Monomeric and Dimeric Forms of Component II of the Anthranilate Synthetase–Anthranilate 5-Phosphoribosylpyrophosphate Phosphoribosyltransferase Complex of *Salmonella typhimurium*. Implications Concerning the Mode of Assembly of the Complex†

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ABSTRACT: The multifunctional anthranilate synthetase–anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase complex of the tryptophan biosynthetic pathway is composed of two molecules each of subunits components I (CoI) and II (CoII). The component II subunit serves two enzymatic functions in the complex: (1) it catalyzes the synthesis of phosphoribosyl anthranilate from anthranilate and phosphoribosyl pyrophosphate (phosphoribosyltransferase activity), an activity that it can also carry out with equal efficiency when existing uncomplexed, and (2) it complements component I in the glutamine-dependent synthesis of anthranilate from chorismate by acting as a glutamine amidotransferase (CoI-complementing activity). The existence of two species of uncomplexed component II molecules has been demonstrated in extracts of mutants lacking the component I subunit. Both species were purified to homogeneity and characterized. Evidence is presented that the two species represent the monomeric ($CoII_M$) and dimeric ($CoII_D$) forms of component II. The molecular weight of $CoII_D$ (~130,000) is twice that of $CoII_M$ (~65,000) as estimated by gel filtration, while their subunit molecular weights, determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, are identical (~65,000). Purified $CoII_M$ was found to aggregate spontaneously during aging at 4° forming $CoII_D$. The dimerization is not readily reversible at 4° but dissociation of $CoII_D$ with the liberation of $CoII_M$ molecules was accomplished by incubation at elevated temperatures. $CoII_D$ and $CoII_M$ display similar kinetic properties when catalyzing the phosphoribosyltransferase reaction. However, $CoII_D$ molecules are not subject to the rapid loss of phosphoribosyltransferase activity upon exposure to low concentrations of trypsin found with $CoII_M$ molecules. $CoII_D$ molecules are not capable of spontaneous assembly with component I subunits and consequently are almost devoid of CoI-complementing activity, possessing only 1–2% of the potential of $CoII_M$. These latter traits strongly suggest that this dimeric form of component II is not a naturally occurring intermediate in the process of assembly of the synthetase–transferase complex.

In *Salmonella typhimurium* the multifunctional anthranilate synthetase–anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase (synthetase–transferase)[1] enzyme complex catalyzes the first and second steps of the tryptophan biosynthetic pathway (Figure 1). This aggregate is a tetramer consisting of two molecules each of subunits component I and component II (Henderson and Zalkin, 1971), the products of the first two genes of the *trp* operon, *trpA* and *trpB*, respectively (Bauerle and Margolin, 1966). The complex as well as the individual subunits can be readily purified and the properties of each have been well studied.

The synthesis of anthranilate from chorismate and glutamine (reaction 1, Figure 1) requires the intact aggregate (Bauerle and Margolin, 1966) but can also be catalyzed with equal efficiency by a partial complex, made up of intact component I and an amino-terminal fragment of component II. This partial complex occurs *in vivo* as a result of mutations internal to *trpB* which cause premature termination of translation (Secor and Bauerle, 1970), but can also be generated *in vitro* by limited proteolysis of the wild type complex (Hwang and Zalkin, 1971; Tamir and Srinivasan, 1969). Neither component I nor component II is active by itself in the glutamine-dependent anthranilate synthetase reaction. The role of component II, or the component II fragment, in reaction 1 (Figure 1) appears to be as a glutamine amidotransferase, donating the amide group of glutamine to a chorismate–component I intermediate, since it has been shown that the binding site for glutamine resides in the component II subunit (Nagano *et al.*, 1970). Consistent with this is the fact that uncomplexed component I is capable of anthranilate synthetase activity when NH_3 replaces glutamine as the amino donor (Zalkin and Kling, 1968).

The conversion of anthranilate to phosporibosyl anthranilate with 5-phosphoribosyl pyrophosphate as co-substrate (reaction 2, Figure 1) is catalyzed with equal efficiency by the intact enzyme complex and by uncomplexed component II (Bauerle and Margolin, 1966; Henderson *et al.*, 1970). Neither free component I nor the partial complex is capable of phosphoribosyltransferase activity. Thus, the component II subunit is a bifunctional molecule possessing glutamine amido-

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[1] Abbreviations used are: synthetase–transferase, the anthranilate synthetase–anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase complex; CoI and CoII, component I and II subunits, respectively.

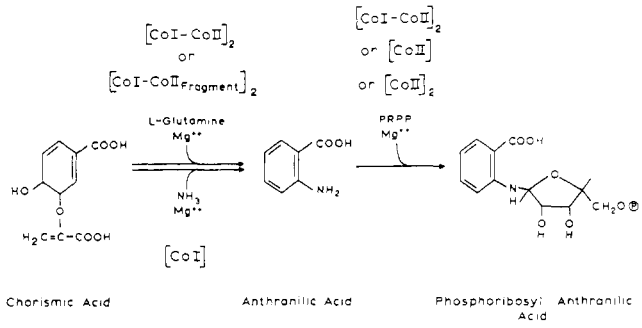

FIGURE 1: The two biosynthetic reactions of the tryptophan pathway catalyzed by the anthranilate synthetase–anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase complex of *Salmonella typhimurium*. Component I and component II represent the two dissimilar subunits of the tetrameric complex.

transferase activity when complexed with component I (referred to herein as CoI-complementing activity) and phosphoribosyltransferase activity. Similar conclusions have been drawn from studies of the homologous component II subunit of *Escherichia coli* (Yanofsky *et al.*, 1971). Genetic and biochemical studies of the fine structure of component II of *S. typhimurium* have established that its CoI-complementing activity resides specifically in the amino-terminal 40% of the polypeptide while the carboxy-terminal 60% is responsible for phosphoribosyltransferase activity (Secor and Bauerle, 1970; Grieshaber and Bauerle, 1972). It is also clear that the recognition sites involved in self-assembly of component II with component I to form the complex reside in the amino-terminal 40% of the component II molecule. This derives from the findings that amino-terminal fragments of component II readily assemble into a complex with component I both *in vivo* and *in vitro* (Grieshaber and Bauerle, 1972), while carboxy-terminal fragments possessing phosphoribosyltransferase activity do not (Margolin and Bauerle, 1966).

Four general mechanisms of assembly are possible for the synthetase–transferase complex: (1) the formation of an [component I–component II] intermediate which in turn dimerizes to yield a [component I–component II]$_2$ tetrameric molecule; (2) preliminary dimerization of both monomeric subunits forming [component I]$_2$ and [component II]$_2$ intermediates followed by their association to yield a [component I]$_2$–[component II]$_2$ tetrameric molecule; (3) the formation of a [component I]$_2$–[component II] trimeric intermediate from the dimeric form of component I and the monomeric form of component II, followed by the addition of the second monomeric component II subunit to yield the tetrameric [component I]$_2$–[component II]$_2$ molecule; and (4) the formation of a [component I]–[component II]$_2$ trimeric intermediate from the monomeric form of component I and the dimeric form of component II, followed by the addition of the second monomeric component I subunit to yield the tetrameric [component I]$_2$–[component II]$_2$ molecule. Mechanisms 2, 3, and 4 demand the occurrence of a dimeric form of either one or both the subunits. There have been suggestions that self-aggregation of component II occurs *in vitro* (Henderson *et al.*, 1970; Marcus and Balbinder, 1972). We report here evidence for the existence *in vitro* of a stable dimeric form of the component II subunit. But we also show that, unlike the monomeric form, this dimer is refractory to spontaneous assembly with component I, which indicates that it is not an assembly intermediate and suggests that assembly mechanisms 2 and 4 are unlikely.

Materials and Methods

Organisms. All strains are derivatives of *S. typhimurium* LT2. Mutant strain *trpA703* which carries a weakly polar nonsense mutation in the *trpA* gene of the *trp* operon and therefore possesses the component II subunit of the synthetase–transferase complex in the uncomplexed state (Smith and Bauerle, 1969) was used for the purification of component II molecules. The following strains were used in specific experiments as indicated in the text: strain *trpA512* which carries a deletion of almost all the *trpA* gene (Wuesthoff and Bauerle, 1970) thereby possessing its component II in the uncomplexed state; deletion strain *trpABEDC167* which lacks the entire *trp* operon; double mutant strain *trpA703 trpB88* which carries in addition to the *trpA703* nonsense site an amber nonsense mutation early in *trpB;* and strain *trpR782 trpA703* which carries in addition to *trpA703* a regulatory mutation (*trpR782*) which leads to constitutive expression of the operon. Deletion strain *trpBEDC43* which possesses only the *trpA* gene intact (Smith and Bauerle, 1969) was the source of uncomplexed component I which was used in the glutamine-dependent CoI-complementation assay for component II.

Chemicals. Anthranilic acid was obtained from Sigma Chemical Co. and recrystallized once from water. Phosphoribosyl pyrophosphate (tetrasodium salt) was purchased from Sigma Chemical Co. and used without further purification. Crystalline chorismic acid was isolated from the culture medium of *Aerobacter aerogenes* strain 62–1 according to the method of Gibson (1970). Dithiothreitol, amino acids, and Tricine were purchased for Calbiochem; Sephadex, DEAE-Sephadex, and Blue Dextran 2000 from Pharmacia Fine Chemicals, Inc.; hydroxylapatite (Bio-Gel HT) from Bio-Rad Labs; UltraPure $(NH_4)_2SO_4$ from Mann Research Labs; acrylamide, bisacrylamide, and Temed from Canalco; trypsin (2× crystallized), soybean trypsin inhibitor, aldolase, arginase, and carboxypeptidase from Worthington Biochemical Corp.; bovine serum albumin, egg albumin, and myoglobin from Pentex, Inc. All other chemicals were reagent grade. Streptomycin sulfate was a generous gift from the Merck Co.

Growth of Cells and Preparation of Cell Extracts. Cells were grown in the minimal salts medium of Davis and Mingioli (1950) modified by the exclusion of citrate and containing 0.25% glucose. Tryptophan was supplied at 4 μg/ml, an amount which limits the growth of *trp* auxotrophs to about 10^9 cells/ml. Upon exhaustion of tryptophan, cell division ceases and the enzymes of the tryptophan pathway become derepressed to a level about 100-fold that of the repressed level of actively growing cells. For analytical purposes the desired strain was grown in 500-ml volumes in 1-l. flasks at 37° with vigorous aeration. The cells were harvested by centrifugation at 2°, washed once with 50 ml of cold 0.9% NaCl, and resuspended in standard buffer (0.05 M potassium phosphate (pH 7.5) containing 10^{-4} M dithiothreitol and 10^{-4} M EDTA) using 4 ml/g wet weight of cells. The cells were disrupted by sonification in an ice-jacketed 50-ml stainless steel beaker using a Branson W185D Sonifier at 70 W for 35 sec. Cell debris was removed by centrifugation for 30 min at 48,000*g* at 2°. The clear supernatant containing 8–10 mg of protein/ml was decanted and constituted the crude extract referred to herein.

Enzyme Assays. The component II subunit is a bifunctional enzyme and can be assayed either by its anthranilate phosphoribosylpyrophosphate phosphoribosyltransferase activity or by its ability to complement the component I subunit in

the glutamine-dependent synthesis of anthranilate from chorismate (CoI-complementing activity).

Phosphoribosyltransferase activity was determined fluorometrically by measuring the rate of disappearance of anthranilic acid at 22° using a Farrand fluorometer as described previously (Smith and Bauerle, 1969). The assay mixture contained 15 μM anthranilic acid, 0.3 mM phosphoribosyl pyrophosphate, 10 mM $MgCl_2$, 100 mM Tricine buffer (pH 7.6), and enzyme preparation in a final volume of 1.0 ml.

CoI-complementing activity of component II was assayed fluorometrically by measuring the formation of anthranilic acid in the presence of a saturating amount of purified component I. The reaction mixture contained 2 mM chorismic acid, 20 mM glutamine, 10 mM $MgCl_2$, 100 mM potassium phosphate buffer (pH 7.0), 10 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 component I subunit, purified from deletion strain *trpBEDC43* (Smith and Bauerle, 1969), was essentially homogeneous and was routinely stored at −20° in 50% glycerol–0.025 M potassium phosphate (pH 7.5) at a concentration of 100 units/ml with little loss of activity.

A unit of activity is defined as the disappearance (phosphoribosyltransferase assay) or formation (CoI-complementation assay) of 1 nmol of anthranilate in 1 min. Specific activity is expressed as enzyme units per milligram of protein, which was determined colorimetrically (Lowry *et al.*, 1951).

Purification of the Monomeric and Dimeric Species of Component II. Monomeric component II ($CoII_M$) and dimeric component II ($CoII_D$) are present together in crude extracts of strain *trpA703* so that it was possible to purify both species in a single procedure.

(1) GROWTH OF CELLS. Strain *trpA703* was grown under the same conditions as above but in a 300-l. batch fermentor. Upon completion of growth the entire culture was cooled to 16–18° by circulation of cold tap water through the fermentor jacket and then pumped through a cooling coil immersed in ice water into a refrigerated continuous flow centrifuge at a flow rate of 1.8 l./min. Harvesting was complete in about 3 hr with the temperature of the cells maintained at 5–8°. The cells (usually about 0.6 kg/300 l. of culture) were immediately washed with 4 l. of cold saline. All subsequent steps were carried out at 5° or less. After each step phosphoribosyltransferase activity, CoI-complementing activity, and protein content were measured.

(2) PREPARATION OF CRUDE EXTRACT. The washed cell pellet was resuspended in standard buffer at 4 ml/g wet weight. The cells were disrupted with the Branson W185D Sonifier at full power in a jacketed continuous-flow Rosette cell cooled by circulation of ice water. The flow rate of the suspension through the cell was such that one cell volume was exchanged in 60 sec. The sonicate was centrifuged at 2° for 2 hr at 12,000*g* and then at 36,000*g* for 1 hr. The clear supernatant was decanted and constituted the crude extract.

(3) TREATMENT WITH STREPTOMYCIN SULFATE. In order to precipitate nucleic acids 10 ml of 20% streptomycin sulfate in standard buffer was added with stirring to each 100 ml of crude extract. After 30 min the precipitate was removed by centrifugation at 24,000*g* for 30 min and discarded.

(4) AMMONIUM SULFATE FRACTIONATION. To each liter of supernatant from step 3 was added slowly with constant stirring 243 g of solid $(NH_4)_2SO_4$ (40% saturation). After 15 min the precipitate was collected by centrifugation at 24,000*g* for 30 min. The pellet which contained the phosphoribosyltransferase and CoI-complementing activity was gently washed *in situ* with standard buffer in order to remove excess supernatant and was then dissolved in modified standard buffer (0.05 M potassium phosphate at pH 7.5 containing 10% glycerol, 10^{-3} M dithiothreitol, and 10^{-4} M EDTA). The preparation was then dialyzed against 4 l. of this buffer for 6–8 hr with two changes. The dialyzed extract was centrifuged at 135,000*g* for 3 hr and the pellet discarded. The glycerol and the increased concentration of dithiothreitol prevented loss of activity during dialysis and centrifugation.

(5) DEAE-SEPHADEX FRACTIONATION. The preparation from step 4 was applied to a column (7.5 × 80 cm) of DEAE-Sephadex A50 which had been swelled and equilibrated with standard buffer containing 10% glycerol. The gel was washed with 6 l. of the same buffer, which removed a bulk of unadsorbed material, and then developed with a 4-l. KCl gradient (0–0.5 M) prepared in the buffer. The flow rate of sample application, washing, and development was 100 ml/hr, maintained by a peristaltic pump. Fractions of 100 ml were collected. Those fractions with at least 15% of the phosphoribosyltransferase activity of the peak fraction were pooled and brought to 60% saturation with $(NH_4)_2SO_4$ (39 g/100 ml). The general procedure was as above in step 4. The protein pellet was dissolved in about $^1/_{20}$th the original volume of standard buffer and dialyzed against 4 l. of the same for 6 hr with one change.

(6) SEPHADEX G-150 GEL FILTRATION. The dialyzed preparation from step 5 was applied to the bottom of a column (10 × 115 cm) of Sephadex G-150 which had been swelled and equilibrated with standard buffer. The column was developed by upward flow with standard buffer at a flow rate of 100 ml/hr. Fractions of 150 ml were collected. The monomeric and dimeric forms of component II were only partially resolved in this fractionation, but could be distinguished by differences in their CoI-complementing activity (see Results). Thus, it was possible to prepare two pools, one by combining fractions possessing predominately the dimeric form of component II, the other by combining those possessing predominately the overlapping monomeric form. The pooled fractions were concentrated to about $^1/_{50}$th their volume by ultrafiltration using an Aminco cell equipped with a type P30 membrane.

(7) HYDROXYLAPATITE FRACTIONATION. The two component II preparations from step 6 were exhaustively dialyzed against 0.01 M potassium phosphate buffer (pH 7.5) containing 10^{-4} M dithiothreitol and 10^{-4} M EDTA. Each was then applied to a column of hydroxylapatite (1.5 × 27 cm) which had been equilibrated with the same buffer. The columns were developed stepwise, at a flow rate of 10 ml/hr, first with 80 ml of the equilibration buffer, followed by 100 ml of 0.05 M potassium phosphate (pH 7.5). Fractions of 4 ml were collected.

Acrylamide Gel Electrophoresis. Standard disc gel electrophoresis was performed in 7.5% polyacrylamide gel using Tris–glycine buffer (pH 9.3) according to the general method of Davis (1964). Electrophoresis in sodium dodecyl sulfate (0.1%) was performed using 5% polyacrylamide gels and 0.1 M $NaPO_4$ buffer (pH 7.2) as described by Weber and Osborn (1969). Proteins (concentration about 1 mg/ml) were denatured by dialysis at 37° for 12 hr against 0.1 M $NaPO_4$ (pH 7.2) containing 0.1% sodium dodecyl sulfate, 8 M urea, and 10^{-3} M dithiothreitol. Electrophoresis was at 8 mA/tube for 3 hr at room temperature, after which the protein bands were fixed by treating the gels with 20% sulfosalicylic acid for 12 hr. The gels were then stained with 0.25% Coomassie Blue for at least 2 hr and destained with 7% acetic acid. The gels were scanned at 280 mμ in the linear transport accessory of the

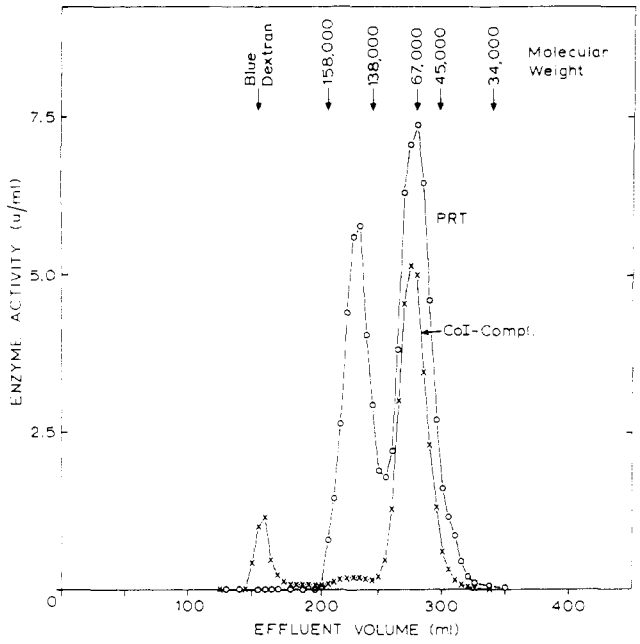

FIGURE 2: Sephadex G-100 fractionation of the component II activities of a crude extract of *S. typhimurium* mutant strain *trpA703*. Growth of cells, preparation of extract, column methodology, and procedure for the assay of phosphoribosyltransferase (○) and CoI-complementing activity (X) are described under Materials and Methods.

Gilford recording spectrophotometer using a slit width of 0.2 mm, transport speed of 2 cm/min, and chart speed of 0.5 min/in. Standard proteins used in the estimation of molecular weight were bovine serum albumin dimer and monomer (mol wt 134,000 and 67,000, respectively), egg albumin (mol wt 45,000), and myoglobin (mol wt 17,000).

Analytical Gel Filtration. Columns of Sephadex G-100 and G-150 of fixed bed volume (2.5 × 85 cm), maintained by flow adaptors, were used for analytical fractionations. The gel column, prepared and equilibrated using standard buffer, was developed by upward flow at a rate of 10 ml/hr using a peristaltic pump. A constant sample volume of 5 ml was applied to the bottom of the column and fractions of 5.0 ml were collected volumetrically. Excluded volume was determined with Blue Dextran 2000. When desired, calibration of the column was carried out with the following standard proteins of indicated molecular weights: aldolase (158,000), arginase (138,000), bovine serum albumin (67,000), egg albumin (45,000), and carboxypeptidase (34,000).

Analytical Ultracentrifugation. Sedimentation analysis was performed using a Spinco Model E analytical ultracentrifuge equipped with monochromater, ultraviolet optics, rotor temperature and indicator control device, and photoelectric scanner. The proteins at a concentration of about 0.7 mg/ml in 0.05 M potassium phosphate buffer (pH 7.5) were sedimented at 48,000 rpm at 20° using a 12-mm double-sector cell. Scanning was by absorbancy at 280 mμ. $s_{20,w}$ was calculated assuming a partial specific volume of 0.725 cm^3/g. Sedimentation equilibrium determinations of molecular weights were performed at 8000 rpm.

Trypsin Digestion of Purified CoII. The proteolytic digestion of the two species of component II by trypsin was performed as described previously (Grieshaber and Bauerle, 1972). The digestion was carried out at 20° in standard buffer using 7 μg/ml of trypsin and 500 μg/ml of component II protein. Samples were removed to enzyme reaction mixtures at the indicated times for the immediate assay of the residual phosphoribosyltransferase and CoI-complementing activities.

Heat Treatment of Purified Component II. Aliquots of 0.2 ml of the two species of purified component II, diluted in standard buffer to 3–4 units/ml, were dispensed to a series of cold matched cuvets which were then sealed with parafilm. At zero time the cuvets were immersed in a circulating water bath maintained at the desired temperature. Two cuvets were removed at the indicated intervals and immediately transferred to an ice water bath. After 3 min, the preparations were warmed to 22° in a water bath and phosphoribosyltransferase and CoI-complementing activities were assayed separately by the addition of 0.8 ml of the appropriate reaction mixture to one of the cuvets.

Results

Demonstration of Two Species of Component II Molecules by Sephadex Gel Filtration. It has been shown that nonsense and deletion mutations of *trpA*, the gene coding for component I of the synthetase–transferase complex, result in the accumulation in the cell of component II molecules in the unaggregated state (Bauerle and Margolin, 1966). Such free component II molecules can be detected by their phosphoribosyltransferase activity or by their ability to complement component I in the glutamine-dependent synthetase reaction (CoI-complementing activity). Gel filtration analysis of the component II activities of extracts of several such mutants has demonstrated that two distinct species of soluble component II molecules exist. Figure 2 depicts the Sephadex G-100 fractionation pattern obtained with an extract of the weakly polar nonsense mutant *trpA703*. Two peaks of phosphoribosyltransferase activity were resolved, one eluting at 235 ml, containing about 45% of the total recovered activity, and the other at 280 ml, containing the remaining 55% of the activity. Overall recovery of phosphoribosyltransferase activity was 75% of that applied to the column. Calibration of the Sephadex column using a set of standard proteins made it possible to extrapolate approximate molecular weights of 130,000 and 65,000 for the two component II molecules. Since a subunit mol wt of 63,000 has been ascertained for purified component II by sodium dodecyl sulfate–polyacrylamide electrophoresis (Henderson *et al.*, 1970) it can be assumed that the peak eluting at 280 ml represents the monomeric form of component II. The mol wt of 130,000 for the larger component II species is consistent with the possibility that it is a homologous dimeric form of the subunit.

Besides the difference in molecular weight, the two component II species differ markedly in their CoI-complementing activity (Figure 2). Unlike the monomeric form the larger molecular species demonstrates little CoI-complementing activity. The ratio of phosphoribosyltransferase to CoI-complementing activity across the peak at 235 ml was about 25 while that across the monomeric peak was about 1.5. It should be noted that the peak of CoI-complementing activity devoid of phosphoribosyltransferase activity which emerged with the excluded volume at 160 ml is characteristic of the fractionation of crude extracts and probably consists of a particulate fraction of component II molecules bound nonspecifically to membrane fragments (Hwang and Zalkin, 1971). This component II fraction will not be considered further in this report.

Although the possibility that the larger soluble component II species is a dimeric form of the monomeric subunit is attractive, other possibilities are equally plausible. Since the

trpA703 nonsense mutation lies well within the *trpA* gene (Wuesthoff and Bauerle, 1970) it is conceivable that the larger component II species might be an aberrant dimeric complex between a single component II molecule and a slightly truncated, but inactive component I molecule. This possibility was eliminated by the finding that the fractionation pattern of the component II activity of deletion strain *trpA512*, which lacks almost the entire *trpA* gene, is essentially identical with that obtained with *trpA703*. Another possibility considered was that the larger component II species might be a fortuitous complex between a component II subunit molecule and a usually unrelated protein which is present in limited supply in the cell and whose interaction with component II is insignificant when component I is available. To test this, the monomeric component II peak from a Sephadex G-100 fractionation of a crude extract of strain *trpA703* was pooled and mixed with crude extract prepared from cultures of either the double mutant strain *trpA703trpB88*, which lacks *trpA* and *trpB* function, or deletion strain *trpABEDC167*, which lacks the entire *trp* operon. When such mixtures were refractionated using the same Sephadex column no significant shift in the position of the monomeric component II species to that of the larger component II species was found, thereby discrediting the possibility that one of the other *trp* enzymes (in the case of *trpA703 trpB88*) or some other non-*trp* protein (in the case of *trpABEDC167*) was being erroneously titrated into an aggregate by the component II monomeric subunit.

A number of variations in experimental conditions had no significant effect on the fractionation pattern shown in Figure 2. Routinely, cultures were grown under conditions of starvation for tryptophan in order to derepress the *trp* operon and provide high levels of component II production. But the same fractionation pattern was found with strain *trpR782 trpA703*, which possesses a high constitutive level of expression of the *trp* operon, whether grown under conditions of tryptophan starvation or excess. Neither an increase in the pH of the extraction and column buffer from 7.5 to 8.6, nor the addition of 0.001 M L-glutamine (one of the ligands of the component II molecule) to the buffer affected the pattern. However, increasing the concentration of dithiothreitol in the standard buffer from 10^{-4} to 10^{-2} M did reduce the proportion of the larger component II species by about 50%. In all these experiments the ratio of phosphoribosyltransferase to CoI-complementing activity of the two component II species was unchanged from that shown in Figure 2.

For convenience the monomeric form of component II will be referred to hereafter as $CoII_M$ and the putative dimeric form as $CoII_D$.

Purification of the $CoII_M$ and $CoII_D$ Entities. Purification of the $CoII_M$ and $CoII_D$ species from *trpA703* was attained in a single procedure according to the methodology outlined under Materials and Methods. The results of the purification are summarized in Table I. A total recovery of 19% of the initial phosphoribosyltransferase activity was obtained, with 7% residing in the purified $CoII_M$ fraction and 12% in the $CoII_D$ fraction. The specific phosphoribosyltransferase activities of the two preparations were similar (3135 units/mg for $CoII_M$ and 3850 units/mg for $CoII_D$), but the specific CoI-complementing activities varied markedly (1844 units/mg for $CoII_M$ and 57 units/mg for $CoII_D$), thereby establishing the characteristically distinct ratios of phosphoribosyltransferase to CoI-complementing activity of 1.7 for $CoII_M$ and 67 for $CoII_D$, values consistent with those determined for the two species resolved in the Sephadex G-100 fractionation of the crude extract of *trpA703* (Figure 2).

TABLE I: Purification of the Two Molecular Species of the Component II Subunit from Mutant *trpA703*.[a]

Fraction	Vol (ml)	Protein Concn (mg/ml)	Total Protein (mg)	Phosphoribosyltransferase Act.				CoI-Complementing Act.				Transferase/CoI-Compl
				Total Act. (Units)	Sp Act. (Units/mg)	Purif	Recov (%)	Total Act. (Units)	Sp Act. (Units/mg)	Purif	Recov (%)	
1. Crude extract	1200	14.8	17,760	498,000	28	1	100	287,000	16	1	100	1.8
2. Streptomycin sulfate supernatant	1340			466,000			93	284,000			99	1.6
3. $(NH_4)_2SO_4$ precipitate (dialyzed)	120	58	6,960	398,000	56	2	78	229,000	32	2	80	1.7
4. 135,000*g* supernatant	117	57	6,670	402,000	59	2	81	213,000	32	2	74	1.8
5. DEAE-Sephadex	40	13.9	556	316,000	590	21	63	124,000	232	14.5	43	2.5
6. Sephadex G-150												
(a) Fraction 1 ($CoII_D$)	725	0.15	109	154,000	1410	50	31	55,000	507	32	19	2.8
(b) Fraction 2 ($CoII_M$)	570	0.16	91	63,000	700	25	13	45,000	494	31	16	1.4
7. Hydroxylapatite of												
(a) 6a ($CoII_D$)	22	0.68	15	59,000	3850	137	12	885	57	(3.6)	3	67
(b) 6b ($CoII_M$)	15	0.74	11	35,000	3135	112	7	20,600	1844	115	7	1.7

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

The $CoII_M$ and $CoII_D$ species behaved similarly during $(NH_4)_2SO_4$ fractionation, the phosphoribosyltransferase and CoI-complementing activities of the streptomycin sulfate-treated supernatant both precipitating with good yield at 40% saturation. Likewise, a single, broad peak of phosphoribosyltransferase activity emerged from the DEAE-Sephadex column at about 0.4 M KCl of the gradient. However, CoI-complementing activity was not uniformly coincidental with the phosphoribosyltransferase peak, being displaced toward its front edge as evidence by an increasing ratio of phosphoribosyltransferase to CoI-complementing activity across the peak. This partial resolution of $CoII_M$ and $CoII_D$ indicates a slightly lower affinity of the $CoII_M$ for DEAE-Sephadex under these conditions.

After concentration, the preparation from the DEAE-Sephadex column was fractionated by gel filtration using Sephadex G-150 which resulted in a partial, but substantial resolution of $CoII_M$ and $CoII_D$. The bulk of the phosphoribosyltransferase activity emerged between 5.8 and 7.0 l. of effluent while the CoI-complementing activity appeared between 6.0 and 7.0 l. The partial resolution of the two species was also indicated by the ratio of phosphoribosyltransferase to CoI-complementing activity of the fractions, which decreased across the peak from a value of 10 at its front edge to 1.5 at its back edge. The results of other experiments have shown that the lack of complete resolution of the two component II species in this instance was due to the large load applied to the gel column. Two pools were made of the Sephadex G-150 fractions, one including those fractions between 5.8 and 6.4 l. (preparation 6a of Table I), containing predominately $CoII_D$, and the other including those fractions between 6.5 and 7.0 l. (preparation 6b in Table I), containing predominately $CoII_M$. A minor peak of phosphoribosyltransferase activity, of larger molecular size, eluted at 5.05 l., and was similar to $CoII_D$ in its low level of CoI-complementing activity. It is possible that this species is a tetrameric form of component II (see also Figure 5b).

The complete resolution of $CoII_M$ and $CoII_D$ was accomplished by chromatography on hydroxylapatite. $CoII_D$ molecules were found not to absorb to the hydroxylapatite at 0.01 M potassium phosphate (pH 7.5), eluting with the void volume of the column, while $CoII_M$ molecules were retained, being subsequently eluted with 0.05 M potassium phosphate (pH 7.5). In the fractionation of preparation 6a of Table I, which contained predominately $CoII_D$ molecules, approximately 65% of the phosphoribosyltransferase activity eluted with the 0.01 M potassium phosphate wash. This fraction constituted the purified $CoII_D$ preparation (preparation 7a of Table I) possessing a ratio of phosphoribosyltransferase to CoI-complementing activity of 67, and a specific activity of 3850 units/mg for phosphoribosyltransferase and 57 for CoI-complementing activity. The remaining phosphoribosyltransferase activity eluted with 0.05 M potassium phosphate and displayed a ratio of phosphoribosyltransferase to CoI-complementing activity characteristic of $CoII_M$. Hydroxylapatite fractionation of preparation 6b of Table I, which contained predominately $CoII_M$ molecules, resulted in the elution of a minor peak of activity with the 0.01 M potassium phosphate wash ($CoII_D$ molecules) and a major peak with the 0.05 M potassium phosphate, the latter containing about 85% of the recovered phosphoribosyltransferase activity and exhibiting a ratio of phosphoribosyltransferase to CoI-complementing activity of 1.7 (preparation 7b of Table I). This constituted the purified $CoII_M$ preparation having a specific activity of 3135 units/mg for phosphoribosyltransferase and of 1844 units/mg for CoI-complementing activity.

Purity and Molecular Weight of the Isolated $CoII_M$ and $CoII_D$ Proteins. Standard disc electrophoresis in polyacrylamide gels demonstrated a single major band for both $CoII_D$ and $CoII_M$. The rates of migration of the two molecules in 7.5% gels (Tris–glycine buffer at pH 9.3) were very similar ($R_F \sim 0.6$). The $CoII_D$ preparation contained one very minor band of lesser mobility while the $CoII_M$ preparation was slightly less pure, possessing several minor bands of both greater and lesser mobilities. Quantitation of spectrophotometric scans of the stained gels, assuming equivalent staining properties of the various protein bands, indicated a purity greater than 95% for $CoII_D$ and 85% for $CoII_M$. When unstained gels were sliced into 1-mm segments immediately after electrophoresis and the slices extracted with standard buffer, a peak of phosphoribosyltransferase activity was readily detected at the position coincidental with the major protein band of both the $CoII_D$ and the $CoII_M$ preparations. CoI-complementing activity was also detected in the $CoII_M$ gel coincidental with the major band, although in low yield when compared to the recovery of phosphoribosyltransferase activity. No CoI-complementing activity was detectable in the $CoII_D$ gel.

Molecular weights for purified $CoII_M$ and $CoII_D$ were estimated by analytical gel filtration using the same Sephadex G-100 column which had been used to fractionate the phosphoribosyltransferase activities of crude extracts (Figure 2). The $CoII_D$ eluted as a single component at 235 ml, in the same position as the $CoII_D$ of crude extracts, with an extrapolated molecular weight of about 130,000. The ratio of phosphoribosyltransferase to CoI-complementing activity across the peak was constant at about 60, unchanged from the value of the applied preparation. The major portion (90%) of the phosphoribosyltransferase activity of the purified $CoII_M$ preparation eluted at 280 ml, in the same position as the $CoII_M$ of crude extracts, with an extrapolated mol wt of about 65,000. The remaining phosphoribosyltransferase activity eluted as a peak at 235 ml, having all the characteristics of $CoII_D$.

Sedimentation analysis in the analytical ultracentrifuge yielded $s_{20,w}$ values of 4.9 for $CoII_M$ and 5.8 for $CoII_D$. Molecular weights of 63,000 and 86,000 were determined by sedimentation equilibrium for $CoII_M$ and $CoII_D$, respectively. The $s_{20,w}$ value and the molecular weight found for $CoII_D$ are not in good agreement with the molecular weight obtained by gel filtration, being depressed probably due to the fact that the centrifugation was performed at 20°. In subsequent studies it was found that dissociation of the $CoII_D$ molecule occurs at elevated temperatures (see section on heat treatment of the $CoII_M$ and $CoII_D$).

Subunit molecular weights of the purified $CoII_M$ and $CoII_D$ were determined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate after denaturation in 0.1% sodium dodecyl sulfate and 8 M urea. Tracings of the stained gels made by spectrophotometric scanning are shown in Figure 3. An identical subunit mol wt of about 65,000 was obtained for both $CoII_M$ and $CoII_D$ (Figures 3a and 3b, respectively). A minor, slowly migrating band with an extrapolated mol wt of about 130,000 was present in the $CoII_D$ gel and probably consisted of undenatured $CoII_D$ molecules. Otherwise the tracings indicate a high degree of homogeneity of the two component II preparations as evidenced by the absence of any major contaminating protein components throughout the gels. When the $CoII_M$ and $CoII_D$ proteins were denatured, mixed, and electrophoresed together, a single,

homogeneous band was formed (Figure 3c), again indicating identical subunit molecular weights for the two species.

Spontaneous Conversion of Pure $CoII_M$ to $CoII_D$. The data of the preceding section strongly suggest that the $CoII_D$ species is a stable dimeric form of the component II monomer. This possibility has been verified by the finding that during storage at 4° the purified $CoII_M$ was gradually converted to the $CoII_D$ species. As mentioned above, even the freshest $CoII_M$ preparation contained a small amount of $CoII_D$. Using fractionation with Sephadex G-150 to monitor the level of $CoII_D$ and $CoII_M$ in the preparation, it was found that after 21 days of storage at 4° the content of $CoII_D$ in the $CoII_M$ preparation increased from an initial level of 15 to 45% of the total phosphoribosyltransferase activity. This shift is due to a net increase in the total units of $CoII_D$ and a concomitant decrease in the total units of $CoII_M$, not to differential decay of the two component II species during storage. During similar storage the purified $CoII_D$ molecules were not converted to the $CoII_M$ form, indicating the equilibrium of the interaction at 4° is strongly toward the dimeric state. This is consistent with our inability to obtain a $CoII_M$ preparation free of $CoII_D$ while homogeneous preparations of $CoII_D$ were readily procured. A detailed study of the effects of experimental variables, such as protein concentration, buffer components, etc., on the conversion of $CoII_M$ to $CoII_D$ has not yet been undertaken.

Interaction of $CoII_M$ and $CoII_D$ with Component I. It has been established that component I and component II subunits are capable of spontaneous assembly *in vitro* forming the fully functional synthetase–transferase complex (Smith and Bauerle, 1969). The appearance of glutamine-dependent synthetase activity in a mixture of component I and component II molecules, for example during the assay of CoI-complementing activity of component II, is concomitant with and requires the establishment of the quaternary complex. It is reasonable to assume from the low CoI-complementing activity of $CoII_D$ that the dimerization of $CoII_M$ to form $CoII_D$ leads to the "masking" of those portions of the molecule involved in CoI-complementation. However, this masking may not necessarily affect the ability of $CoII_D$ molecules to assemble into a complex with component I subunits. Such a complex, however, must be considered abnormal since it attains only a fractional amount of the synthetase activity expected relative to its phosphoribosyltransferase activity. To test this, an excess of purified component I molecules was added to aliquots of the purified $CoII_M$ and $CoII_D$ preparations. After incubation at 4° for 1 hr to permit assembly of the subunits (Smith and Bauerle, 1969), enzymatic assays demonstrated, as expected, the appearance of a high level of glutamine-dependent synthetase activity in the $CoII_M$–component I mixture (synthetase units equal to about 50% of the units of phosphoribosyltransferase activity) and of a very low level (synthetase units equal to about 1.5% of the units of phosphoribosyltransferase activity) in the $CoII_D$–component I mixture. Phosphoribosyltransferase activity remained unchanged in both cases. The mixtures were subsequently fractionated by gel filtration using Sephadex G-150.

As expected, it was found that most of the $CoII_M$ molecules readily assembled with component I into a fully active wild-type complex which eluted at 200 ml and possessed equivalent levels of both phosphoribosyltransferase and glutamine-dependent synthetase activity (Figures 4c and 4d). A small fraction of molecules possessing phosphoribosyltransferase activity did remain unaggregated and was distributed broadly among the column fractions between 250 and 350 ml. This fraction appears to have contained both

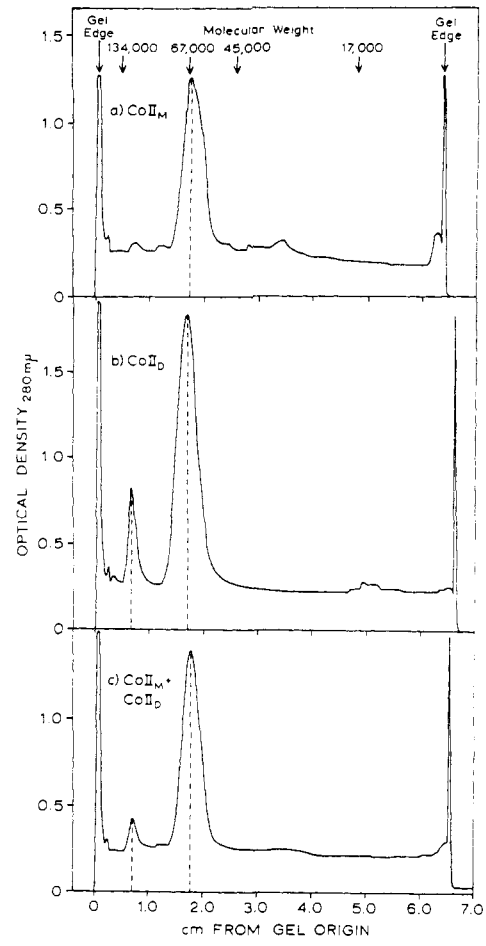

FIGURE 3: Spectrophotometric scan of sodium dodecyl sulfate–polyacrylamide gels of the purified $CoII_M$ and $CoII_D$ proteins. Denaturation of the proteins and procedures for electrophoresis, staining, and scanning are described under Materials and Methods. A total of approximately 15 μg of protein was electrophoresed in each instance: (a) purified $CoII_M$; (b) purified $CoII_D$; (c) equivalent mixture of $CoII_M$ and $CoII_D$.

$CoII_M$ and $CoII_D$ molecules as estimated by the profile of CoI-complementing activity. The reason for the incomplete assembly of the $CoII_M$ molecules is not clear but was not due to a limiting supply of functional component I molecules since a large excess of the latter, assayed by their synthetase activity in the presence of saturating amounts of purified $CoII_M$ subunit (broken curve in Figure 4d), was demonstrable, eluting characteristically at 330 ml.

The $CoII_D$ molecules, on the other hand, did not assemble into a complex with the added component I subunit (Figure 4b). The phosphoribosyltransferase activity of the mixture eluted as a single major peak at 275 ml, the same characteristic position found with the control $CoII_D$ preparation (Figure 4a). The small phosphoribosyltransferase peak at 200 ml, although being of the expected molecular size, is not a minor peak of wild-type complex since it lacks completely synthetase activity. Rather it appears to be identical with the peak observed in the large scale Sephadex G-150 fractionation used in the purification of the component II species, where there was no contact with component I molecules. It is possible that this peak represents a tetrameric form of component II.

Kinetic Properties of $CoII_M$ and $CoII_D$. Determination of V_{max} and K_m values for anthranilic acid, phosphoribosyl pyrophosphate, and Mg^{2+} in the phosphoribosyltransferase re-

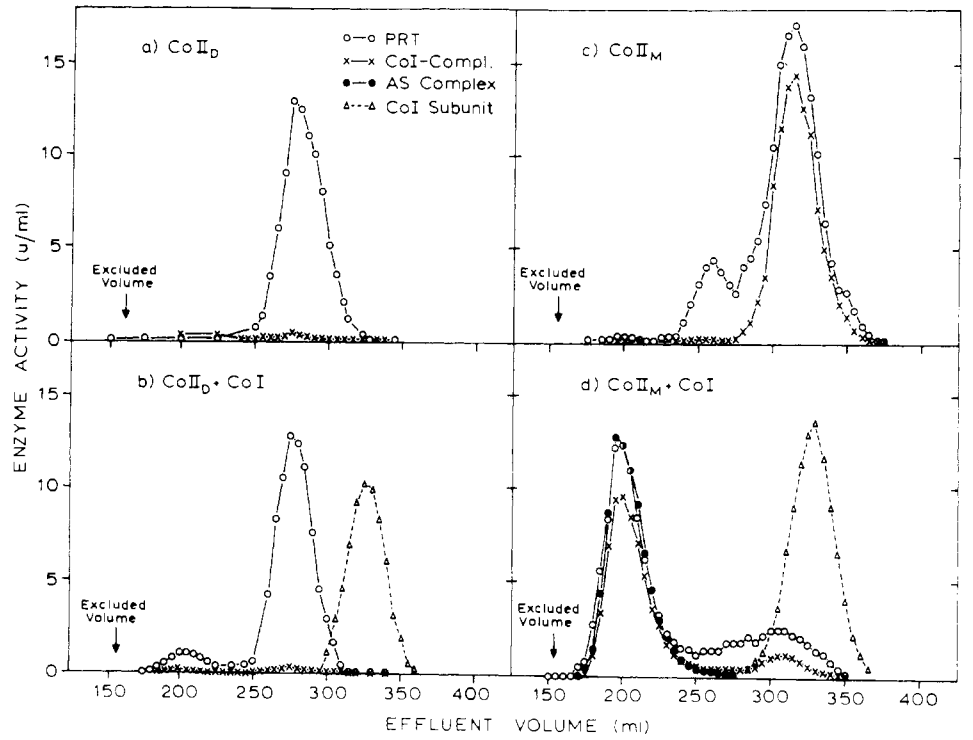

FIGURE 4: Sephadex G-150 fractionation of purified CoII$_M$ and CoII$_D$ proteins alone and after mixing with component I subunit. Assay procedures and column methodology are described under Materials and Methods. The preparations applied to the column in each case were as follows: (a) 500 units of CoII$_D$ subunit (phosphoribosyltransferase activity); (b) 500 units of CoII$_D$ subunit (phosphoribosyltransferase activity) premixed with 1000 units of component I subunit (synthetase activity, when fully saturated with component II); (c) 500 units of of CoII$_M$ subunit (phosphoribosyltransferase activity); (d) 500 units of CoII$_M$ subunit premixed with 1000 units of component I as in (b); (○) phosphoribosyltransferase activity; (X) CoI-complementing activity; (●) synthetase complex activity; (△) component I subunit activity.

action was made for both CoII$_M$ and CoII$_D$ using classical methodology. Initial reaction velocities were measured in all the assays where, routinely, only one substrate was present in limiting amounts. The data were subject to significant variations from experiment to experiment due probably to the fact that the phosphoribosyltransferase reaction remains linear for only a short time (usually no more than 2 min). This problem was especially troublesome at low concentrations of anthranilic acid and phosphoribososyl pyrophosphate and was more marked in assays of CoII$_D$ than of CoII$_M$. The kinetic constants obtained (Table II) indicate no significant catalytic differences between the two component II species in their phosphoribosyltransferase activity. In addition, both molecules displayed a strict requirement for Mg^{2+} for phosphoribosyltransferase activity, as well as a broad pH dependence with an optimum at pH 7.6 (data not shown). Phosphoribosyltransferase activity was higher and linear for a longer period when Tricine buffer (pH 7.6) was used in the assay rather than Tris buffer (pH 7.8) which had been used previously (Smith and Bauerle, 1969).

TABLE II: Kinetic Constants of CoII$_M$ and CoII$_D$ in the Phosphoribosyltransferase Reaction.[a]

Constant	CoII$_M$	CoII$_D$
V_{max}	4700 units/mg	5800 units/mg
K_M for anthranilic acid	6×10^{-6} M	4×10^{-6} M
K_M for phosphoribosyl pyrophosphate	1×10^{-5} M	1×10^{-5} M
K_M for Mg^{2+}	3×10^{-5} M	3×10^{-5} M

[a] The constants were determined by Lineweaver–Burk treatment of the data. The phosphoribosyltransferase assay procedure is described under Materials and Methods.

Inhibition by Tryptophan. One characteristic of the component II subunit is an increased sensitivity of its phosphoribosyltransferase activity to end product inhibition by tryptophan when it exists in the complex with component I, compared to when it exists in the uncomplexed state (Bauerle and Margolin, 1966; Henderson *et al.*, 1970). This is presumably due to an indirect effect of the binding of tryptophan to the feedback site on the component I subunit on the conformation of one or both of the component II molecules of the complex. High concentrations (*i.e.*, >1 mM) of tryptophan do eventually diminish the phosphoribosyltransferase activity of the uncomplexed component II subunit, possibly indicating the existence of a binding site for tryptophan on the component II molecule itself, although the metabolic significance of this effect is questionable. Figure 5 shows the effect of tryptophan on the phosphoribosyltransferase activity of CoII$_M$ and CoII$_D$ both in the presence and absence of saturating amounts of component I subunit. The component II–component I mixtures used here were the same ones analyzed by Sephadex G-150 gel filtration (Figure 4). The free CoII$_M$ and CoII$_D$ molecules behaved identically, exhibiting only a slight sensitivity to tryptophan inhibition, even at concentrations as high as 5 mM. The level of inhibition of the phosphoribosyltransferase of CoII$_D$ in the mixture with component I remained essentially unchanged. However, the phosphoribosyltransferase of CoII$_M$ inherited a marked sensitivity to tryptophan inhibition in the mixture with component I. The failure of the level of inhibition to exceed 55%, even at the highest concentrations of tryptophan, is consistent with earlier reports of this phenomenon (Bauerle and Margolin, 1966;

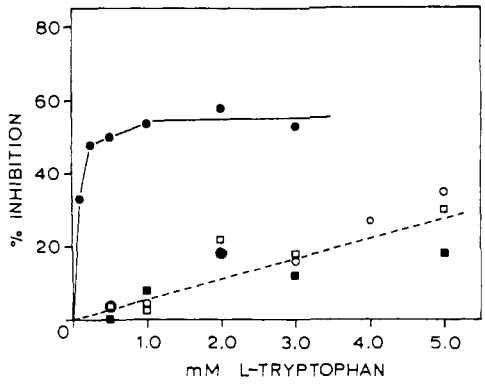

FIGURE 5: Inhibition by tryptophan of phosphoribosyltransferase activity of $CoII_M$ and $CoII_D$ proteins alone and after mixing with component I subunit. Methods of assay are described under Materials and Methods: (○) $CoII_M$ alone; (●) $CoII_M$ + component I; (□) $CoII_D$ alone; (■) $CoII_D$ + component I.

Henderson *et al.*, 1970). The data of Figure 5 are in full accord with the earlier observation that, in contrast to $CoII_M$, $CoII_D$ is incapable of assembling into a complex with component I (Figures 4b and 4d).

Sensitivity of $CoII_M$ and $CoII_D$ to Digestion by Trypsin. It has been shown that native monomeric component II molecules are very susceptible to digestion by trypsin *in vitro* (Greishaber and Bauerle, 1972), being degraded from the carboxy terminus in a defined, stepwise fashion. Under controlled conditions the digestion yields a fairly homogeneous preparation of component II fragments, composed of the amino-terminal 40% of the molecule. Like $CoII_M$ molecules, such fragments possess the ability to assemble spontaneously with component I, but form only a partial complex which possesses full synthetase activity but is completely devoid of phosphoribosyltransferase activity. Thus, trypsinolysis of component II leads to a rapid loss of phosphoribosyltransferase activity while incurring a negligible loss of CoI-complementing activity. Tryptic digestion of the purified $CoII_M$ preparation being studied here (Figure 6a) proceeded exactly as reported earlier (Grieshaber and Bauerle, 1972). Phosphoribosyltransferase activity was rapidly lost (half-life of about 1.5 min) while CoI-complementing activity decayed much more slowly (half-life about 57 min).

Tryptic digestion of $CoII_D$ carried out under identical conditions was strikingly different (Figure 6b). Both phosphoribosyltransferase and CoI-complementing activity were degraded slowly and at about the same rate (half-life of more than 80 min). Thus, it can be concluded that dimerization has led to an occlusion of those sites on the component II molecule which were particularly vulnerable to attack by trypsin.

Effect of Heat Treatment on the Activities of $CoII_M$ and $CoII_D$. The relative effects of heat treatment on the enzymatic activities of the purified $CoII_M$ and $CoII_D$ were examined and again dramatic differences were found (Figure 7). Both phosphoribosyltransferase and CoI-complementing activities of $CoII_M$ were lost rapidly at 46°, decaying exponentially with half-lives of about 4 and 3 min, respectively. In the case of $CoII_D$, however, while a slow decay of phosphoribosyltransferase activity occurred during the heat treatment, the CoI-complementing activity *increased* sharply with time, attaining a level of over five times the initial activity after 5 min at 46°. This phenomenon was examined in more detail using temperatures of 36, 46, and 50° (Figure 8). The emergence of new CoI-complementing activity was most rapid at 50° reaching its peak at 2 min, after which time the activity began to show

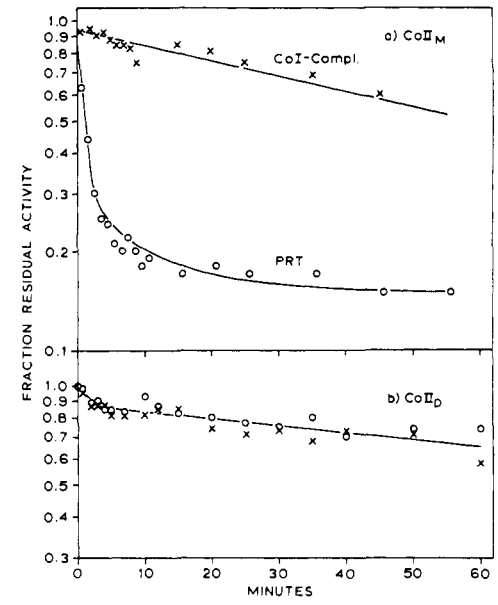

FIGURE 6: Sensitivity of the $CoII_M$ and $CoII_D$ proteins to trypsin digestion. Details of the trypsin treatment and procedures for assay of phosphoribosyltransferase (○) and CoI-complementing activity (X) are described under Materials and Methods; (a) $CoII_M$; (b) $CoII_D$.

a net decrease. Decay of phosphoribosyltransferase activity at 50° began immediately and was rapid (half-life of about 3 min). Thus, it appears that the perturbation of the $CoII_D$ molecule effected by the heat and resulting in the increased CoI-complementing activity was accompanied by the simultaneous thermal denaturation of other areas of the molecule leading eventually to a net decay of the activity with time.

Loss of phosphoribosyltransferase activity of $CoII_D$ was

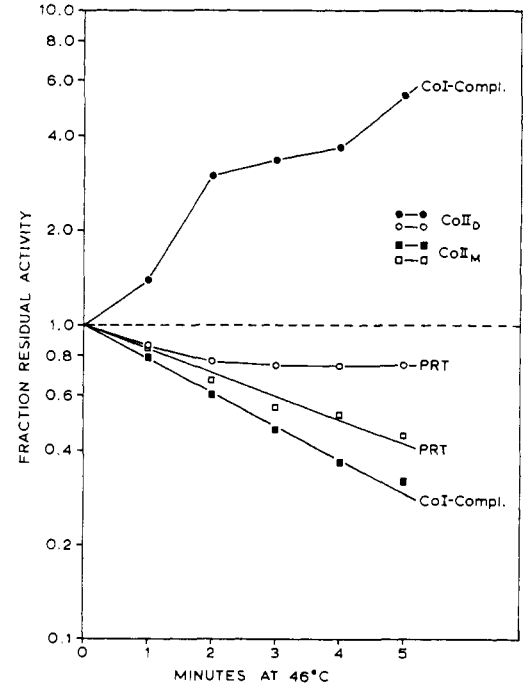

FIGURE 7: The effect of heat treatment at 46° on the enzymatic activities of the $CoII_M$ and $CoII_D$ proteins. Details of the heat treatment and procedures for assay of phosphoribosyltransferase (open symbols) and CoI-complementing activity (closed symbols) of the $CoII_M$ (□, ■) and $CoII_D$ (○, ●) proteins are described under Materials and Methods.

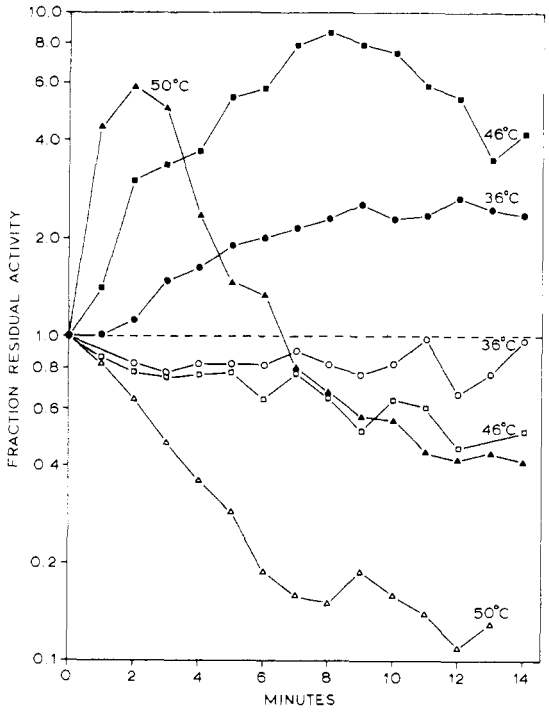

FIGURE 8: The effect of the heat treatment at 36, 46, and 50° on the enzymatic activities of the $CoII_D$ protein. Details of the heat treatment and procedures for assay of phosphoribosyltransferase (open symbols) and CoI-complementing activity (closed symbols) are described under Materials and Methods: (○, ●) 36°; (□, ■) 46°; (△, ▲) 50°.

less rapid at 36 and 46° but the appearance of new CoI-complementing activity was likewise slower. The largest increase in activity (8.5-fold) was attained after 8 min at 46°, after which time the simultaneous thermal decay became predominant. Although a variety of conditions of heat treatment have been tested it has not been possible to attain from a $CoII_D$ preparation that amount of CoI-complementing activity (*i.e.*, a 40-fold increase) which would result in a ratio of phosphoribosyltransferase to CoI-complementing activity characteristic of the $CoII_M$ species.

In order to ascertain whether the heat treatment of the $CoII_D$ preparation led to the release of free $CoII_M$ molecules, as might be expected due to the emergence of CoI-complementing activity, a sample was treated at 46° and fractionated by gel filtration using the same Sephadex G-150 column described earlier (see Figure 4). It was found that the heat treatment did lead to the appearance of a small peak of CoI-complementing activity at 330 ml, the position characteristic of $CoII_M$ (see Figure 4c). There was also a significant shoulder in the phosphoribosyltransferase profile at this position and the ratio of phosphoribosyltransferase to CoI-complementing activity across this peak was that characteristic of the $CoII_M$ species. The heat-induced increase in the CoI-complementing activity of the preparation applied to the column was about fourfold. Consistently, the total recovered CoI-complementing activity under the peak at 330 ml was approximately 3–4 times that under the $CoII_D$ peak at 280 ml.

Attempts were made to increase the yield of $CoII_M$ molecules released from the $CoII_D$ preparation during heat treatment by "trapping" them with component I molecules which had been added in excess prior to the treatment. However, no beneficial effect of the presence of the component I molecules was noted.

Discussion

Evidence has been presented for the existence of a stable dimeric form of the component II subunit of the synthetase–transferase complex of *S. typhimurium*. Both the monomeric ($CoII_M$) and dimeric ($CoII_D$) forms of the subunit have been purified to near homogeneity using a mutant lacking the companion component I subunit of the complex. The mol wt of the purified $CoII_D$ molecule (~130,000) was found to be twice that of the purified $CoII_M$ molecule (~65,000) as estimated by gel filtration. Subunit molecular weights of the two proteins determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 3) are identical (~65,000). The $CoII_M$ was found to undergo spontaneous self-aggregation during aging at 4°, forming the dimer and possibly also a homologous tetrameric form (Figure 4). The equilibrium of the interaction at 4°, under the conditions employed, is strongly toward dimerization since no reversibility was demonstrable by analytical gel filtration. Dissociation of $CoII_D$, liberating $CoII_M$ molecules, was shown to occur at elevated temperatures, but due to the sensitivity to general thermal inactivation of both species of component II molecules, it was not possible to establish conditions leading to the complete dissociation of $CoII_D$ while retaining all the $CoII_M$ molecules in a catalytically active state (Figures 7 and 8).

The comparative study of the properties of the purified $CoII_M$ and $CoII_D$ molecules revealed many similarities as well as differences and possibly offered some insight into the mechanism of assembly of component II with component I to form the intact synthetase–transferase complex. Dimerization does not appear to lead to major differences in the net electrical charge of the component II molecule since during the purification process the two species were not significantly resolved during DEAE-Sephadex chromatography. Also, both molecules migrated nearly identically during standard polyacrylamide gel electrophoresis. On the other hand $CoII_D$ did display a reduced affinity for hydroxylapatite, possibly indicating a reduction in the availability of carboxyl groups for interaction with this adsorbent (Bernardi, 1971).

The capabilities of $CoII_M$ and $CoII_D$ in the phosphoribosyltransferase reaction are very similar. The V_{max} and K_m values for anthranilic acid, phosphoribosyl pyrophosphate, and Mg^{2+}, pH optima for activity, and sensitivity to tryptophan inhibition of both are essentially indistinguishable (Table II and Figure 5). Although these data indicate that the catalytic sites involved in the phosphoribosyltransferase reaction are not functionally affected by dimerization, it is clear from other lines of evidence that the segment of the component II molecule wherein these sites are located does undergo some conformational alteration. As pointed out in the introductory statement, it has been established that the binding sites involved in phosphoribosyltransferase activity lie in the carboxy-terminal 60% of the molecule, the same portion of the $CoII_M$ molecule which is especially susceptible to trypsinolysis. The lack of this sensitivity to trypsin digestion in the $CoII_D$ species (Figure 6) indicates that some structural occlusion of the trypsin-sensitive sites in the carboxy-terminal arm of the molecule has occurred in the process of dimerization. This finding would seem to exclude a simple head-to-tail or head-to-head interaction of the subunits of the dimer, suggesting instead extensive interactions along the surface of the two sister molecules.

This idea is further supported by the finding that the dimerization greatly reduces the ability of the component II molecule to assemble with component I (Figure 4) and to

complement component I in the glutamine-dependent synthetase reaction, where $CoII_D$ is only about 2% as active as $CoII_M$ (Table I). It is known that the sites responsible for assembly of the component II subunit with component I as well as for elaboration of glutamine amidotransferase activity are located in the amino-terminal 40% of the molecule (Secor and Bauerle, 1970; Grieshaber and Bauerle, 1972). Thus it must be concluded that the dimerization of component II also involves some interaction of its amino-terminal arm, causing an occlusion of these functional sites. It is possible that the low level of CoI-complementing activity of $CoII_D$ might not be actually intrinsic to the dimeric molecule itself. Rather it might reflect a limited thermal dissociation of the molecule, which might possibly unmask the CoI-binding sites and/or release active monomer under the conditions of assay (22°). This possibility has not been tested carefully but is supported by the finding of an unexpectedly low sedimentation coefficient and molecular weight for $CoII_D$ in the sedimentation analysis which was performed at 20°.

Since the intact complex is a tetramer containing two molecules each of component I and component II, four general routes of assembly are possible: (1) 2[component I] + 2[component II] → 2[component I–component II] → [component I–component II]$_2$; (2) 2[component I] + 2[component II] → [component I]$_2$ + [component II]$_2$ → [component I]$_2$–[component II]$_2$; (3) 2[component I] + 2[component II] → [component I]$_2$ + 2[component II] → [component I]$_2$–[component II] + [component II] → [component I]$_2$–[component II]$_2$; (4) 2[component I] + 2[component II] → 2[component I] + [component II]$_2$ → [component I] + [component I]–[component II]$_2$ → [component I]$_2$–[component II]$_2$. The lack of the ability of the $CoII_D$ studied here to assemble with component I strongly suggests that this molecule is not an intermediate in the assembly process of the complex, as required for mechanisms 2 and 4 above. Furthermore, in analogous studies of the component I subunit, using mutants of *trpB* which lack the component II subunit and possess component I in the unaggregated state, no indication of the existence of a stable dimeric form of component I or of a tendency of component I to self-aggregate has been noted (Zalkin and Kling, 1968; R. H. Bauerle, unpublished observations). Thus, assembly mechanism 1 appears to be the favored one at this time. A detailed physical study of the assembly process whch is now feasible since both monomeric subunits are readily available in pure form will give an unequivocal answer to this question.

The occurrence of the $CoII_D$ species in solution is thus probably artifactual, arising under conditions of relatively high concentration of the subunit when its usual companion component I subunit is absent. The dimeric form of component II does not occur in extracts which contain both component I and component II in a functional state (R. H. Bauerle, unpublished observations). This is undoubtedly a reflection of the fact that the associative affinity of component II is significantly greater for component I than for itself. Thus, the component I–component II interaction, as measured by the establishment of linearity in the assay for synthetase activity, is complete in a matter of minutes, and appears to be essentially irreversible (Yanofsky *et al.*, 1971; R. H. Bauerle, unpublished observations), while the formation of $CoII_D$ from $CoII_M$ requires days and is readily reversible at physiological temperatures (Figure 8). Earlier studies of the component II subunit (Henderson *et al.*, 1970) were undoubtedly carried out with a mixture of the $CoII_M$ and $CoII_D$ species since the purification methods used were not sufficient to resolve the two. This explains the high molecular weight determined for the native subunit as well as the erratic ratio of phosphoribosyltransferase to CoI-complementing activity noted during purification by these investigators.

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