Biochimica et Biophysica Acta, 445 (1976) 464—474

BBA 67910

ANTHRANILATE SYNTHASE-ANTHRANILATE 5-PHOSPHORIBOSYLPYROPHOSPHATE PHOSPHORIBOSYLTRANSFERASE FROM *SALMONELLA TYPHIMURIUM*

PURIFICATION OF THE ENZYME COMPLEX AND ANALYSIS OF MULTIPLE FORMS

THOMAS H. GROVE * and H. RICHARD LEVY

Biological Research Laboratories, Department of Biology, Syracuse University, 130 College Place, Syracuse, N.Y. 13210 (U.S.A.)

(Received April 5th, 1976)

Summary

1. The anthranilate synthase-anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase enzyme complex (chorismate pyruvate-lyase (amino-accepting), EC 4.1.3.27) · (*N*-(5′-phosphoribosyl)-anthranilate:pyrophosphate phosphoribosyltransferase, EC 2.4.2.18), from *Salmonella typhimurium* has been purified with high yields to homogeneity. Sodium dodecyl sulfate gel electrophoresis of the purified enzyme complex revealed one major band containing 96% of the protein. The final yield of enzyme complex activity ranged from 30 to 60%. The absorbance spectrum of enzyme complex showed a peak at 280 nm and fine structure with peaks at 253, 259, 266 and 269 nm. These latter wavelengths correspond closely with the known absorbance maxima of phenylalanine.

2. When purified enzyme complex was subjected to standard gel electrophoresis, a four band pattern of protein peaks was consistently observed. The major enzyme complex band was apparently the native tetramer, having a molecular weight of 280000 and containing ammonia- and glutamine-dependent anthranilate synthase activity. The other three bands were molecular weight isomers of the major enzyme complex band. Two forms of molecular weight isomers were present: dimers and an aggregate of the native enzyme complex. The molecular weight isomers of the enzyme complex may represent forms generated by aggregation and denaturation of the native enzyme complex.

3. A new and highly sensitive spectrophotometric assay for phosphoribosyltransferase is described. The method is based upon the difference in extinction coefficients between anthranilate and *N*-(5′-phosphoribosyl)anthranilate.

* Present address: Dept. of Laboratory Medicine, University of Connecticut School of Medicine, Farmington, Conn. 06032 (U.S.A.).

Introduction

In *Salmonella typhimurium*, the complex between anthranilate synthase (chorismate pyruvate-lyase (amino-accepting), EC 4.1.3.27) and anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase (*N*-(5′-phosphoribosyl)-anthranilate:pyrophosphate phosphoribosyltransferase, EC 2.4.2.18) catalyzes the first two specific steps in the biosynthesis of tryptophan [1]. Anthranilate synthase catalyzes the reaction shown in Eqn. 1.

$$\text{Chorismate} + NH_3 \text{ (or L-glutamine)} \xrightarrow{Mg^{2+}} \text{Anthranilate} + \text{pyruvate} + H^+ \text{ (+L-glutamate)} \quad (1)$$

When anthranilate synthase is not associated with phosphoribosyltransferase, only NH_3 can be used as the amino donor in eqn. 1. Association of anthranilate synthase with phosphoribosyltransferase allows either NH_3 or glutamine to be utilized as a substrate. Phosphoribosyltransferase associated or unassociated with anthranilate synthase catalyzes the reaction shown in Eqn. 2.

$$\text{Anthranilate} + \text{5-phosphoribosyl 1-pyrophosphate} \xrightarrow{Mg^{2+}} (N\text{-5′-Phosphoribosyl})\text{anthranilate} + PP_i \quad (2)$$

The enzyme complex has been purified by classical [2] and affinity column [3] procedures. In our hands, the classical procedure did not give reproducible results and had a low yield (10%). The affinity column procedure resulted in an alteration of the glutamine amidotransferase activity of the enzyme complex. The present study describes a purification procedure characterized by a high yield and reproducible results. Electrophoretic analysis of the purified enzyme complex revealed the presence of multiple forms, differing in molecular weight and/or charge.

Methods

Enzyme purification

Growth of *Salmonella typhimurium trpE2* (ATCC 25566), disruption of cells and preparation of the ultracentrifuge supernatant have been described [3]. An important modification of the procedure is that 0.05 M potassium phosphate, pH 7.4, containing 0.1 mM EDTA, 0.4 mM 2-mercaptoethanol and 30% glycerol (Buffer 1) is now used in all purification steps except (a) when washing the cells with 0.05 M potassium phosphate, pH 7.4, and (b) for dissolving protamine sulfate in 0.1 M potassium phosphate, pH 7.0. The first 0—40% ammonium sulfate precipitate was centrifuged at 24000 × *g* (average) for 15 min. These modifications resulted in a lesser proportion of small proteins (<200000 daltons) compared with large proteins in the ultracentrifuge supernatant. The enzyme complex was further purified by gel-filtration on a 5 × 90 cm column of Biogel A1.5m arranged for upward elution (Fig. 1A). A flow rate of 50—60 ml per h was maintained with the use of a peristaltic pump. Fractions of 8 ml were taken. Fractions of peak glutamine-dependent anthranilate synthase activity were pooled and concentrated to 0.5 mg protein per ml using an Aminco Model 212 stirred pressure cell with a PM10 membrane. The solution was de-gassed in vacuo.

Anion exchange chromatography was done with DEAE-Biogel A. The resin was washed with 0.01 M NaOH and then equilibrated with Buffer 1 by following the conductivity and pH of the suspension. The sample was applied to a 1.6 × 26 cm column of DEAE-Biogel A and eluted using a 300 ml linear gradient of 0—0.3 M NH_4Cl in Buffer 1 (Fig. 1B). Fractions of 3 ml were taken. Fractions of peak glutamine-dependent anthranilate synthase specific activity were pooled and ammonium sulfate was added to 50% saturation. After stirring for 45 min, the suspension was centrifuged at 24000 × *g* (average) for 45 min. The pellet was dissolved in Buffer 1 and stored at −18° C.

Enzyme complex assay

L-Glutamine-dependent and NH_3-dependent anthranilate synthase assays were performed as previously described [3]. Phosphoribosyltransferase activity was assayed fluorometrically according to the method of Henderson et al. [4] with modification [3]. Assays were performed with a Hitachi Perkin-Elmer MPF-3 spectrofluorimeter equipped with a thermostatically temperature-con-

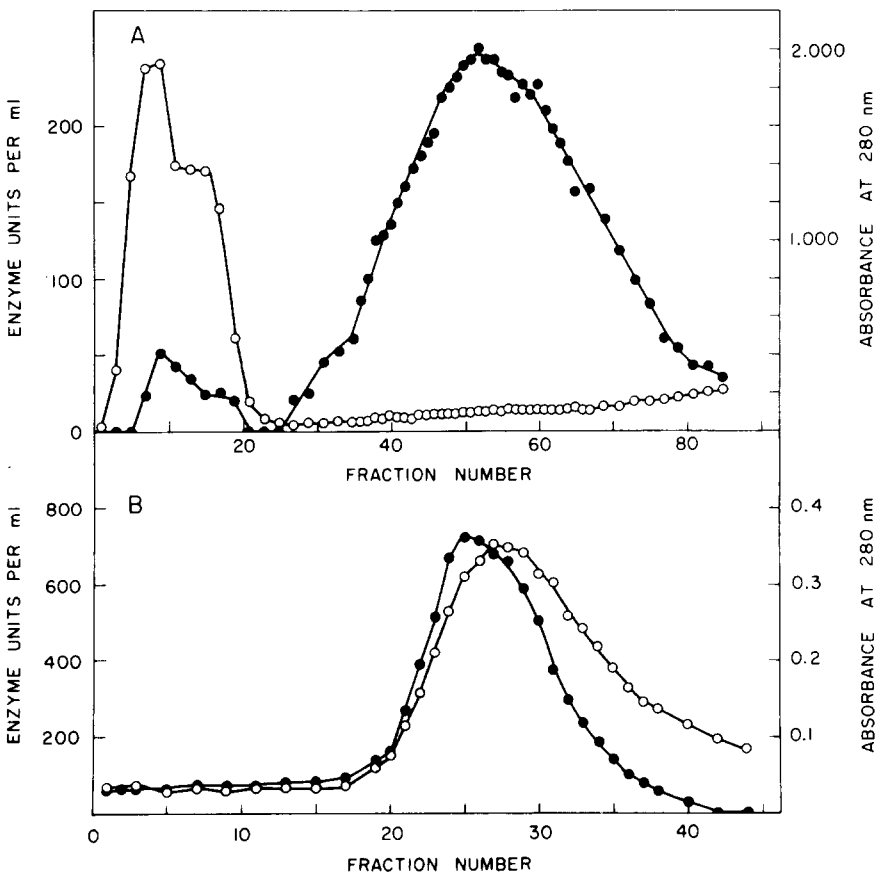

Fig. 1. Elution profiles of columns used in the enzyme complex purification. (A) Biogel A1.5m fractionation. (B) DEAE-Biogel A fractionation. Enzyme assays and column methodology are described under Methods. ●, glutamine-dependent anthranilate synthase activity; ○, 280 nm absorbance.

trolled sample compartment (excitation 320 nm; emission 400 nm).

Phosphoribosyltransferase activity in purified samples of enzyme complex was assayed by a new spectrophotometric procedure. The method, based on that used by Kronenberg et al. [5] for ATP-phosphoribosyltransferase, depends on the difference in extinction coefficients at 250 nm or 320 nm between anthranilate and *N*-(5′-phosphoribosyl)anthranilate [6] and results in an increase in absorbance as the reaction proceeds. The spectrophotometric assay procedure is more sensitive than the fluorimetric procedure as it measures an increase in absorbance rather than a decrease in fluorescence. Saturating amounts of the fluorescent substrate anthranilate can be used in the spectrophotometric assay. The reaction mixture contained: 0.2 mM anthranilate, 0.4 mM 5-phosphoribosyl 1-pyrophosphate, 2.5 mM $MgCl_2$, 1 mM 2-mercaptoethanol and either 20 mM barbitol, pH 8.9 (for the 320 nm assay) or 50 mM triethanolamine · HCl, pH 8.9 (for the 250 nm or 320 nm assay). The assay was linear for the first 2 min and then curved off slightly. The 320 nm assay procedure is only 35% as efficient as the 250 nm procedure but circumvents much of the problem of background absorbance. The assay procedure gives a false low reading for partially purified preparations of enzyme complex. This may be due to the presence of co-derepressed indoleglycerol phosphate synthase, which utilizes *N*-(5′-phosphoribosyl)anthranilate as a substrate. One unit of phosphoribosyltransferase activity assayed spectrophotometrically is defined as the quantity of enzyme catalyzing a change of 0.001 absorbance units per min at 25°C at either 250 nm or 320 nm. The instability of *N*-(5′-phosphoribosyl)anthranilate precludes an accurate determination of its extinction coefficient. Assays were performed with a Gilford 240 spectrophotometer equipped with a thermostatically temperature-controlled cell compartment. The baseline of the Gilford spectrophotometer was offset to compensate for the background absorbance of anthranilate.

Even though the pH optimum for phosphoribosyltransferase activity is 7.4 [4], a more reliable enzyme activity is obtained at pH 8.9. This is because the product *N*-(5′-phosphoribosyl)anthranilate is unstable at neutral and acid pH, rapidly decomposing into anthranilate and 5-phosphoribose [6]. *N*-(5′-phosphoribosyl)anthranilate was synthesized according to the method of Creighton [6], partially purified by precipitating with 5 M lithium chloride in ethanol and analyzed for stability at various pH values and at 25°C by observing the fluorescence of anthranilate (excitation 320 nm; emission 400 nm). At pH 8.9, *N*-(5′-phosphoribosyl)anthranilate was found to be stable over the time course of the enzyme assay.

Protein analysis

Protein was determined by a microbiuret procedure [7], by the method of Warburg and Christian [8] or by the extinction coefficient determined below. Specific activity refers to units per mg of protein.

The extinction coefficient at 280 nm was determined on a dry weight basis with the use of a salt-free enzyme complex solution. The enzyme complex solution was dialyzed with 3 changes against 4000 vols. of distilled water for 36 h. The protein precipitated as a result of this dialysis and was redissolved by dialyzing against 3000 vols. of 1 mM NaOH for 24 h. An aliquot was then dis-

solved in 0.048 mM potassium phosphate, pH 7.5 and the absorbance at 280 nm determined. The NaOH treatment did not interfere with the 280 nm absorbance of the protein. A volume of dialyzed sample containing approximately 2 mg of protein was dried onto a preweighed metal planchet. The planchet was dried at 105—110°C in an oven to constant weight and the amount of dry weight protein was calculated.

Electrophoresis

Standard gel electrophoresis was done according to the method of Davis [9] on 5% acrylamide, with a Tris/glycine (pH 8.3) reservoir buffer. Either ammonium persulfate (1.4 mg/ml) or riboflavin (2.5 μg/ml) was used as the catalyst. Gels were pre-run for 30—60 min before layering on of the sample. Protein in the gels was stained using Coomassie Brilliant Blue. The percent of the total protein in each band was estimated by scanning the stained gel, cutting out the peak tracings and weighing the paper. Native enzyme complex was located on the gels by assaying for anthranilate synthase activity as previously described [10]. Urea gel electrophoresis was performed by modifying the standard gel electrophoresis system with the inclusion of 8 M urea and 10 mM 2-mercaptoethanol in the upper reservoir buffer and in the gels. The sample was prepared by incubating the enzyme complex in 8 M urea containing 10 mM 2-mercaptoethanol for 21 h at room temperature. Sodium dodecyl sulfate gel electrophoresis was performed according to the method of Weber and Osborn [11]. The gels were fixed and stained as described elsewhere [3]. The distances of migration and intensities were measured using a linear transport attachment on a Gilford 240 spectrophotometer.

Chemicals

Sodium dodecyl sulfate, riboflavin, 5-phosphoribosyl 1-pyrophosphate, L-glutamine, and barium chorismate were from Sigma Chemical Co. Barium chorismate was converted to its potassium salt before use. Enzeme grade ammonium sulfate and ultrapure urea came from Mann Research Labs; once-recrystallized anthranilate, acrylamide and bisacrylamide from Eastman Chemical Co.; DEAE-Biogel A and Biogel A1.5m from Bio-Rad Labs; *N,N,N'N'*-tetramethylethylenediamine from Matheson, Coleman and Bell; and ammonium persulfate from E-C Apparatus Corp. All other chemicals were of reagent grade.

Results and Discussion

Purification

A summary of a typical purification is shown in Table I. The final yield of enzyme complex activity ranged from 30 to 60% and represents a 3- to 5-fold improvement over the yields of previously purified enzyme complex [2]. The procedure resulted in a 34-fold purification of the glutamine-dependent anthranilate synthase activity and an 58-fold purification of the phosphoribosyltransferase activity. Using the spectrophotometric assay procedure the specific activity of purified phosphoribosyltransferase was 15300 units per mg of protein when assayed at 250 nm and 5300 units per mg of protein when assayed at 320 nm.

TABLE I

SUMMARY OF PURIFICATION OF ENZYME COMPLEX

Fraction	Volume (ml)	Protein (mg/ml)	Anthranilate synthase			Phosphoribosyltransferase		
			Total activity (units)	Specific activity (units/mg)	Yield (%)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Extract	135	22 *	111 900	37	—	135 900	45	—
Ultracentrifuge supernatant	32	7.7 **	62 000	250	55	96 400	390	70
Biogel A 1.5 m	308	0.1 **	37 900	1230	33	80 100	2600	59
DEAE-Biogel A	42	0.52 **	31 900	1460	28	71 400	3270	53
Ammonium sulfate	1.3	22.6 ***	37 300	1270	33	76 300	2600	56

* Protein determined by microbiuret procedure.

** Protein determined by the method of Warburg and Christian [8].

*** Protein determined by extinction coefficient determined in this paper. When the protein concentration was determined as in footnote **, the protein concentration was 15.2 mg/ml and the specific activity was 1890 for anthranilate synthase and 3860 for phosphoribosyltransferase.

The absorbance spectrum of the enzyme complex (Fig. 2) showed a peak at 280 nm and fine structure with peaks at 253, 259, 266 and 269 nm. These latter wavelengths correspond closely with the known absorption maxima of phenylalanine [12]. The absorption spectrum tails off from 300 to 475 nm (data not shown). Dixon et al. [16] have speculated that this tail is diagnostic of a tightly bound transition metal ion, possibly involved in the binding of ammonia at an active site. The A_{280}:A_{260} ratio was 1.5. The extinction coefficient at 280 nm and in 0.048 M potassium phosphate, pH 7.5 is 6.78 for a 1.0% solution.

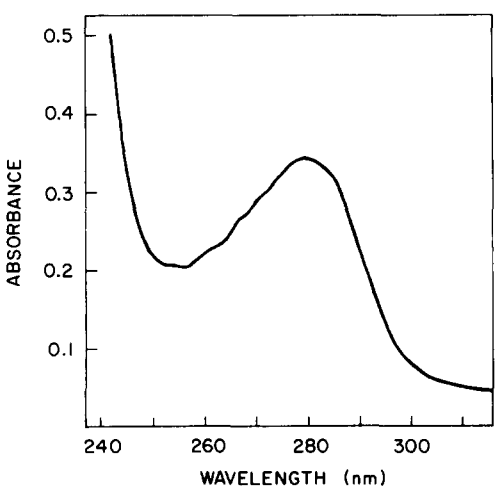

Fig. 2. Absorbance spectrum of purified enzyme complex. The enzyme complex (50 μg/ml) was in 50 mM potassium phosphate, pH 7.4 at room temperature. Spectral measurements were taken with a Cary Model 15 spectrophotometer.

Electrophoresis of enzyme complex

When purified enzyme complex was subjected to standard gel electrophoresis using either ammonium persulfate or riboflavin as the catalyst, a four-band pattern of major peaks was consistently observed (Fig. 3A). Minor peaks, representing less than 5% of the total protein, were not consistently present in the various final preparations. Sodium dodecyl sulfate gel electrophoresis of the en-

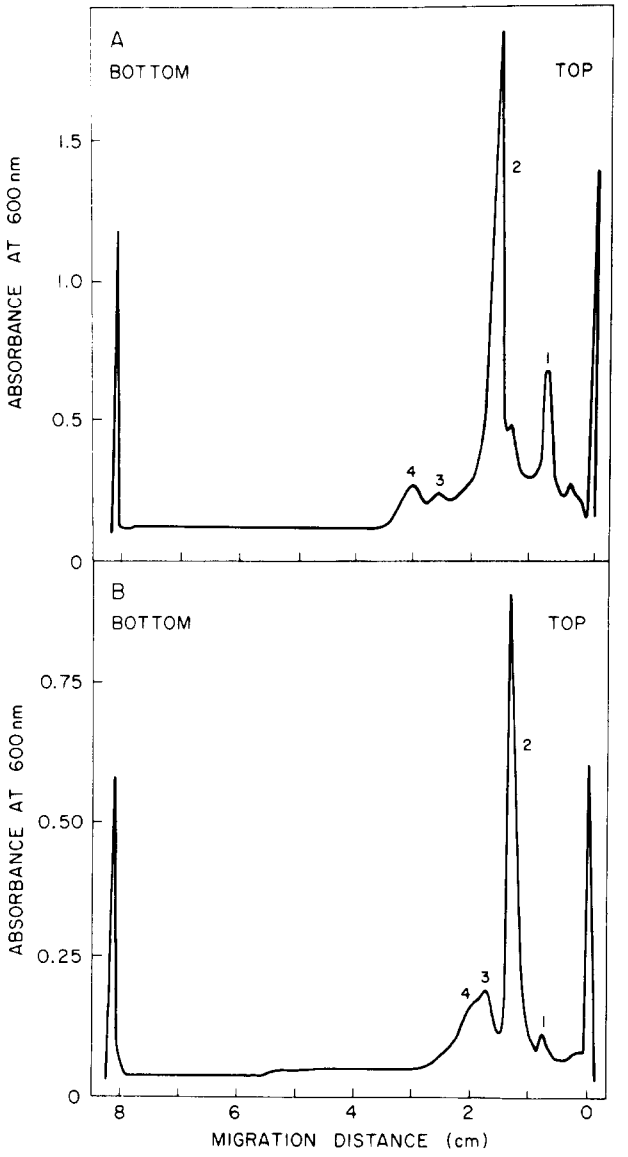

Fig. 3. Scans of purified enzyme complex electrophoresed on 5% standard polyacrylamide gels. The protein bands are numbered in order of increasing mobility with band 1 being near the top. (A) Scan of once-run enzyme complex. The gel contained 20 μg of protein and was stained for protein. (B) Scan of rerun enzyme complex. Band 2 of once-run enzyme complex was rerun and stained for protein.

zyme complex revealed one major band containing 96% of the protein and trace amounts of 3—5 other protein bands. Urea gel electrophoresis resolved the enzyme complex into two bands. The faster moving band has been identified as the anthranilate synthase subunit and the slower moving band as the phosphoribosyltransferase subunit [13]. The ratio of protein staining intensities of anthranilate synthase to phosphoribosyltransferase subunits is 1.10. Assuming the enzyme complex subunits absorb protein stain with equal intensity, there is an equal amount of anthranilate synthase and phosphoribosyltransferase subunits in the purified enzyme complex. A picture of representative standard, sodium dodecyl sulfate and urea electrophoresis gels is shown in Fig. 4. Bands 1 and 2 of purified enzyme complex have both glutamine- and NH_3-dependent anthranilate synthase activities when assayed directly on the gels. Bands 3 and 4 did not show either of these activities. Activity bands corresponding to bands 1 and 2 are also present in crude extract samples subjected to standard gel electrophoresis.

Rerun experiment

Standard electrophoresis gels were run using 100 μg of purified enzyme complex per gel. Band 2 was located by the anthranilate synthase gel assay, sliced from the gel and eluted by placing the minced slices in 200 μl of Buffer 1. When isolated band 2 was rerun on standard electrophoresis gels, it separated into the same four band pattern as the once-run enzyme complex (Fig. 3B). There was an equilibration between band 2 and bands 1, 3 and 4. Table II

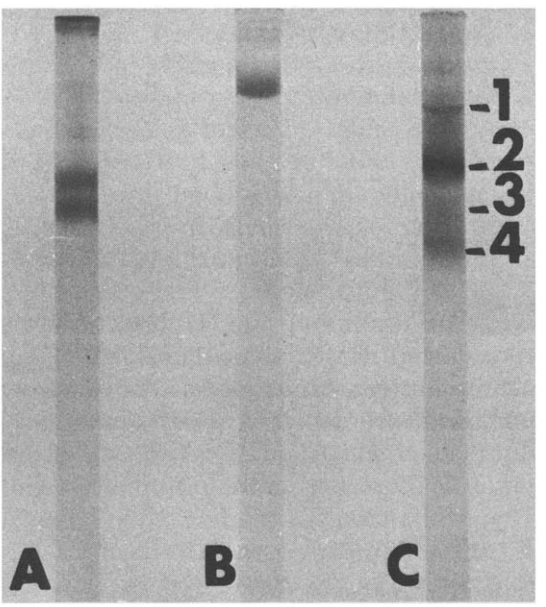

Fig. 4. Representative gels of purified enzyme complex electrophoresed under various conditions. A, Urea electrophoresis gel. B, Sodium dodecyl sulfate electrophoresis gel. C, Standard polyacrylamide electrophoresis gel. Each gel contained 20 μg of protein. The band nomenclature for C is described in the legend for Fig. 3.

TABLE II

ELECTROPHORETIC COMPARISON OF MULTIPLE FORMS OF ENZYME COMPLEX

Band	Once-run protein		Rerun protein	
	R_m	Percentage of total enzyme complex per band	R_M	Percentage of total enzyme complex per band
1	0.15	13	0.16	4
2	0.24	71	0.27	65
3	0.35	8	0.36	16
4	0.40	8	0.41	15

compares the R_M values and percent distribution of the various bands of once-run and rerun enzyme complex.

Properties of the multiple forms

An analysis of the multiple bands was done by the method of Hedrick and Smith [14] by plotting varying gel concentration versus 100 log ($R_M \times 100$). The results shown in Fig. 5 were obtained. Band 1 is a molecular weight isomer of band 2, as are bands 3 and 4; bands 3 and 4 are charge isomers of each other. The slope of the Hedrick and Smith plot is related to the molecular weight of the protein [14]. A standard curve was generated using bovine serum albumin monomer (mol.wt. 68000), dimer (mol.wt. 136000) and trimer (mol.wt. 204 000), rat mammary gland glucose-6-phosphate dehydrogenase monomer (mol. wt. 120 000) and dimer (mol. wt. 235 000), and apo-ferritin monomer (mol.wt. 450000) and dimer (mol.wt. 900000). The standard curve sloped downward at high molecular weight values. The following preliminary estimates of molecular weights were found for the multiple forms: band 2, 280000; band 3, 150 000; and band 4, 130000. Band 1 was an aggragate of band 2, probably with a molecular weight of 520000.

The enzyme complex is a tetrameric structure (mol.wt. 280000) composed of two monomers each of anthranilate synthase (mol.wt. 62000) and phosphoribosyltransferase (mol.wt. 62000) [15]. It thus appears that band 1 is an

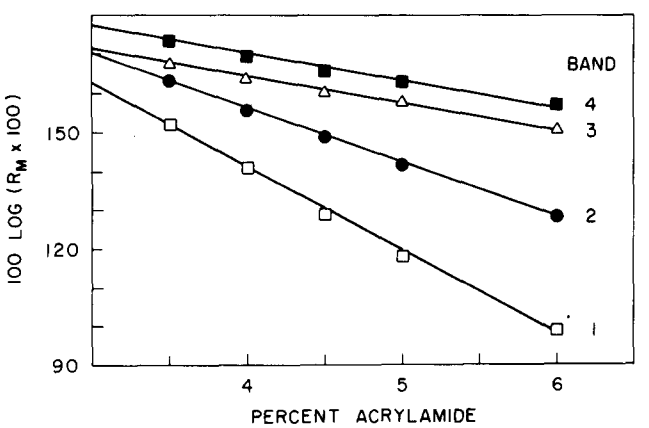

Fig. 5. Plot of 100 log ($R_M \times 100$) vs. acrylamide concentration for purified enzyme complex. Each gel contained 25 μg of protein and was stained for protein. R_M values were determined relative to the migration of the Bromophenol Blue dye front. The band nomenclature is described in the legend for Fig. 3.

octamer, band 2 is the native tetramer, and bands 3 and 4 are dimers of the enzyme complex subunits.

Protein in bands 1 and 2 was extracted from the gels as outlined in the standard gel electrophoresis rerun experiment. The individual bands were then subjected to urea gel electrophoresis to separate the enzyme complex subunits. The urea gel band pattern of rerun bands 1 and 2 was identical to the urea gel pattern of once-run enzyme complex. The ratio of anthranilate synthase to phosphoribosyltransferase subunits was 1.17 for band 2 and 1.02 for band 1. Thus, the tetramer and octamer are both composed of equal numbers of the two enzyme complex subunits. No attempt was made to rerun the diffuse and anthranilate synthase-inactive bands 3 and 4.

No regulatory significance is ascribed to the presence of the multiple forms. Rather, the band pattern is believed to be characteristic of enzyme complex purified by this procedure. It is not known whether the various forms are present in solution or are generated upon standard gel electrophoresis. The enzyme complex does show a tendency to aggregate into high-molecular-weight forms (>1500000). Active enzyme complex fractions were detected in the void volume of Biogel A1.5m (Fig. 1A) and Biogel A5m [3] gel-filtration columns. Brewer [17] has reported multiple banding in urea gel electrophoresis of yeast enolase, a protein containing no cysteine or cystine. Mitchell [18] observed similar results in standard electrophoresis of clostridiopeptidase B as did Yue et al. [19] with creatine kinase. The apparent cause of all of these multiple band patterns was the oxidizing effect of ammonium persulfate, used as a catalyst in gel polymerization. A large percentage of the protein aggregated and denatured during electrophoresis. The problem was overcome in the examples cited by using riboflavin as the catalyst in gel polymerization. In this study, the multiple forms of the enzyme complex were present whether ammonium persulfate or riboflavin was used as the catalyst. The presence or absence of an electrophoretic pre-run also did not change the banding pattern. The origin of the multiple forms is still not clear. Bands 1, 3 and 4 may represent forms generated by aggregation and denaturation of the enzyme complex in band 2. The apparent homogeneity of purified enzyme complex on urea and sodium dodecyl sulfate electrophoresis gels supports this view.

Acknowledgements

This work was supported by Grant GM19839 from the United States Public Health Service. The authors thank Roberta Sheffer for her excellent technical assistance.

References

1 Bauerle, R.H. and Margolin, P. (1966) Cold Spring Harbor Symp. Quant. Biol. 31, 203—214
2 Henderson, E.J., Nagano, H., Zalkin, H. and Hwang, L.H. (1970) J. Biol. Chem. 245, 1416—1423
3 Grove, T.H. and Levy, H.R. (1975) Biochim. Biophys. Acta 397, 80—93
4 Henderson, E.J., Zalkin, H. and Hwang, L.H. (1970) J. Biol. Chem. 245, 1424—1431
5 Kronenberg, H., Vogel, T. and Goldberger, R. (1975) Anal. Biochem. 65, 380—388
6 Creighton, T. (1968) J. Biol. Chem. 243, 5605—5609
7 Zamenhof, S. (1957) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds.), Vol. 3, p. 702, Academic Press, New York

8 Warburg, O. and Christian, W. (1941) Biochem. Z. 310, 384—421
9 Davis, B. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
10 Grove, T.H. and Levy, H.R. (1975) Anal. Biochem. 65, 458—465
11 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
12 Beaven, G.H. and Holiday, E.R. (1952) Adv. Protein Chem. 7, 319—386
13 Nagano, H., Zalkin, H. and Henderson, E.J. (1970) J. Biol. Chem. 245, 3810—3820
14 Hedrick, J.L. and Smith, A.J. (1968) Arch. Biochem. Biophys. 126, 155—164
15 Henderson, E.J. and Zalkin, H. (1971) J. Biol. Chem. 246, 6891—6898
16 Dixon, N.E., Gazzola, C., Blakeley, R.L. and Zerner, B. (1976) Science 191, 1144—1150
17 Brewer, J.M. (1967) Science 156, 256—257
18 Mitchell, W. (1967) Biochim. Biophys. Acta 147, 171—174
19 Yue, R.H., Jacobs, H.K., Okabe, K., Kentel, H.J. and Kuby, S.A. (1968) Biochemistry 7, 4291—4298