3 = 1.35 l./g in eq 2. F_1 as a function of concentration was obtained using Steiner's equation (see Appendix).

gained simply by extrapolating M_w^a values obtained by eq 1 to zero concentration. These values generally fell in the range 43,000 to 45,000 (Figure 1) but were given little weight because of the uncertainty inherent in the extrapolation. A somewhat better value was obtained using number- and weight-average molecular weights and eq 6. This value, at best, is probably good to about 5% (Yphantis, 1964). M_1 was also obtained by sedimentation equilibrium under denaturing conditions, and by sodium dodecyl sulfate–acrylamide gel experiments. (On sodium dodecyl sulfate–polyacrylamide, two lighter bands representing approximately 10–15% of the total stainable material appeared in positions corresponding to molecular weights of 29,000 and 14,000 daltons. These bands had never been observed previously in regular or urea-containing gels at several acrylamide concentrations.) In addition, it was discovered that the apoenzyme dissociates completely in 0.1 M Tris-Cl buffer above pH 9 at protein concentrations up to 0.2% (Figure 5). The results of these experiments are summarized in Table I. We estimated M_1 as 44,500 and used this value in subsequent calculations.

Demonstration of Equilibria. The concentration dependency of M_w^a can be demonstrated by achieving sedimentation equilibrium at two rotor speeds or by varying the initial concentration of protein at a single speed (Yphantis, 1964; Hathaway *et al.*, 1969). The latter experiment must be performed at constant column height and constant radial distance, *i.e.*, $(b - a)$, and a must not vary between samples, except in the special case of negligible concentration at the meniscus. By choosing to vary the speed of the experiment (in a single run) one eliminates these requirements. Figure 2

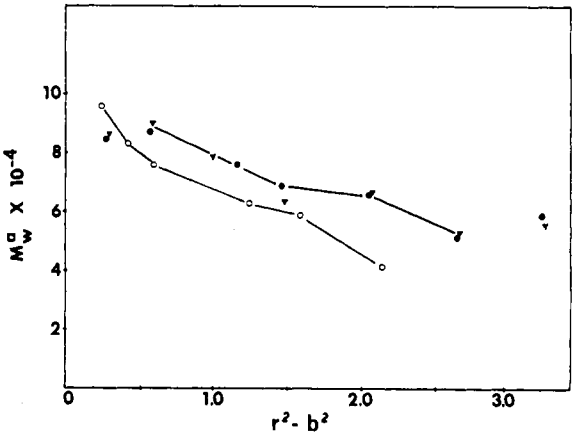

FIGURE 2: Sedimentation equilibrium was obtained at two rotor speeds, 13,000 and 18,000 rpm: equilibrium at 13,000 (—▼—), (—●—); 18,000 (—○—). Solvent was 0.2 M potassium phosphate buffer, pH 7.4, plus 10^{-3} M β-mercaptoethanol and 5 μg/ml of pyridoxal phosphate. Temperature was 16°. Total run time was 56 hr.

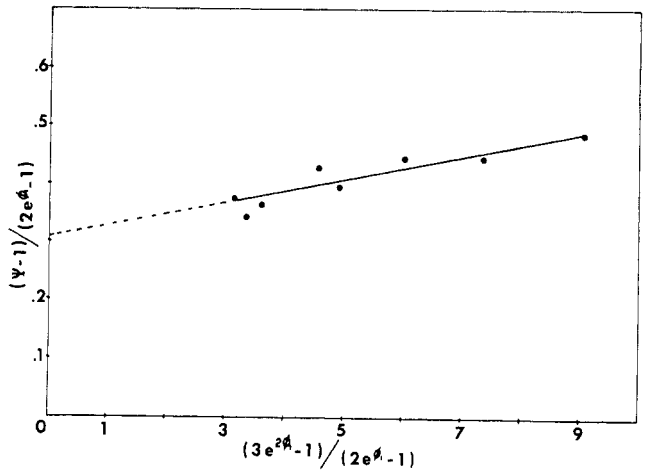

FIGURE 3: Test for the nature of the association: B holoenzyme in 0.2 M potassium phosphate buffer, pH 7.5, containing 10^{-3} M β-mercaptoethanol, 5 μg/ml of pyridoxal phosphate, and 0.5% sucrose. A monomer–dimer association would have given a horizontal line.

TABLE II: Variation of Monomer–Dimer (K_2) and Monomer–Dimer–Trimer (K_3) Association Constants with pH.

Enzyme	pH	K_2 (l./g)	K_3 (l./g)
Holoenzyme[a]	7.3	10.4[b]	1.4[b]
Apoenzyme[c]	6.7	14.5	14.0
Apoenzyme	7.1	8.3	8.8
Apoenzyme	7.5	3.1	7.3
Apoenzyme	8.1	2.6	5.6
Apoenzyme	8.5	2.0	4.5
Apoenzyme	8.8	1.8	1.1
Apoenzyme	9.5	*d*	*d*

[a] Concentrations determined using 280 nm specific absorbancy $\epsilon^{1\%}_{1cm} = 6.5$. Data from Figure 1. [b] A best fit to data was made using eq 2 and assuming various values of K_2 and K_3. [c] Concentrations determined using 280 nm specific absorbancy $\epsilon^{1\%}_{1cm} = 5.8$. Conditions may be found in the legend to Figure 5. [d] Not observable.

shows the results of an experiment in which equilibrium was achieved at a relatively low rotor speed followed by a shift to a higher radial velocity. After equilibrium was attained at the higher speed, the rotor was decelerated to the first speed and the solution allowed to reach equilibrium again. This experiment should demonstrate both concentration dependence of M_w[a] and reversibility provided no alteration of the enzyme or loss of mass occurs during the long time required for the experiment, and provided effects from buffer redistribution can be neglected. Irreversible molecular weight changes should result in three nonsuperimposable curves. Reversibility would be expected to give rise to two identical curves and one nonidentical curve.

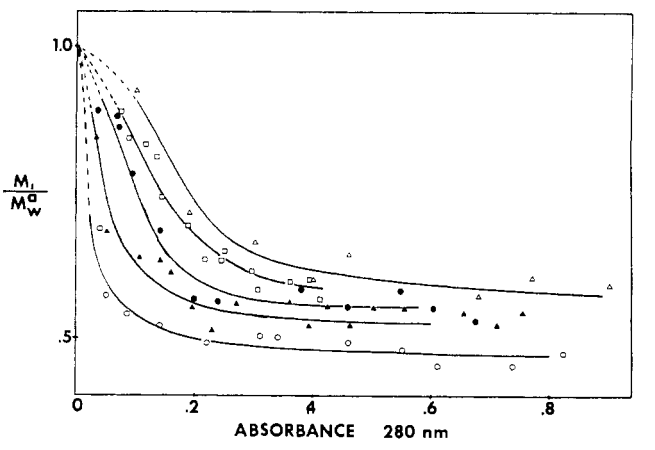

FIGURE 4: Molecular weight of B holoenzyme as a function of concentration from pH 6.5 to pH 9.6: 0.1 M potassium phosphate buffer, 10^{-3} M β-mercaptoethanol, and 5 μg/ml of pyridoxal phosphate; pH 6.5 (—○—), pH 7.5, (—▲—), pH 8.0 (—●—), pH 9.1 (—□—). Potassium carbonate buffer (0.1 M) pH 9.6 containing 10^{-3} M β-mercaptoethanol and 10 μg/ml of pyridoxal phosphate (—△—).

Characterization of the Type of Equilibrium. Adams (1965) has shown that it is possible to distinguish monomer–dimer, monomer–dimer–trimer, and monomer–*n*-mer equilibria by use of eq 4. Application of eq 4 also gives a measure of the various weight fractions ($f_1, f_2, f_3 \ldots$) evaluated at the limits defined by ϕ. Combined with the total concentration at this limit, these yield the apparent association constants. Values of K_2 and K_3 obtained in this way were generally in good agreement with those obtained from eq 2 and 3. Results are shown in Figure 3 and suggest the presence of a monomer–dimer–trimer equilibrium. Data from 12 experiments performed at varying protein concentrations yielded the same qualitative results. A similar plot of the data as suggested by Adams eliminated the possibility of a monomer–trimer equilibrium.

Effect of pH on the Association. The apparent weight-average molecular weight of the B protein varies with pH and concentration as shown in Figure 4. Estimating the apparent binding constant (K_2) for the holoenzyme was complicated somewhat owing to the enhancement of 280 nm absorption by the binding of pyridoxal phosphate (Goldberg and Baldwin, 1967; Wilson, 1965). The value 6.5 obtained for the specific absorbancy under conditions similar to those of Figure 1 (see Methods) was used to estimate K_2 and K_3 for the holoenzyme at pH 7.3 (Table II).

The discovery that dissociation of the holoenzyme is enhanced in 0.1 M Tris-Cl at pH 9.0 and that under these conditions it is probably an apoenzyme (see below) led us to examine the effect of pH on the protein resolved of pyridoxal phosphate. From Figure 5 it is clear that the loss of cofactor greatly increases the dissociation at alkaline pH, to the point of virtual completion (Table II). Data supporting the conclusion that the pH effect is reversible are shown in Figure 6.

Effects of Temperature and Ionic Strength. Temperatures in the range 8–37.5° had no effect on the values of K_2 and K_3 obtained with holoenzyme in 0.2 M potassium phosphate, pH 7.5, supplemented with pyridoxal phosphate and β-mercaptoethanol. Similarly, apoenzyme examined at 6° and

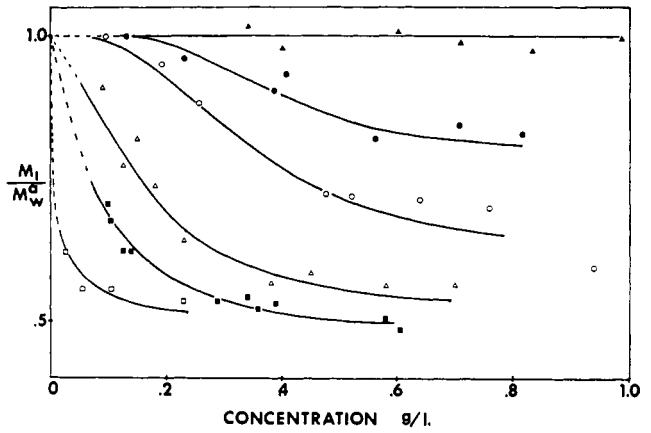

FIGURE 5: Apoenzyme molecular weight as a function of pH: all buffers were 0.1 M Tris-Cl plus 10^{-3} M β-mercaptoethanol; pH 6.7 (—□—), pH 7.1 (—■—), pH 8.1 (—△—), pH 8.8 (—○—), pH 9.0 (—●—), pH 9.4 (—▲—); speed 18,000 rpm, temperature 20°.

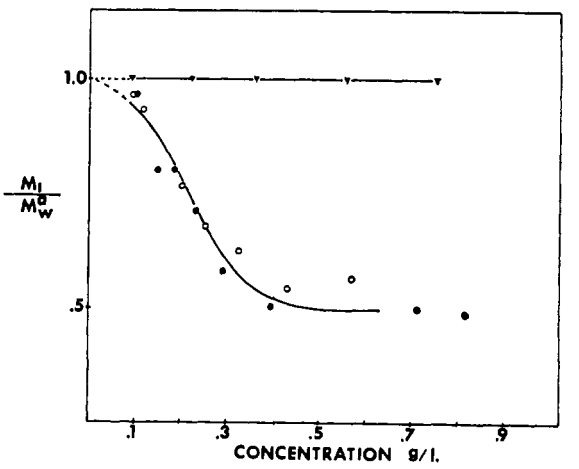

FIGURE 6: Demonstration of the reversibility of the effects of pH. Apo-B-protein dialyzed 36 hr against 0.1 M Tris-Cl buffer, pH 7.74 (—●—). Apoprotein dialyzed 36 hr against the same buffer at pH 9.45 (—▼—). Apoprotein dialyzed 18 hr at pH 9.45 and then 18 hr at pH 7.74 (—○—).

20° in 0.1 M potassium phosphate at pH 6.7, 7.9, and 8.8 showed no influence of temperature on association. Although there was no increase in dispersity, molecular weight values showed slight changes which we interpret as a temperature dependence of the partial specific volume (Hunter, 1966). A change of 0.0005 ml/deg g sufficed to account for the observed change in M_w^a.

The holoenzyme was studied in supplemented potassium phosphate buffer, pH 7.5, at ionic strengths from 0.11 to 0.46. No dependency of K_2 and K_3 was observed within this range of ionic strengths.

Cation and Anion Effects on Apoenzyme. We have examined the effects of K^+, NH_4^+, and $Tris^+$ cations and Cl^- and glycinate anions on the association of apoenzyme subunits. Figure 7 shows the molecular weight distributions in various buffers in the pH range 7.9–9.0. The data show that ammonium ions, particularly, have a strong effect on the apparent molecular weight by enhancing association. In ammonium borate buffer, at pH 9.0, K_2 was 1.8 by eq 3. At pH 7.9 in 0.1 M K^+, NH_4^+, and Tris phosphate, results indicated an effectiveness in increasing K_2 in the following order: $NH_4^+ > K^+ > Tris^+$. Glycine and chloride had no apparent effect.

Molecular Weight Distribution of Various Spectral Forms. The ultraviolet absorption spectra of the B apo- and holoenzyme were reported previously (Wilson and Crawford, 1965). The enzyme is somewhat unusual in possessing absorption at both 415 and 335 nm. These are absent when the protein is resolved of its cofactor or when dialyzed 36 hr against 0.1 M Tris-Cl, pH 9.0. In an attempt to obtain direct physical evidence regarding the active enzyme species, the equilibrium distribution in the ultracentrifuge was measured, scanning at wavelengths 280, 300, 335, and 415 nm. The ratio of absorbances in our sample of holoenzyme was found to be: 280:300 = 5.6, 280:335 = 9.2, 280:414 = 5.7. These ratios were measured both in a spectrophotometer and in the centrifuge (at the start of centrifugation) yielding the same values within ±2%. Figure 8 shows the variation of the logarithm of protein concentration over common radii using the different wavelengths. The slope of this plot gives $1/rc\ dc/dr$ and is proportional to the molecular weight average for the phase measured at that particular radius. The preparation was monodisperse at 415 nm up to about 1.0 g/l., whereas over the same portion of the solution column the protein appeared paucidisperse both at 280 and 300 nm. The absorption at 335 nm gave a distribution identical with that obtained at 415 nm. A subsequent experiment showed that under these conditions M_w^a ranged from 89,000 to 136,000 daltons over the protein concentration range 0–10 g/l. when scanned at 335 or 415 nm.

The Molecular Weight of the AB Complex and the Effect of α Protein on β–β Binding. The estimated molecular weight

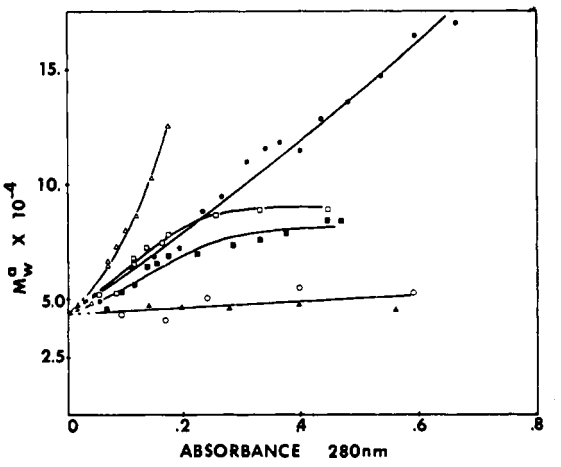

FIGURE 7: Effect of ammonium, potassium, and Tris ions on the sedimentation of B apoenzyme: enzyme in 0.1 M ammonium borate buffer, pH 9.0 (—●—); 0.1 M ammonium phosphate, pH 7.9 (—△—); 0.1 M Tris-phosphate, pH 7.9 (—■—); 0.1 M potassium phosphate, pH 7.9 (—■—); 0.1 M Tris-Cl, pH 8.9 (—○—); and 0.1 M Tris-glycine, pH 8.9 (—▲—); temperature 15°, speed 18,000 rpm.

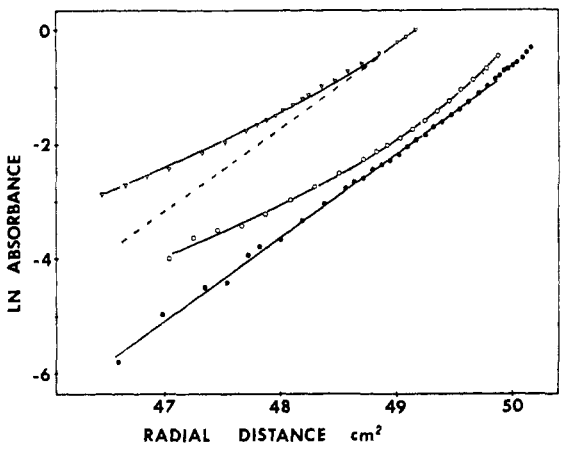

FIGURE 8: Sedimentation of holoenzyme in 0.1 M potassium carbonate buffer, pH 9.6, containing 10^{-3} M β-mercaptoethanol and 10 μg/ml of pyridoxal phosphate. Distributions were recorded for the same sample over common radii at 280 nm (—▽—); 300 nm (—○—), and 415 nm (—●—). Molecular weight recorded at 415 nm was 88,900 ± 2700. The dashed line has a slope [d ln optical density/d(r^2)] corresponding to this molecular weight and is shown as a visual aid.

of the $\alpha\beta_2$ and $\alpha_2\beta_2$ complex from known component molecular weights is 118,700 and 147,000 daltons, respectively. The distribution of tryptophan synthetase complex (selected as the fast eluting peak from Sephadex G-150) appeared essentially monodisperse at sedimentation equilibrium, even at concentrations below 70 μg/ml, and gave an apparent molecular weight of 120,900 ± 6000 (assuming $\bar{v}$ complex = $f_\alpha\bar{v}_\alpha + f_\beta\bar{v}_\beta$ = 0.739 ml/g). When 0.15 M DL-serine and 0.1 M NaCl were included in the procedure for complex preparation, M_w^a was a function of concentration and ranged from 86,000 to 147,000 g/mole.

Discussion

One of the most interesting findings obtained in these experiments is that β-chain monomers do not show the absorption spectrum characteristic of bound pyridoxal phosphate. Although it is possible that the cofactor remains bound to the monomer, but changes its spectrum, it seems more likely that free monomer does not bind the cofactor at all. It is plausible that the binding site exists only after association takes place. There is evidence that a similar situation exists for the binding of threonine to homoserine dehydrogenase (Mankovitz and Segal, 1969). In either case, the monomer's activity in reactions involving serine is likely to be significantly altered since a Schiff base complex between enzyme and cofactor would seem to be a prerequisite for efficient catalysis (Cordes and Jencks, 1962). As yet only indirect evidence exists concerning this point, however.

The primary binding constant (K_2) for the β-chain monomers at optimal pH is 2–20 times greater than that reported for several other proteins (α-chymotrypsin, lysozyme, and β-lactoglobulin) (Adams and Filmer, 1966; Adams and Lewis, 1968). The tendency for β chains to form dimers and higher multimers is spontaneous, as measured by the standard free energy change, under almost every conditions we have studied (Rao and Kegeles, 1958). Furthermore, it is apparent that conditions which enhance the association of β chains, such as the presence of α chains, cofactor, ammonium ions, etc., also evoke increased catalytic activity (Hatanaka *et al.*, 1962).[1] This evidence supports the conclusion that B protein exists *in vivo* as part of an $\alpha_2\beta_2$ complex (Wilson and Crawford, 1965; Creighton and Yanofsky, 1966). The associations we have measured are likely to have been influenced by the nonphysiological conditions employed (absence of α subunits and cofactor, extremes of pH, etc.). In this regard, the formation of trimer and higher order aggregates may be of little importance under normal conditions in the cell. Nevertheless, we feel there are certain advantages to studying equilibria of this type, particularly in that they provide a sensitive system for probing the chemical nature of groups involved in dimer assembly, and protein assembly in general.

At present we cannot identify the specific group(s) responsible for the dramatic pH effect on association. Difficulties were encountered in measuring K_2 near the enzyme's isoelectric point due to the tendency of the protein to precipitate. For this reason, a maximum value of K_2 could not be obtained. Still there were sufficient data to allow a rough estimate of the pH at which K_2 was half-maximal. For the apoenzyme, this was pH 6.5 ± 0.5. Further work will be necessary to judge the significance of this result.

Error in the estimation of the true β-chain binding constant arises from errors in determining M_w as a function of activity. In our experiments this error may be due to (1) the presence in the protein solution of molecules of molecular weight less than M_1, (2) the generation of nonequilibrating, denatured monomer, (3) error in the partial specific volume, (4) sensitivity to base-line error of the absorption measurements in the analytical ultracentrifuge, and (5) nonideal behavior. At very low 280 nm absorbances (<0.05 optical density) we often obtained anomalously low M_w^a values which result in peculiar S-shaped plots as seen in Figures 4, 5, and 6. These values could be due to a contaminant of low molecular weight, but extensive tests for such heterogeneity in our purified preparations have been negative. It is possible that a low molecular weight protein is generated by denaturing β chains whose polypeptide backbone has been cleaved during purification and storage. The minor bands which appeared on sodium dodecyl sulfate–acrylamide gel electrophoresis tend to support this. However, enzyme assays performed on our samples before and after equilibrium centrifugation showed only a slight (<5%) decrease in specific activity. Since we found only about 10–15% small fragments on sodium dodecyl sulfate–acrylamide gels, all of the denatured enzyme would have to be of the altered form to affect the results significantly. It seemed more likely that the uncertainty at low absorbance arose from the sensitivity of the method to base-line error. For this reason, we have not used values of M_w^a obtained at protein concentrations less than 0.17 g/l. in calculating equilibrium constants. While this did not eliminate the problem, it minimized the error in estimating K_2, which was

[1] In the relationship of cofactor and ions to subunit structure and activity, there are many similarities between this enzyme and *E. coli* tryptophanase (Morino and Snell, 1967). At the same time there are differences, such as the strong temperature dependence of the equilibrium shown by that enzyme.

weighted heavily by values of M_w^a obtained at low optical density.

In this work we depended upon a value for the partial specific volume obtained by pycnometry under conditions similar to those employed in most of our experiments (Wilson, 1965). Rigorously, $\bar{v}$ should be determined under all of the varied conditions employed in this study for both monomer and polymer. However, it is felt that the degree of departure from the conditions under which $\bar{v}$ has been experimentally determined is unlikely to introduce serious error into our calculations (Creeth and Pain, 1967). Our assumption that $\bar{v}$ is equal for both holoenzyme and apoenzyme as well as all associating species was supported somewhat by the agreement between the value of M_1 found for the dissociated apoenzyme under relatively mild conditions (Tris-Cl buffer) and values obtained by other procedures (Table I).

The amount of error introduced into the evaluation of binding constants from nonideal behavior can be considerable, even at the dilute protein concentrations employed in this study. We have attempted to evaluate the quantity BM_1 from eq 5 by the method of successive approximations. For this we used data obtained in the concentration range 0–4 g/l. A value of ± 0.03 provided the best fit to the observed data. From eq 3, it can be seen that nonideal behavior of this magnitude introduces negligible error into the association constant K_2. Our reported values for K_2 and K_3 may therefore be considered equivalent to the true binding constants within the limits of experimental error.

We regard tryptophan synthetase as a model for protein self-assembly which does not involve the formation of interchain disulfide bonds (Hathaway *et al.*, 1969, and unpublished results). While the present studies are preliminary, it is hoped that further investigations on this enzyme may lead to a general understanding of the mechanism of recognition and cohesion between the protein subunits of complex structures. It may also become apparent why biological function often appears only after association takes place. Further investigations on the effects that chemical modifications and specific amino acid substitutions have on the association process are in progress.

Appendix

Designation	Definition	Units
M_w	True weight-average molecular weight	daltons
M_w^a	Apparent weight-average molecular weight	daltons
M_1	Monomer molecular weight	daltons
M_n^a	Apparent number-average molecular weight	daltons
a	Radial distance to the solution meniscus	cm
b	Radial distance to the solution–fluorocarbon interface	cm
r	Radial distance	cm
c	Protein concentration in grams/liter. With a subscript denotes concentration of monomer, dimer, etc., or concentration at a particular radial distance	g/l.
α	The apparent monomer concentration	g/l.
f	The apparent weight fraction. With subscript (1, 2, 3) denotes value for monomer, dimer, trimer, etc.	
y	Activity coefficient on the grams per liter scale	
σ_w	The apparent, weight-average, effective, reduced molecular weight	cm^{-2}
$\bar{v}$	Partial specific volume	ml/g
ρ	Solution density	g/ml
ω	Radial velocity	sec^{-1}
R	Universal gas constant	erg/deg mole
T	Absolute temperature	°K
B	The second virial coefficient	l. mole/g^2
K_n	The apparent association constant, $n = 2, 3 \ldots i$	l./g

The general equation for the description of a two component system (showing no pressure dependence) has been derived by Van Holde and Baldwin (1958).

$$M_w = \frac{RT[1 + c(\partial \ln y/\partial c)_{P,T}]}{(1 - \bar{v}\rho)\omega^2} \frac{1}{rc}\frac{dc}{dr} \quad (1)$$

For ideal solutions, the concentration dependency of activities is zero and the above equation becomes

$$M_w^a = \frac{RT}{(1 - \bar{v}\rho)\omega^2} \frac{1}{rc}\frac{dc}{dr} = \frac{A\, d \ln c}{d(r^2)} \quad (1')$$

For an associating system, the difference (eq 1 − eq 1′) is then

$$\frac{M_1}{M_w^a} - \frac{M_1}{M_w} = BM_1c \text{ (Adams, 1965)}$$

provided $\ln y_i = iBM_1 + (0)\, c^2$ and BM_1 is therefore a measure of nonideality.

The association constants in this work were defined in the following manner

$$K_2 = c_2/c_1^2; \quad K_3 = c_3/c_1c_2; \quad K_3' = c_3/c_1^3$$

$$c = c_1 + K_2c_1^2 + K_3'c_1^3$$

From the definition of weight-average molecular weight

$$M_w = \Sigma c_iM_i/\Sigma c_i$$

$$M_w = \frac{c_1M_1 + c_2M_2 + c_3M_3}{c} = \frac{M_1(c_1 + 2c_2 + 3c_3)}{c}$$

substituting $K_2c_1^2$ for c_2 and $K_3c_1c_2$ for c_3 and introducing f_1 we obtain (Fujita, 1962)

$$M_w^a = \frac{M_1}{c}[(f_1c) + 2K_2(f_1c)^2 + 3K_2K_3(f_1c)^3] \quad (2)$$

where $\ln f_1 = \int_0^c (M_1/M_w^a - 1)\, dc/c$ (Steiner, 1952)

and

$$\lim_{c\to 0} (M_1/M_w^a - 1)/c = -K_2 + BM_1 \text{ (Adams, 1965)} \quad (3)$$

Adams and Williams (1964) have shown that for a monomer–dimer–trimer association

$$\psi - 1 = f_{2,a}(2e^{\phi_1} - 1) + f_{3,a}(3e^{2\phi_1} - 1) \quad (4)$$

where

$$\psi = \left(\frac{1}{2AM_1c_a}\frac{1}{r}\frac{dc}{dr}\right)\Big/ e^{\phi_1}$$

it is easily shown that

$$\psi = \frac{c_r M_{w,r}^a}{c_a M_1} e^{-\phi_1}$$

where

$$A = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \text{ and } \phi_1 = AM_1(r^2 - a^2)$$

The following equation holds for a monomer–dimer–trimer association (Adams, 1965)

$$\frac{6cM_1}{M_n^a} - 5c = 2\alpha e^{-BM_1c} + 3BM_1c^2 - \frac{1}{(M_1/cM_w^a) - BM_1} \quad (5)$$

where

$$M_n^a = \frac{cM_1}{\int_0^c M_1/M_w^a dc} \text{ and } \alpha = ce^{\ln f_1}$$

$$M_1 = \frac{JM_{n,r}^a - M_{w,r}^a}{J - 1} \text{ Yphantis (1964)} \quad (6)$$

Added in Proof

Because centrifugal experiments done in 0.1 M Tris-Cl buffer at high pH in the absence of a supporting electrolyte involve low ion strengths, charge effects are possible. We therefore examined the apparent molecular weight of B protein to which the cofactor had been covalently affixed with sodium borohydride. This form of the enzyme is known to be more highly charged than the apoenzyme at pH 9.3 (Hathaway *et al.*, 1969). No decrease in molecular weight was observed for the modified protein in the pH range 6.9–9.6, while under the same conditions apoenzyme decreases to one-half the dimer molecular weight. We conclude that the apoenzyme is dissociated in Tris above pH 9.

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