The Anthranilate Synthetase–5-Phosphorylribose 1-Pyrophosphate Phosphoribosyl Transferase Complex of the Tryptophan Pathway in *Salmonella typhimurium*. Purification by the *in Vitro* Assembly of Its Subunits*

Derald Smith and Ronald H. Bauerle†

ABSTRACT: A bifunctional enzyme aggregate catalyzes the first two steps of tryptophan biosynthesis in *Salmonella typhimurium*. The complex is composed of an arrangement of two nonidentical protein components, the anthranilate synthetase subunit and the 5-phosphorylribose 1-pyrophosphate phosphoribosyl transferase subunit. A method of purification of the complex has been devised which makes use of two properties of the system: (1) when mixed *in vitro*, the uncomplexed subunit proteins undergo a rapid and spontaneous self-assembly forming the active complex, and (2) auxotrophic mutant strains of *S. typhimurium* are available which lack either the anthranilate synthetase component or the 5-phosphorylribose 1-pyrophosphate phosphoribosyl transferase component and thus can serve as sources of uncomplexed 5-phosphorylribose 1-pyrophosphate phosphoribosyl transferase and anthranilate synthetase subunits, respectively. Partially purified extracts of two such mutant strains were subjected to preliminary, parallel fractionation by Sephadex G-100 gel filtration. These two subunit preparations were subsequently combined permitting spontaneous aggregation of the subunits into the complex. The pooled preparation was then refractionated by repeating the Sephadex G-100 gel filtration. The newly formed anthranilate synthetase–5-phosphorylribose 1-pyrophosphate phosphoribosyl transferase complex emerged from the column characteristically in the excluded volume preceding the residual uncomplexed subunits and the other nonspecific components which were present in the mixture. A high degree of purity of the isolated complex was indicated by ultracentrifugational analysis and disc gel electrophoresis. This technique of "complementation-coupled gel filtration" might readily be applied to the purification of other enzyme or protein aggregates.

The first two reactions of the biosynthetic pathway leading to tryptophan in *Salmonella typhimurium* are catalyzed by a bifunctional enzyme complex composed of the proteins coded by the first two genes of the tryptophan (*trp*) operon (Bauerle and Margolin, 1966). The gene–enzyme relationships and the enzymatic steps involved in the synthesis of tryptophan are shown in Figure 1. Both component I, the product of gene *trpA*, and component II, the product of gene *trpB*, are required subunits for the synthesis of anthranilic acid from chorismic acid when L-glutamine, contributing its amide group, serves as the amino donor. It appears, though, that all the catalytic sites for the synthesis of anthranilic acid are found on component I since the reaction proceeds efficiently *in vitro* in the absence of component II if NH_4^+ ions replace glutamine as the amino donor (Bauerle and Margolin, 1967) and since L-glutamine competitively inhibits the utilization of NH_4^+ (Zalkin and Kling, 1968). Thus the role of component II in the *in vivo* reaction can be considered a type of activation of component I, possibly by effecting through its binding, conformational changes which are necessary for the glutamine-dependent reaction and which do not exist when the subunit is uncomplexed. Besides this function in the first reaction, component II also catalyzes the second reaction of the pathway, namely the transfer of the 5-phosphoribosyl moiety of 5-phosphorylribose 1-pyrophosphate to anthranilic acid forming *N*-(5-phosphoribosyl)anthranilate. This activity proceeds with equal efficiency whether component II is complexed with component I or is free. Owing to the distinct intrinsic activities of the two proteins we will refer herein to component I as the anthranilate synthetase (ASase) subunit of the complex and component II as the phosphoribosyl transferase (PRTase) subunit.

The anthranilate synthetase–5-phosphorylribose 1-pyrophosphate phosphoribosyl transferase complex is interesting for several reasons: first, it possesses a unique quaternary structure (as described above) and second, its activities are subject to negative feedback regulation by tryptophan (Bauerle and Margolin, 1966). We have been interested in pursuing a study of both these features and it occurred to us that a unique method for purifying the complex is possible. Mutant strains of *S. typhimurium* are available which lack the function either of *trpA* or of *trpB*. Such mutants can be

* From the Division of Biology, Southwest Center for Advanced Studies, P. O. Box 30365, Dallas, Texas. *Received November 5, 1968.* This work was supported by Research Grant GM14206 and Divisional Grant GM13234 from the U. S. Public Health Service.

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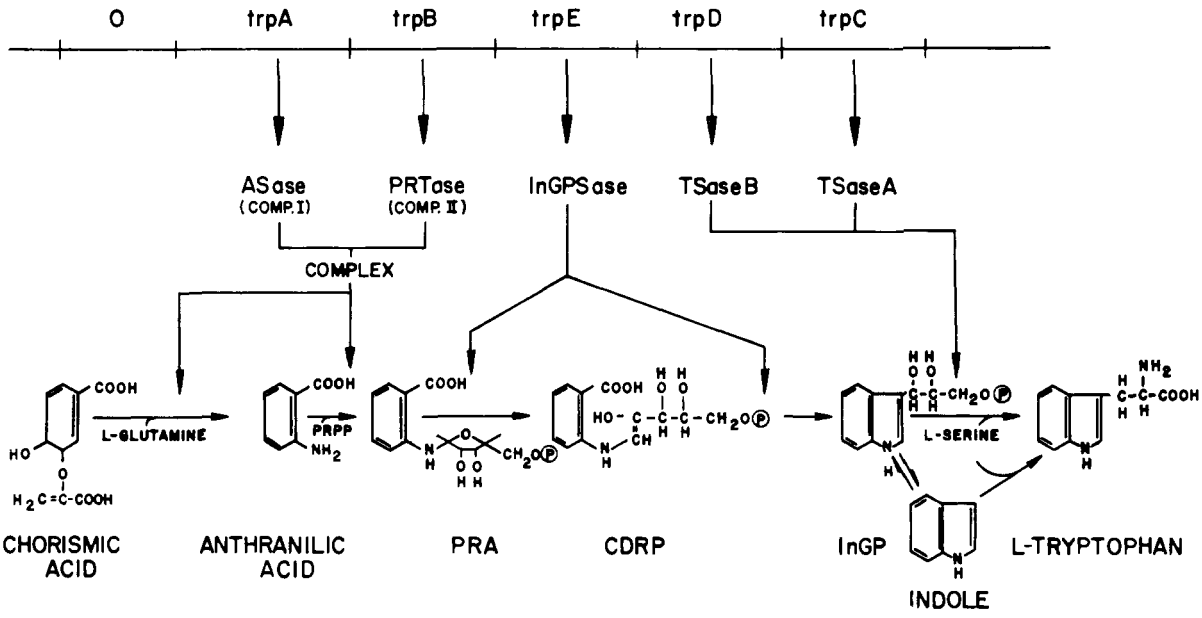

FIGURE 1: The genetic map of the *trp* operon and the tryptophan biosynthetic pathway.

used, then, as sources of the uncomplexed PRTase and ASase subunits, respectively. It has already been shown that when mixed together *in vitro* the ASase and PRTase subunits spontaneously and efficiently interact forming the active complex (Bauerle and Margolin, 1966). Taking advantage of these facts and applying gel filtration techniques, we have been able to obtain the ASase–PRTase complex in a highly purified state. This report, while concerned specifically with the ASase–PRTase complex, presents a purification procedure which could be appropriately applied to other enzyme or protein agregates where sources of uncomplexed subunits are available and where conditions for spontaneous self-assembly are feasible.

Materials and Methods

Organisms. The tryptophan auxotrophs of *S. typhimurium* used as sources of the enzyme subunits were strains *trpBEDC43* and *trpA703*. The properties of these mutants have been described previously (Bauerle and Margolin, 1966). Strain *trpBEDC43* carries a deletion mutation which removes all of genes *trpE*, *trpD*, and *trpC* and part of *trpB* but not gene *trpA* (see Figure 1) and, therefore, produces the ASase subunit in the uncomplexed form. Strain *trpA703* carries a probable nonsense mutation in the *trpA* gene which exerts a weak polar effect (44%) on the activity of the remaining operator distal genes (Bauerle and Margolin, 1966). Its PRTase subunit has been shown to exist in the uncomplexed form.

Chemicals. Chorismic acid was isolated and purified from the growth medium of *Aerobacter aerogenes strain 62-1* as described by Gibson (1964). Anthranilic acid was obtained from Sigma Chemical Co. and recrystallyzed from hot water. The other chemicals were purchased from the following sources: PRPP and dithiothreitol from Calbiochem; L-tryptophan, L-glutamine, and Tris from Sigma Chemical Co.; enzyme grade $(NH_4)_2SO_4$ from Mann Biochemicals; Sephadex and DEAE-Sephadex from Pharmacia Fine Chemicals, Inc.; sucrose from Merck; acrylamide, *N,N'*-methylenebisacrylamide, and *N,N,N',N'*-tetramethylethylenediamine from Eastman Organic Chemicals. All other chemicals were reagent grade.

Growth of Cells and Preparation of Cell-Free Extracts. Each organism (100 l.) was grown in glass carboys in 20-l. batches at 37° with forced aeration. The culture medium was the minimal salts medium of Davis and Mingioli (1950) modified by the exclusion of citrate and containing 0.25% glucose. L-Tryptophan was supplied at 5 μg/ml, an amount sufficient to limit the population at about 10^9 cells/ml. At the time the tryptophan supplement becomes nearly exhausted, the pathway enzymes are released from end product repression control and derepress to a level approximately 100-fold that of the repressed level. The cells were harvested by centrifugation at 2° and stored frozen as pellets at −75°. When ready for use the cells were slowly thawed and resuspended in standard buffer (0.05 M phosphate buffer (pH 7.5) containing 10^{-4} M EDTA and 10^{-4} M dithiothreitol) at 5 ml/g wet weight of cells. The cells were disrupted by ultrasonic oscillation using a Branson sonifier (Model W185C) operated at a meter setting of 70 A. Disruption was carried out in a jacketed continuous-flow Rosette cell which was cooled by constant circulation of coolant maintained at 2°. The flow rate of the cell suspension through the Rosette cell was adjusted such that one cell volume was exchanged in 1 min. Cell debris was removed by centrifugation at 2° for 45 min at 48,000*g*. The pellet was discarded and the supernatant used as the source of the enzyme.

Enzyme Assays. ASase was assayed by measuring fluorometrically the formation of anthranilic acid from

chorismic acid. *In vitro* both complexed and uncomplexed ASase are able to utilize NH_4^+ as amino donor in the reaction. However, it is possible to distinguish the ASase subunit in the complexed state from that in the uncomplexed state by virtue of the former's ability and the latter's inability to utilize L-glutamine, the physiologically active amino donor. The reaction mixture for the NH_4^+-dependent assay contained 0.25 μmole of chorismic acid, 10 μmoles of $MgCl_2$, 50 μmoles of $(NH_4)_2SO_4$, and 50 μmoles of Tris-HCl buffer (pH 9.0) and enzyme preparation in a final volume of 1.0 ml. The reaction mixture for the glutamine-dependent assay was identical except that the $(NH_4)_2SO_4$ was replaced by 20 μmoles of L-glutamine and 100 μmoles of phosphate buffer (pH 7.0) was used in place of the Tris buffer.

PRTase was assayed by measuring fluorometrically the disappearance of anthranilic acid. The reaction mixture contained 0.025 μmole of anthranilic acid, 0.3 μmole of PRPP, 10 μmoles of $MgCl_2$, 100 μmoles of Tris-HCl (pH 7.8), and enzyme preparation in a final volume of 1.0 ml.

The appearance or disappearance of anthranilic acid was followed directly at 22° using a Farrand photoelectric fluorometer (Model A3) equipped with a Sargent Model SRL recorder. Activating light of 313 mμ was selected by incorporation of an interference filter (half-band-width of 5 mμ) in the primary filter system of the fluorometer. The secondary filter system for the fluorescence emission selected light of wavelength between 380 and 480 mμ. Enzyme activity in each assay was derived from the initial velocity of the reaction. Activity was directly proportional to enzyme concentration and, except in the case of the uncomplexed ASase subunit in the NH_4^+ assay, proceeded linearly with time of incubation for at least 20 min. The activity of the ASase subunit remained linear for only 2–3 min.

One unit of enzyme activity is defined as the formation or removal of 0.1 μmole of anthranilic acid in 20 min at 22°. Specific activity is expressed as enzyme units per mg of protein. Protein was measured colorimetrically (Lowry *et al.*, 1951).

Purification of the ASase and PRTase Subunits. The preliminary steps in the purification of the ASase and PRTase subunits from crude extracts of the two mutant strains were identical. All procedures were carried out at 5° or less. After each step subunit enzyme activity (ASase or PRTase) and the protein content of the preparation were measured.

1. TREATMENT WITH STREPTOMYCIN SULFATE. To each liter of crude extract was added 50 ml of a 20% solution of streptomycin sulfate. The solution was stirred for 15 min and the precipitate which formed was removed by centrifugation at 23,000*g* for 20 min and discarded.

2. AMMONIUM SULFATE FRACTIONATION. To each liter of the supernatant from step 1 was slowly added with stirring 243 g of solid $(NH_4)_2SO_4$ (40% saturation at 25°). The preparation was then stirred for 15 min. The precipitate was collected by centrifugation at 23,000*g* for 20 min and the supernatant was discarded. The precipitate was dissolved in enough standard buffer to give a protein concentration of about 30 mg/ml. The preparation was then dialyzed for 6 hr against four changes (1 l. each) of standard phosphate buffer.

3. DEAE-SEPHADEX FRACTIONATION. The dialyzed preparation was applied to a column (6 × 15 cm) of DEAE-Sephadex (type A50) which had been swelled and equilibrated with standard buffer. After the solution had entered the gel bed, the column was washed with 200 ml of standard buffer and then eluted with a 1-l. KCl gradient of 0–0.5 M prepared in standard buffer. The flow rate was about 50 ml/hr and fractions of 10 ml were collected. Those fractions possessing enzyme activity greater than 5% that of the peak fraction were pooled and solid $(NH_4)_2SO_4$ was added with stirring to achieve 40% saturation. After 15 min the precipitate was collected by centrifugation at 23,000*g* for 20 min and dissolved in 15 ml of standard buffer, and the solution was dialyzed as in step 2.

4. SEPHADEX G-100 GEL FILTRATION. The dialyzed preparation of step 3 was applied to the bottom of a Sephadex G-100 gel column (5 × 140 cm) which had been equilibrated with standard buffer. Development of the column was by upward flow with standard buffer at a flow rate of 100 ml/hr maintained with a Harvard peristaltic pump (Model 600-1200V). Fractions of 20 ml were collected. Those fractions possessing enzyme activity greater than 5% that of the peak fraction were pooled. Solid $(NH_4)_2SO_4$ was added to the preparation to achieve 40% saturation. The precipitate was collected by centrifugation at 23,000*g* for 20 min and dissolved in 5 ml of standard buffer. The preparation was then desalted by passing through a Sephadex G-25 gel column (2 × 15 cm) using standard buffer.

Formation of the ASase–PRTase Complex. Up to this point the ASase and PRTase subunits were subjected to identical but separate fractionation procedures. The desalted preparations from step 4 were combined at 2° and allowed to stand for 30 min to permit aggregation of the subunits forming the active ASase–PRTase complex. It had been found in other experiments that complex formation takes place rapidly and efficiently under these conditions with the equilibrium favoring the complexed state.

Isolation of the ASase–PRTase Complex by Sephadex G-100 Gel Filtration. The pooled enzyme mixture containing the partially purified ASase and PRTase subunits was fractionated by gel filtration using the same Sephadex G-100 gel column and procedure as in step 4. As in all the previous steps, assay of each fraction for the ASase and PRTase subunits was performed. In addition, enzyme assay was also carried out for the ASase activity characteristic of the complex (see assay methods above) in order to distinguish the ASase activity of the complex from that of any residual, uncomplexed ASase subunit.

Disc electrophoresis was performed in 7.5% polyacrylamide gel in Tris–glycine buffer (pH 9.3) at 22° using the Buchler Polyanalyst apparatus according to the general procedure of Davis (1964).

Sucrose density gradient centrifugation was carried out in 5-ml linear sucrose gradients (5–20%) prepared in standard buffer. Centrifugation was at 36,500 rpm for 17 hr in a Spinco L2 ultracentrifuge at 2° using a

TABLE I: Preliminary Purification of the Anthranilate Synthetase Subunit of the Anthranilate Synthetase–5-Phosphorylribose 1-Pyrophosphate Phosphoribosyl Transferase Complex.[a]

Fraction	Vol (ml)	Protein Concn (mg/ml)	Total Protein (mg)	Total Act. (units)	Sp Act. (units/mg)	Recov (%)	Purificn
1. Crude extract	967	10	9,800	53,000	5.4	100	
2. Streptomycin sulfate supernatant	997			51,400		97	
3. $(NH_4)_2SO_4$ precipitate (dialyzed)	100	26	2,600	44,000	16.8	83	3
4. DEAE-Sephadex	221	2.6	566	22,800	40.3	43	7.5
5. Sephadex G-100	251	0.7	174	14,150	81	30	15

[a] Details of the procedures are described in Materials and Methods. Enzyme activity was determined using the NH_4^+ assay procedure.

SW50L rotor. Fractions of 10 drops were collected.

Sephadex G-200 gel filtration was used in the determination of the molecular weight of both the ASase–PRTase complex purified by the method described herein and the unpurified, naturally occurring complex present in a crude extract of wild-type cells. The gel column (1.5 × 94 cm), prepared and equilibrated with standard buffer, was developed by upward flow at a rate of 5 ml/hr. A sample volume of 2 ml was used and fractions of 2 ml were collected. The void volume of the column, determined using Blue Dextran 2000, was 62 ml. Calibration of the column was carried out with the following standard proteins at 2 mg/ml: bovine fibrinogen (molecular weight 330,000) and human γ-globulin (molecular weight 176,000), purchased from Pentex, Inc.; catalase (molecular weight 250,000), yeast alcohol dehydrogenase (molecular weight 150,000), and D-amino acid oxidase (molecular weight 115,000), purchased from Worthington Biochemical Corp. Protein was determined colorimetrically (Lowry *et al.*, 1951).

Results

Preliminary Purification of the ASase Subunit. The source of the ASase subunit was 207 g of cells (wet weight) of mutant strain *trpBEDC43* which yielded upon breakage 9.8 g of protein. Partial purification of the subunit from the crude extract was accomplished by the fractionation series shown in Table I. Details of the procedures are described in Materials and Methods. These preliminary steps resulted in an over-all 15-fold purification with 30% recovery of the initial enzyme activity.

The majority of the ASase subunit activity was eluted from the DEAE-Sephadex column (step 4, Table I) at 0.37 M KCl. In addition, a minor peak of ASase activity emerged in the wash volume before the gradient elution was started. This activity, which represented less than 1% of the total recovered activity, was discarded. The fractions collected between 0.32 and 0.43 M KCl were pooled. After concentration the enzyme preparation was applied to the Sephadex G-100 column (step 5). The void volume of the column, determined using Blue Dextran 2000, was 840 ml. The ASase activity emerged in two peaks. The minor peak, representing less than 2% of the recovered activity, was eluted at 1100 ml and was discarded. The majority of the activity appeared as a symmetrical peak at 1380 ml. The fractions spanning 1300–1560 ml were pooled and used as the source of the ASase subunit in subsequent steps. At this point the ASase preparation was not homogeneous when judged by disc gel electrophoresis.

Preliminary Purification of the PRTase Subunit. The source of the PRTase subunit was 178 g of cells (wet weight) of mutant strain *trpA703*, which yielded upon breakage 7.3 g of protein. The same preliminary fractionation procedures which were applied to the ASase subunit were also used in purification of the PRTase subunit. The results are shown in Table II. The steps yielded an over-all 58-fold purification of the enzyme with 20% recovery of the initial enzyme activity.

As was the case with the ASase subunit, a minor peak of PRTase activity, representing about 10% of the recovered activity, was eluted from the DEAE-Sephadex column with the wash volume and was discarded. The remaining PRTase activity was eluted from the column as a single peak at 0.38 M KCl. The fractions collected between 0.31 and 0.46 M KCl were pooled, concentrated, and then subjected to gel filtration, on the same Sephadex G-100 column used for fractionation of the ASase subunit.

The PRTase activity emerged from the Sephadex G-100 column at two positions, a minor peak representing about 10% of the recovered activity at 940 ml, and the major peak at 1160 ml. The fractions collected between 1080 and 1320 ml were pooled, concentrated, and used for subsequent complex formation with the partially purified ASase subunit. At this time, the PRTase subunit protein appeared to be nearly homogeneous as judged by disc gel electrophoresis.

It should be mentioned that in several experiments the behavior of the PRTase subunit upon gel filtration on Sephadex G-100 has been quite erratic. With some preparations a *minor* peak emerged at 1160 ml (the position of the major peak obtained with the preparation used here), with the major peak at 1380 ml (the position where the ASase subunit emerges and which, from the results of previous work (Bauerle and Margolin, 1966), would also have been expected of the

TABLE II: Preliminary Purification of the 5-Phosphorylribose 1-Pyroposphate Phosphoribosyl Transferase Subunit of the Anthranilate Synthetase–5-Phosphorylribose 1-Pyroposphate Phosphoribosyl Transferase Complex.[a]

Fraction	Vol (ml)	Protein Concn (mg/ml)	Total Protein (mg)	Total Act. (units)	Sp Act. (units/mg)	Recov (%)	Purificn
1. Crude extract	630	11.5	7,300	123,300	16.9	100	
2. Streptomycin sulfate supernatant	654			101,700		83	
3. $(NH_4)_2SO_4$ precipitate (dialyzed)	57	31.5	1,800	76,800	42.6	62	2.5
4. DEAE-Sephadex	302	0.9	263	41,700	159	34	9.5
5. Sephadex G-100	230	0.11	25	24,800	982	20	58

[a] Details of the procedures are described in Materials and Methods.

TABLE III: Formation and Isolation of the Anthranilate Synthetase–5-Phosphorylribose 1-Pyroposphate Phosphoribosyl Transferase Complex.[a]

Fraction	Enzyme Act.	Vol (ml)	Total Protein (mg)	Total Act. (units)	Sp Act. (units/mg)	Recov (%)	Purificn
1. Crude Complex	Anthranilate synthetase–NH_4^+ assay	18	241[b]	16,900	70	36	13
	5-Phosphorylribose 1-pyroposphate phosphoribosyl transferase	18	241[b]	23,000	95	19	5.6
	Anthranilate synthetase–glutamine assay	18	241[b]	5,900	24	(100)	
2. Sephadex G-100	Anthranilate synthetase–NH_4^+ assay	80	23	6,400	283	13.7	
	5-Phosphorylribose 1-pyroposphate phosphoribosyl transferase	80	23	11,300	497	9.1	29
	Anthranilate synthetase–glutamine assay	80	23	4,600	202	(78)	(8)

[a] Details of the procedures are described in Materials and Methods. The recovery and purification values in parentheses refer to the results obtained in the formation of the "crude complex" (step 1 above) from the partially purified subunits from step 5 of Tables I and II, and in the Sephadex G-100 fractionation of the "crude complex" (step 2 above).
[b] It is assumed that the discrepancy between this value and the value expected from the combination of the preparations of step 5 of Tables I and II is due to experimental error in the protein assay procedure.

PRTase subunit). It would appear that this phenomenon reflects some association–dissociation behavior of the PRTase subunit, probably with itself, which is affected by conditions which are not yet clear and therefore have not been properly controlled.

In Vitro Formation of the ASase–PRTase Complex. As mentioned earlier formation of the ASase–PRTase complex occurs rapidly and spontaneously when preparations of the uncomplexed subunits are mixed (Bauerle and Margolin, 1966). Accordingly, a simple mixture of the partially purified ASase preparation (containing a total of 174 mg of protein) with the PRTase preparation (containing 25 mg of protein) was made. At this step therefore, the specific activity of each subunit should decrease since the total protein content of the pooled preparation increased although the total enzymatic activity of each subunit remained the same. Since the ASase preparation contained seven times as much protein as the PRTase preparation, it would be expected that the specific activity of the ASase subunit should decrease by only 12% while that of the PRTase subunit should suffer a drastic eightfold reduction. Such was the case (Table III). The specific activity of the ASase subunit of the mixture decreased from 81 to 70 units per mg of protein while that of the PRTase subunit decreased from 982 to 95 units per mg. Assay for the ASase activity characteristic of the complex (the glutamine reaction) demonstrated that the *in vitro*

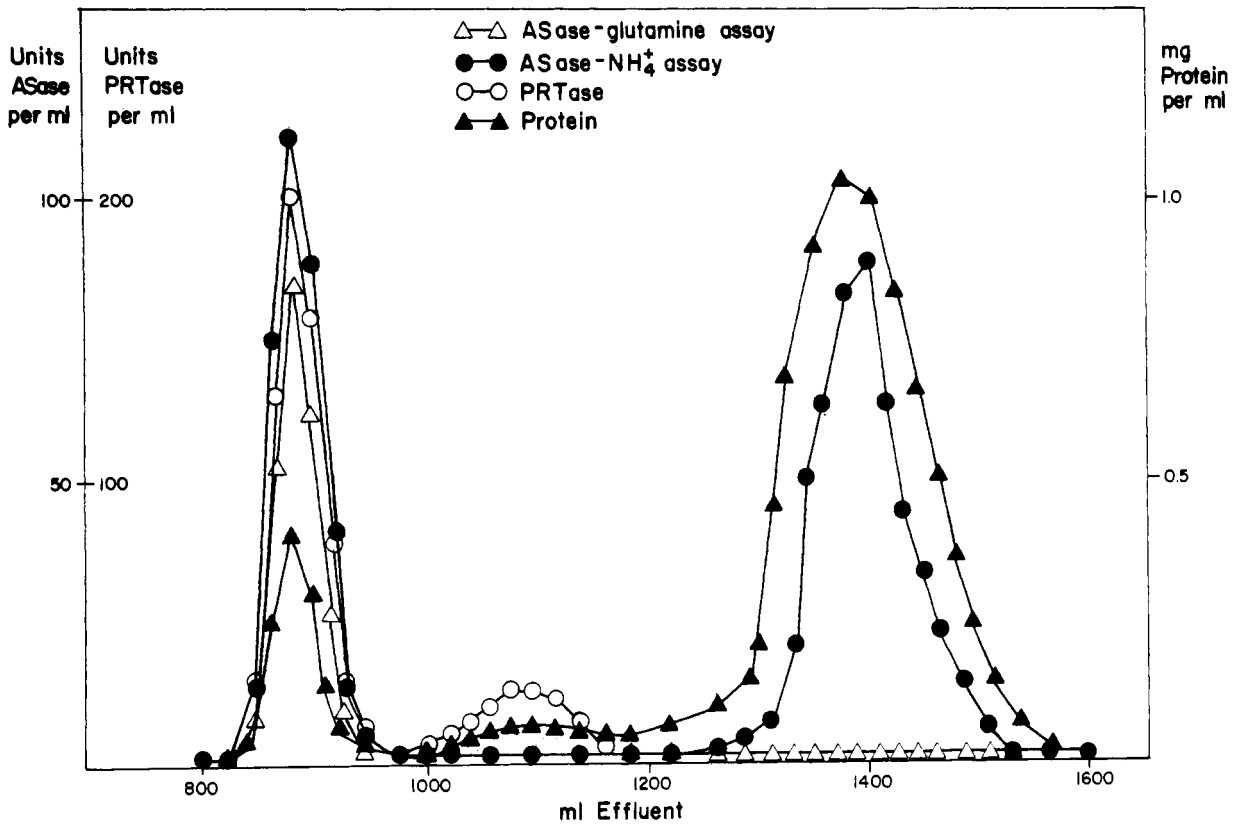

FIGURE 2: Isolation of the anthranilate synthetase–5-phosphorylribose 1-pyrophosphate phosphoribosyl transferase complex from a mixture of its partially purified subunits by Sephadex G-100 gel filtration. Conditions were described in Materials and Methods.

assembly of the two subunits did take place. The specific activity of ASase in this reaction was found to be 24 units/mg (Table III).

Isolation of the ASase–PRTase Complex. Sephadex G-100 gel filtration was used to separate the newly formed complex from the residual uncomplexed subunits of the pooled preparation. The elution pattern of the column is shown in Figure 2. The complex emerged from the column as a symmetrical peak between 800 and 1000 ml, a position quite distinct from those obtained earlier with the two uncomplexed subunits. The ASase activity (detectable by both the NH_4^+ and glutamine assay procedures) and the PRTase activity were exactly coincidental, peaking at 880 ml. A symmetrical protein peak was present at this same position coincidental with the enzyme activities. Since the material present at this position in the column effluents in the two preliminary gel filtration treatments was discarded, it can be concluded that this new enzyme species represents a specific aggregation of the smaller subunit molecules. The bulk of the protein (as expected) and a large peak of ASase subunit activity (detectable only by the NH_4^+ assay procedure) emerged at 1400 ml. A small broad peak of PRTase subunit activity was found at about 1100 ml, near the position found previously for one species of the subunit (see above). The elution profile of the Sephadex G-100 column indicates then that a large excess of the ASase subunit was present in the pooled ASase–PRTase preparation. A major fraction of the PRTase subunit molecules was converted to the complex form by association with ASase subunit molecules.

The fractions collected between 840 and 920 ml were pooled and analyzed (Table III). The gel filtration resulted in about a fivefold increase in specific activity of the PRTase component. When assayed using the NH_4^+ assay procedure the ASase component showed a fourfold increase in specific activity. However, using the glutamine assay, an eightfold purification was indicated. This apparent discrepancy reflects the fact that whereas all of the ASase subunit molecules in the pooled preparation (crude complex of Table III) possessed activity in the NH_4^+ assay, only a portion possessed activity in the glutamine assay since the PRTase subunit was present in limiting amount. This was not so in the case of the purified complex.

The over-all purification of the ASase subunit of the isolated complex cannot be calculated directly from the specific activity of the crude extract since the subunit is significantly more active in the NH_4^+ assay when functioning in the complexed state. However the final purification can be considered to be the product of the purification obtained in the final gel filtration of the crude complex (eightfold) and the over-all purification of the subunit obtained in the procedures prior to this final step (13-fold), or about 100-fold. On the other hand the degree of purification of the PRTase subunit of the complex can be calculated directly from the activity

TABLE IV: Specific Activities of the Anthranilate Synthetase–5-Phosphorylribose 1-Pyroposphate Phosphoribosyl Transferase Complex in the Peak Fractions of the Final Sephadex G-100 Gel Filtration.[a]

Fraction	Vol (ml)	Total Protein (mg)	Total Activity (units)		Specific Activity (units/mg)	
			Anthranilate Synthetase	5-Phosphorylribose 1-Pyroposphate Phosphoribosyl Transferase	Anthranilate Synthetase	5-Phosphorylribose 1-Pyroposphate Phosphoribosyl Transferase
43	20	5.1	1020	2510	200	493
44	20	8.4	1720	4110	205	490
45	20	6.1	1260	3070	206	504
46	20	3.1	580	1590	187	514

[a] Details of the procedures are described in Materials and Methods. The anthranilate synthetase activity was determined using the glutamine assay procedure. The four fractions are from the Sephadex G-100 gel filtration fractionation shown in Figure 2 and contain the column effluent between 860 and 920 ml.

of the crude extract since the subunit is equally active in the complexed and uncomplexed states. Accordingly, the PRTase subunit has been purified 29-fold. This result can be reconciled with the finding that the preliminary fractionation of the PRTase subunit yielded an essentially homogeneous enzyme after 58-fold purification (Table II) if it is assumed that (a) the ASase–PRTase complex is composed of equal numbers of each subunit and (b) the molecular weight of each protomer is about the same. Previous data (Bauerle and Margolin, 1966) indicate that at least the latter assumption is correct. Thus, the over-all purification of the PRTase in the complex (29-fold was obtained) would be expected to be half that attained in the homogeneous subunit preparation (58-fold).

Purity of the Isolated ASase–PRTase Complex. A reasonably high degree of purity of the isolated complex is indicated by the results of several types of analysis. (1) The occurence in the final gel filtration step (Figure 2) of coincident protein, ASase and PRTase peaks, suggests a high degree of homogeneity of the isolated complex. This is supported by the fact that the specific activities of both the ASase and PRTase components were essentially constant in each fraction throughout the activity peak (Table IV). (2) Analytical ultracentrifugational analysis using boundary sedimentation also indicates a high degree of homogeneity (Figure 3). It can be concluded from the tracing that the preparation contains one major component ($s_{20,w}$ value calculated to be 13.2 assuming a partial specific volume of 0.725 cm^3/g) plus a minor, more slowly sedimenting component, which represents about 10% of the total protein content (assuming equivalent extinction coefficients for each component). (3) Disc electrophoresis in acrylamide gels demonstrated the presence of one major protein band and several very minor bands which possessed higher mobilities.

The minor protein components which have been revealed by analytical ultracentrifugation and disc gel electrophoresis may not represent impurities in the preparation but in fact may have arisen by partial dissociation of the enzyme complex. This seems likely since sucrose gradient analysis of the purified enzyme has revealed a minor peak of uncomplexed ASase trailing the predominant, rapidly sedimenting ASase–PRTase complex. In addition, when the acrylamide gel column on which the purified complex has been electrophoresed was sliced into thin cross sections and the gel slices extracted with standard buffer, traces of ASase activity could be demonstrated coincident with the weakly staining protein bands as well as with the major band. Although it cannot be said that all of the apparent nonhomogeneity can be attributed to dissocia-

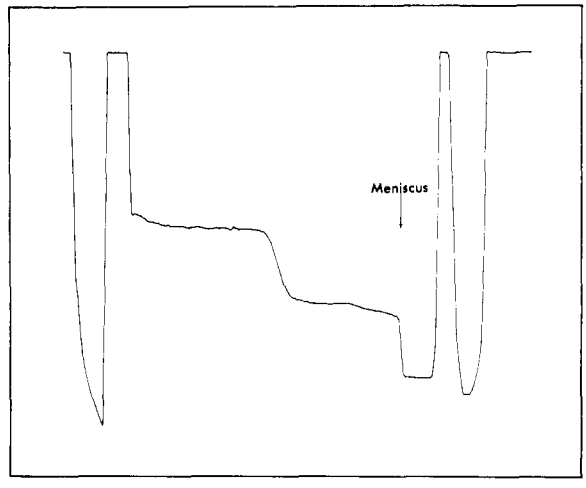

FIGURE 3: Sedimentation analysis of the purified anthranilate synthetase–5-phosphorylribose 1-pyrophosphate phosphoribosyl transferase complex. A Spinco Model E analytical ultracentrifuge equipped with ultraviolet optical system and a monochromator was used. The enzyme, at a concentration of 1.1 mg/ml ($OD_{280m\mu}$ 0.75), in 0.05 M phosphate buffer (pH 7.5) was sedimented at 56,000 rpm at 20° using a single-sector, 12-mm cell. Sedimentation was followed by monitoring the absorbance at 280 mμ. The tracing shown is of the exposure taken 24 min after full speed was attained, and was made using a Joyce-Loebel Mark IIIc microdensitometer.

TABLE V: Kinetic Constants of the Anthranilate Synthetase–5-Phosphorylribose 1-Pyroposphate Phosphoribosyl Transferase Complex in the Anthranilate Synthetase Reaction.[a]

Enzyme	K_M (M) Chorismic Acid	K_M (M) Glutamine	V_{max} (units)
Purified anthranilate synthetase–5-phosphoryl-ribose 1-pyroposphate phosphoribosyl transferase (*in vitro* assembled complex)	4×10^{-6}	9×10^{-4}	308
Crude anthranilate synthetase–5-phosphoryl-ribose 1-pyroposphate phosphoribosyl transferase (natural complex)	5×10^{-6}	8×10^{-4}	335

[a] The constants were determined by Lineweaver–Burk treatment of the data. The crude anthranilate synthetase–5-phosphorylribose 1-pyroposphate phosphoribosyl transferase complex was a cell-free extract of wild-type cells grown in unsupplemented minimal medium. The extract was passed through a Sephadex G-25 gel column (2×15 cm), using standard buffer, in order to remove low molecular weight components.

tion of the complex, it would not be too unreasonable to assume this.

The Nature of the Isolated ASase–PRTase Complex. One important factor which must be considered is whether the complex purified in the manner described here is identical in structure and function with the complex occurring naturally. This question is especially relevant since the complex isolated here was assembled from a nonstoichiometric mixture of its subunits, a situation which most probably does not occur *in vivo*. An exhaustive, comparative study of the subunit structure and kinetic properties of purified preparations of the *in vitro* assembled and of the naturally occurring complex is required in order to clarify this issue. This is not possible presently since we have not yet completely purified the natural complex. However some experiments have been carried out comparing certain properties of the complex purified as described here with those of an unpurified preparation of the natural complex, obtained from wild-type cells.

The purified and the unpurified, natural complex were found to be essentially identical in molecular weight as judged by zone sedimentation in sucrose gradients (5–20%) as well as by gel filtration analyses using a Sephadex G-200 column (1.5×94 cm) calibrated using a set of standard proteins. In the gel filtration procedure the ASase–PRTase complex of both preparations emerged in the included volume, each peaking at 86 ml (void volume of the column was 62 ml). From the results obtained with standard proteins, a molecular weight of about 290,000 was estimated for the purified and crude complexes. Limited kinetic analysis of the ASase reaction of the complex has also revealed no great difference in properties of the two complex preparations (Table V). In addition both appeared to be equally sensitive to end product inhibition by L-tryptophan. Thus on the basis of this preliminary evidence, it is reasonable to assume tentatively that the ASase–PRTase complex isolated here does represent the natural species of the enzyme.

Discussion

The unique feature of the enzyme purification procedure described here is the use of what might be called "complementation-coupled gel filtration." The pivotal factor is the spontaneous self-assembly, after mixing, of the ASase and PRTase subunits, which had been partially purified by separate yet parallel gel filtration methods, forming the active ASase–PRTase enzyme complex. Refractionation of the mixture using the same gel filtration treatment readily sorts out the newly formed, larger complex from the protein components remaining uncomplexed. The purified product appears to possess a high degree of homogeneity. The streptomycin sulfate, $(NH_4)_2SO_4$, and DEAE-Sephadex fractionation procedures preliminary to the actual gel filtration steps were incorporated into the purification procedure only to reduce the protein load on the Sephadex G-100 column. We have found that if crude extracts containing less protein are used these preliminary fractionation steps can be omitted with no reduction in the effectiveness of the gel filtration technique. Thus, the limiting factor in the isolation of preparative amounts of the complex by this procedure is the capacity of the gel column that is used. Of course any technique which separates proteins on the basis of molecular size or weight, such as preparative sucrose gradient centrifugation, could be substituted for the gel filtration procedure. It can be imagined that the use of this method could be extended to the isolation and purification of any macromolecular aggregate where sources of the unaggragated components are available and where conditions for self-assembly of the aggregate from its subunits are attainable.

It is clear that purification of the ASase–PRTase complex as such could also be accomplished by fractionation, using standard techniques, of an extract of a bacterial strain possessing both subunits already bound in the complex. Partial purification of the analogous complex in *Escherichia coli* has been accomplished in such a way (Baker and Crawford, 1966).

Isolation of the complex by complementation-coupled gel filtration offers an alternate method which is unique, is able to be carried out with facility, and renders a very pure product.

A study of the kinetic, allosteric, and subunit properties of the purified ASase–PRTase complex is now under way.

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Kinetic Mechanism of Maltodextrin Phosphorylase*

Julie Chao, George F. Johnson, and Donald J. Graves†

ABSTRACT: The kinetic mechanism of *Escherichia coli* maltodextrin phosphorylase has been investigated by (1) initial velocity experiments, (2) studies with inhibitors, and (3) isotopic exchange measurements at equilibrium. Rate equations have been derived for various mechanisms. These equations are different from the common two substrate systems because one of the substrates, polysaccharide, serves as a reactant in both the forward and reverse reactions. Double-reciprocal plots of initial velocity measurements were linear and showed converging line patterns. Inhibition by maltotetraose was competitive with respect to maltoheptaose and mixed with respect to P_i; uridine diphosphate glucose was competitive with respect to P_i and mixed with respect to maltoheptaose. The equilibrium exchange rates for $^{32}P_i \rightleftarrows$ glucose 1-phosphate and [^{14}C]glucose 1-phosphate $\rightleftarrows$ maltoheptaose increased when the concentrations of P_i and glucose 1-phosphate were increased (in constant ratio) at a fixed concentration of maltoheptaose. The exchanges also increased as a function of maltoheptaose concentration at a constant glucose 1-phosphate and P_i. The data are consistent with a rapid equilibrium random Bi-Bi kinetic mechanism for *E. coli* phosphorylase.

Maltodextrin phosphorylase of *Escherichia coli* has been purified and found to possess enzymatic properties similar to animal and plant polysaccharide phosphorylases (Schwartz and Hofnung, 1967). This enzyme was shown to contain 1 mole of pyridoxal phosphate/mole of protein indicating that maltodextrin phosphorylase is a simple monomeric system. Detailed kinetic studies of the complex multisite system of liver and muscle glycogen phosphorylases have been done (Lowry *et al.*, 1967; Maddaiah and Madsen, 1966), but their actual kinetic mechanisms have not yet been completely established. Mechanistic studies have been initiated on the simpler bacterial phosphorylase to firmly establish (1) its kinetic mechanism and (2) provide a framework for further studies, *e.g.*, pH dependence of kinetic parameters, to elucidate the role of specific functional groups and/or pyridoxal phosphate in phosphorylase catalysis.

The mechanism of action of maltodextrin phosphorylase has been derived from initial velocity experiments, inhibition studies, and isotopic exchange measurements and is consistent with a rapid equilibrium random Bi-Bi kinetic mechanism. A preliminary account of this work has been given (Chao and Graves, 1968).

Experimental Section

Materials. Glucose 6-phosphate dehydrogenase, phosphoglucomutase, potassium glucose 1-phosphate, uridine diphosphoglucose, and carrier-free [^{32}P]phosphoric acid in 1 N HCl were obtained from Calbiochem. TPN was purchased from Sigma Chemical Co. The substrate, maltoheptaose, and the inhibitor, maltotetraose, were gifts of Dr. Walter Verhue and Dr.

* From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50010. *Received September 16, 1968.* Journal Paper J-6121 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Project No. 1510. This research was supported by a research grant (GM-09587) from the National Institutes of Health, U. S. Public Health Service.

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