Immunochemical Analysis of the Anthranilate Synthase–Anthranilate 5-Phosphoribosylpyrophosphate Phosphoribosyltransferase Aggregate of *Escherichia coli*†

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ABSTRACT: The first two reactions of tryptophan synthesis in *Escherichia coli* are catalyzed by an aggregate of two proteins each about 60 000 in molecular weight and designated component I (CoI) and component II (CoII). The aggregate formula is $(CoI)_2(CoII)_2$ and the binding is provided by the interaction of component I with a region of component II, the glutamine amidotransferase region, that comprises about one-third of the amino acid chain. We have prepared goat and rabbit antisera to $(CoI)_2(CoII)_2$ in order to examine some of the structural properties of the aggregate as well as the structural relationship between the free and bound forms of the constituent subunits. By means of quantitative microcomplement fixation and precipitation of radiolabeled antigens, evidence was obtained for the following conclusions. The tertiary structure of component I differs little whether it is in the free or bound form. On the other hand, free component II undergoes a notable modification in tertiary structure, displaying extensive heterogeneity. The glutamine amidotransferase region, the linkage region of component II, was found to contribute no antibodies to the several antisera. Since antibodies to this region are made when the unbound component II is injected into rabbits, it appears that the entire surface of the glutamine amidotransferase region is concealed when the aggregate is formed. The immunochemical data thus help to explain properties of the proteins such as the strong, irreversible bond between the aggregate components and, with respect to the components, the relative ease with which component I is purifiable, whereas the purification of component II has not yet been reported.

In *Escherichia coli* the chemical reactions of L-tryptophan biosynthesis are catalyzed by the products of five consecutively linked structural genes designated *trp*E, D, C, B, A, with the E gene proximal to the control elements of the operon. Under normal conditions, the polypeptide products of the E gene, component I (CoI),[1] and the D gene, component II (CoII), form the enzyme complex, $(CoI)_2(CoII)_2$, with two enzyme activities, anthranilate synthase (EC 4.1.3.27) and anthranilate 5-phosphoribosylpyrophosphate phosphoribosyltransferase (EC 2.4.2.18). The complex catalyzes the hydrolysis of glutamine and the following reactions of which 2 and 3 are the first two physiological reactions specific to the pathway.

$$\text{chorismate} + NH_3 \xrightarrow{Mg^{2+}} \text{anthranilate} + \text{pyruvate} \quad (1)$$

$$\text{chorismate} + \text{glutamine} \xrightarrow{Mg^{2+}} \text{anthranilate} + \text{pyruvate} + \text{glutamate} \quad (2)$$

$$\text{anthranilate} + \text{PRPP} \xrightarrow{Mg^{2+}} N\text{-(5′-phosphoribosyl)anthranilate} + PP_i \quad (3)$$

From studies with mutant strains it is known that component I, by itself, can catalyze reaction 1 with NH_3 as the amino donor (Ito et al., 1969). The other subunit, component II, catalyzes reaction 3 when by itself (Ito and Yanofsky, 1969). In addition, component II is bifunctional. A region comprising about 40% of the polypeptide, beginning with the NH_2 terminus, provides the site of activation of glutamine, the glutamine amidotransferase function (GADT). The bifunctional nature of component II appears to be restricted to *E. coli* and a few related organisms (Zalkin and Hwang, 1971; Grieshaber and Bauerle, 1972; Jackson and Yanofsky, 1972; Crawford, 1975). In all other bacteria studied, the phosphoribosyltransferase activity is unaggregated, the glutamine amidotransferase activity is encoded by a separate gene, and anthranilate synthase has the formula $(CoI)_2(GADT)_2$. It has been proposed that a fusion of the genes coding for the two functions could account for the bifunctional CoII (Zalkin and Hwang, 1971; Robb and Belser, 1972; Grieshaber and Bauerle, 1972).

In recent years, a great deal of information has been obtained about the properties of anthranilate synthase in various microorganisms (Zalkin, 1973). However, for the enzyme complex in *E. coli*, other than the stoichiometry, little is known regarding the form of the aggregate and the structural relationships among the bound and free forms of each subunit. In order to obtain such information, we carried out an immunochemical analysis of the proteins and present the results in this report.

Materials and Methods

Bacterial Strains and Growth Conditions. The *E. coli* strain used for the purification of the $(CoI)_2(CoII)_2$ and $(CoI)_2(GADT)_2$ aggregates was A2/F′A2, a strain with a chain-termination mutation in the *trpA* gene on the chromosome and an episome. Component I was purified from

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[1] Abbreviations used are: CoI, component I; CoII, component II; GADT, glutamine amidotransferase subunit; EDTA, (ethylenedinitrilo)tetraacetic acid; DTT, dithiothreitol; DEAE, diethylaminoethyl; Ab, antibody; Ig, immunoglobulin; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride.

D9778/F'D9778 with a chain-termination mutation in the *trpD* gene on the chromosome and an episome. This strain produces no component II. Component II was obtained from ΔE1417/F'ΔE1417trpR$^-$, a strain with a deletion covering the *trpE* gene (Bertrand et al., 1975). For the radioimmunoassay experiment, the *E. coli* glutamine amidotransferase fragment was also purified from this strain. For other experiments, the same fragment was produced from the double mutant E5972D10481 with the chain-terminating mutations in the operator distal portion of the *trpE* gene and in the operator distal portion of *trpD*. The glutamine amidotransferase subunit from *Serratia marcescens* was obtained from trpE1, a strain with a mutation in *trpE* that produces the unaggregated subunit.

All strains except ΔE1417/F'ΔE1417trpR$^-$ were grown in salts (Vogel and Bonner, 1956) supplemented with acid-hydrolyzed casein, 1 g/L; D-glucose, 2.5 g/L; and L-tryptophan, 0.005 g/L. For strain ΔE1417/F'ΔE1417trpR$^-$, the casein was decreased to 0.1 g/L. All cells were grown at 37 °C with vigorous aeration in 15-L glass bottles. The cells were harvested 5–8 h after exhaustion of tryptophan to allow maximum synthesis of the enzymes.

Enzyme Assays. Enzyme assays were performed as described by Jackson and Yanofsky (1972). Units are defined as nanomoles of product formed or substrate utilized per minute. Protein was determined by the method of Lowry et al. (1951). Specific activity is expressed as units/mg of protein.

Protein Purifications: $(CoI)_2(CoII)_2$ Aggregate. The aggregate was purified by a modification of the procedure of Pabst et al. (1973). All operations were carried out at 0 to 5 °C. Cells of A2/F'A2 (approximately 50 g) were suspended in 150 mL of 0.05 M potassium phosphate buffer, pH 7.5, supplemented with 20% (v/v) glycerol, 0.1 mM EDTA, 1.0 mM DTT, and 1 mM $PhCH_2SO_2F$ (buffer A), and disrupted ultrasonically. The crude extract was made 1% (w/v) in streptomycin sulfate by the dropwise addition of a 10% (w/v) solution in buffer A. After centrifugation at 48 000*g* for 30 min to remove debris, the aggregate was precipitated by the addition of 196 mg of finely divided ammonium sulfate to 1.0 mL of the supernatant. The precipitate was collected by centrifugation and then redissolved in a minimal volume of buffer A. This solution was dialyzed for 16 h against 100 volumes of buffer A, centrifuged at 150 000*g* for 90 min, applied to a DEAE-cellulose column (2 × 50 cm), and eluted with a 2-L linear gradient of NH_4Cl (0–0.25 M) in buffer A. Fractions of highest specific activity were pooled, precipitated by the addition of 243 mg of solid ammonium sulfate per mL, redissolved in a minimal volume of buffer A, and applied to a Sephadex G-200 column (2.5 × 120 cm). Fractions of constant specific activity (7500–8000) were pooled and 50 μg of protein was subjected to polyacrylamide disc gel electrophoresis (Davis and Ornstein, 1964) which revealed a single protein band upon staining with Coomassie brilliant blue. Acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate also revealed a single band migrating with a molecular weight of 64 000 as compared with several proteins of known molecular weight (Weber and Osborn, 1969). This was expected as components I and II are of identical molecular weight.

Component I. Component I was purified by a modification of the procedure of Ito and Yanofsky (1969). All operations were carried out at 0–5 °C. Cells of D9778/F'D9778 (approximately 50 g) were suspended in 150 mL of 0.05 M Tris-HCl buffer, pH 7.8, at 4 °C, supplemented with 0.1 mM EDTA, 10 mM 2-mercaptoethanol, and 10% (v/v) glycerol (buffer B) and disrupted, and nucleic acids were precipitated with streptomycin sulfate as described above. Solid ammonium sulfate was added to a final concentration of 243 mg/mL to the supernatant and stirred for 1 h. The precipitate was collected by centrifugation, redissolved in buffer B, dialyzed for 16 h against 100 volumes of buffer B, applied to a DEAE-cellulose column (2 × 50 cm), and eluted with a 2-L, linear KCl gradient (0–0.2 M) in buffer B. Fractions of highest specific activity were combined and concentrated by the addition of solid ammonium sulfate (313 mg/mL). The precipitate was resuspended in buffer B and applied to a Sephadex G-100 superfine column (2.5 × 120 cm) eluted in the upward mode. Fractions of constant specific activity (approximately 3500) were pooled. Fifty micrograms of this pool was judged homogeneous by the presence of single bands in disc gel electrophoresis performed with or without sodium dodecyl sulfate.

$(CoI)_2(GADT)_2$. All steps were the same as described for the purification of the $(CoI)_2(CoII)_2$ aggregate up to and including the ammonium sulfate precipitation. The precipitate was redissolved in buffer A without $PhCH_2SO_2F$ (buffer C). The solution was centrifuged at 150 000*g* for 2 h to sediment the particulate fraction of $(CoI)_2(CoII)_2$. The pellet was resuspended in buffer C to about 15 mg of protein/mL. Crude pancreatic lipase (B grade Calbiochem)[2] was suspended at 10 mg/mL in buffer C and the insoluble material was removed by centrifugation at 48 000*g* for 15 min. Fifty microliters of lipase solution was added per mL of the resuspended membrane pellet. The mixture was incubated at 37 °C for 60 min and recentrifuged at 150 000*g* for 2 h. The supernatant solution contained all the glutamine-dependent anthranilate synthase activity and no phosphoribosyltransferase activity. The enzyme was precipitated by the addition of 300 mg of ammonium sulfate per mL, redissolved in buffer C, and applied to a Sephadex G-200 column equilibrated in the same buffer. The enzyme, upon elution from this column, was judged pure by polyacrylamide disc gel electrophoresis.

Component II. The buffer utilized was buffer C. Extract preparation, removal of nucleic acids, ammonium sulfate precipitation, and DEAE-cellulose chromatography were as described above for component I. The enzyme was about 20% pure, as estimated by disc gel electrophoresis, and was used as an immunogen for the production of neutralizing antisera.

Glutamine Amidotransferase Fragment. The procedure was exactly as described above for component II up to and including DEAE-cellulose chromatography. The enzyme was then digested as described above for the lipase treatment in the purification of $(CoI)_2(GADT)_2$, except that the incubation period was decreased to 20 min. With this decreased time for the digestion, proteolysis of the CoII molecule is not complete. Approximately 30 to 40% of the phosphoribosyltransferase activity remains while all the glutamine amidotransferase activity remains (glutamine amidotransferase activity was defined as the conferral of glutamine-dependent anthranilate synthase activity on component I). Longer digestion times produced significant inactivation of the amidotransferase activity. The digested extract was applied to a Sephadex G-100 superfine column (2.5 × 70 cm). Fractions eluting at a position characteristic of a 20 000 molecular weight protein were collected and pooled. The enzyme was about 50% pure, as estimated by polyacrylamide disc gel electrophoresis, with no single contaminant comprising more than 5%. This preparation

[2] The proteolytic activity of the crude lipase solution is responsible for its action on the anthranilate synthase complex.

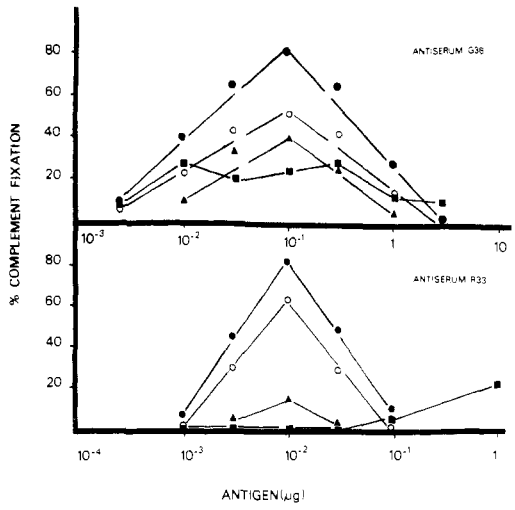

FIGURE 1: Microcomplement fixation with antiserum ASG36 at a dilution of 1/900 and ASR33 at 1/11 000. The symbols employed for the antigens are: (●—●) $(CoI)_2(CoII)_2$; (○—○) $(CoI)_2(GADT)_2$; (▲—▲) CoI; (■—■) CoII.

was used for radiolabeling in the double-precipitation experiments. For the enzyme-neutralization assays, dialyzed crude extracts of trpE5972D10481 were used. Crude extracts of *S. marcescens* trpE1 were the source of the *S. marcescens* glutamine amidotransferase subunit.

Preparation of Antisera. A goat was injected at several intramuscular sites with 2 mg of purified $(CoI)_2(CoII)_2$ emulsified in Freund's complete adjuvant. Three weeks later, the goat was boosted with 500 μg of the complex in Freund's incomplete adjuvant and bled 1 week later. This antiserum is referred to as ASG36. All rabbit antisera, with the exception of ASR523, were prepared by injecting intravenously 1 mg of pure antigen with precipitated alum (Kabat and Mayer, 1961). Six weeks later, the rabbits were boosted with 500 μg of protein. Seven and ten days later, the animals were bled. Antisera ASR33 and ASR34 were prepared against purified $(CoI)_2(CoII)_2$ and ASR957 against purified component I. Antiserum ASR523 was prepared against a partially purified preparation of component II. Seven milligrams of protein in complete Freund's adjuvant were injected at several intramuscular sites, the same amount reinjected 4-weeks later and the serum was collected the following week. Rabbits 33 and 34 received two booster courses.

Lactoperoxidase Labeling. A small tube contained 0.95 mL of 0.15 M NaCl buffered with 0.05 M potassium phosphate, 10^{-2} μM KI, 5 μL of lactoperoxidase (0.5 mg/mL), and 5 μL of ^{125}I (50 mCi/mL specific activity, New England Nuclear), and 500 μg of the protein to be labeled was cooled to 4 °C. The reaction was initiated with 10 μL of H_2O_2 (stock 0.03%) and, after 30 min, was stopped with 50 μL of 2% NaN_3 and applied to a Sephadex G-25 column (1 × 25 cm) to separate free ^{125}I and labeled antigen. No attempt was made to eliminate the lactoperoxidase as it was only 0.1% of the total protein in the labeling mixture.

Chloramine T Labeling. A small tube containing 0.05 M potassium phosphate (pH 7.0), 0.15 M NaCl, 200 μg of protein, and 0.5 mCi of carrier-free $Na^{125}I$ in a total volume of 1.0 mL was kept in an ice bath. Four micrograms of chloramine T in the phosphate–NaCl buffer was added, the tube was mixed and, after 30 s, 100 μL of $NaHSO_3$ (10 mg/mL) was injected to stop the reaction. The solution was then applied to a Sephadex G-25 column (1 × 25 cm) equilibrated in phosphate–NaCl buffer + 10 mM 2-mercaptoethanol. One-milliliter fractions were collected and counted, and the appropriate fractions were pooled.

Double Antibody Experiments. Labeled antigen and purified IgG either from goat ASG36 or rabbit ASR33 antiserum were incubated at 4 °C for 24 h. Goat antiserum to rabbit immunoglobulin or rabbit antiserum to goat immunoglobulin was then added in amounts sufficient to precipitate all the immunoglobulin the reactions incubated for 24 h, and the precipitate was collected, washed by centrifugation, and counted directly in a Beckman BioGamma counter.

Enzyme Neutralization. Preparations of glutamine amidotransferase in buffer B were mixed with varying amounts of antiserum to a final volume of 0.5 mL. After 10 min at 37 °C, 50 μL was removed, mixed with a previously determined concentration of component I, and assayed in the glutamine-dependent anthranilate synthase reaction. In certain experiments, component I was added prior to antiserum and incubated for 5 min, and then antiserum was added as above.

Microcomplement Fixation. Microcomplement fixation was done by the method of Wasserman and Levine (1961) with the modifications described by Murphy and Mills (1968).

Precipitation and Agar Diffusion Reactions. These were carried out as previously described (Mills et al., 1973).

Results

Specificity of Antisera. The antisera used in these experiments were examined for antibodies to contaminating proteins by double diffusion and quantitative inhibition. (1) In double diffusion in agar, the three antisera to the $(CoI)_2(CoII)_2$ enzyme aggregate, ASG36, ASR33, and ASR34, gave single sharp bands against either purified aggregate or crude bacterial extracts containing the aggregate. There was no evidence of spur formation between crude and purified forms of the enzyme. Similar results were obtained for the antiserum to component I, ASR957, when tested against crude and purified forms of component I. We have no antisera against purified component II. (2) For inhibition experiments, we used a strain of *E. coli* with deletions of genes *trpE* and *trpD* but otherwise identical to the strains from which the immunogens were purified (*trpE* and *trpD* specify CoI and CoII). Extracts of the deletion strain were obtained from cells grown under conditions of derepression identical to those for the other *E. coli* strains. These extracts gave no direct reaction with any of the antisera in microcomplement fixation or the precipitin reaction; nor did the extracts inhibit any of the quantitative reactions between the antisera and their corresponding antigens. By these criteria each of the antisera was free of antibodies to contaminating proteins.

The Reactions of the Enzyme Aggregate and Its Subunits with Antisera to the Aggregate. Of the various subunits and derivatives of the anthranilate synthase–phosphoribosyltransferase complex, three were available in electrophoretically homogeneous form: the complex itself, $(CoI)_2(CoII)_2$; component I; and the proteolytically derived complex $(CoI)_2(GADT)_2$ where, as previously noted, GADT stands for the functional glutamine amidotransferase region of the component II polypeptide chain. Two others, component II and the independent glutamine amidotransferase subunit from *Serratia marcescens,* were available as partially purified preparations. We examined the reactions of these molecules in microcomplement fixation with the goat antiserum, ASG36 (290 μg of Ab/mL), and the two rabbit antisera, ASR33 (3.34 mg of Ab/mL) and ASR34. The data obtained with ASR34, a slightly weaker antiserum than ASR33, will not be given, as

they were qualitatively identical to those obtained with ASR33.

The reactions with ASG36 are shown in Figure 1. To obtain optimum fixation, about 80% at maximum, 1.0 mL of a 1/900 dilution of ASG36, 0.32 μg of antibody, was used per reaction mixture. With $(CoI)_2(CoII)_2$), $(CoI)_2(GADT)_2$, and component I, complement fixation curves were obtained that are normally seen with monodisperse, globular proteins. The curves were symmetrical, with well-defined maxima and relatively little trailing in antigen excess. The partial aggregate $(CoI)_2(GADT)_2$ gave, reproducibly, the maximum cross-reaction, about 63% with this antiserum; the cross-reaction of component I was about 50%. With all antisera to the aggregate, the partial aggregate invariably gave higher cross-reaction than did component I, irrespective of the absolute extent of cross-reaction.

The form of the curve with component II was entirely different. There was no definite maximum; the curve staggered over the range of antigen shown. This is characteristic of a molecule with extensive heterogeneity; one that, in effect, is a mixture of antigens each with a subset of the determinants of the original molecule. The consequence of the simultaneous presence of multiple cross-reacting antigens to a given antiserum is discussed by Cohn (1952) and illustrated for the precipitin reaction by Cohn et al. (1950) and for the microcomplement fixation reaction by Bethell et al. (1968). The heterogeneity displayed by component II did not appear to be irreversible. When component II in crude extracts was titrated with purified component I, a subunit ratio was reached such that the formed aggregate gave a curve indistinguishable from the naturally occurring complex. This indicated that significant amounts of the unbound components did not coexist with the newly formed complex, since it could easily be shown that the presence of either component I or II in excess had the expected effect of lowering maximum fixation through competition for the antibody.

The final molecule tested in direct fixation, the amidotransferase subunit, gave no detectable reaction at the 1/900 dilution used above or at a dilution of 1/200, the maximum amount of antiserum that could be used before it became prohibitively anticomplementary.

The same set of reactions was run with the rabbit antisera. In Figure 1 is a plot of the data obtained with the much more powerful rabbit antiserum ASR33. For the $(CoI)_2(CoII)_2$ aggregate, about 80% fixation was obtained with 1.0 mL of 1/11 000 dilution or 0.31 μg of Ab/mL, as compared with the 1/900 dilution required for the goat antiserum. The data offer precisely the same relationships and inferences with respect to $(CoI)_2(GADT)_2$ and component I. The curve for component II, which is not shown completely, was again unorthodox. Fixation rose almost indefinitely with increasing concentrations of component II containing extracts and remained high until the reaction could not be measured because of the anticomplement effect of the high levels of extract. This finding also suggests that component II is heterogeneous when divorced from the complex.

As was true with the goat antiserum, the amidotransferase subunit either from *E. coli* or *S. marcescens* gave no reaction. With ASR33, the antiserum concentration could be increased 40-fold and there was still no detectable reaction.

Complement Fixation in the Presence of Excess Amounts of the Various Subunits. An advantage offered by the microcomplement fixation assay is the ease with which an antiserum may, in effect, be absorbed with a given antigen simply by putting the test dilution of antiserum into antigen excess.

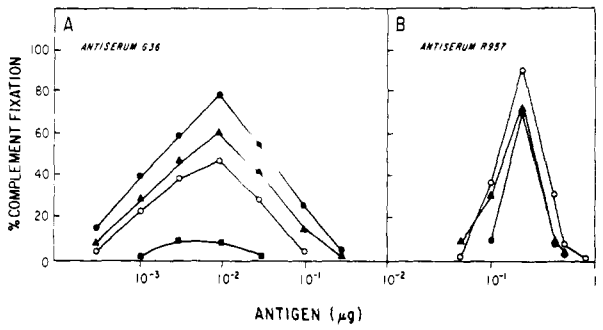

FIGURE 2: (A) Microcomplement fixation with antiserum ASG36 at 1/900 and $(CoI)_2(CoII)_2$ as antigen for all curves. Each of the blocking antigens was added in antigen excess and incubated with the antiserum for 1.0 h at 4 °C prior to the addition of $(CoI)_2(CoII)_2$. The symbols are: (●—●) no addition; (▲—▲) 6 μg of CoI per reaction mixture; (○—○) 6 μg of $(CoI)_2(GADT)_2$ per reaction mixture; (■—■) 6 μg of CoII per reaction mixture. (B) Microcomplement fixation with anti-CoI antiserum ASR957 at a dilution of 1/800. The symbols employed are: (●—●) CoI; (○—○) $(CoI)_2(GADT)_2$: (▲—▲) $(CoI)_2(CoII)_2$.

This procedure offers a sensitive test for the possible presence of antibodies to an antigenic species, in this case, the amidotransferase subunit, that may not be detectable by direct precipitin or complement fixation reactions with the subunit itself but which participate in reactions when the subunit is part of a larger structure. The antisera were therefore put into antigen excess with each of the derivatives of the complex (relatively large amounts of partially purified component II were required) then reacted with the complex. The experimental data are recorded in Figure 2A for the goat antiserum. The data offered additional information on the antigenic structure of the complex and its subunits. First, $(CoI)_2(GADT)_2$ caused a greater diminution in complement fixation activity than did component I. Second, the absorption data showed that a considerable fraction of the complement-fixing antibody in the goat antiserum was directed against component II, whereas with the two rabbit antisera, for which the curves are not shown, the bulk of the antibody appeared to be made to component I determinants. The erratic nature of the curves given by component II in direct fixation had made it impossible to estimate the relative contribution to fixation of antibodies to component II in the antisera. Third, with the several antisera there was no detectable inhibition of any curve by the amidotransferase subunit. The data further reveal that irrespective of the specific subset of reacting antibodies in the antiserum, whether directed against component I or component II, the curves given with the $(CoI)_2(CoII)_2$ aggregate were standard in shape and had a normal maximum. (Where necessary, higher concentrations of antiserum were used to give curves with maximum fixation between 50 and 70%.) These data reinforce the conclusion that the shape of the curve given by isolated component II with the anti-component II antibody subset was not a consequence of some singularity of the antibodies but rather of the structure of the component II antigen.

Reactions with Antiserum to Purified Component I. The various subunits were examined in fixation with ASR957. Component II gave no reaction with the antiserum. As expected, component I, $(CoI)_2(GADT)_2$, and $(CoI)_2(CoII)_2$ all reacted (Figure 2B) at about the same level and gave curves of standard form. Of the three forms $(CoI)_2(GADT)_2$ gave the highest extent of fixation. The difference was small but invariable. By cross-absorption and precipitation with component I, we could detect no residual antibodies to $(CoI)_2(GADT)_2$

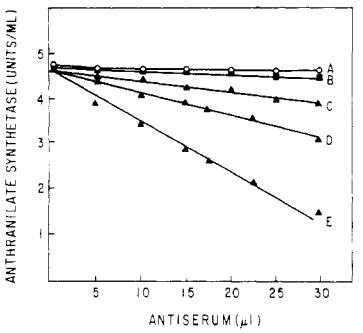

FIGURE 3: Neutralization of amidotransferase binding to component I by antiserum to component II, ASR523. Details are in Methods. (A) Reaction with rabbit 523 preimmune serum; (B) amidotransferase + 5 μg of component I mixed and incubated for 5 min prior to antiserum addition; (C, D, E) amidotransferase mixed with antiserum followed by component I at 10, 5, and 1 μg.

in the antiserum. We interpret the higher fixation to be a consequence of the difference in quaternary structure between $(CoI)_2(GADT)_2$ and component I for which there is experimental precedence (Bethell et al., 1968; Mills et al., 1973).

Does the Amidotransferase Subunit Contribute to the Surface of $(CoI)_2(CoII)_2$? The interaction of components I and II to form the tetrameric enzyme takes place between component I and the amidotransferase portion of component II. The interaction is strong, essentially irreversible (Pabst and Somerville, 1973), but is composed primarily of noncovalent forces, since the components are separable by urea–detergent treatment.

One of the striking features of the evidence obtained in the above experiments was that no antibody was demonstrable to the amidotransferase subunit in the three antisera examined. We considered two possibilities: that the amidotransferase region ($\overline{M} \simeq 20\,000$) was not immunogenic or that the region was not a significant contributor to the surface of the complex. To test the first hypothesis, component II was partially purified and injected into rabbits. The antiserum was then examined for neutralization of the reaction between component I and the amidotransferase subunit derived from *E. coli* and *S. marcescens*. It was found that the antiserum did neutralize, indistinguishably, both forms of the amidotransferase subunit in glutamine-dependent anthranilate formation. The data for the fragment derived from *E. coli* are in Figure 3. Normal serum had no effect on the reaction and there was no neutralization by the antiserum of NH_3-dependent anthranilate formation, a property of unaggregated component I. The simplest interpretation of the data is that anti-component II antibody and component I are competitive ligands for the amidotransferase subunit.

With evidence that the amidotransferase was immunogenic, the second possibility was examined. It was assumed there were antibodies directed to amidotransferase determinants in the antisera to the aggregate and that our previous methods had missed them. The following experiment was designed to look at the primary reaction between antibody and the amidotransferase determinants. The partially purified amidotransferase subunit was labeled with ^{125}I and reacted with the goat and rabbit antisera to the complex. To the reaction was added either anti-goat immunoglobulin or anti-rabbit immunoglobulin. Since the anti-immunoglobins were directed against light as well as heavy chains they could precipitate antibody to the amidotransferase irrespective of the type of immunoglobulin. The presence of antibody to amidotransferase determinants should thus result in the precipitation of ^{125}I by the anti-immunoglobulin.

TABLE I: Precipitation of ^{125}I-Labeled GADT Fragment by Antiserum to Component I.[a]

Antigen	μg of Antigen	Antiserum	Antiserum vol (μL)	Normal serum vol (μL)	Ppt (cpm)
CoI	2	R33	10	10	275 840
CoI	5	R33	10	10	616 488
CoI	10	R33	10	10	1 244 880
CoI	2			10	6 615
CoI	5			10	14 672
CoI	10			10	30 146
GADT	50	R33	200	10	3 522
GADT	100	R33	200	10	5 117
GADT	50	R33	500	10	4 693
GADT	50	R33	—	10	4 023
GADT	100	R33	—	10	4 927
CoI	2	G36	20	10	239 958
CoI	5	G36	20	10	590 530
CoI	10	G36	20	10	967 988
CoI	2			10	7 832
CoI	5			10	13 212
CoI	10			10	20 392
GADT	50	G36	200	10	4 838
GADT	100	G36	200	10	6 305
GADT	50	G36	500	10	8 972
GADT	50			10	4 434
GADT	100			10	6 239

[a] Component I and GADT fragment prepared from *E. coli* were incubated for 15 min at 37 °C in the presence of 10 μL of normal rabbit serum. To each mixture was then added antiserum and the appropriate anti-IgG, and the resulting precipitate was treated as described under Methods.

Our attempts to radioiodinate the amidotransferase subunit with the relatively mild lactoperoxidase procedure were unsuccessful, although by this method we labeled component I to high specific activity. Although the amidotransferase subunit contains tyrosine (C. Yanofsky, personal communication), these residues were apparently not accessible to lactoperoxidose iodination. With the chloramine T method, we were able to label the amidotransferase to high specific activity. The labeled subunit was then incubated with either normal serum or antiserum to $(CoI)_2(CoII)_2$, followed by the appropriate anti-immunoglobulin. The results, given in Table I, show conclusively that the goat and rabbit antisera contained no measurable antibody to the amidotransferase subunit. The table also included the fact that the method worked well with ^{125}I-labeled component I and gave appropriate proportionality between counts precipitated and amount of antigen added. The conclusions drawn from this experiment would be invalid if the ^{125}I-labeling procedure denatured the amidotransferase subunit. This was examined by reacting the labeled amidotransferase with component I. The labeled subunit conferred glutamine-dependent anthranilate synthase activity upon component I. Further, when antiserum to component I was added to the mixture of radioiodinated amidotransferase and component I, at least 90% of the total label could be precipitated (Table II). Thus, as measured by its function, the amidotransferase subunit was not appreciably denatured.

Discussion

Three antisera against the anthranilate synthase–phos-

phoribosyltransferase aggregate were used in these experiments: a goat antiserum of moderate strength made by injection of the immunogen in Frend's adjuvant, and two potent third course rabbit antisera made by repeated injection of the purified complex absorbed into aluminum hydroxide gel, a method shown to favor the expression of surface antigenic determinants (Murphy and Mills, 1968). In none of these antisera was it possible to show, by sensitive procedures, the presence of antibodies to determinants of the glutamine amidotransferase region of the *E. coli trpD* gene product, component II. The same result was obtained with the autonomous glutamine amidotransferase subunit, the *trpG* gene product of *S. marcescens*. The *S. marcescens* subunit is about the same size as the *E. coli* amidotransferase region, 20 000 daltons, and also combines irreversibly with *E. coli* component I to give fully active glutamine-dependent anthranilate synthase (Robb and Belser, 1972). Since the amidotransferase region of the *E. coli* molecule was found to be immunogenic (Figure 3), the data imply that the region is buried in the complex. The results also imply that antisera to the purified amidotransferase region (or subunit) would not react with $(CoI)_2(CoII)_2$. Such additional evidence awaits the purification of the amidotransferase subunit. We also have some evidence that in the smaller complex, $(CoI)_2(GADT)_2$, there is little if any contribution by the amidotransferase subunit to the protein surface. The studies reported here were originally prompted by our observation that potent antiserum to the *S. marcescens* $(CoI)_2(GADT)_2$ complex showed weak cross-reactivity and little enzyme neutralization of *E. coli* $(CoI)_2(CoII)_2$ and $(CoI)_2(GADT)_2$ aggregates. This was puzzling in view of the extensive amino acid and functional homology shown by the corresponding amidotransferases; the above data resolve this dilemma. The immunochemical data suggest that a large portion of the surface of the GAT region contributes to the binding between the subunits of the enzyme aggregate, a conclusion consistent with the fact that only by harsh treatment, for example, 8 M urea–dodecyl sulfate or 1 M formic acid, is it possible to separate, irreversibly, the subunits (Pabst et al., 1973; Li et al., 1974).

The immunochemical data also rationalize some of the biochemical information available about the individual components of the enzyme complex. The separate component I is a stable molecule and presented no unusual or troublesome features during purification. The same is true of the $(CoI)_2(GADT)_2$ complex from *E. coli*. Both proteins show the typical curves in microcomplement fixation that are expected of homogeneous proteins. Component II has not been purified from *E. coli*. During our unsuccessful attempts, we found the molecule to be unstable. All purification procedures were accompanied by large losses and little increase in specific activity, properties associated with a polydisperse population. The complement fixation analysis reflected these properties. The serological behavior of component II is not characteristic of phosphoribosyltransferase molecules in general. We have purified the naturally unaggregated enzyme from *S. marcescens* and from *Erwinia carotavora*, two other members of the Enterobacteriaceae, and both enzymes are stable and homogeneous.

The biochemical and genetic evidence obtained with the *trp*D gene and component II have suggested a protein composed of two domains independently folded and connected by a hinge region (Jackson and Yanofsky, 1972). The immunochemical evidence is consistent with this model and the antibodies may provide us with a convenient method of purifying the protein and obtaining direct evidence on this point by methods that have been successfully employed in structural studies with immunoglobulin (Yguerabide et al., 1970).

TABLE II: Precipitation of [^{125}I]GADT by Unlabeled Component I and Antiserum ASR957 to Component I.[a]

Antigen[b] (μg)	Antiserum ASR957 (μL)	Normal serum (μL)	Ppt (cpm)
GADT (20)	20	10	3 890
GADT (20) + CoI (50)	20	10	232 600
GADT (50) + CoI (50)	20	10	562 300
GADT (50) + CoI (50)	0	10	4 790

[a] Labeled GADT was mixed with component I, normal serum, and antiserum, followed by the appropriate anti-IgG as described under Methods. The data in the table were obtained with the enzymatically active amidotransferase fragment made from *E. coli* component II. [b] Microgram quantities are in parentheses.

Acknowledgment

We thank Professor Charles Yanofsky of Stanford University for his generous gift of the bacterial strains used in this work.

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Interaction of Adipic Acid Dihydrazide Analogue of ATP with Myosin. Involvement of the Essential Sulfhydryl Groups†

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ABSTRACT: The hydrolysis by myosin of a soluble ATP analogue, adipic acid dihydrazide-ATP, is shown to proceed in a fashion similar to the hydrolysis of ATP by myosin modified at either of the two essential sulfhydryl groups. In both systems, the Mg^{2+}-activated hydrolysis of the nucleotide is increased, whereas the EDTA-stimulated activity is inhibited. Blocking of one of the two essential sulfhydryl groups of myosin, SH_1 or SH_2, leads to a complete inhibition of the analogue hydrolysis. The analogue is unable to expose the essential sulfhydryl group SH_2 for modification by thiol reagents. Evidence is presented to show that the nucleotide derivative does not label irreversibly the protein. It is concluded that adipic acid dihydrazide-ATP (ADH-ATP) need interact with only one of the two essential thiol sites of myosin. The hydrolysis of MgADH-ATP by myosin is inhibited by large excess of actin and does not result in contraction of actomyosin threads. MgADH-ATP is also a rather weak dissociating agent of the acto-heavy meromyosin complex. These properties of the ATP analogue are discussed in conjunction with the previous modification studies of myosin and the mechanism of ATP hydrolysis derived from them (Burke, M., Reisler, E., and Harrington, W. F. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3793; Reisler, E., Burke, M., and Harrington, W. F. (1974a), *Biochemistry 13,* 2014).

Recently it was suggested that the low rate of ATP cleavage in the resting state of muscle results from formation of a stable complex structure involving the two essential thiol sites (SH_1 and SH_2) on each myosin head and MgATP (Burke et al., 1973; Reisler et al., 1974a). Activation was thought to occur by interaction of actin with myosin in the vicinity of one of the two essential thiol sites, thus breaking the stable complex and leading to a rapid dissociation of split products. In a way the action of actin was viewed as analogous to the chemical blocking of SH_1 sites of myosin; it was noted, in earlier studies, that such modification resulted in significant activation of myosin ATPase in the presence of Mg^{2+} ions (Sekine and Kielley, 1964) and the concomitant loss of the ability of actin to activate this hydrolysis reaction (Silverman et al., 1972). In terms of the proposed model (Burke et al., 1973; Reisler et al., 1974a), these two observations are corollary of the inability of the chemically modified SH_1 site to participate in the formation of the stable complex structure with MgATP. It is assumed implicitly, in this context, that the above two effects of modification of SH_1 sites of myosin are linked and stem from the same mechanistic reason.

If indeed true, such explanation would imply that the cyclic formation and opening of the inhibitory MgATP-myosin complex is an essential requirement of the contractile process. The last conclusion is supported within the qualifications set above, by the recent finding that the modification of SH_1 sites of myosin precludes contraction of actomyosin threads (Harrington et al., 1975).

The above suggestions rely, however, upon a reasonable but unproved thesis, that chemical modification does not introduce structural changes, which may produce the observed alterations in the enzymatic properties of myosin. Thus, it would be desirable to find experimental conditions which lead to, or support, the same conclusions, without involving a chemical assault on the protein. In principle, such a goal could be achieved by employing an appropriate ATP analogue, which could not form the assumed cyclic complex with the two thiol sites. Such an analogue should display hydrolytic behavior (with native myosin) comparable to that of ATP with myosin modified at SH_1 or SH_2 (i.e., activated MgATPase, inhibited EDTA(K^+) ATPase, very small, or no actin activation, etc.). We require, thus, a limited structural modification of ATP, which would preclude the precise interaction with the two thiol sites, but would not impair the hydrolysis reaction.

To our knowledge, none of the numerous ATP analogues previously tested with myosin meets the rather restrictive and specific requirements listed above (for a comprehensive review on ATP analogues and their interaction with myosin, see Yount, 1975).

The comprehensive studies of Tonomura et al. (1967) have shown a tolerance of modification of the ribose ring of ATP for cleavage of phosphate and much less for contraction. In view of this, our attention focused on ribose modified ATP derivatives prepared by Lamed et al. (1973) and successfully employed in affinity chromatography of myosin fragments (Lamed and Oplatka, 1974; Oplatka et al., 1975; Muhlrad et al., 1975). We have chosen to study the interaction and hydrolysis by myosin of adipic acid dihydrazide-ATP (ADH-

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