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Evidence for the Presence of Two Nonidentical Subunits in Carbamyl Phosphate Synthetase of *Escherichia coli*†

Susan L. Matthews and Paul M. Anderson*

ABSTRACT: A preparation of carbamyl phosphate synthetase which appears to be homogeneous by acrylamide gel electrophoresis can be obtained by carrying out as the last purification step gel filtration on Sephadex G-200 twice, once in presence of UMP (enzyme existing as a monomer) and then again in the presence of ornithine (enzyme existing as oligomer). Two protein peaks are obtained when carbamyl phosphate synthetase is subjected to gel filtration on Sepharose 4B in the presence of 6 M guanidine hydrochloride. Estimation of their molecular weights by relating their elution volumes (distribution coefficients) with those of proteins of known molecular weight gave values of 145,000 and 48,000 for the large and small peaks, respectively (referred to as the α and β subunits, respectively). Summation of these two molecular weight values yields a value which is consistent with the range of 170,000 to 200,000 established previously for the molecular weight of this enzyme. The ratio of protein in the two peaks is 2.7 to 1; the amino acid compositions of the protein in the two peaks are significantly different, but summation of the amino acid compositions, assuming that the enzyme is composed of one of each of the two subunits with molecular weights of 140,000 and 48,000, respectively, yields values very close to those of the native enzyme. The presence of two nonidentical subunits was confirmed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; two protein bands are obtained, the slower and faster moving bands corresponding to the α and β subunits, respectively. Estimation of the molecular weight of the two components corresponding to the α and β subunits by relating their relative mobilities with those of proteins of known molecular weight gave values of 138,000 and 48,000, respectively. Previous studies have shown that at least three different reactive SH groups are present in carbamyl phosphate synthetase and that the availability of two of these SH groups for reaction with *N*-ethylmaleimide is dependent on the presence or absence of different ligands. Reaction of these SH groups individually with *N*-[^{14}C]ethylmaleimide gave the following results. (1) The single SH group which reacts under all conditions and which is the only SH group which reacts when ornithine is present is located in the α subunit. (2) The SH group which reacts only when ATP–$MgCl_2$ and bicarbonate are present (resulting in a 50% loss in the synthetase and ATP synthesis activities) is located in the B subunit. (3) The SH group which can be reacted with *N*-[^{14}C]ethylmaleimide after the above two SH groups have been reacted with *N*-ethylmaleimide (resulting in nearly complete loss of synthetase activity, but a 100% increase in ATP synthesis activity) is located in the α subunit.

At least three different SH groups can be identified in carbamyl phosphate synthetase from *Escherichia coli* B by showing that the availability of each for reaction with *N*-ethylmaleimide (NEM)[1] or DTNB is dependent on the presence (or absence) of different substrates or allosteric effectors and that different effects on the catalytic activities of the enzyme are observed as a result of reaction of each of the differ-

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[1] Abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; Gdn·HCl, guanidine hydrochloride.

ent SH groups with these reagents (Foley *et al.*, 1971). One SH group reacts under all conditions studied with no effect on the catalytic activities of the enzyme and this is the only SH group which reacts when ornithine, a positive allosteric effector (Anderson and Marvin, 1968), is present. A second SH group reacts when ATP–$MgCl_2$ and bicarbonate are present resulting in a 50% decrease in both the carbamyl phosphate synthetase and ATP synthesis activities of the enzyme; the presence of ornithine enhances the effect of ATP–$MgCl_2$ and bicarbonate, while the presence of UMP, a negative allosteric effector (Anderson and Meister, 1966a), inhibits this reaction. A third SH group can be titrated in the presence of higher concentrations of the SH reagents; the presence of UMP increases the rate of this reaction when the enzyme concentration is high (Anderson and Marvin, 1970), while the presence of ornithine and/or ATP–$MgCl_2$ plus bicarbonate prevent the reaction, and the reaction is accompanied by virtually complete loss of the carbamyl phosphate synthetase activity, but 100% activation of the ATP synthesis activity.

Previous studies on the stoichiometry of the reaction bebetween NEM or DTNB and these SH groups have provided evidence that there is only one of each of the different SH groups per enzyme molecule and that the molecular weight of the enzyme (monomeric unit) is in the range of 170,000–200,000 (Foley *et al.*, 1971; Anderson and Marvin, 1970). The majority of enzymes with molecular weights this large are composed of a small even number of identical subunits which occupy equivalent positions (Klotz and Langerman, 1970). The presence of only one of each of the different SH groups per monomeric unit of carbamyl phosphate synthetase, however, would suggest that this enzyme is composed of a single large polypeptide chain or has an unusual (nonsymmetrical) subunit structure. In this paper evidence is presented showing that carbamyl phosphate synthetase is composed of two nonidentical subunits with approximate molecular weights of 140,000 and 48,000, respectively, and that of the three different SH groups described above, two are located in the large subunit and one in the small subunit.

A modification of the purification procedure for obtaining highly purified carbamyl phosphate synthetase is also reported.

Materials and Methods

Carbamyl phosphate synthetase was isolated from *E. coli* B as described by Anderson *et al.* (1970b), except for the modification described in this paper. *E. coli* cell paste (washed, 0.75 log phase, grown on enriched medium) was purchased from Grain Processing Corp. Enzyme concentration was measured by its absorbance at 280 mμ (Anderson and Marvin, 1970).

L-Ornithine, EDTA, Tris, NEM, Gdn·HCl, sodium dodecyl sulfate, and DNP-alanine were obtained from Sigma Chemical Co.; Gdn·HCl was purified by the method of Nozaki and Tanford (1967); sodium dodecyl sulfate was recrystallized from ethanol. [^{14}C]NEM was purchased from Schwarz Bio-Research. Sepharose 4B, Sephadex G-200, and Blue Dextran 2000 were products from Pharmacia. Human γ-globulin, ovalbumin, bovine serum albumin, and Coomassie Brilliant Blue were obtained from Schwarz/Mann. Phosphorylase *a* and carboxypeptidase were obtained from Worthington Biochemicals.

Acrylamide and methylenebisacrylamide were obtained from Eastman; all other reagents used for electrophoresis were from E-C Apparatus Corp.

The standard Model EC474 vertical gel apparatus from E-C Apparatus Corp. was used for all electrophoresis experiments. The Model EC489 electrophoretic destainer from E-C Apparatus Corp. was used for destaining. The procedure used for acrylamide gel electrophoresis in sodium dodecyl sulfate was essentially the same as that described by Dunker and Rueckert (1969). Relative mobilities were calculated by dividing each migration distance (measured directly from the destained and cleared gel) by that of the small (β) subunit of carbamyl phosphate synthetase; the samples were placed on the gel slab so that carbamyl phosphate synthetase was always located in the sample compartments on both sides of the sample compartment containing the reference protein(s).

The procedures employed for gel filtration on Sepharose 4B in the presence of Gdn·HCl were essentially the same as those described by Davison (1968) and Fish *et al.* (1969). The protein in the fractions was measured by a turbidimetric procedure similar to that reported by Davison (1968); to 0.2 ml of sample was added 0.5 ml of 4% trichloroacetic acid and the absorbance at 500 mμ was read after standing for 5 min with a Beckman DBG spectrophotometer equipped with a Beckman 10-in. recorder. The standard denaturing solution used for equilibrating and eluting the columns of Sepharose 4B contained Gdn·HCl (6 M)–KCl (0.05 M)–EDTA (0.01 M)–mercaptoethanol (0.01 M), pH 7.0. Protein samples were dialyzed against this standard denaturing solvent for at least 24 hr before application to the column. The void volume of the column, V_0, was determined by measuring the elution volume of Blue Dextran 2000 (located by measuring its absorbance at 640 mμ); the total volume accessible to solvent, V_i, was determined by measuring the elution volume of DNP-alanine (located by measuring its absorbance at 330 mμ). When [^{14}C]-labeled proteins were employed the radioactivity was located by counting 0.2-ml aliquots of each fraction with a Nuclear-Chicago gas flow planchet counter. The distribution coefficient, K_d, is defined as $K_d = (V_e - V_0)/(V_i - V_0)$ where V_e is the elution volume of solvent corresponding to the peak concentration of the protein.

The molecular weights assigned to the reference proteins for use in establishing the calibration curves for molecular weight estimations were those cited by Dunker and Rueckert (1969).

Carbamyl phosphate synthetase activity was routinely determined by measuring the rate of ADP formation. The aliquot containing enzyme was added to 0.3 ml of a standard reaction mixture containing ATP (20 mM)–$MgCl_2$ (20 mM)–glutamine (10 mM)–$NaHCO_3$ (10 mM)–ornithine (10 mM)–KCl (0.1 M)–Tris-HCl buffer (0.1 M, pH 8.2) at 37°; the ADP formed after 10 min at 37° was determined by coupling with lactate dehydrogenase and pyruvate kinase as previously described (Anderson and Meister, 1966b).

Results

Enzyme Purification. Although the purification procedure previously used for isolating carbamyl phosphate synthetase yielded a very pure preparation, 3–5 faint additional bands (representing less than 5% of the total protein) were often observed when the purified preparation was subjected to acrylamide gel electrophoresis under a variety of conditions (P. Anderson, unpublished data). The discovery that the molecular weight of carbamyl phosphate synthetase observed in the presence of ornithine is approximately twice as high as that observed in the presence of UMP (Anderson and Marvin, 1968, 1970) suggested that a completely homogeneous

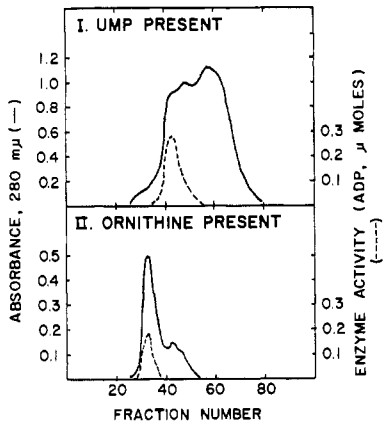

FIGURE 1: Chromatography of carbamyl phosphate synthetase on Sephadex G-200. Fractions containing enzyme activity after chromatography on DEAE-Sephadex A-50 as previously described (Anderson *et al.*, 1970b) were combined and the protein precipitated by adding $(NH_4)_2SO_4$ (45 g/100 ml) followed by centrifugation at 10,000*g* for 15 min (four enzyme preparations carried through the DEAE-Sephadex step were combined for this particular experiment); the protein (approximately 400 mg) was subsequently dissolved in a small volume (20–30 ml) of buffer A [0.2 M potassium phosphate (pH 7.6)–0.5 mM EDTA, and 0.2 mM UMP] and this solution was then applied to a column (5 × 90 cm) of Sephadex G-200 equilibrated with buffer A. The protein was eluted at a rate of 30 ml/hr and collected in 10-ml fractions. The enzyme activity was located by measuring the micromoles of ADP as described in the text which were formed when a 5-μl aliquot of the fraction was incubated with the standard assay mixture for 5 min at 37° (upper graph). The fractions containing most of the activity (39–45) were combined and the protein precipitated with $(NH_4)_2SO_4$ as described above. The precipitated protein was dissolved in a small volume (10–20 ml) of buffer B (same as buffer A, but with 2 mM ornithine in place of UMP) and the solution subjected to chromatography on the same column as described above, but now equilibrated with buffer B (lower graph). After this step the enzyme was usually concentrated [by $(NH_4)_2SO_4$ precipitation as described above or by dialysis against a solution containing 0.2 M potassium phosphate (pH 7.6)–0.5 mM EDTA–polyethylene glycol (20% w/v)] and dialyzed exhaustively against 0.2 M potassium phosphate buffer, pH 7.6, containing 0.5 M EDTA. After centrifugation at 10,000*g* for 10 min the enzyme could be stored at 4° for several weeks without loss of activity.

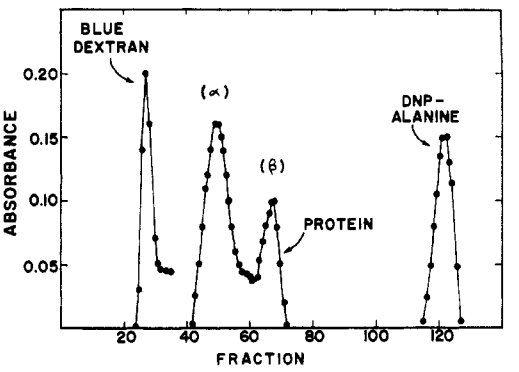

FIGURE 2: Chromatography of carbamyl phosphate synthetase on Sepharose 4B in the presence of Gdn·HCl. Chromatography of the denatured enzyme was carried out as described in the text. The column measured 2.5 × 45 cm and the sample volume applied to the column was 1 ml (approximately 13 mg of protein/ml). Fractions of 1.7 ml were collected at a rate of 5 ml/hr. The protein, Blue Dextran, and DNP-alanine in each fraction were measured as described in the text. The K_d values for the protein peaks labeled α and β were 0.23 and 0.44, respectively.

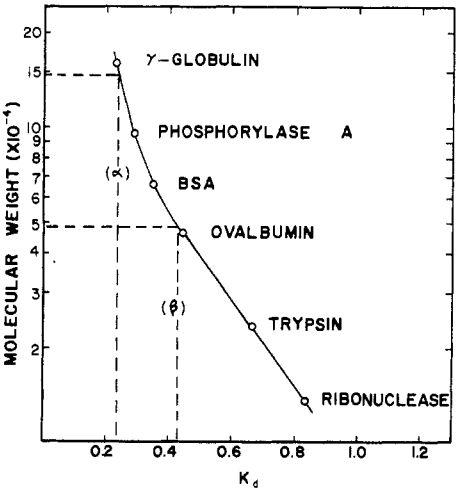

FIGURE 3: Chromatography of proteins of known molecular weight in 6 M Gdn·HCl on Sepharose 4B. The data are graphed as a semilogarithmic plot of the molecular weight of the protein *vs.* K_d. The K_d values were determined as described in the text (essentially as described by Davison, 1968). The column used for these experiments measured 1 × 55 cm; elution positions were measured by weight rather than volume to obtain maximum precision (Fish *et al.*, 1969). Human γ-globulin was denatured and chromatographed in the same way except mercaptoethanol was not present. The K_d values determined with this column for the α and β subunits of carbamyl phosphate synthetase are indicated by the dashed lines.

preparation might be obtained if the last step normally employed in the purification, *i.e.*, gel filtration on Sephadex G-200, was carried out twice, once in the presence of UMP, and then again in the presence of ornithine. The elution profiles of two such consecutive gel filtration chromatography steps are shown in Figure 1; in the second step the enzyme activity is eluted earlier, as expected, and in a zone which should be essentially free of contaminating proteins, since these were presumably removed during the first elution step carried out in the presence of UMP. Only one band of protein could be observed after electrophoresis of as much as 160 μg of this enzyme preparation on 5% polyacrylamide gels (Tris–borate–EDTA buffer, pH 9.2) at 20°.

Gel Filtration in the Presence of Gdn·HCl. It has been shown that in the presence of 6 M Gdn·HCl most (reduced) proteins lose all residual noncovalent structure and are dissociated to their constituent polypeptide chains, and that the elution volume (distribution coefficient, K_d) of the resulting randomly coiled polypeptide chains from columns of cross-linked gels (such as Sepharose 4B) is a function of their molecular weight (Davison, 1968; Fish *et al.*, 1969; Tanford, 1968). The elution profile obtained when carbamyl phosphate synthetase is subjected to gel filtration on Sepharose 4B in the presence of 6 M Gdn·HCl is shown in Figure 2. Assuming that the enzyme is homogeneous and that under these conditions it is fully denatured, the data in Figure 2 indicate that the enzyme is composed of (at least) two subunits of different molecular weights. The two protein subunits are designated α and β for the larger and smaller molecular weight protein peaks, respectively (*i.e.*, with distribution coefficients of 0.23 and 0.44, respectively). The molecular weights of these two protein components were estimated by measuring the distribution coefficients of several proteins of known molecular weights and plotting these values *vs.* the log of their molecular weights as described by Davison (1968). As shown in Figure 3, the

TABLE I: Comparison of the Amino Acid Composition of Carbamyl Phosphate Synthetase with That of the α and β Subunits.

	Residues/Molecule[a]			
Amino Acid	α (100,000)	β (100,000)	α (140,000) + β (48,000)	Native Enzyme (188,000)
Lysine	50	52	95	94
Histidine	13	31	33	33
Arginine	61	43	106	101
Aspartic acid	85	106	169	177
Threonine	53	62	104	99
Serine	46	43	84	72
Glutamic acid	114	100	207	220
Proline	39	46	77	73
Glycine	72	87	143	147
Alanine	103	103	193	196
Valine	83	60	145	134
Methionine	28	21	49	46
Isoleucine	62	56	114	105
Leucine	74	87	145	131
Tyrosine	26	20	45	52
Phenylalanine	31	37	61	59

[a] Amino acid analysis was carried out as previously described (Foley *et al.*, 1971), except hydrolysis was carried out for 24 hr only and values for tryptophan and half-cystine were not determined. The α and β subunits were isolated by chromatography on Sepharose 4B in 6 M Gdn·HCl as described in Figure 5. The molecular weights used for the calculations are indicated in parentheses; the value of 100,000 used in the first two columns is for the purpose of comparison.

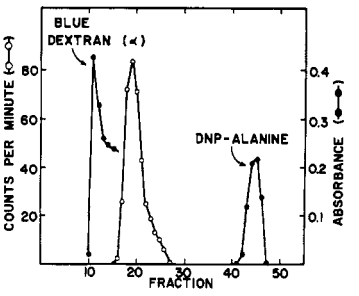

FIGURE 4: Chromatography on Sepharose 4B in 6 M Gdn·HCl of carbamyl phosphate synthetase reacted with [^{14}C]NEM in the presence of ornithine. A reaction mixture (0.12 ml) containing enzyme (27 mg/ml), potassium phosphate buffer (0.13 M, pH 7.6), EDTA (0.3 mM), [^{14}C]NEM (0.5 mM, 10.3 mCi/mmole), and ornithine (17 mM) was incubated at 17° for 16 min at which time the reaction was essentially complete and no loss in synthetase activity had occurred; the reaction of the [^{14}C]NEM with the enzyme was followed by measuring the incorporation of radioactivity into the protein as previously described (Foley *et al.*, 1971). The reaction was stopped by adding 10 μl of a solution containing 0.2 M cysteine to the 95 μl of reaction solution which remained. After 5 min an aliquot (0.9 ml) of the standard denaturing solution containing 6 M Gdn·HCl was added and the resulting solution dialyzed exhaustively and chromatographed as described in the text. The column size was 0.9 × 25 cm and the sample volume applied to the column was 0.2 ml. Fractions of about 0.5 ml were collected at a rate of about 1 ml/hr. DNP-alanine, Blue Dextran, and radioactivity were located in the fractions as described in the text. The peak of radioactivity was eluted at a position with a K_d value of 0.23 which corresponds to the α subunit described in Figure 2.

results yielded values for the α and β subunits of 145,000 and 48,000, respectively. (It should be recognized that estimation of the molecular weight of the larger subunit by this procedure, as well as by sodium dodecyl sulfate acrylamide gel electrophoresis as described below, is probably subject to more error than that of the smaller subunit, particularly because of the lack of reference proteins without disulfide bonds in this molecular weight range.) Estimation of the total protein in each of the two protein peaks yields a protein ratio of 2.7:1 for the α and β subunits, respectively, which is consistent with these molecular weight values if the enzyme is composed of equal numbers of each subunit. Summation of the above estimated molecular weight values for each of the two subunits gives a value of about 190,000, which is in close agreement with the estimated molecular weight of the native enzyme (Anderson and Marvin, 1970; Foley *et al.*, 1971), suggesting that the monomeric unit of carbamyl phosphate synthetase is composed of one of each of these two different subunits. Amino acid analysis of the protein in each of the two different protein peaks was carried out and the results are given in Table I. The amino acid compositions of the protein in the two peaks are significantly different; however, the sum of the amino acid compositions for the α and β subunits, assuming that the molecular weights are 140,000 and 48,000, respectively, agree reasonably well with the values for the native enzyme, providing further indirect evidence that the enzyme is composed of one of each of the two different subunits.

Virtually the same results are obtained when gel filtration is carried out as described in Figure 2 but in the absence of mercaptoethanol, indicating that the two subunits are probably not linked by disulfide bonds (see Figure 5). Similar results have also been obtained when 8 M urea is used as a denaturant instead of 6 M Gdn·HCl.

Gel Filtration in the Presence of Gdn·HCl of Carbamyl Phosphate Synthetase with Different SH Groups Labeled by Reaction with [^{14}C]NEM. As indicated in the introduction above, previous studies have shown that the availability of at least three different SH groups in carbamyl phosphate synthetase for reaction with NEM is dependent on the presence or absence of different ligands so that they can be titrated individually. The possibility that one of these SH groups might be located in the protein represented by one of the protein peaks described in Figure 2 while the other SH groups were located in the protein represented by the other peak was therefore investigated, since this would provide additional and more direct evidence that the presence of the two different protein peaks described in Figure 2 is the result of the enzyme being composed of two nonidentical and functional subunits.

The results of these studies are summarized in Figures 4–7. As shown in Figure 4, the SH group which is available for reaction with [^{14}C]NEM when only ornithine is present is located only in the protein representing the first peak, or α subunit, since virtually all of the radioactivity associated with this SH group after reaction with [^{14}C]NEM is eluted as a sharp peak with a K_d value of 0.23. The additional single SH group which reacts with [^{14}C]NEM when ATP-$MgCl_2$ and bicarbonate are present (with or without ornithine present) is apparently located in the protein representing the second peak, or β subunit, since two radioactive peaks corresponding

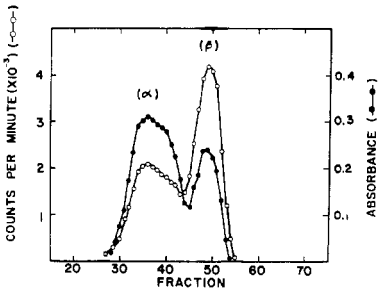

FIGURE 5: Chromatography on Sepharose 4B in 6 M Gdn·HCl of carbamyl phosphate synthetase reacted with [^{14}C]NEM in the presence of ATP–$MgCl_2$ and bicarbonate. The reaction mixture contained enzyme (13.5 mg/ml), [^{14}C]NEM (0.36 mM, 1.8 mCi/mmole), ATP (16 mM), $MgCl_2$ (16 mM), and HCO_3^- (16 mM) in a final volume of 0.75 ml; the reaction of [^{14}C]NEM with the enzyme was followed with time by measuring the decrease in synthetase activity as previously described (Foley *et al.*, 1971). The reaction was complete after 60 min at which time the reaction was stopped by adding 10 μl of 0.2 M cysteine to the 0.71 ml of reaction solution which remained. After 5 min, 0.1 ml of 0.1 M NEM was added and the resulting solution dialyzed exhaustively against the standard denaturing solution which contained 0.01 M NEM but no mercaptoethanol. The entire dialyzed sample was applied to the column (1 × 55 cm) and chromatographed as described in the text except mercaptoethanol was not present. Fractions of about 0.4 ml were collected at a rate of about 1 ml/hr. DNP-alanine and Blue Dextran were omitted from this experiment because the protein in each peak was to be used for other purposes. The protein and radioactivity in the fractions were located as described in the text.

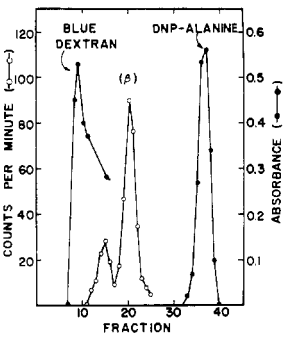

FIGURE 6: Chromatography on Sepharose 4B in 6 M Gdn·HCl of carbamyl phosphate synthetase reacted with NEM in the presence of ornithine and then with [^{14}C]NEM in the presence of ATP–$MgCl_2$ and bicarbonate. The enzyme was first reacted with NEM in the presence of ornithine as described in Figure 4, except unlabeled NEM (0.025 M) was used in place of [^{14}C]NEM (the enzyme concentration was 37 mg/ml and the volume of the reaction mixture was 0.4 ml). The reaction was stopped after 16 min at 17° by cooling to 4° and separating the enzyme from the reaction components by gel filtration on a small column of Sephadex G-50 (0.9 × 25 cm) equilibrated with 0.2 M potassium phosphate buffer, pH 7.6 containing 0.5 mM EDTA. The fractions containing the highest enzyme activity were combined and the enzyme was reacted with [^{14}C]NEM essentially as described in Figure 5; the reaction mixture contained enzyme (3.7 mg/ml), ATP (14 mM), $MgCl_2$ (14 mM), HCO_3^- (14 mM), ornithine (7 mM), and [^{14}C]NEM (0.34 mM, 10.3 mCi/mmole) in a final volume of 0.35 ml at 17°. The reaction was stopped after 16 min (at which time the reaction was nearly complete, the synthetase activity having decreased more than 80%) by adding 10 μl of 0.2 M cysteine to the 290 μl of reaction solution which remained. After 5 min, 0.45 ml of the standard denaturing solution was added and the resulting solution was dialyzed exhaustively. An 0.2-ml aliquot of the dialyzed solution was chromatographed as described in the text. The column size was 0.9 × 28 cm. Fractions of about 0.5 ml were collected at a rate of about 1 ml/hr. DNP-alanine, Blue Dextran, and radioactivity were located in the fractions as described in the text. The large peak of radioactivity was eluted in a position with a K_d value of 0.42 which corresponds to the β subunit as indicated above and described in Figure 2. The small peak of radioactivity presumably corresponds to the α subunit.

to the two protein peaks are obtained (see Figure 5). These results are consistent with previous studies which have shown that treatment of carbamyl phosphate synthetase with NEM in the presence of ATP–$MgCl_2$ and bicarbonate involves reaction of the same SH group which reacts when only ornithine is present (located in the α subunit as indicated above) plus an additional single SH group which reacts only in the presence of ATP–$MgCl_2$ and bicarbonate (apparently located in the β subunit as demonstrated in Figure 5) (Foley *et al.*, 1971). Additional evidence that this latter SH group is associated with a different subunit (β subunit) was obtained by separating by gel filtration the subunits of an enzyme preparation in which only this SH group had been reacted with [^{14}C]NEM; this was accomplished by reacting the enzyme with unlabeled NEM in the presence of ornithine followed by isolation and reaction of this derivative with [^{14}C]NEM in the presence of ATP–$MgCl_2$ and bicarbonate (Figure 6). As shown in Figure 6, the majority of the radioactivity is associated with the β subunit (K_d = 0.42). Some radioactivity is associated with the α subunit in this experiment; the significance of this observation is not known, but it is possibly related to previously published data which showed that a small amount of reaction occurred when DTNB was reacted in the presence of ornithine with enzyme which had been isolated after it had been previously allowed to react with DTNB (until the reaction was considered to be complete) also in the presence of ornithine (Foley *et al.*, 1971). The ratio of protein in the two peaks (α and β, respectively) in Figure 5 is about 2.7:1, which agrees with the data in Figure 2, while the ratio of radioactivity in the same two peaks is about 1:1.2. The latter ratio would be expected to be 1:1 if the enzyme was composed of two nonidentical subunits, each containing a single SH group which is labeled by reaction with [^{14}C]NEM. However, an exact ratio of 1:1 has not always been obtained; although further study is required on this point, a possible explanation for this apparent lack of exact stoichiometry is the observation which we have made that the availability of the SH group which has been reported to react stoichiometrically with NEM or DTNB in the presence of ornithine (Foley *et al.*, 1971) varies with time after isolation when the purification procedure described in this paper is employed, the ratio becoming closer to 1 after the enzyme has been stored for several weeks after isolation. This observation as well as the additional observation that the labeling of the α subunit in the experiment illustrated in Figure 6 can be minimized by carrying out the reaction with [^{14}C]NEM immediately after isolating the enzyme which has been reacted with unlabeled NEM in the presence of ornithine might indicate that the enzyme is subject to rather slow and complex conformational changes other than those which have been reported previously (Anderson and Marvin, 1970).

The third SH group which has been identified is located exclusively in the α subunit (K_d = 0.21), as shown in Figure 7. This SH group was labeled specifically as described in Figure 7 by first reacting the enzyme with unlabeled NEM in the presence of ornithine, ATP–$MgCl_2$, and bicarbonate; this enzyme derivative was then isolated and reacted with [^{14}C]-NEM (Foley *et al.*, 1971).

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate. As shown in Figure 8, a sample of carbamyl phosphate synthetase which yields only one band

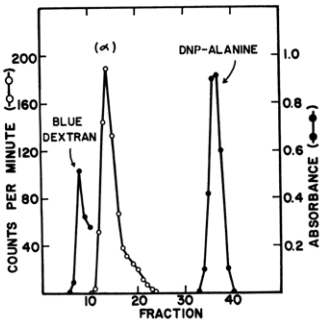

FIGURE 7: Chromatography on Sepharose 4B in 6 M Gdn·HCl of carbamyl phosphate synthetase reacted with NEM in the presence of ATP–$MgCl_2$ and bicarbonate and then with [^{14}C]NEM. The two SH groups which are available for reaction with SH reagents when ATP–$MgCl_2$ and bicarbonate are present were first reacted with NEM; the reaction mixture contained enzyme (34 mg/ml), NEM (1.1 mM), ornithine (11 mM), ATP (11 mM), $MgCl_2$ (11 mM), EDTA (0.4 mM), and potassium phosphate buffer (0.17 M, pH 7.8) in a final volume of 0.37 ml. The reaction was essentially complete after 15 min at 17° [the reaction was followed with time by measuring the synthetase activity in aliquots removed from the reaction mixture as previously described (Foley *et al.*, 1971)]; after 30 min of reaction time the enzyme was separated from the other components of the reaction mixture by gel filtration on a small column (0.9 × 25 cm) of Sephadex G-50 equilibrated with 0.1 M potassium phosphate buffer, pH 7.8, at 4°. The fractions containing the highest enzyme activity were combined and the enzyme was reacted with [^{14}C]NEM; the reaction mixture contained enzyme (6.7 mg/ml), [^{14}C]NEM (0.6 mM, 10.3 mCi/mmole), EDTA (0.1 mM), and potassium phosphate buffer (0.08 M, pH 7.8) in a final volume of 0.32 ml at 17°. The reaction was stopped after 90 min (at which time the reaction was nearly complete, the synthetase activity having decreased to less than 2% of the value when the reaction was started) by adding 20 μl of 0.2 M cysteine to the 0.28 ml of reaction solution which remained. After 5 min, 0.15 ml of the standard denaturing solution was added and the resulting solution dialyzed exhaustively. An 0.2-ml aliquot of the resulting dialyzed solution was chromatographed as described in the text. The column size was 0.9 × 28 cm. Fractions of about 0.4 ml were collected at a rate of about 1 ml/hr. DNP-alanine, Blue Dextran, and radioactivity were located in the fractions as described in the text. The peak containing radioactivity was eluted at a position with a K_d value of 0.21 which corresponds to the α subunit as indicated above and described in Figure 2.

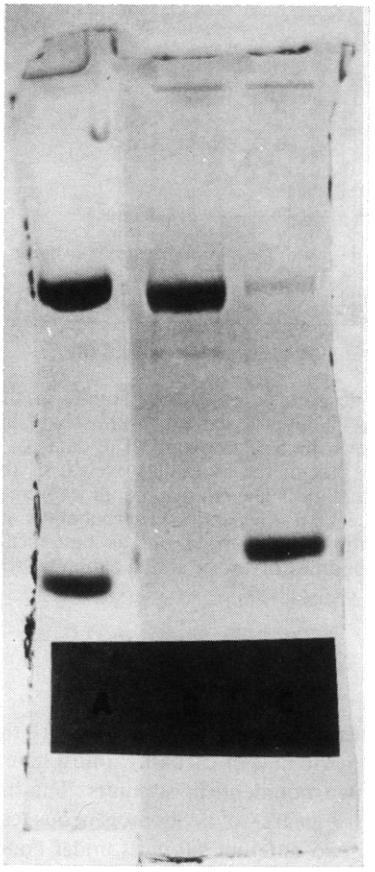

FIGURE 8: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of carbamyl phosphate synthetase and the α and β subunits of carbamyl phosphate synthetase. Vertical gel electrophoresis was carried out on a 5% polyacrylamide gel slab of 6-mm thickness [prepared by dissolving 14.51 g of acrylamide, 0.5 g of bisacrylamide, 0.3 ml of *N,N,N′,N′*-tetramethylethylenediamine, and 0.2 g of ammonium persulfate in 300 ml of buffer (0.04 M sodium phosphate, pH 7.2, containing 0.1% sodium dodecyl sulfate)] at 200 V for 4 hr at 20°. After electrophoresis, the protein was fixed by soaking in solvent A [methanol–water–acetic acid (5:5:1, v/v/v)] overnight and then stained by immersing the slab in 0.2% Coomassie Brilliant Blue in solvent A for 5 hr. The gel slab was destained by immersing in solvent A overnight followed by electrophoretic destaining. The destained gel slab was cleared by soaking in 5% glycerol overnight. The α and β subunits were isolated by chromotography on Sepharose 4B essentially as described in Figure 5. The enzyme or respective subunit was added to a solution containing urea (4 M), sodium dodecyl sulfate (1%), and iodoacetamide (0.01 M) and incubated for 2 hr at 37° just prior to electrophoresis; the sample applied to the gel compartment was composed of 10 μl of this solution mixed with 10 μl of a solution containing 40% sucrose and Bromothymol Blue which served as a tracking dye. Pattern A was obtained in a different experiment than B and C, but the conditions were identical in both experiments except that the running time was slightly longer in A. A: native enzyme (50 μg); α subunit (approximately 20 μg); C: β subunit (approximately 20 μg).

of protein after polyacrylamide gel electrophoresis can be separated into two protein components by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; under these conditions the enzyme should be fully denatured and dissociated into its constituent polypeptide chains, and separation is achieved on the basis of molecular weight (Fish *et al.*, 1970). The results in Figure 8 also show that the α and β subunits identified and separated by gel filtration in the presence of Gdn·HCl as described above correspond to the slower and faster migrating bands, respectively. The molecular weights for each subunit were estimated as described by Dunker and Rueckert (1969) by comparing the relative migration distances of marker proteins of known molecular weight with those of the α and β subunits. The data are given in Figure 9 as a plot of the relative migration distance *vs.* the log of the molecular weights for each of the reference proteins (Dunker and Rueckert, 1969); molecular weights of 138,000 and 48,000 were estimated from this data for the α and β subunits, respectively. It should be recognized that the molecular weight of the larger (α) subunit estimated by this method is probably subject to considerable error, since, as in the case with the calibration curves used to estimate the molecular weight of the subunits by gel filtration in the presence of Gdn·HCl (Figure 3), unreduced proteins containing more than one polypeptide chain linked by disulfide bonds have necessarily been used as reference proteins for this subunit; Fish *et al.* (1970) have pointed out that gel electrophoresis of unreduced proteins in sodium dodecyl sulfate solution cannot generally be used to obtain accurate estimates of molecular weight. Nevertheless, the data in Figures 8 and 9 are consistent with the data obtained above by gel filtration in the presence of Gdn·HCl and provide confirming evidence that the enzyme is composed of two nonidentical subunits.

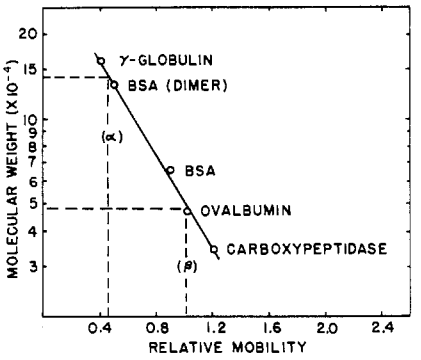

FIGURE 9: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate of carbamyl phosphate synthetase and proteins of known molecular weight. The data are graphed as a semilogarithmic plot of the molecular weight of the proteins *vs.* their relative mobilities. The relative mobilities were determined as described in the text. The entire electrophoretic procedure and sample preparation were carried out as described in Figure 8. The relative mobilities of the α and β subunits, respectively, are indicated by the dashed lines.

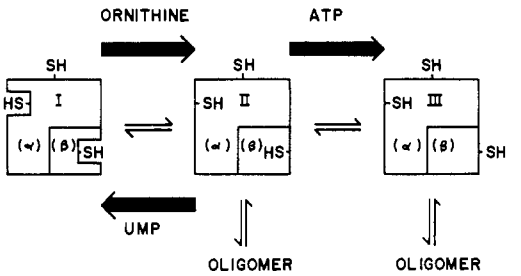

FIGURE 10: Scheme illustrating the existence of two nonidentical subunits in carbamyl phosphate synthetase. Except for the division into subunits, this scheme is the same as those previously proposed as a possible mechanism for the allosteric effector- and ATP–$MgCl_2$-dependent aggregation and inhibition by NEM of carbamyl phosphate synthetase (Anderson and Marvin, 1970), and for summarizing the conditions under which the different SH groups which have been identified are available for reaction with NEM or DTNB (Foley *et al.*, 1971).

Discussion

The data presented above strongly support the conclusion that the monomeric unit of carbamyl phosphate synthetase is composed of two nonidentical subunits. The highly purified enzyme which appears to be homogeneous can clearly be separated into two different subunits under conditions where the enzyme is probably fully denatured, and the sum of the molecular weights estimated for each subunit (188,000) is in close agreement with the estimated molecular weight range of 170,000–200,000 for the monomeric unit of carbamyl phosphate synthetase determined from sedimentation constants obtained from sucrose density gradient studies (Anderson and Marvin, 1970) and the results of studies on the stoichiometry of the reaction between NEM or DTNB and the different SH groups which have been identified in the enzyme (Foley *et al.*, 1971). The value of 2.7:1 obtained for the ratio of total protein in the large and small subunits, respectively, and the approximately equal labeling of each subunit obtained when the enzyme is reacted with [^{14}C]NEM in the presence of ATP–$MgCl_2$ and bicarbonate are also consistent with a model in which the monomeric unit of the enzyme is composed of one of each of the two different subunits with molecular weights of about 140,000 and 48,000, respectively.

Evidence has been previously reported supporting the scheme shown in Figure 10 in which carbamyl phosphate synthetase is considered to exist in at least three different conformational states designated monomer-I, monomer-II, and monomer-III, respectively, and to possess at least three different SH groups which are available for reaction with SH reagents under different conditions (indicated by their location outside the enclosures representing the different conformational states in the illustration in Figure 10) (Anderson and Marvin, 1970; Foley *et al.*, 1971). The scheme shown in Figure 10 has been modified to the extent that carbamyl phosphate synthetase is now considered to be composed of two nonidentical subunits and that the three SH groups previously identified are located in only one or the other of the subunits as shown. It is particularly significant that the SH group which is labeled by reaction with [^{14}C]NEM in the presence of ATP–$MgCl_2$ and bicarbonate is present in the small subunit whereas the SH group which is labeled by reaction with excess [^{14}C]NEM (in the absence of substrates or allosteric effectors, or in the presence of UMP) is located in the large subunit, since, as previously reported (Foley *et al.*, 1971), these reactions each result in marked alteration(s) of different catalytic activities of the enzyme and therefore implicate a functional role for each subunit.

These results confirm previous reports that carbamyl phosphate synthetase from *E. coli* is composed of two nonidentical subunits (Anderson *et al.*, 1970, and Trotta *et al.*, 1971b). They are also in agreement with the recently reported studies by Trotta *et al.*, 1971a, who have provided further convincing evidence for the presence of two nonidentical subunits by successfully separating the two subunits under conditions where the catalytic activities of the two subunits are not destroyed. The larger subunit was shown to retain ammonia-dependent (but not glutamine-dependent) carbamyl phosphate synthesis activity as well as the activities of two other partial reactions catalyzed by the native enzyme, *i.e.*, the bicarbonate-dependent hydrolysis of ATP and the synthesis of ATP from ADP and carbamyl phosphate; the smaller subunit was shown to possess only glutaminase activity. Glutamine-dependent carbamyl phosphate synthesis activity was recovered when the two separated subunits were mixed. Thus, a functional role for both subunits, suggested in this paper indirectly by the observation that two unique SH groups which can be reacted with NEM or DTNB resulting in different effects on the catalytic activities of the enzyme are located on different subunits, is directly confirmed.

Acknowledgments

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Multiple Forms of Colicin E_3 from *Escherichia coli* CA-38 (col E_3, col I)†

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ABSTRACT: Multiple forms of colicin E_3 have been isolated from the extracellular fluid of an induced culture of *Escherichia coli* CA-38 (col E_3, col I). After precipitation with ammonium sulfate the protein was collected by centrifugation and passed through a Sephadex G-50 column. Chromatography on a DEAE-cellulose column effected the separation of three forms of colicin E_3, designated E_3-I, E_3-II, and E_3-III. The specific activity of E_3-I was 2–5 $\times$ 10^6 units/mg while that of E_3-II was 1–3 $\times$ 10^6 units/mg and that of E_3-III was 6 $\times$ 10^5 units/mg. These three forms of colicin E_3 have identical molecular weights (60,000) as determined by equilibrium ultracentrifugation and by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The amino acid compositions of E_3-I and E_3-II are very similar (E_3-III was not examined because of contaminating protein material). All three forms have glycine as the N-terminal amino acid. Rechromatography of E_3-I or E_3-II on DEAE-cellulose revealed that these forms are not stable; E_3-I can convert to E_3-II and E_3-III, while E_3-II can convert only to E_3-III. These conversions are irreversible. Studies using isoelectric focusing gradients have indicated that the three forms are converted to progressively more acidic molecules by steps of approximately one charge unit with a concomitant loss of biological activity. Furthermore, these studies have demonstrated that the conversion leads to at least two additional forms, E_3-IV and E_3-V.

Colicins are highly specific antibiotic proteins produced by certain strains of intestinal bacteria which kill other, usually closely related, strains of bacteria. The potential to produce colicin (colicinogeny) is conferred by the presence of extrachromosomal DNA called a col factor (DeWitt and Helinski, 1965; Clewell and Helinski, 1969). Colicin production can be induced by treatment of colicinogenic cells with ultraviolet radiation or mitomycin C. The colicin produced by induced cells is released into the medium where it kills sensitive bacteria by adsorbing to specific receptor sites on the cell surface rather than by penetrating and acting from within the cell (Fredericq, 1948; Maeda and Nomura, 1966). A colicinogenic cell is immune to the specific colicin it is capable of producing.

Colicins have been classified into groups according to their specificity for receptor sites (Fredericq, 1948); each group of colicins binds to a unique receptor. The three members of the E group of colicins, E_1, E_2, and E_3, all bind to a common receptor site. They were originally differentiated by the fact that a cell colicinogenic for one of the three was immune to its own colicin but sensitive to the other two. It was also shown that these three colicins have different biochemical targets; E_1 causes disruption of all macromolecular synthesis, presumably by interfering with oxidative phosphorylation (Luria, 1964), E_2 causes degradation of DNA (Jacob *et al.*, 1952), while E_3 causes cessation of protein synthesis (Nomura, 1963). Recently, the chemical lesion caused by colicin E_3 has been shown to be the specific cleavage of the 16S rRNA, resulting in inactive ribosomes (Senior and Holland, 1971; Bowman *et al.*, 1971).

Colicin E_3 has been purified and characterized by Herschman and Helinski (1967), who showed this colicin to be a

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