On the Evolution of an Oligocephalic Enzyme. Glutamine-Chorismate-Amidotransferase-Free Anthranilate Phosphoribosyltransferases from Mutant Strains of *Salmonella typhimurium*

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Oligocephalic Enzymes, Enzyme Purification, Glutamine-Chorismate-Amidotransferase-Free Anthranilate Phosphoribosyltransferases, Subunit Assembly, Enzyme Evolution

(1) A procedure has been described for the purification of two glutamine-chorismate-amidotransferase-free anthranilate phosphoribosyltransferases from mutant strains TAX6*trpR782* and *trpAB1653trpR782* of *Salmonella typhimurium*.

(2) The native enzymes tend to aggregate forming polymers of molecular weights 333,000 in the case of TAX*trpR782* and 220,000 and larger than 1×10^6 in the case of *trpAB1653trpR782*. In the presence of sodium dodecyl sulfate the polymer of *trpAB1653trpR782* dissociates into a single component with molecular weight of 72,000.

(3) In contrast to anthranilate phosphoribosyltransferase of the wild type component II, the glutamine-chorismate-amidotransferase-free proteins do not complex with component I. They do however show catalytical similarities with the wild type with respect to anthranilate phosphoribosyltransferase activity.

In *Salmonella typhimurium* the first two reactions of tryptophan biosynthesis are catalyzed by the multifunctional anthranilate synthase (EC 4.1.3.27)-anthranilate phosphoribosyltransferase (EC 2.4.2.18). This enzyme complex is a tetramer composed of two molecules each of subunits component I and component II coded for by genes *trpA* and *trpB* of the tryptophan operon [1].

The intact aggregate having the composition (component I – component II)$_2$ is required to catalyze the first reaction

(a) chorismate + L-glutamine $\xrightarrow{Mg^{2+}}$ anthranilate + pyruvate + H_2O.

Neither component I nor component II alone can mediate this reaction. But if glutamine is replaced by ammonia only component I is necessary for the formation of anthranilate. The second reaction

(b) anthranilate + 5-phospho-α-D-ribose-1-diphosphate $\xrightarrow{Mg^{2+}}$ N-(5′-phosphoribosyl)-anthranilate + PP_i

is catalyzed by either the complex or component II alone (Fig. 1).

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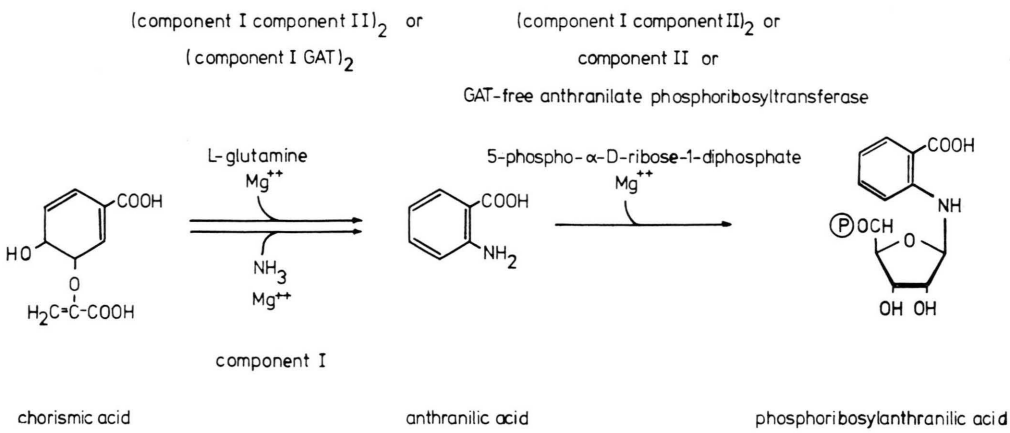

Fig. 1. The first two biosynthetic reactions of the tryptophan pathway catalyzed by the anthranilate synthase-anthranilate-5-phosphoribosylpyrophosphate phosphoribosylthransferase complex of *Salmonella typhimurium*. (Abbreviation used GAT = glutamine-chorismate-amidotransferase).

Previously it was demonstrated that anthranilate phosphoribosyltransferase activity can be removed from either the entire aggregate [2] or from the uncomplexed component II [3] by proteolytic digestion. In the former case a partial complex is obtained that can catalyze reaction (a) in the presence of glutamine. The degradation of uncomplexed component II yields a protein that restores glutamine utilization of component I in reaction (a). This protein (molecular weight 24.000) functions as a glutamine-chorismate-amidotransferase and its role in reaction (a) has been called "component I – complementing activity" *. From this it is evident that component II of the antranilate synthase-anthranilate phosphoribosyltransferase is an oligocephalic enzyme which possesses within one polypeptide glutamine-chorismate-amidotransferase activity when complexed with component I and anthranilate phosphoribosyltransferase when existing free or complexed.

In addition to these biochemical experiments the examination of the fine structure (Fig. 2) of the gene *trpB* revealed that glutamine-chorismate-amidotransferase is coded for by the operator proximal 40% (region 1) of the gene while the information for anthranilate phosphoribosyltransferase resides in its operator distal 60% (region 2) [3]. These results led to the conclusion that gene *trpB* of *S. typhimurium* has arisen during evolution by the fusion of formerly separated gens coding for anthranilate phosphoribosyltransferase. Consequently the protein coded for by this fused gene consists of a glutamine-chorismate-amidotransferase moiety and an anthranilate phosphoribosyltransferase which are covalently linked. Later this hypothesis was supported by Li *et al.* [4] who found a striking homologous primary structure of linked and naturally occuring unlinked glutamine-chorismate-amidotransferases of three different species of *Enterobiaceae*.

But in order to completely consolidate the concept of heteromeric subunit linkage by covalent binding of formerly separate subunits the complete independence of both enzymatic functions of component II in *S. typhimurium* should be demonstrated. So far, however, in none of the *in vitro* experiments mentioned was it possible to recover a catalytically active anthranilate phosphoribosyltransferase that is separated from glutamine-chorismate-amidotransferase.

Already in 1966 Margolin and Bauerle [5] reported the isolation of mutants in *S. typhimurium* which are able to utilize anthranilic acid despite the fact that they are lacking the *trp* operator, *trpA* and the operator proximal part of *trpB*. Preliminary

* The nomenclature of this protein poses some difficult questions. In former papers [3, 9] it was called "glutamine-amido-transferase", or "component I — complementing activtiy". This term needs supplementation since it functions only as acceptor for glutamine thereby providing the ε-amidogroup for the amination of chorismate. Therefore the term "glutamine-chorismate-amidotransferase" has been introduced here. It must be kept in mind, however, that the enzymatic activity described by this term is given only when this peptide is complemented with component I.

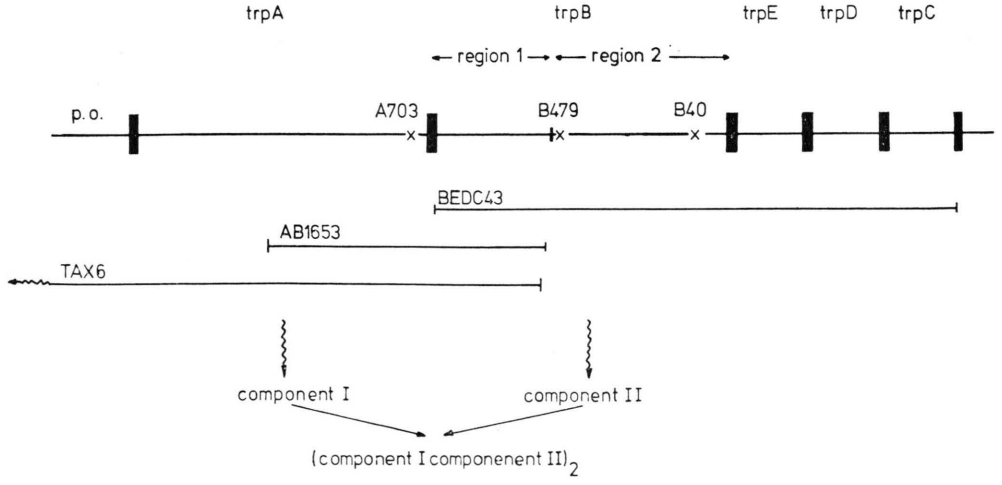

Fig. 2. Genetic fine structure of the trp operon and the mutants used of *Salmonella typhimurium*. (This map is drawn from the data obtained by Bauerle and Margolin [1, 5] and by Tanemura [6].)

experiments conducted then suggested that these strains possess a glutamine-chorismate-amidotransferase-free anthranilate phosphoribosyltransferase synthesized from a partial *trpB* gene fused to a gene of a foreign operon. Recently several other strains with deletions extending from *trpA* into *trpB* of the otherwise intact *trp* operon were isolated and demonstrated to have functional anthranilate phosphoribosyltransferase [6].

In this paper the properties of two glutamine-chorismate-amidotransferase-free anthranilate phosphoribosyltransferase proteins of different origin are described and their behavior with respect to glutamine-chorismate-amidotransferase activity and assembly with component I is examined.

Materials and Methods

(a) Organisms

All strains are derivatives of *Salmonella typhimurium* LT2. Mutant strain TAX6 is an anthranilate using revertant of *supX33* which carries a deletion extending from outside the *trp* operon past the operator element and *trpA*, in *trpB* removing region 1 [5]. In strain *trpAB1653* [6] approximately half of *trpA* and almost the complete region 1 of *trpB* of the otherwise intact operon are deleted. The double mutant strain *trpA703trpB479* carries nonsense mutations in *trpA* and *trpB* and gives rise to amino terminal fragments of component II having glutamine-chorismate-amidotransferase activity.

The following strains were used as sources for auxiliary proteins needed for the complementation experiments: *trpA703* for uncomplexed monomeric component II and *trpBEDC43* for uncomplexed component I. Strain *trpB40* was used as a source for phosphoribosylanthranilate isomerase [N-(5′-phosphoribosyl)-anthranilate isomerase, EC 4.1.1.48] and indole-3-glycerolphosphate synthase (EC 4.1.1.48). All strains carried the constitutive mutation *trpR*$^-$, *trpR782*.

(b) Chemicals

Anthranilic acid was obtained from Sigma, St. Louis and recrystallized once from water. Phosphoribosyldiphosphate (5-phospho-α-D-ribose-1-diphosphate) was also purchased from Sigma. Since it is extremely labile the actual concentration of this compound was estimated prior to every K_m determination, using the anthranilate phosphoribosyltransferase reaction of the wild type anthranilate synthase-anthranilate phosphoribosyltransferase complex. Crystalline chorismic acid was prepared according to the method of Gibson [7]. Ultrogel AcA34 was obtained from LKB, Stockholm. The proteins used for the calibration of the analytical Ultrogel column were purchased from Boehringer, Mannheim. All other chemicals were reagent grade.

(c) Growth of cells and preparation of cell extracts

Cells were grown in the minimal salts medium of Davis and Minigioli [8] modified by the exclusion of citrate. Tryptophan was supplemented at 8 μg/ml and glucose at 25 μg/ml. For analytical purposes as well as for purification experiments the cells were grown in 1 liter cultures using a New Brunswick Incubator thermostated at 37 °C. After harvesting the cells at an OD_{550} of 1.3 they were treated as described previously [9] with the exception that standard phosphate buffer was replaced by 0.05 M Tris (pH 7.3) containing 10^{-4} M EDTA, 10^{-4} M dithiothreitol and 10% (v/v) glycerol.

The procedure for the purification of both proteins was similar to that described earlier [9]. Modifications designed to reduce the denaturation of the anthranilate phosphoribosyltransferase are outlined in the section "results". During the final step of the purification procedure of anthranilate phosphoribosyltransferase from *trpAB1653* a preparative polyacrylamid gel electrophoresis "Ultrophor" (Colora Meßtechnik, Lorch) was used. The electrophoresis itself was performed as recommended by Colora for anionic systems.

(d) Enzyme assays

Glutamine-chorismate-amidotransferase-free anthranilate phosphoribosyltransferase activity was determined fluorimetrically by measuring the rate of disappearence of anthranilate. The assay mixture contained 10 nmol of anthranilic acid, 0.3 μmol of phosphoribosyldiphosphate, 10 μmol $MgCl_2$, 100 μmol of Tricine buffer (pH 7.0), 0.1 ml of a Sephadex G-25 treated extract of strain *trpB40* corresponding to approximately 300 units of indole-3-phosphoglycerol synthase, and enzyme preparation in a final volume of 1.0 ml.

The amination of chorismate in the presence of NH_3 (hitherto called ammonia activity) of uncomplexed component I was determined in an assay

mixture that contained 0.25 μmol chorismic acid, 10 μmol $MgCl_2$, 50 μmol $(NH_4)_2SO_4$, and 100 μmol Tricine buffer (pH 8.2) in a final volume of 1.0 ml.

Since the glutamine-chorismate-amidotransferase shows its enzymatic activity only in the presence of component I of the anthranilate synthase the addition of saturating amounts of highly purified component I to the assay mixture is necessary. The reaction mixture for glutamine-chorismate-amidotransferase contained 2 μmol chorismic acid, 20 μmol glutamine, 10 μmol $MgCl_2$, 100 μmol potassium phosphate buffer (pH 7.0), 600 units (*i. e.*, when fully saturated with component II) of component I subunit, and enzyme preparation in a final volume of 1.0 ml. The formation of anthranilate was monitored fluorimetrically using a primary filter of 313 nm and a secondary filter of 390 ± 20 nm.

A unit of activity is defined as the disappearance or formation of 1 nmol anthranilate in 1 sec. Specific activity is expressed as enzyme units per milligram of protein which was determined colorimetrically [10].

(e) Analytical gel filtration

The 48,000 × *g* supernatant (5 ml) was applied to an Ultrogel AcA34 colum (2.5 × 86 cm) previously equilibrated with 0.05 M Tris (pH 7.3) containing 10^{-4} M dithiothreitol, 10^{-4} M EDTA and 10% glycerol. The column was developed with equilibration buffer by upward flow at a rate of 35 ml/h. Fractions of 5 ml were collected. The column was calibrated with the following proteins, each applied at 10 mg in 5 ml buffer: ferritin (540,000), catalase (240,000), aldolase from rabbit (158,000), bovine serum albumin (molecular weight of the dimer 134,000), uncomplexed component I of the anthranilate synthase-anthranilate phosphoribosyltransferase complex (65,000) and chymotrypsinogen A (25,000).

Results

(a) Glutamine-chorismate-amidotransferase-free anthranilate phosphoribosyltransferase activity

Assay of crude extracts of strains TAX6 and *trpAB1653* revealed anthranilate phosphoribosyltransferase activity for both. Glutamine-chorismate-amidotransferase activity was absent. As indicated in Table I specific activities for anthranilate phosphoribosyltransferase are markedly lower in mutant strains TAX6 and *trpAB1653* than in wild type and *trpA703*.

Gel filtration analyis of these extracts demonstrated a single peak of anthranilate phosphoribosyltransferase activity (molecular weight 320,000) in the case of TAX6 (Fig. 3 a). Anthranilate phosphoribosyltransferase from *trpAB1653* separated into two peaks (Fig. 4 a). The first species was eluted in the vicinity of the excluded volume indicating a molecular weight greater than 1×10^6 and the second appeared at 275 ml which corresponds to a molecular weight of 220,000. Repeated gel filtration of either peak did not result in a renewed separation into two peaks. As expected from results of Table I anthranilate phosphoribosyltransferases of both strains completely lacked glutamine-chorismate-amidotransferase activity after Ultrogel filtration.

Early experiments on the subunit complementation of the anthranilate synthase-anthranilate phosphoribosyltransferase complex suggested that not the entire component II protein but only the glutamine-chorismate-amidotransferase moiety (then called the "sticky" region) might be required for complex formation [1]. In order to confirm this

Table I. Enzymatic activities of different mutant strains of *S. typhimurium*. A unit of activity is defined as the disappearence or formation of 1 nmol anthranilate in 1 sec.

Mutant	Amination of chorismate in the presence of NH_3		Amination of chorismate in the presence of glutamine		Anthranilate phosphoribosyltransferase	
	U/mg protein	%	U/mg protein	%	U/mg protein	%
trp$^+$	0.67	100	4.85	100	3.2	100
trpA703trpR782	0	0	0	0	2.7	84
TAX6*trpR782*	0	0	0	0	0.5	16
trpAB1653trpR782	0	0	0	0	0.5	18

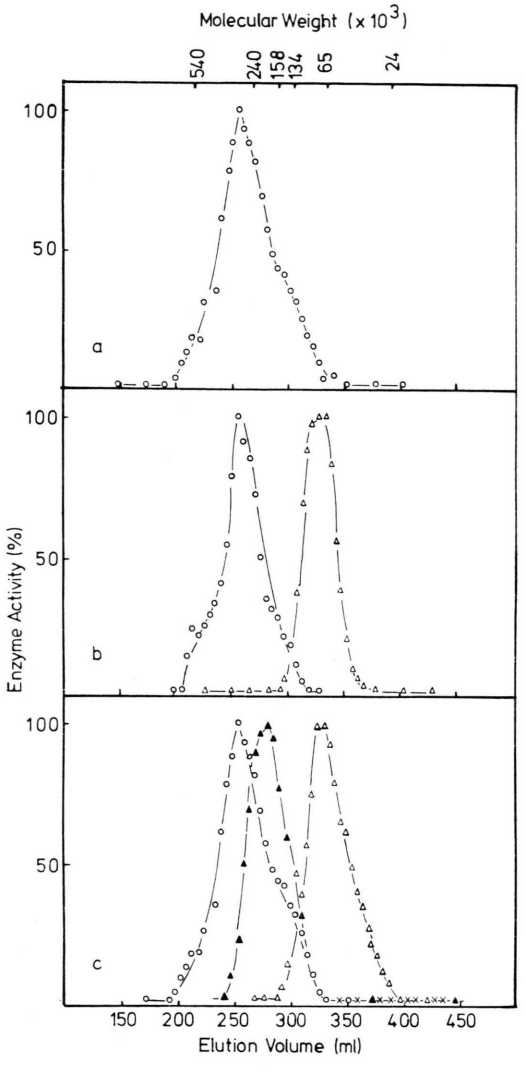

Fig. 3. Ultrogel AcA 34 fractionation of anthranilate phosphoribosyltransferase of TAX6. a) Fractionation of crude extracts; anthranilate phosphoribosyltransferase activity (○—○) and glutamine dependent anthranilate synthase activity in the presence of purified component I in the assay mixture (×—×). b) Partially purified TAX6 anthranilate phosphoribosyltransferase (○—○) and ammonia dependent anthranilate synthase activity (△—△). c) Elution profile of a protein mixture containing TAX6 anthranilate phosphoribosyltransferase, component I and glutamine amidotransferase from strain *trpA703trpB479*. Anthranilate phosphoribosyltransferase activity (○—○), glutamine dependent anthranilate synthase activity with no subunit added (▲—▲), ammonia dependent anthranilate synthase (△—△) and component I complementing activity assayed in the glutamine dependent anthranilate synthase reaction (×—×).

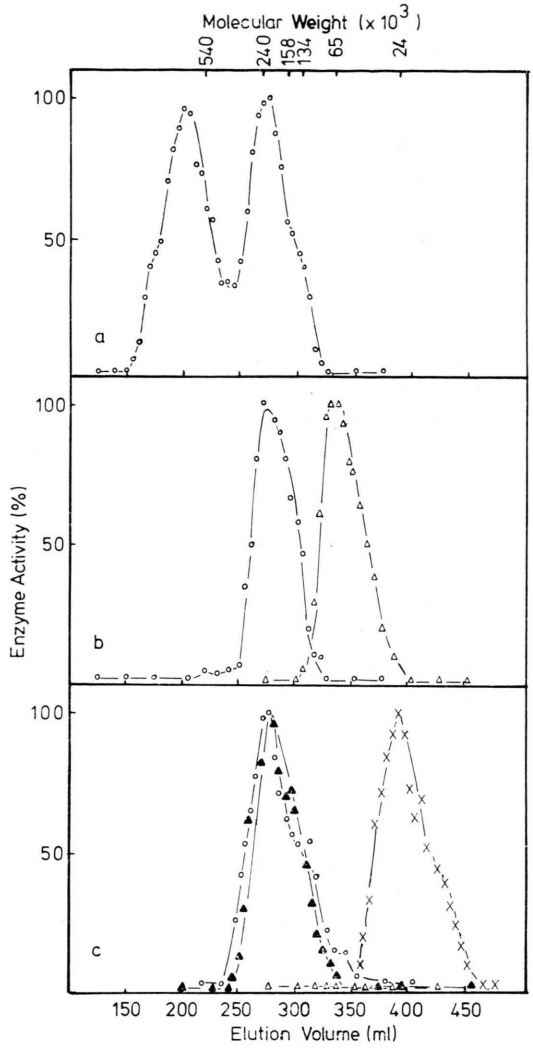

Fig. 4. Ultrogel AcA 34 fractionation of anthranilate phosphoribosyltransferase of *trpAB1653*. a) Fractionation of crude extracts; anthranilate phosphoribosyltransferase activity (○—○) and glutamine dependent anthranilate synthase activity in the presence of purified component I in the assay mixture (×—×). b) Elution pattern of a mixture of anthranilate phosphoribosyltransferase peak II of (a) above (○—○) and component I. Ammonia dependent anthranilate synthase activity (△—△). c) Elution profile of a mixture containing anthranilate phosphoribosyltransferase peak II of (a) above, monomeric component I and glutamine-chorismate-amidotransferase from strain *trpA703trpB479*. Anthranilate phosphoribosyltransferase activity (○—○), glutamine dependent anthranilate synthase activity with no subunit added in the assay mixture (▲—▲), component I complementing activity assayed in the glutamine dependent anthranilate synthase reaction by adding component I to the assay mixture (×—×), ammonia dependent anthranilate synthase activity (△—△).

conclusion anthranilate phosphoribosyltransferase from strain TAX6 or anthranilate phosphoribosyltransferase from peak II of *trpAB1653* were incubated with purified component I for 12 h at 4 °C. This preparation was again subjected to gel filtration on Ultrogel AcA34. If an aggregate formation of the two proteins had occured both proteins should elute from the column at volumes different from those of the original protein. From Fig. 3 b and 4 b it is evident that anthranilate phosphoribosyltransferase from TAX6 and the second peak of anthranilate phosphoribosyltransferase from *trpAB1653* showed their original elution pattern. The added component I fractionated in both cases as the monomeric subunit and no glutamine-dependent anthranilate synthase activity appeared. Fig. 4 b also serves to demonstrate that, as mentioned above, the *trpAB1653* anthranilate phosphoribosyltransferase from peak II does not separate again into two peaks upon refractionation.

Glutamine-chorismate-amidotransferase-free anthranilate phosphoribosyltransferase from TAX6, purified component I and partially purified glutamine-chorismate-amidotransferase from strain *trpA703trpB479* were then mixed and incubated 12 h at 4 °C. Subsequent gel filtration of this mixture eluted anthranilate phosphoribosyltransferase at the same volume as in previous runs. (Fig. 3 c). In addition a new enzyme species appeared at 275 ml corresponding to a molecular weight of 220,000. This protein possessed inherent glutamine-chorismate-amidotransferase activity but no anthranilate phosphoribosyltransferase and is identical with the previously described partial complex having the composition (component I-glutamine-chorismate-amidotransferase)$_2$ [3]. Since the protein mixture contained twice as much component I than glutamine-chorismate-amidotransferase, the residual uncomplexed component I was eluted from the column as expected at 330 ml.

In the case of anthranilate phosphoribosyltransferase from *trpAB1653* similar results were obtained. Anthranilate phosphoribosyltransferase from this strain did not change the elution pattern of component I (Fig. 4 b). The simultaneous fractionation of a mixture of anthranilate phosphoribosyltransferase, component I and glutamine-chorismate-amidotransferase showed overlapping enzymatic activities for anthranilate phosphoribosyltransferase-, inherent glutamine-chorismate-amidotransferase- and ammonia activity between 250 and 350 ml (Fig. 4 c). But this pattern is fortuitous since anthranilate phosphoribosyltransferase and the partial complex (component I-glutamine-chorismate-amidotransferase)$_2$ have the same molecular weight. This was established by a separate fractionation of the partial complex alone which eluted again with the same volume (results not shown). In addition any competition between the anthranilate phosphoribosyltransferase and the glutamine-chorismate-amidotransferase for assembly with component I should have resulted in a complex with a molecular weight larger than 220,000. No evidence for this was seen in the elution patterns obtained.

An excess of glutamine-chorismate-amidotransferase was used in this experiment and was detected in the effluent between 350 and 450 ml (molecular weight 24,000) by assay of its activity in the presence of component I in the reaction mixture (Fig. 4 c).

(b) Purification of anthranilate phosphoribosyltransferase from mutant strains TAX6 and trpAB1653

Purification to homogeneity of the enzymes of both strains appeared to be difficult because of the lability of the anthranilate phosphoribosyltransferases. A rapid two step purification procedure was developed for TAX6. The first step involved ion-exchange chromatography on Sephadex A-50 (2.5 × 20 cm) equilibrated with 10 mM Tris (pH 7.4), 0.1 mM EDTA, 0.1 mM dithiothreitol and 10% glycerol. The column was exhaustively washed with the same buffer and then developed with 400 ml KCl gradient (0 – 0.5 M KCl in the above buffer). Fractions containing at least 20% of the anthranilate phosphoribosyltransferase activity of the peak fraction were concentrated and then subjected to gel filtration on Ultrogel AcA34. The elution profile of this column (5 × 100 cm) showed a single peak of anthranilate phosphoribosyltransferase activity eluting between 1.1 and 1.3 liter. The combined and concentrated fractions of this step contained anthranilate phosphoribosyltransferase which showed a tenfold increase in specific activity. This preparation was used to characterize anthranilate phosphoribosyltransferase from TAX6.

The purification procedure used for the anthranilate phosphoribosyltransferase of *trpAB1653* is outlined in Table II. The first three steps were per-

Table II. Purification of glutamine-chorismate-amidotransferase-free anthranilate phosphoribosyltransferase from strain *trpAB1653*. A unit of activity is defined as the disappearence of 1 nmol anthranilate in 1 sec.

Fraction	Vol [ml]	Protein concentration [mg/ml]	Total protein [mg]	Activity [U/ml]	Specific activity [U/mg protein]	Recovery [%]
1. Crude extract	128	15.5	1984	90.1	5.8	100
2. Streptomycin sulfate supernatant	134	14.7	1970	74.6	5.0	87
3. $(NH_4)_2SO_4$ precipitate (dialyzed)	17	37.0	629	428.8	11.6	63
4. Ultrogel AcA34 (peak II)	8.5	9.2	78	105.6	11.5	8
5. DEAE-Sephadex	3.9	7.5	29	89.5	12.0	3
6. Preparative gel electrophoresis	1.1	1.5	1.7	27.3	17.6	0.3

formed as described earlier [9] with the exception that standard phosphate buffer was replaced by 50 mM Tris (pH 7.3) containing 0.1 mM EDTA, 0.1 mM dithiothreitol and 10% glycerol. Filtration of Ultrogel AcA34 (5 × 100 cm) resolved two peaks of anthranilate phosphoribosyltransferase activity. The first peak which partially eluted with the excluded volume contained 76% of the total activity. So far further purification of peak I protein has proven impossible.

Fractions of peak II containing the remaining anthranilate phosphoribosyltransferase activity were concentrated. This preparation was then applied to DEAE-Sephadex A-50 (2.5 × 15 cm) previously equilibrated with 50 mM Tris (pH 7.3) containing 0.1 mM EDTA, 0.1 mM dithiothreitol and 10% glycerol. The column was washed with 500 ml of the same buffer and then developed with a 400 ml gradient of KCl (0 – 0.5 M in the above buffer). Anthranilate phosphoribosyltransferase activity appeared in a single peak at 0.4 M KCl. This preparation which was about 50% homogeneous was used to characterize anthranilate phosphoribosyltransferase from *trpAB1653*.

In order to determine the subunit composition and the molecular weight of the protomers further purification was necessary. The only method found which did not destroy anthranilate phosphoribosyltransferase activity completely was preparative poylacrylamid gel electrophoresis. Two milliliters of the preparation of step 5 (Table I) were separated on a 7.5% slab gel. Anthranilate pohsphoribosyltransferase activity eluted in a single peak which contained about 10% of the antranilate phosphoribosyltransferase activity applied to the gel. Analytical polyacrylamid gel electrophoresis of this preparation revealed two major bands with R_F values of 0.40 and 0.47. The fractionation of unstained gels into 1 mm slices and subsequent extraction showed anthranilate phosphoribosyltransferase activity that coincided with both stained with both stained bands.

SDS gel electrophoresis [11] of the anthranilate phosphoribosyltransferase eluted from the preparative gel electrophoresis revealed one major band corresponding to a molecular weight of 72,000 and a few faint bands. The major band was about 90% pure as judged by the intensity of Coomassie blue staining.

(c) Effect of pH on the enzyme activity

Similar pH optima of 6.9 were obtained for the anthranilate phosphoribosyltransferase activity of both enzymes. However, anthranilate phosphoribosyltransferase of strain TAX6 displayed a broader optimum than anthranilate phosphoribosyltransferase from *trpAB1653*. The pH optimum of anthranilate phosphoribosyltransferase activity of unaggregated wild type monomer component II was found to be pH 7.4.

(d) Inhibition by phosphoribosylanthranilate, Na-pyrophosphate, and tryptophan

It was routinely found that the anthranilate phosphoribosyltransferase activity of the partially purified anthranilate phosphoribosyltransferase of both mutant strains remains linear for only a short period of time, normally less than 30 seconds. It

was suspected that this might be due to product inhibition by pyrophosphate or by phosphoribosylanthranilate. Unfortunately one product of the anthranilate phosphoribosyltransferase reaction, phosphoribosylanthranilate, is extremely unstable and therefore cannot be used for product inhibition studies. However, the addition of crude preparations of phosphoribosylanthranilate isomerase and indole-3-glycerolphosphate synthase, the following enzymes of the tryptophan pathway, completely relieved the inhibition of the anthranilate phosphoribosyltransferase reaction. In the presence of these enzymes the reaction remained linear for at least four minutes. The influence of pyrophosphate on the anthranilate phosphoribosyltransferase activity was also studied in the presence of phosphoribosylanthranilate isomerase and indole-3-glycerolphosphate synthase. Increasing amounts of pyrophosphate caused a significant inhibition of anthranilate phosphoribosyltransferase activity. In order to achieve a 50% inhibition of the enzymatic activity of anthranilate phosphoribosyltransferase of TAX6 20 mM pyrophosphate was required whereas 5 mM pyrophosphate was sufficient to inhibit half of the enzymatic activity obtained from the mutant *trpAB1653* (Fig. 5 a).

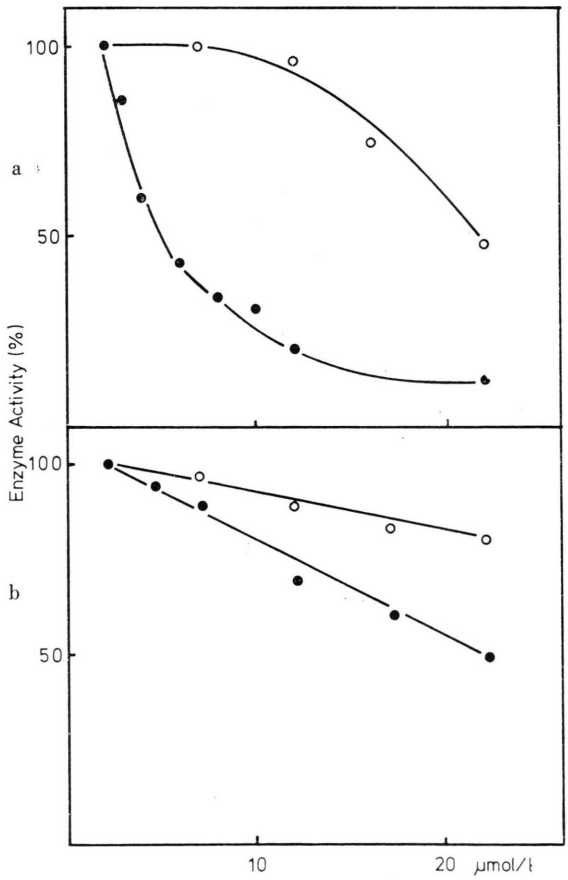

Fig. 5. Inhibition of anthranilate phosphoribosyltransferase activity by (a) sodiumpyrophosphate and (b) L-tryptophane. TAX6 (○—○); *trpAB1653* (●—●).

End product inhibition by tryptophan of anthranilate phosphoribosyltransferase activity is a well established characteristic of the wild type anthranilate synthetase-anthranilate phosphoribosyltransferase complex. However, monomeric unaggregated component II is not affected by tryptophan concentrations sufficient to inhibit 50% of the anthranilate phosphoribosyltransferase activity in complexed component II [9]. Increasing amounts of tryptophan eventually diminish anthranilate pohsphoribosyltransferase activity, but even at high concentrations of tryptophan (20 mM) inhibition is only partial. Fig. 5 b shows the effect of tryptophan on anthranilate phosphoribosyltransferase of TAX6 and *trpAB1653*. Enzymatic activity of TAX6 was reduced by 20% in the presence of 22 mM tryptophan while that of *trpAB1653* was reduced by 50%. Inhibition was concentration dependent.

(e) Effect of heat treatment on anthranilate phosphoribosyltransferase

Heat inactivation was used as an additional descriptive parameter to characterize glutamine-chorismate-amidotransferase-free anthranilate phosphoribosyltransferase. Fig. 6 demonstrates that anthra-

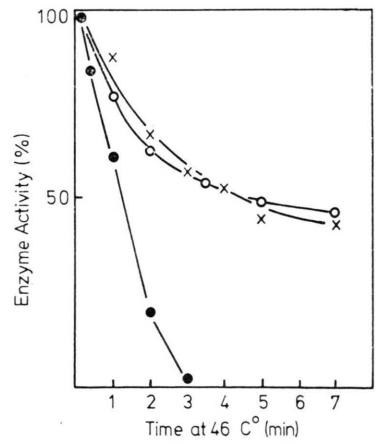

Fig. 6. The effect of heat treatment at 46 °C on the enzymatic activity of anthranilate phosphoribosyltransferase from TAX6 (●), *trpAB1653* (○) and uncomplexed wild type monomeric component II (×).

nilate phosphoribosyltransferase of TAX6 lost half its enzymatic activity within 1.5 min, while anthranilate phosphoribosyltransferase activity of *trpAB1653* and of wild type component II was more stable showing half lives of 5 min.

(f) Kinetics

The Michaelis-Menten constants were determined by measuring the anthranilate phosphoribosyltransferase activity at saturating concentrations of anthranilate and phosphoribosylpyrophosphate respectively. Initial velocities could only be determined without significant variation if phosphoribosylanthranilate isomerase and indole-3-glycerolphosphate synthase from straind *trpB40* was present in the assay mixture.

The K_m for phosphoribosylpyrophosphate using 10 μM anthranilate was 1×10^{-5} M for wild type monomeric component II, 1.3×10^{-5} M for TAX6 and 6×10^{-5} M for *trpAB1653*.

The K_m for anthranilate using 200 μM phosphoribosylpyrophosphate of monomeric component II was estimated to 6×10^{-6} M, of TAX6 to 3×10^{-6} M and of *trpAB1653* to 4×10^{-6} M. Preliminary experiments with the two enzymes at varying concentrations of anthranilate and phosphoribosylpyrophosphate and their kinetic treatment by double reciprocal plots yielded converging lines, thereby confirming the results obtained by Zalkin for component II [13].

Discussion

The anthranilate synthase-anthranilate phosphoribosyltransferase of *Salmonella typhimurium* provides a good example for the evolution of an oligocephalic enzyme. The enzyme of wild type strains is composed of the protomers component I and component II, each present twice in the aggregate. Component I catalyzes the amination of chorismate. Component II is endowed with several functions: (a) it provides the glutamine binding site faciliating the transamidation reaction between glutamine and chorismate, (b) it is required for the formation of the enzyme aggregate, and (c) it catalyzes the addition of the phosphoribosyl moiety of 5-phosphoribosyl-1 pyrophosphate to the aminogroup at the 3-position of anthranilate. Thus, component II of the anthranilate synthase-anthranilate phosphoribosyltransferase complex is an oligocephalic enzyme that contains three functions within one polypeptide.

A complete understanding of the component II protein can only be achieved if both fragments, the glutamine-chorismate-amidotransferase and the anthranilate phosphoribosyltransferase, are available as separate entities. It has already be shown that the glutamine-chorismate-amidotransferase activity of component II can be separated from the anthranilate phosphoribosyltransferase activity *in vitro* by proteolysis of either the entire complex [2] or of component II alone [3]. However, this proteolytic procedure completely destroyed the anthranilate phosphoribosyltransferase activity. Since these *in vitro* experiments yielded only glutamine-chorismate-amidotransferase, the isolation of proteins possessing only anthranilate phosphoribosyltransferase activity was approached by the use of specific mutant strains.

The results of genetic mapping indicate that the anthranilate phosphoribosyltransferase from TAX6 consists of the carboxyterminal two thirds of component II in addition to a section of a foreign protein which presumably is attached to its amino terminal end. On the other hand, the anthranilate phosphoribosyltransferase from *trpAB1653* consists of the amino terminal half of component I fused to the carboxy terminal two thirds of component II. Therefore both proteins must lack glutamine-chorismate-amidotransferase [), 6]. It has been shown here that the biochemical properties of these proteins agree with their genetic deduced composition.

Examination of catalytically active anthranilate phosphoribosyltransferase by gel sieve chromatography indicated that the TAX6 enzyme has a molecular weight of 330,000 and that the *trpAB1653* enzyme exists in two form with molecular weights of 220,000 and larger than 1×10^6. These results suggest that both proteins tend to aggregate. In the case of homogeneous anthranilate phosphoribosyltransferase from *trpAB1653* a subunit molecular weight of 72,000 was determined by SDS gel electrophoresis. This value is in good agreement with the genetic evidence which predicts a molecular weight of 75,000 for the monomeric protein [6]. Therefore, three protomers should be present in the smaller aggregate of anthranilate phosphoribosyltransferase from *trpAB1653*. But the method for the determination of the molecular

weights using Ultrogel AcA34 has large standard deviations as estimated with standard proteins.

The behaviour of anthranilate phosphoribosyltransferase from both mutant strains in the presence of purified component I ascertains the role of the amino terminal portion of component II in complex formation of the wild type anthranilate synthase-antranilate phosphoribosyltransferase. The results obtained by gel filtration experiments of mixtures of component I and glutamine-chorismate-amidotransferase-free anthranilate phosphoribosyltransferase clearly demonstrate the absence of any interaction between these two proteins. Glutamine-chorismate-amidotransferase of *trpA703trpB479* (that is, glutamine-chorismate-amidotransferase unfused to anthranilate phosphoribosyltransferase) on the other hand complexes readily with component I to form a partial aggregate but also fails to aggregate with anthranilate phosphoribosyltransferase from TAX6 and *trpAB1653*. Therefore it seems obvious that the covalent linkage between glutamine-chorismate-amidotransferase and anthranilate phosphoribosyltransferase which is due to the fusion of formerly separated genes was antecedent to the evolutionary addition of anthranilate phosphoribosyltransferase activity to the wild type complex.

The comparative study of the physical properties of glutamine-chorismate-amidotransferase-free anthranilate phosphoribosyltransferase and the anthranilate phosphoribosyltransferase of uncomplexed monomeric component II reveals many similarities. The K_m values for anthranilic acid, phosphoribosylpyrophosphate, and Mg^{2+} are similar for the anthranilate phosphoribosyltransferase for all three proteins. Both species of anthranilate phosphoribosyltransferase are sensitive to feedback inhibition only at unphysiologically high concentrations of tryptophan. This is also true for anthranilate phosphoribosyltransferase activity of uncomplexed wild type monomeric component II [9, 13]. Product inhibition by pyrophosphate and presumably by phosphoribosylanthranilate are essentially indistinguishable. pH optima are slightly lower for the glutamine-chorismate-amidotransferase-free anthranilate phosphoribosyltransferase than for the wild type monomeric component II. From this set of data it is assumed that the catalytic site of anthranilate phosphoribosyltransferase is the same in all three species of proteins.

The only descriptive parameter which distinguishes the varied forms of anthranilate phosphoribosyltransferase is their different sensitivities to heat inactivation. This indicates some structural changes in the tertiary structure possibly induced by the covalently linked foreign protein in the case of TAX6 or by the amino terminal half of component I attached to anthranilate phosphoribosyltransferase from *trpAB1653*.

Further study of mutationally altered enzyme forms, such as the monofunctional anthranilate phosphoribosyltransferase reported as here, should continue to offer significant detail of the relationship of structure and function in enzymes and of some of the mechanisms by which enzymes evolve.

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