Purification of Anthranilate 5-Phosphoribosylpyrophosphate Phosphoribosyltransferase from Salmonella typhimurium Using Affinity Chromatography: Resolution of Monomeric and Dimeric Forms

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Summary

Anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase (PRT) as the unaggregated enzyme, has been purified to 90-95 percent homogeneity using affinity and ion-exchange chromatography. The affinity matrix consisted of anthranilate coupled to succinylamidohexamethylimino-Sepharose via its free amino group. Electrophoresis of the purified PRT on gels of varying polyacrylamide concentrations resolved a faster-moving minor band at concentrations above 8 percent. Both the major and minor bands were found to possess PRT activity. The minor band was found to have a molecular weight one-half that of the major band. Electrophoresis of the enzyme in the presence of sodium dodecyl sulfate resulted in almost complete conversion to the lower molecular weight form.

In Salmonella typhimurium, the second reaction in the tryptophan biosynthetic pathway is catalyzed by anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase (PRT), the product of the trp B gene of the tryptophan operon (1). The substrates of PRT are anthranilic acid and 5-phosphoribosyl 1-pyrophosphate (PRPP). PRT alone or aggregated with anthranilate synthetase (the product of the trp A gene) catalyzes the following reaction:

$$\text{Anthranilate} + \text{PRPP} \xrightarrow{Mg^{++}} \text{N-(5'-phosphoribosyl-)anthranilate} + \text{PPi}$$

PRT has been purified to near-homogeneity using classical means by Henderson et al (2). In this paper we describe a new method for the purification of PRT and evidence of the existence of monomeric and dimeric forms of the enzyme.

[1] This work is taken, in part, from a dissertation submitted in partial fulfillment of the requirements for the Ph.D. degree.

Purification of PRT

The unaggregated form of PRT was obtained from S. typhimurium strain trp A703, which carries a nonsense mutation at the distal end of trp A and produces the free trp B gene product (2).

Anthranilic acid was coupled to succinylamidohexamethylimino-Sepharose by procedures described elsewhere (3). Cells were cultured as described elsewhere (3), harvested, washed once in 0.05M potassium phosphate, pH 7.4, containing 1 mM 2-mercaptoethanol, 0.2 mM anthranilate, and 20 percent glycerol (v/v). Cells were then resuspended in 3 volumes of wash buffer and broken by ultrasonic disruption. Cell debris was pelleted at 27,000 X g for 45 minutes and discarded. All steps were performed at 0-4°. The extract was placed on a column of the anthranilate-Sepharose derivative previously equilibrated with wash buffer, and washed until no protein was observed in the effluent fractions. Enzyme activity was eluted using 0.05M triethanolamine-HCl, pH 9.5, containing 1 mM 2-mercaptoethanol and 20 percent glycerol. Peak PRT fractions were pooled and immediately concentrated by the addition, with stirring, of 0.24 grams of ammonium sulphate per ml of pooled eluate. Precipitated protein was centrifuged at 27,000 X g for 10 minutes. The pellet was dissolved in a minimal volume of wash buffer and dialysed against 160 volumes of wash buffer for 8 hours. The dialysate was placed on a column of DEAE-cellulose equilibrated with wash buffer and eluted using a linear gradient of 0-0.25M NaCl in wash buffer. Peak PRT fractions were pooled, dialysed overnight against 70 volumes of wash buffer, and placed on a column of DEAE-Sephadex previously equilibrated with wash buffer. PRT activity was eluted with the same linear gradient used for the DEAE-cellulose step. Peak PRT fractions were pooled and concentrated using an Amicon ultrafiltration apparatus with a UM-10 filter. A summary of the purification procedure is given in Table 1. Purity was determined by polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue, scanned using a Gilford gel scanner, and the area under each peak determined.

Table 1

Purification of PRT

Step of purification	Total protein (milligrams)	Total activity (units)	Specific activity (units/mg protein)	Per cent yield
1. Crude extract	1052	52,000	50.8	---
2. Ammonium sulfate concentrate of affinity column eluate	50.5	20,800	412.0	40
3. DEAE-cellulose	8.8	7,920	900.0	15.2
4. DEAE-Sephadex (after concentration)	1.77	2,800	1540.0	5.4

Assays were performed as previously described (2), except that a 3-ml volume was used and 2-mercaptoethanol was substituted for dithiothreitol. One unit of enzyme activity represents the disappearance of 1 nmole of anthranilate per minute. Specific activity is expressed as units per milligram of protein. Protein determinations were performed according to the method of Lowry et al (9).

Molecular Weight Determinations

The molecular weight of the active, purified PRT was estimated using the procedure of Hedrick and Smith (4). Polyacrylamide gels and Tris-glycine buffer (pH 8.2) were prepared according to Lipshitz and Lebowitz (5).

At gel concentrations below 8 percent, PRT migrated as a single band, although 8 percent gels showed a slight haze in front of the dense band (Figure 1). At gel concentrations above 8 percent a discrete minor band was seen running just ahead of the major band (bands 2 and 1 in Figure 1, respectively). Scans of stained 10 percent gels showed that band 2 contained approximately 20-25 percent of the protein in band 1. Fifty µg of PRT were electrophoresed on 10 percent polyacrylamide gels and each gel was then sliced into 1.3 mm sections. Each slice was macerated in equal volumes

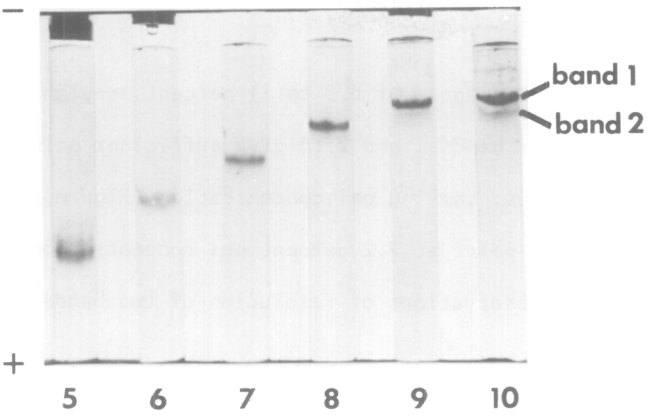

Figure 1. Resolution of PRT bands 1 and 2 on gels of increasing acrylamide concentration. Fifty µg of protein were applied to each gel. Migration is toward the + pole.

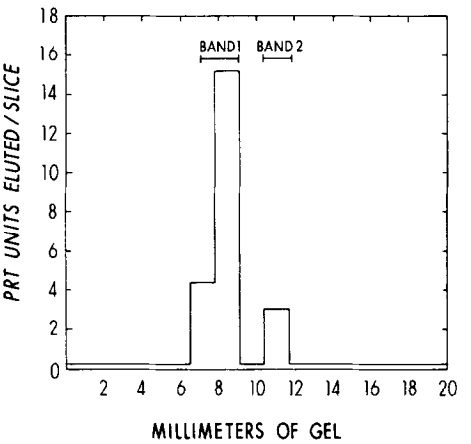

Figure 2. Histogram of PRT activity eluted from 1.3 mm slices of gels containing 10 percent acrylamide. Fifty µg of protein were applied to each gel. Duplicate gels were fixed and stained to determine the location of bands 1 and 2. Distance was measured from the top of each gel.

of wash buffer at 4°C and PRT activity assayed after 24 hours. Identical gels were stained for localization of protein bands. Results indicated that both bands 1 and 2 contained PRT activity in amounts roughly equivalent to their degree of staining (Figure 2); band 2 contained 18-25 percent of the PRT activity found in band 1.

Electrophoresis using gels of 8.5 to 11 percent acrylamide allowed the measurement of Rm's for bands 1 and 2 to give sufficient points for a plot of 100 log (Rm X 100) vs. varying gel concentration (Figure 3). Such plots showed an intersection point at 7.2 percent gel concentration, in good agreement with our visual observations of resolution of two bands above 8 percent

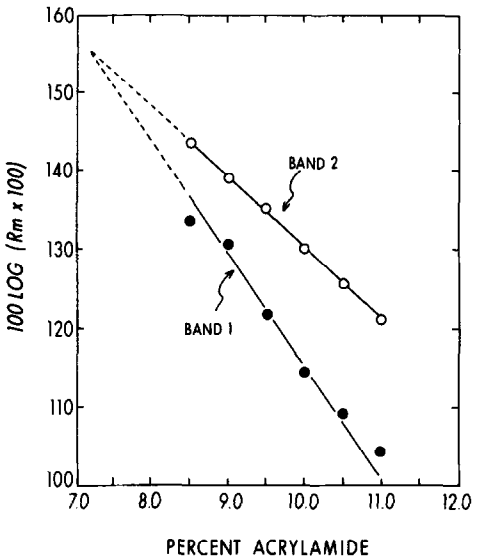

Figure 3. Plot of 100 log(Rm X 100) vs. acrylamide concentration for PRT bands 1 and 2. Rm values were determined relative to the migration of the bromophenol blue dye front. Each point represents the average of data collected from at least two gel runs. Dashes indicate extrapolation to an intersection point.

gel concentration. Molecular weight estimates of bands 1 and 2 using the slopes of the above plots and relating them to a standard curve (5) were 150,000 ± 15,300 for band 1, and 70,000 ± 6,500 for band 2.

Sodium-dodecyl-sulfate gel electrophoresis was performed according to the method of Weber and Osborn (6). After treatment with SDS, PRT ran as one major band at 64,000 molecular weight (Figure 4), and a minor band appeared at 107,000 ± 11,000 molecular weight which may represent undissociated dimer. The molecular weight of the major band agrees with the previously published value of 63,000 (2).

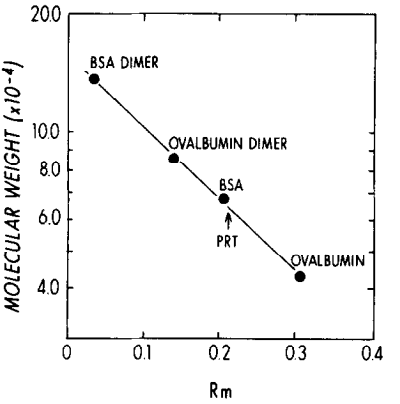

Figure 4. Molecular weight estimation of the PRT polypeptide chain using SDS-gel electrophoresis (6). Rm values were calculated relative to the bromophenol blue dye front. Each point is the averaged data from at least four determinations. BSA, bovine serum albumin. Values for the molecular weights of the reference polypeptide chains (2): BSA (68,700), ovalbumin (43,000).

Discussion

Purified PRT appears to exist in both monomeric and dimeric forms under the conditions used for analysis. These two forms were resolved using polyacrylamide gel electrophoresis on gels above 8 percent in acrylamide. Henderson et al have reported the molecular weight of PRT (using gel filtration) to vary from 75,000 to 100,000, with an average value of 87,000 ± 9,600. Their values for the PRT polypeptide chain using SDS-gel electrophoresis agree well with ours. Our results suggest that previously observed molecular weight estimates of the active form of PRT obtained using gel filtration may represent an intermediate value arising from the presence of a monomer-dimer equilibrium. Disc gel electrophoresis on gels of varying acrylamide concentrations allows separation of proteins not only by size but also by charge (4), and thus provides a more powerful technique by which the two forms of PRT can be resolved. PRT monomer molecular weight estimates obtained using the methods of Hedrick and Smith and SDS-gel electrophoresis differ by approximately 10 percent. This discrepancy is consistent with the currently accepted views that all protein-SDS complexes assume the same rod-like shape, varying

only in the length of the rod (7), whereas migration of a native protein through a gel depends on the radius and surface area of the specific protein being studied (6).

The existence of a dimer form of PRT may have some significance in light of recent findings (8) that the native anthranilate synthetase-PRT aggregate may be composed of two subunits of each component. Our results raise the question of whether PRT is packaged into the dual-enzyme aggregate as the monomer or dimer.

Acknowledgments

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