THE MOLECULAR AGGREGATION OF ANTHRANILATE SYNTHASE IN BACILLUS SUBTILIS

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Abstract. Anthranilate synthase in Bacillus subtilis was found to be a molecular complex consisting of subunit-E (the product of trpE) and subunit-X (the product of a new genetic locus, trpX). Subunit-X was partially purified by gel chromatography. It has a molecular weight of about 16,000; its synthesis is repressible by tryptophan; and it has no anthranilate synthase activity in isolation from its complex. Its possible identity as all or part of another biosynthetic enzyme of tryptophan synthesis is discussed.

Introduction. Anthranilate synthase has been characterized as a molecular aggregate of two non-identical subunits in at least four microorganisms (1-6). In Escherichia coli component I catalyzes the formation of anthranilate in the presence of ammonia and chorismate, but it cannot utilize glutamine as an amino donor reactant. Component II is also phosphoribosyl (PR) transferase, the second enzyme in the pathway of tryptophan biosynthesis. Only components I and II in association are capable of forming anthranilate from chorismate and glutamine.

The following experiments led us to the acquisition of mutants which show that the enzyme of B. subtilis also consists of non-identical subunits. A regulatory mutant of B. subtilis (NP 100) which excretes tryptophan has an attending partial requirement for phenylalanine as the result of the inhibition of prephenate dehydratase by tryptophan (7). One can select for spontaneous, secondary mutations at 50^{0} which result in the loss of the partial requirement for phenylalanine. All such mutants examined thus far carry a second mutation in which the original regulatory mutation (probably an R^{-} constitutive) is retained. Many of these mutants possess a partial deficiency for one of the enzymes of tryptophan biosynthesis, and accordingly, these mutants produce much less tryptophan than the parent strain NP 100. Strains I-12 and I-15 are two such mutants which lack in vitro activity for anthranilate synthase when glutamine is used as the amino donor (ant syn-gln). However, dramatic in vitro

complementation for ant syn-gln occurs upon mixing extracts of strains I-12 and I-15. These strains were examined further in order to study the subunit organization of anthranilate synthase in B. subtilis.

Experimental. Our strains are all mutant derivatives of strain 168 (8). Strain NP 40 is a prototroph (9). Strains BS 46 and BS 48 carry the his_2 locus (10) and nonsense mutations in trpE which are suppressible by the Luria sus-3 (Crawford, personal communication).

All of the strains were grown on a minimal medium (11) and, where indicated, were supplemented with 50 µg per ml each of the aromatic amino acids: tyrosine, phenylalanine, and tryptophan. The preparation of extracts and assays for ant syn-gln and for PR transferase were done as before (11). The assay for anthranilate synthase using ammonia as the amino donor (ant syn-amn) was carried out at pH 8.55 in the presence of 50 µmoles of Tris-HCl, 10 µmoles $MgCl_2$, 50 µmoles NH_4Cl, 0.2 µmole of potassium chorismate, and enzyme in a final volume of 1 ml. Barium chorismate with a purity of 91% was obtained from Sigma.

Results. Table 1 shows that strains I-12 and I-15 retain the regulatory mutation of NP 100, as illustrated by the derepressed levels of PR transferase relative to the repressed levels observed in strains BS 46, BS 48, and the reference prototroph NP 40. Although both strains I-12 and I-15 lack ant syn-gln, they differ in activity for ant syn-amn. Strain I-15 has enzyme activity using ammonia as a reactant; strain I-12 does not. Two trpE nonsense mutants, BS 46 and BS 48, resemble strain I-12 as follows: all three lack ant syn-gln and ant syn-amn, and all three complement strain I-15 in vitro to reconstitute ant syn-gln activity. Hence, these mutants provide the basis for a subunit assay system using in vitro complementation. Strain I-12 is our source of subunit-X, while strain I-15 is our source of subunit-E.

Fig. 1 illustrates the fractionation of extract protein from strain NP 100 by gel chromatography. The elution of ant syn-gln and ant syn-amn activities were coincident. When ant syn-gln activity was assayed in the presence of extract protein from strain I-15, free subunit-X with a molecular weight of about 16,000 was revealed. Extract of strain I-15 (i.e., subunit-E) does not further stimulate the activity of the ant syn-gln complex, molecular weight 84,000. The addition of partially purified subunit-X (100-fold) from other chromatography runs stimulated ant syn-gln activity in the 84,000 molecular weight fraction as did the addition of crude extract of strain I-12.

Gel chromatography of extract from strain I-15 produces the 84,000 molecular weight peak of activity for ant syn-amn, no activity for ant syn-gln, and no 16,000

Table 1

Comparison of strain activities for PR transferase and anthranilate synthase

Strain	Specific Activities[a]			
	PR transferase[b]	ant syn-amn[c]	ant syn-gln[c]	ant syn-gln[c] + I-15 extract[d]
NP 40	< 0.01	< 0.01	< 0.01	0.22
NP 100	4.10	2.00	9.40	12.00
I-12	1.83	< 0.01	< 0.01	3.64
I-15	4.00	2.00	< 0.01	-
BS 46	< 0.01	0	0	0.17
BS 48	< 0.01	0	0	0.19

[a] Specific activities noted as less than 0.01 are barely detectable activities yielding rates near the sensitivity limits of the assay. Strains were grown under conditions of repression: minimal medium plus aromatic amino acids. BS 46 and BS 48 also contained 50 μg per ml of histidine. When strain NP 40 was grown in a minimal medium, specific activity values for the enzyme activities listed from left to right above were 0.05, 0.07, 0.2 and 0.8. Specific activities in constitutive strains NP 100, I-15 and I-12, on the other hand, were identical in extracts made from cultures grown in minimal medium.

[b] Specific activity is defined as nmoles anthranilate utilized/min/mg protein.

[c] Specific activity is defined as nmoles anthranilate formed/min/mg protein.

[d] 480 μg of extract protein from strain I-15 were added/ml of reaction mixture.

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molecular weight fraction of subunit-X. However, the assay for ant syn-gln in the presence of extract from strain I-12 (i.e., subunit-X) reveals a peak of activity at the molecular weight position of 84,000. Gel chromatography of extract from strain I-12 results in no elution of activities for ant syn-gln or ant syn-amn. When such I-12 eluate fractions were assayed for ant syn-gln activity in the presence of extract from strain I-15, the 84,000 molecular weight peak of ant syn-gln activity as well as the 16,000 molecular weight peak of subunit-X was found.

A profile similar to that shown in Fig. 1, but having much lower enzyme activities, was obtained upon fractionation of extracts from NP 40. The amount

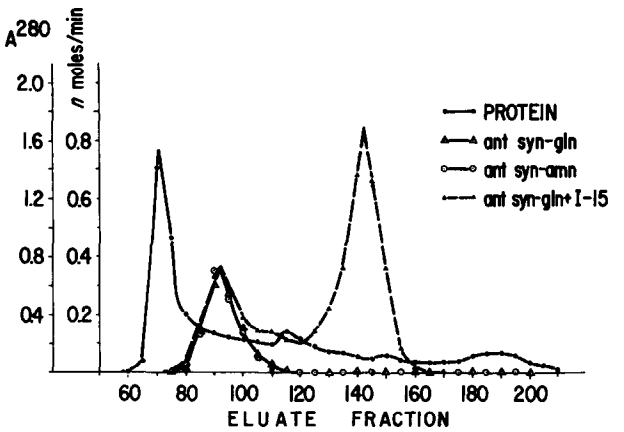

Figure 1. The sephadex G-100 column was equilibrated with 0.1 M potassium phosphate buffer at pH 7.2 containing 0.5 M KCl, 0.1 mM EDTA, 6 mM mercaptoethanol, 10 mM glutamine and 30% glycerol. An extract of NP 100 was brought to 70% saturation with ammonium sulfate. The precipitate was resuspended in one half of the original volume of buffer. The resulting 25 mg of extract protein were put on the column. The flow rate was 12 ml per hour at 4° and 2.5 ml fractions were collected. Eluate protein was estimated as absorbance at 280 nm. Standards used as molecular weight markers were alkaline phosphatase (E. coli) and cytochrome C (horse heart). Activity for ant syn-amn was measured as described in methods. Activity for ant syn-gln was determined in the presence and absence of 480 µg of extract protein from strain I-15.

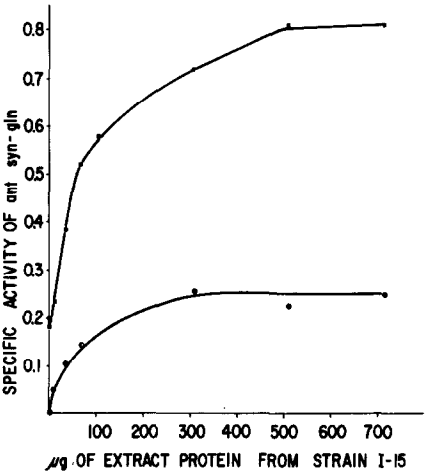

Figure 2. Ant syn-gln activity was determined using 644 µg of extract protein from strain NP 40 grown in minimal (■ - ■) or minimal plus tryptophan (● - ●). The amount of protein present from strain I-15 is indicated along the bottom of the chart.

of subunit-X present in a given extract can be titered by assaying the activity of ant syn-gln after the addition of saturating levels of subunit-E from extracts of strain I-15. Such a saturation curve is illustrated in Fig. 2. A culture of NP 40 grown in the presence of tryptophan had one-third the level of subunit-X as did a culture grown in minimal medium. The amount of subunit-X present in the constitutive strain NP 100 is about 18-fold higher than in extracts made from minimal-grown cultures of wild type strain NP 40.

Discussion. Mutant I-15, which lacks functional subunit-X, defines a second subunit of anthranilate synthase in B. subtilis. Its ability to complement known trpE mutants cannot be an instance of intracistronic complementation since extracts of strain I-15 complement two trpE mutants which have been identified as nonsense mutations. We suggest that trpX mutants would not be selected as tryptophan auxotrophs, as are trpE mutants, because the remaining ammonia-dependent activity (which would probably be physiologically derepressed) would permit a rate of tryptophan synthesis sufficient for growth (12). Our results indicate that the trpX locus of strain I-15 codes for a non-functional subunit-X which, however, still functions to complex with subunit-E. Thus, the ant syn-amn activity which remains in strain I-15 retains the same molecular weight position of 84,000 that is seen in strain NP 100. Subunit-E has not yet been isolated free of the complex. Either it is entirely associated with subunit-X, which is in excess, or free subunit-E may be labile. In our complementation system, we conclude that active subunit-X exchanges readily with the defective subunit-X that is complexed with subunit-E in strain I-15.

Subunit-X is definitely stable as a dissociated entity after purification of 100-fold. It seems to exist in excess of subunit-E (Fig. 1) and can be recovered as the free 16,000 molecular weight subunit from wild type as well as from mutants which synthesize the enzymes of tryptophan biosynthesis constitutively (NP 100 and I-12). The synthesis of subunit-X is specifically repressible by tryptophan, being 18-fold lower in extracts from minimal-grown cultures of wild type strain NP 40 than in constitutive strains such as NP 100. The addition of tryptophan to growing cultures of strain NP 40 results in a further 3-fold repression. It will be of considerable interest to know if trpX maps with the other structural gene loci of the tryptophan pathway (a probable operon) and whether its gene product, subunit-X, is synthesized coordinately with the other gene products (13). It seems likely that trpX does not have identity with another

of the structural genes of tryptophan biosynthesis, as is the case with PR transferase in E. coli. If the gene product of trpX relates to any of the other tryptophan biosynthetic enzymes, it must not be obligatory for that activity in vitro. Although many mutants have been isolated and studied (13,14), no mutants pleiotrophically lacking both anthranilate synthase and another activity have been isolated.

Since the ant syn-gln activity of the 84,000 molecular weight complex is stimulated further by addition of subunit-X but not by subunit-E, we tentatively suggest that a higher molecular weight species of enzyme exists in the presence of additional molecules of subunit-X. This association presumably is unstable, eluding fractionation under our experimental conditions. This postulate is consistent with our previous observation (15) that the activity of ant syn-gln is not a linear function of protein concentration in crude extracts. It is interesting in this connection that under the same conditions activity for ant syn-amn is a linear function of protein concentration.

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