exclude the presence of an Ra.3 region on the antigen E molecule. Such a region could be relatively small in size, of the order of a penta- or hexapeptide, as has been found for antigenic determinants (Kabat, 1966). The immunogenicity of this region might be such as to require prolonged immunization in rabbits or other animal species for the production of specific antibody.

Acknowledgments

We are greatly indebted to Dr. Bram Rose for his constant interest and for facilitating the progress of this study. We thank Dr. M. W. Chase for donations of antigen E and rabbit antiserum to antigen E. We gratefully acknowledge the capable technical assistance of Mr. D. Woodard, Mr. M. Lanner, and Mr. W. Rankowicz.

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Subunit Structure of the B Component of *Escherichia coli* Tryptophan Synthetase*

G. M. Hathaway, S. Kida, and I. P. Crawford

ABSTRACT: Sedimentation equilibrium studies demonstrate that the B subunit of tryptophan synthetase dissociates into two polypeptides upon treatment of the protein with concentrations of urea greater than 4 M or with performic acid. Some dissociation is observed even in dilute solution in phosphate buffer. C-Terminal amino acid analysis and gel electrophoresis experiments support the hypothesis that the native enzyme is a dimer composed of two identical or very similar subunits whose molecular weight is 45,000 g/mole. The urea-dissociated enzyme can be reconstituted with 80% of the original activity if thiol concentrations are maintained at the proper level during removal of urea. A hybrid enzyme, one of whose subunits contains pyridoxal phosphate reduced onto the enzyme with sodium borohydride, exhibits one-half the specific activity of the native enzyme in catalyzing the conversion of indole into tryptophan.

Tryptophan synthetase (*Escherichia coli*) is a multimeric enzyme composed of two proteins which differ greatly in their physical and catalytic properties (Yanofsky, 1960). The complex dissociates readily into two separable components, termed the A and B proteins, each carrying an active site for an enzymatic half-reaction (Crawford and Yanofsky, 1958).

The A component, which carries the active site for the aldolysis of indole-3-glycerol phosphate to indole and glyceraldehyde 3-phosphate, is a single polypeptide chain of about 29,000 molecular weight (Henning *et al.*, 1962). The B component (Wilson and Crawford, 1965) binds two pyridoxal phosphate molecules; it is capable of performing a variety of catalytic functions, (Miles *et al.*, 1968), but catalyzes best the condensation of L-serine and indole to L-tryptophan. The B protein is

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considerably larger than the A protein and is thought to be a homologous dimer (Wilson and Crawford, 1964). One B protein molecule (β_2) combines with two A protein molecules (α) to form an $\alpha_2\beta_2$ complex (Wilson and Crawford, 1965; Goldberg *et al.*, 1966). Upon combining, each component has a pronounced stimulatory effect on the other's half reaction. The over-all reaction leading to a tryptophan from indole-3-glycerol phosphate occurs at a rate significantly faster than the rate of formation of indole from indole-3-glycerol phosphate. Moreover, indole is not liberated from the enzyme during the catalysis of the overall reaction (Yanofsky, 1960). These results indicate a close functional integration of the active sites of the two components.

The present studies are concerned with the structure of the B protein and the effects of B-chain interbonding on catalytic activity. Previously some evidence was obtained indicating that the enzyme dissociates in urea solutions or when treated with formic acid (Wilson and Crawford, 1964). This work was begun to confirm this suggestion and to explore methods for studying the dissociation of the B protein into subunits as well as the association to active enzyme.

Materials and Methods

Pyridoxal 5′-phosphate, β-alanine, sodium borohydride, lactate dehydrogenase (heart), and bovine plasma albumin were obtained from Sigma Chemical Co. Dithioerythritol was purchased from Nutritional Biochemicals Corp. and ethylene diacrylate was from the Borden Chemical Co. Carboxypeptidase A was obtained from Worthington Biochemical Corp.; pepsin and L-asparagine were from General Biochemicals, Inc. Acrylamide, *N,N′*-methylenebisacrylamide, and 2-mercaptoethanol were from Eastman Organic Chemicals. Urea and imidazole were purchased from Matheson Coleman Corp. All chemicals were reagent grade and were used without further purification unless otherwise specified.

Preparation and Purification of B Protein. B protein was prepared from strain A2/F′A2 (lacking A protein) of *E. coli* following the procedure of Wilson and Crawford (1965). The enzyme was found to be more than 95% pure by the criteria of acrylamide gel electrophoresis at two pH and several gel concentrations (Sulitzeanu *et al.*, 1967; Hedrick and Smith, 1968).

Reduced B protein[1] was prepared from enzyme dialyzed several hours against 0.1 M potassium phosphate buffer (pH 7.8) containing 5 μg/ml of pyridoxal 5′-phosphate and millimolar mercaptoethanol. The dialysis bag was transferred to another flask containing 0.1 M potassium phosphate buffer (pH 7.8) and sodium borohydride at 0.4 mg/ml, and rotated gently for 10 min, or until the yellow color disappeared. A third dialysis against reservoir buffer diluted fourfold was carried out prior to purification by preparative gel electrophoresis.

Apoenzyme was prepared by dialyzing the holoenzyme several hours against 0.1 M potassium phosphate buffer (pH 7.5) containing 0.1 M mercaptoethanol and 0.05 M DL-serine (Miles *et al.*, 1968). Excess serine was then removed by further dialysis in the absence of the amino acid. Performic acid oxidized B protein was prepared by dissolving 50 mg of the lyophilized holoenzyme in 1.5 ml of 99% formic acid at 0°. Performic acid (1 ml)[2] at 0° was added and the mixture was allowed to react 2.5 hr. The reaction mixture was then either dialyzed against deionized water 6 hr followed by lyophilization or diluted with ten volumes of deionized water and lyophilized directly.

Enzyme Assay. Assay of B activity was carried out by measuring both its ability to catalyze the conversion of indole into tryptophan in the presence of A protein, and its ability to stimulate the conversion of indoleglycerol phosphate into indole by the A protein (Smith and Yanofsky, 1962). Salt-free hydroxylamine was prepared according to Beinert *et al.* (1953).

Protein Determination. Protein was estimated by the Lowry *et al.* (1951) method using bovine plasma albumin as standard; a factor of 0.91 was used to reduce the absorbance of the B protein solutions to that of an equivalent weight of albumin (Wilson, 1965). Apoenzyme was estimated by its specific absorbancy $E^{1\%}_{278}$ 5.7 (Wilson, 1965).

C-Terminal Amino Acid Analysis. Carboxypeptidase A (ammonium sulfate suspension) was added at a 1:50 ratio (w/w) to the performic acid oxidized B protein (9.6 mg/ml) in 0.1 M Tris-HCl buffer (pH 8.5) and incubated at 37°. Samples (0.5 ml) were removed just prior to CP addition and at intervals thereafter, placed in centrifuge tubes containing an equal volume of 10% trichloroacetic acid, and centrifuged at 39,000*g* for 15 min. The supernatant was decanted and the precipitate was washed two times with 10% trichloroacetic acid. Supernatants and washings were added to 2.0-ml volumetric flasks and brought to volume with 0.2 N citrate buffer (pH 2.2). Aliquots (1 ml) of each sample were analyzed with a Spinco Model 120 C amino acid analyzer.

Acrylamide Gel Electrophoresis. Analytical disc electrophoresis was carried out using both the pH 9.3 Tris–glycine and the pH 8.5 Tris–asparagine discontinuous systems (Sulitzeanu *et al.*, 1967; Hedrick and Smith, 1968). Ferricyanide was found unnecessary provided the component solutions were kept at 0° prior to mixing. Urea gels were prepared by adding solid urea (recrystallized from 95% ethanol) to each of the several component solutions before bringing to volume. Samples were dialyzed overnight against four-times-diluted reservoir buffer. Specific conditions can be found in the legends to the figures. Samples (50 μl) of a solution

[1] The term "reduced B protein" refers to the molecule in which the azomethine bond between the cofactor, pyridoxal 5′-phosphate, and the enzyme is reduced to form a covalent linkage (see Discussion). The term used thus does not refer to the oxidation state of the sulfhydryl or any other functional groups on the enzyme.

[2] Performic acid was prepared by mixing 0.5 ml of 30% H_2O_2 with 9.5 ml of 99% formic acid and allowing the reaction mixture to stand 2 hr in the dark.

containing B protein at 1.0 mg/ml and 10% sucrose were applied to the stacking gels by layering under the reservoir buffer. Current was applied at 2 mA/tube until the dye front approached the end of the stacking gels. The current was then raised to 4 mA/tube. As the dye front neared the end of the gels, the current was reduced to 1 mA/tube and maintained until the individual gels were removed. The dye front was marked with no. 26 gauge copper wire and the gels were stained 2–24 hr with 1% aniline blue black dye in 7.5% acetic acid. The gels were destained electrophoretically and stored in 7.5% acetic acid.

For estimating relative protein concentrations in the bands, gels contained ethylene diacrylate as cross-linking agent (Cain and Pitney, 1968). After destaining, gels were frozen in liquid nitrogen and the bands were cut from the gels using a razor blade. The sections of gels were dissolved in 1.0 ml of 1 N NH_4OH and the absorbance was measured at 610 mμ. Molecular weights were determined following the method of Hedrick and Smith (1968) but using the pH 9.3 Tris–glycine system of Sulitzeanu *et al.* (1967).

In order to purify reduced B protein and isolate the half-reduced hybrid, preparative gel electrophoresis was carried out with a Shandon apparatus. The buffer system was a modification of the pH 8.5 Tris–asparagine system of Hedrick and Smith (1968). The reservoir buffer contained 30.0 g/l. of L-asparagine brought to pH 7.3 with 2.0 M Tris. The elution buffer contained 0.45 M Tris-HCl (pH 7.4). Pyridoxal phosphate (5 μg/ml) and millimolar β-mercaptoethanol were added to the elution buffer. It was necessary to adjust the stacking gel pH to 6.7 when separating the hybrid enzyme because of apparent instability at pH 5.7. Otherwise, the running and stacking gels were prepared as outlined by Hedrick and Smith (1968). Small pore gel (20 ml) was sufficient for the upper gel column when purifying the reduced enzyme, but a 30-ml column was necessary to separate the hybrid enzyme. Current was initially applied at 30 mA until the protein entered the small pore gel and then increased to 50–60 mA. The column coolant was maintained at 6–8°. Elution buffer was pumped through the annular collection ring at a flow rate of 40 ml/hr. The absorbance of the eluent was monitored at 280 mμ. Fractions (3.0/ml) were assayed for enzyme activity and protein concentration and the peak fractions were pooled. The enzyme was precipitated with solid ammonium sulfate (30 g/100 ml), centrifuged at 39,000*g* for 15 min and taken up in a small volume of 0.1 M phosphate buffer (pH 7.5). Portions were then analyzed by disc electrophoresis.

Dissociation and Reaggregation of B Protein. All procedures were carried out at 0–3°. B protein (1–2 mg/ml) was dialyzed overnight against 0.01 M Tris-HCl buffer (pH 7.8) containing 6 M urea, 0.1 M NaCl, and millimolar mercaptoethanol. Reaggregation was accomplished either by diluting the denatured enzyme into ten volumes of 0.1 M potassium phosphate buffer (pH 7.8) containing 0.1 M mercaptoethanol and 30 μg/ml of pyridoxal 5′-phosphate, or by placing it in a 0.25-diameter Visking dialysis tubing and dialyzing 60 min against the supplemented 0.1 M potassium phosphate buffer, then 90 min against 0.05 M potassium phosphate (pH 7.8). Half-reduced (hybrid) enzyme, containing only 1 mole of pyridoxal 5′-phosphate/90,000 g of B protein, was observed after disaggregating a mixture of equal amounts of reduced and holoenzyme or reduced and apoenzyme, then reaggregating by the dialysis procedure described above. Electrophoretic analysis of the reaggregated protein gave similar patterns whether the starting mixture had contained holo- or apoenzyme (*e.g.*, Figure 10, center gel).

Ultracentrifugation. Sedimentation equilibrium experiments were performed in a Spinco Model E analytical ultracentrifuge. In all experiments, the distribution of the macromolecule was measured by ultraviolet absorption. A scanner, photoelectric detector, and recorder were used to obtain absorbance as a function of radial distance (Lamers *et al.*, 1963). Multiple-cell operation using a four-place rotor and multiplexer switching device ensured identical experimental conditions for up to three samples per centrifuge run. The reference sector of the double-sector centerpiece was filled with dialysate while the solution sector (sector A) received fluorocarbon 43 (0.05 ml) and enough protein solution to give a 3-mm column height (usually 0.09 ml). Both high- and low-speed techniques were employed. Overspeeding was used with the low-speed technique following the procedure of Richards *et al.* (1968), and the high-speed or meniscus-depletion method of Yphantis (1964) was followed. For the high-speed runs, times to equilibrium were estimated according to Yphantis (1964). A given sample was taken to be essentially at equilibrium when successive absorption traces, taken over a span of several hours, did not vary more than experimental error. Samples to be centrifuged were dialyzed overnight against two changes (500 volumes) of cold 0.1 M potassium phosphate buffer (pH 7.5) containing 5 μg/ml of pyridoxal 5′-phosphate and millimolar mercaptoethanol unless stated otherwise. The solution was then centrifuged 30 min at 39,000*g* and diluted with the dialysis buffer to obtain the desired starting 280-mμ absorbance. After equilibrium was attained in the centrifuge, scans at 280 mμ were made. The centrifuge speed was then increased to 44,000 rpm and maintained for 1–2 hr after which the speed was rapidly reduced to the experimental value and a final scan made to correct for any base-line differences. Solvent densities were obtained by pycnometry. The value 0.748 for the partial specific volume obtained by Wilson (1965) was used in most calculations; however, the value 0.737, obtained from amino acid analysis was also employed as noted in the legends and in the text.

Results

Electrophoresis and Molecular Weight Determination. Figure 1 shows the results of gel electrophoresis of the B protein under several conditions. A series of bands was observed when B protein previously dialyzed against 6 M urea was electrophoresed out of the urea in the absence of thiol. The molecular weight of these species was determined by the method of Hedrick and

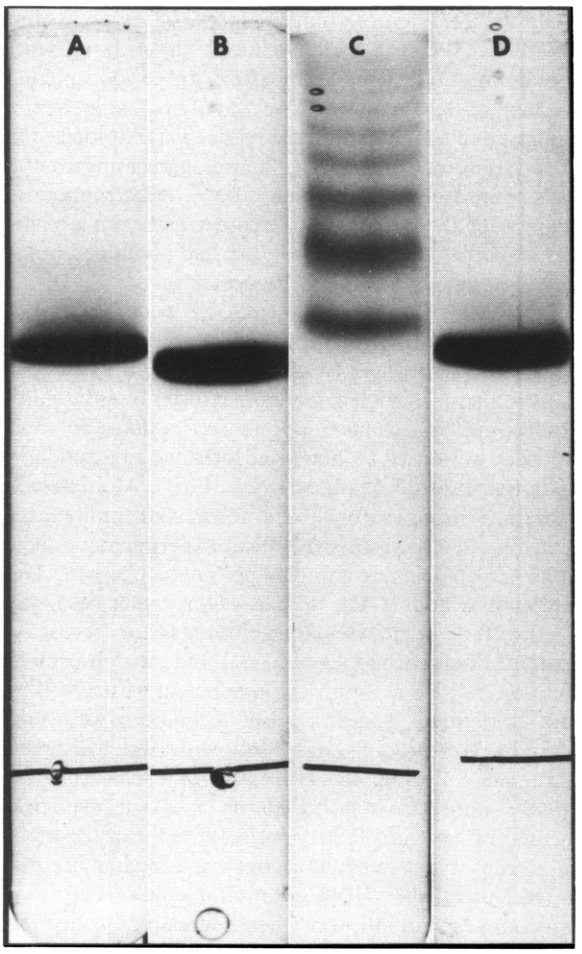

FIGURE 1: Electrophoresis of B protein on 10% polyacrylamide gels using the pH 9.3 Tris–glycine system. (a) Native B protein. (b) Sample as in a plus 0.01 M dithioerythritol. (c) B protein dialyzed against 6 M urea. (d) Sample as in c plus 0.01 M dithioerythritol. The gels contained no added urea or reducing agent. The direction of electrophoresis (anode) was downward.

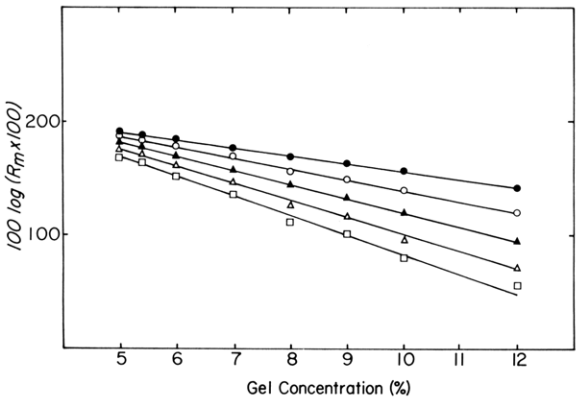

FIGURE 2: Plot of log relative mobility (R_m = distance of protein migration/distance of dye migration) *vs.* per cent acrylamide (see Hedrick and Smith, 1968). Slopes were determined by the method of least squares. The lower curve, —□—□—, represents the most cathodic band of Figure 1c, while the upper curve, ●—●, is the most anodic band.

Smith (1968). The bands were generated on polyacrylamide gels of increasing pore size, and the rate of change of their mobility relative to the tracking dye was measured (Figure 2). Proteins of known molecular weight were used to generate a standard graph which was used to determine the size of the protein species in the various bands (Figure 3). Molecular weight values obtained in this manner were 45, 90, 140, 180, and 204 $\times 10^3 \pm 3\%$ for the successive bands, starting with the most anodic. Holo- and apoenzyme showed identical migration, suggesting that noncovalently bound pyridoxal 5′-phosphate is removed during electrophoresis (Figure 4).

C-Terminal Amino Acid Analysis. The results obtained upon treatment of the performic oxidized enzyme with carboxypeptidase are shown in Table I. The reaction was rapid and nearly 2 moles of isoleucine was released within 15 min after carboxypeptidase addition. An amount of isoleucine equivalent to 2 ± 0.1 moles/90,000 g of enzyme was released after 240 min. These results are in agreement with those of Wilson (1965) who reported 2.5 moles of isoleucine/108,000 g. No significant amounts of amino acids other than those shown in Table I were found.

Sedimentation Equilibrium Experiments. Figure 5 shows the curved plot obtained when B holoenzyme is centrifuged in phosphate buffer. In contrast, straight-line plots were obtained when the protein was centrifuged in 5 M urea. The limiting slope near the bottom of the column yielded an apparent weight-average molecular weight of 89,000 ± 2700. In 5 M urea, this value was 43,000 ± 2000. To investigate the possibility that a dissociation phenomenon might explain the curvature in these plots, high-speed runs were made (Yphantis, 1964), and graphs of log absorbance *vs.* the square of the radial distance, x, were analyzed by a least-squares fit to each five successive data points

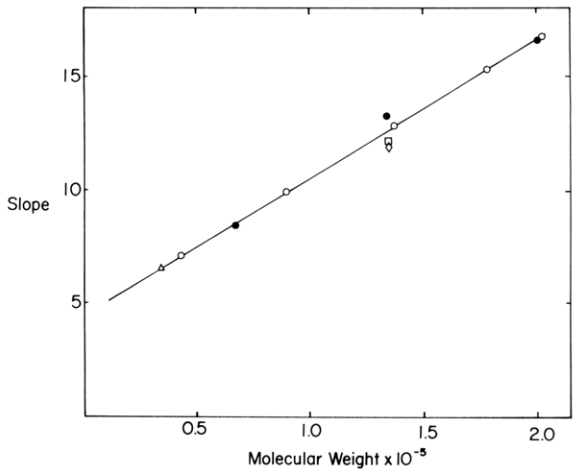

FIGURE 3: Standard curve of slopes obtained from plots similar to those of Figure 2, using pepsin (—△—) (34,000), lactate dehydrogenase (—□—, —◇—) (140,000), and bovine plasma albumin (—●—) (68,000, 136,000, and 204,000) monomer, dimer, and trimer, as standard proteins. The slopes obtained for the urea-generated bands of B protein are represented as open circles.

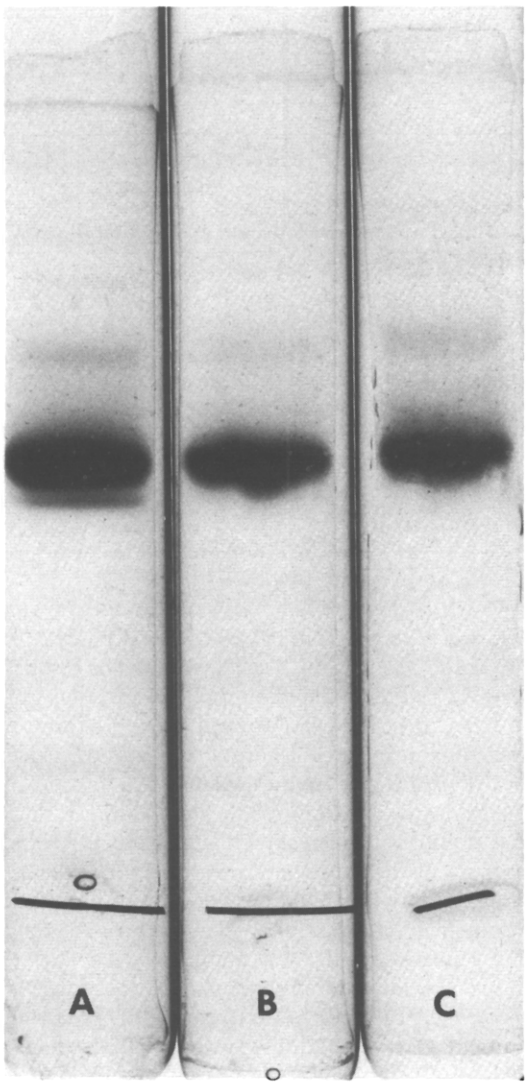

FIGURE 4: Electrophoresis of apo- and holoenzyme in 10% acrylamide gels. (a) Apoenzyme, (b) holoenzyme, and (c) mixture of apo- and holoenzyme. The pH 9.3 Tris–glycine system was used.

TABLE 1: Carboxypeptidase Digestion of Performate-Oxidized B Protein.

	Amount of Amino Acid Observed[a]	
min at 37°	Glycine (nmoles)	Isoleucine (nmoles)
0[b]	0	0
0[c]	1.0	1.6
15[d]	2.2	49.3
30	2.5	49.9
60	2.6	50.2
120	3.9	51.2
240	5.9	52.3

[a] Each analysis was performed on the trichloroacetic acid supernatant from 2.4 mg of protein (25.6 nmoles on the basis of a molecular weight of 90,000). [b] Before the addition of carboxypeptidase. [c] Immediately after the addition of carboxypeptidase. [d] For this and subsequent entries, the amount of amino acid shown is the difference between the observed and the zero time plus carboxypeptidase value.

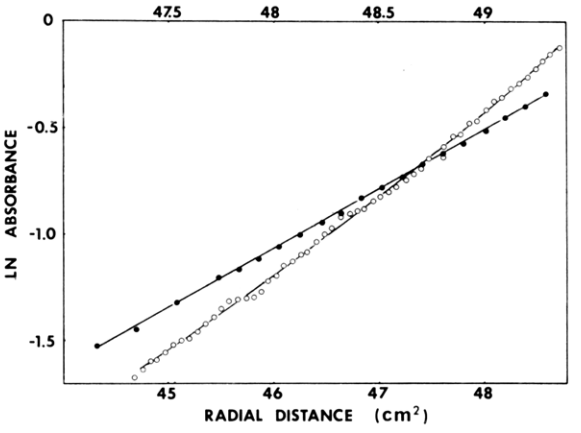

FIGURE 5: Equilibrium centrifugation of holoenzyme. The low-speed technique was used with overspeeding, —○—○— (upper abscissa). The usual phosphate buffer contained 0.01 M mercaptoethanol and pyridoxal 5′-phosphate (5 μg/ml). The sample was accelerated to 14,000 rpm, held for 170 min, rapidly decelerated to 7000 rpm, and held for 9 min followed by acceleration to 10,000 rpm where it was held to equilibrium. High-speed centrifugation (18,000 rpm) in 0.1 M phosphate buffer containing 5 M urea and 0.01 M mercaptoethanol, —●—●— (lower abscissa).

The slopes obtained are proportional to the apparent molecular weight at x provided preferential interaction with solvent components may be neglected (Van Holde and Baldwin, 1958). These slopes, $(d \ln OD)/d(x^2)$, were plotted as a function of absorbance in Figures 6 and 7. The limiting value in the region near the meniscus corresponded to the molecular weight 44,000 ± 3000, while data obtained near the bottom of the column yielded 88,000 ± 2600. The mean value of molecular weight obtained after centrifugation of the holoenzyme in 5, 6, 7, and 8 M urea solutions was 44,500 ± 3000 g/mole, using the value 0.748 cc/g for the partial specific volume. If the value 0.737 cc/g obtained from amino acid analysis was used (Ullman *et al.*, 1968) the average value of the molecular weight obtained in these solutions decreased to 42,000. Oxidation of the enzyme's sulfhydryl groups by treatment with performic acid resulted in both an altered electrophoretic mobility and a reduction in the molecular weight to 45,000 g/mole. In all these studies preferential binding of small ions or additives has been neglected and the macromolecular component has been considered to be ideal at the concentrations used.

Because of the apparent concentration dependency of molecular weight, an attempt was made to distinguish whether the observed decreases in molecular weight were due to a dissociating monomer–polymer system or to the presence of polymers not in chemical equilibrium. Subsequently, the starting concentration of B

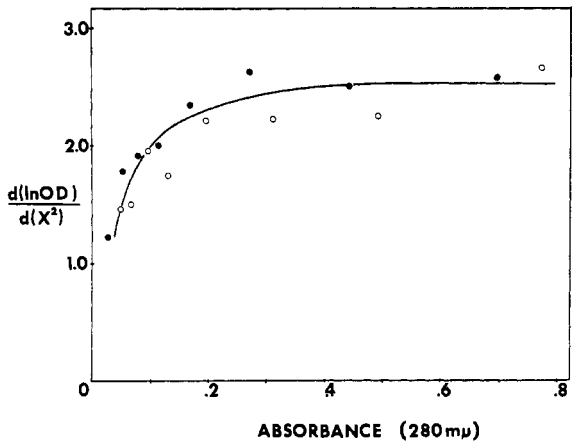

FIGURE 6: Equilibrium centrifugation of holoenzyme in phosphate buffer (pH 7.5), containing 5 μg/ml of pyridoxal 5′-phosphate and 0.1 mM (—○—○—) or 1 mM (—●—●—) dithioerythritol. Speed was 22,000 rpm.

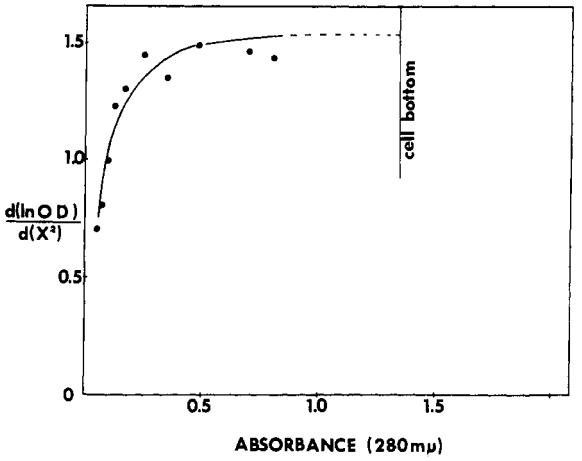

FIGURE 7: Equilibrium centrifugation of apoenzyme in the usual buffer with 0.01 M mercaptoethanol. Speed was 18,000 rpm.

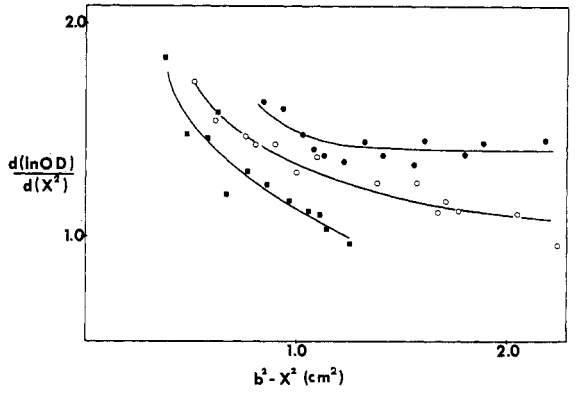

FIGURE 8: Plot of sedimentation equilibrium data using three starting concentrations of B holoenzyme: 0.2 (—■—■—), 0.8 (—○—○—), and 1.7 mg/ml (—●—●—). Solvent was 0.1 M potassium phosphate buffer (pH 7.0) supplemented with 5 μg/ml of pyridoxal 5′-phosphate and 0.001 M mercaptoethanol. Speed was 18,000 rpm. The two higher concentrations were recorded using the 0–2 optical density scale. *b* represents the radial distance to the bottom of the column. *x* is the radial distance at which the slope is measured.

TABLE II: Effect of Mercaptoethanol on Recovery of Enzymatic Activity after Dissociation and Reaggregation.

Mercaptoethanol (M) during Dissocn[a]	Mercaptoethanol (M) during Reaggregation[a]			
	0.1	0.01	0.001	0.0001
0.1	1138[b]	824	NT	NT
0.01	1130	518	260	NT
0.001	997	NA	NA	NA

[a] Dissociation and reactivation were carried out at 0° by the dialysis technique described in Materials and Methods. [b] Values are given as specific activity (units per milligram). The specific activity of the starting material before dissociation was 1322. NT = not tested, NA = no activity.

enzyme was varied from 0.2 to 1.7 mg per ml. A four-place rotor was used at a single speed. The results are shown in Figure 8. Generation of three distinct curves and the shift in the curve toward the cell bottom with lower initial protein concentration suggest the presence of a dynamic equilibrium (Yphantis, 1964).

Reaggregation of Urea-Dissociated B Protein. Various concentrations of mercaptoethanol were used during the urea dissociation and reaggregation process to determine optimal conditions for restoration of enzymatic activity. It was found that dialysis for 60 min against urea-free buffer was optimal for reaggregation, and the per cent restoration of activity was independent of protein concentration over the range 100–2000 μg/ml. With reaggregation and dissociation buffers containing 0.0001, 0.001, 0.01, and 0.1 M mercaptoethanol, it was observed that 0.1 M thiol allowed the greatest restoration of activity (Table II). Pyridoxal 5′-phosphate was not essential for restoration of enzymatic activity, but it protected the enzyme somewhat against inactivation.

B protein treated with 6 M urea in the presence of

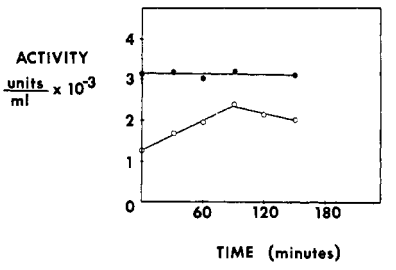

FIGURE 9: Time course of reactivation of urea-dissociated B protein. The enzyme was dialyzed against 6 M urea buffer containing 0.001 (—○—○—) and 0.1 M (—●—●—) mercaptoethanol. At zero time it was diluted into cold phosphate buffer containing 0.1 M thiol (1:10 dilution) and then added to the assay mixture at the time indicated. Final urea concentration was 0.06 M. A control incubated in 0.6 M urea and added directly to the assay solution showed no inactivation during 150 min.

either 0.001 or 0.1 M mercaptoethanol can also be reactivated by dilution at 0° into reaggregation buffer containing 0.1 M mercaptoethanol. No activity was observed when the dilution was carried out at 25°. When the thiol concentration remained at 0.1 M throughout the procedure, activity returned immediately. If dissociation was done in 0.001 M mercaptoethanol, 50% of the activity returned instantaneously, but 90 min was required to obtain maximal activity (Figure 9).

Hybrid Formation. Figure 10 shows that apoenzyme and reduced B protein have different mobilities on polyacrylamide gels. Equal amounts of apoenzyme and reduced B protein were combined, dialyzed against 6 M urea buffer, and then reaggregated. Figure 10 shows that a band with mobility intermediate between apoenzyme and reduced B enzyme appeared. The ratio of stainable material in the three bands shown in the center gel was measured by carrying out electrophoresis on ethylene diacrylate gels. Absorbance at 610 mμ was found in the ratio 1:2:1 as predicted for a random association of reduced and apoenzyme subunits.

Activity of the Hybrid Enzyme. Preparative gel electrophoresis of a mixture similar to that of Figure 10, but made on a larger scale, allowed us to estimate the ability of each of the three protein species to participate in the indole to tryptophan reaction or stimulate the conversion of indoleglycerol phosphate into indole. The results indicated that fully reduced B protein (the fastest moving band) has lost the ability to catalyze the first reaction, but retains its stimulatory effect in the second. These properties had been observed earlier (Wilson and Crawford, 1965). Table III shows the relative activity of the hybrid enzyme (the middle band) in these two reactions. Its specific activity is only half that of the normal enzyme in tryptophan formation, but it retains full ability to stimulate the indoleglycerol phosphate to indole reaction. In fact, there is no great difference in the efficiency with which any of the three protein species stimulate the latter reaction, provided a large excess of the A protein is present in the assay.

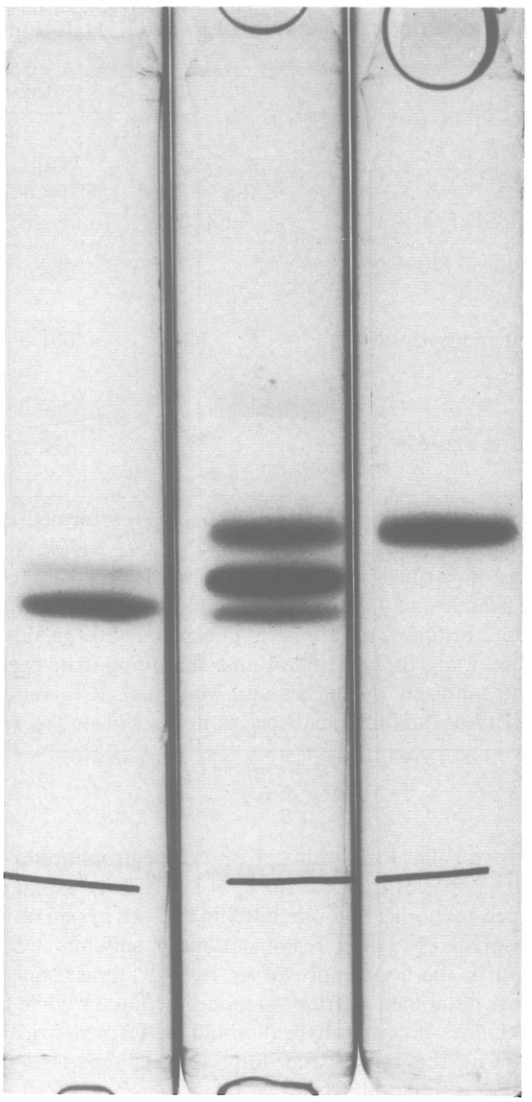

FIGURE 10: Electrophoresis of (left) reduced B protein, (right) apo B protein, and (center) a reaggregated mixture of reduced and apoenzyme. Gel concentration was 7.5%. Running pH was 8.5 using the Tris–asparagine system.

Discussion

The finding that dissociation with urea or performic acid leads to a reduction in the molecular weight of the B protein to about 45,000 has confirmed earlier predictions that the enzyme exists as a dimer (Wilson and Crawford, 1964). In conjunction with the sedimentation equilibrium experiments reported here this has led us to propose a value of 90,000 ± 3000 for its molecular weight. The reason for the disagreement between the molecular weight and values reported previously (Wilson and Crawford, 1965; Goldberg *et al.*, 1966) is not immediately clear. It seems unlikely that the value used for the apparent partial specific volume, $\bar{v}$, can account for the large differences observed. Previous workers used partial specific volumes smaller than 0.748 cc/g (Wilson, 1965); this would give a lower instead of a higher molecular weight. A 1% error in $\bar{v}$ introduces about a 3% error in the calculated molecular weight. This is far less than the difference observed (8000–18,000 g/mole). Workers have cited the ability of thiols to alter the state of aggregation of proteins in order to explain erroneously high molecular weight values (Boyer, 1958; DeVincenzi and Hedrick, 1967). The Archibald technique employed in previous measurements of the B protein's molecular weight would give high values if multimers larger than dimers were present in the cell. The present study demonstrated the production of such multimers under special conditions (Figure 1). The dissociation we have observed in solution (Figures 5–8) may lead secondarily to multimer formation under some conditions. The fact that dissociation was not observed previously is probably due to the fact that concentrations higher than 0.5 mg/ml were used in molecular weight determinations in the ultracentrifuge, or possibly to the inclusion of the substrate, L-serine, in the solvent (Creighton and Yanofsky, 1966).

The observation that dissociation in urea of a mixture of apo- and reduced enzyme followed by reaggregation

TABLE III: Enzymatic Activity of Reduced, Half-Reduced, and Native B Protein Species.

PGE Fraction[a]	Ind → Trp[b] (units/mg)	InGP → Ind[c] With NH_2OH (units/mg)	InGP → Ind[c] With Pyridoxal 5′-Phosphate (units/mg)	Protein (μg/ml)	% Each Species[d]	Cor Sp Act.[e] (units/mg)
Reduced (40–41)	67	482	27.8	68.5	95.5 (R) 4.5 (HR)	35
Half-reduced (46–47)	548	601	27.7	208	31.3 (R) 62.0 (HR) 6.7 (N)	730
Native (56–57)	1280	472	20.0	195	14.0 (HR) 86.0 (N)	1380

[a] Preparative gel electrophoresis was performed as described in Materials and Methods. The fractions shown in parenthesis were combined before assay. [b] Indole + L-serine → L-tryptophan reaction, done in the presence of 102 units of purified component A. [c] Indoleglycerol phosphate → indole + glyceraldehyde phosphate reaction, done in the presence of 358 Ind → Trp units of purified component A. Salt-free NH_2OH (1 M final concentration) was added in one instance, pyridoxal 5′-phosphate (20 mg/ml) in the other. [d] After assay, the fractions were concentrated to about 1 mg of protein/ml and the amount of each species present was determined by analytical acrylamide gel electrophoresis (Materials and Methods). R = reduced, HR = half-reduced, N = native. [e] The calculated specific activity of the individual species in the Ind → Trp reaction is shown.

leads to the formation of three electrophoretically distinct species in the ratio 1:2:1 is readily explained if each monomer has one binding site for pyridoxal 5′-phosphate. Random reassociation of subunits would result in the appearance of a "hybrid" dimer, one of whose monomers carries the reduced cofactor while the other does not; this hybrid would be formed twice as often as the native and fully reduced species. The nature of the group to which the cofactor is linked has not been definitely established, but work on other enzymes has implicated the ε-amino group of lysine (Morino and Snell, 1965; Snell, 1962; Fischer *et al.*, 1958). The reduced form of the B protein has an absorption spectrum similar to that of other reduced pyridoxal 5′-phosphate proteins (Wilson and Crawford, 1965), and label from tritiated borohydride is associated with the fluorescence characteristic of ε-pyridoxyllysine in peptide maps of a tryptic digest of the B protein (R. Fluri and L. Jackson, personal communication).

Investigations into the relationship of subunit aggregation to catalytic function often involve the question of the independence or interaction of active sites. Our experiments in which catalytic activity was measured after one or two cofactors had been reduced onto the B protein suggest to us that there exists one active site per monomer, and that in the hybrid one active site remains functional even though the other has been inactivated by reduction. To this extent the sites seem to act independently, although it is not known whether the reduced subunit retains the ability to bind substrate. This does not mean, however, that function necessarily would persist in the dissociated monomer. Our results also indicate that both the half-reduced and the reduced forms of the enzyme are capable of stimulating the A protein in the conversion of indoleglycerol phosphate into indole.

The ability to dissociate and reassociate the B protein *in vitro* allows investigation of the ability of some mutant subunits to complement the deficiencies of others. A wide variety of altered proteins is available (Crawford and Johnson, 1964), and preliminary experiments have already uncovered several instances of activity increases following the formation of hybrid molecules between certain mutant pairs. These experiments and a complete description of the association phenomenon will be the subject of future communications.

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Studies of the Conformation and Interaction in Dinucleoside Mono- and Diphosphates by Proton Magnetic Resonance*

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ABSTRACT: Proton magnetic resonance data (100 Mc) on 15 nucleosides and nucleotides and on 25 dinucleoside mono- and diphosphates (23 (3′–5′)-ribosyl dimers, TpT, and (2′–5′)-ApC) are reported. The data consist of the chemical shifts, the peak assignments, the line widths, and the coupling constants of all the base protons and the H-1′ protons, observed at two temperatures, 28–30 and 60°, and at varying concentrations in D_2O. These data were analyzed on the basis of two basic guide lines established in studies on the monomers, *i.e.*, the shielding effect of the ring current on the neighboring bases and the specific deshielding effect of the 5′-phosphate on the H-8 proton of purine nucleotides and the H-6 proton of the pyrimidine nucleotides. From these studies, a general conformational model for all the dimers is constructed. In this model the nucleosidyl units all have the *anti* conformation with respect to the sugar–base torsion angle, and the turn of the (3′–5′) screw axis of the stack is right handed.

The general features of this model derived mainly from the data from the ribosyl dimers are similar to those of the single strand in the DNA helix. Within the temperature range of 30–60°, no major change in the ribose conformation or the sugar–base torsion angle of the dimer can be detected. The data suggest that the main effect of temperature elevation on the conformation of the dimer probably comes from the rotation of the phosphorus–oxygen bond (–P–O–) in the ester linkage.

The significance of the conformation of dinucleoside mono- or diphosphates has been well recognized as indicated by the extensive studies on this subject by several techniques from various laboratories. The importance of this problem can be envisaged from two points of view. Firstly, when the monomers are linked together covalently in varying degrees of polymerization, the properties of the dimer must have the closest relationship to those of the monomer as compared with other members in the series. The conformation of the dimer is relatively simple and perhaps can be known to a high degree of completeness and precision. This knowledge is obtained usually through a careful and systematic comparison between the monomer and the dimer. From such a study, the influence of a neighboring unit exerted on the linked monomer may be deduced. Secondly, the dimer can be viewed also as the fundamental unit for the building of the polymer. The

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