Carbamyl Phosphate Synthetase. A Biotin Enzyme*

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ABSTRACT: Purified carbamyl phosphate synthetase of *Escherichia coli* is inhibited by avidin, and such inhibition does not occur when the avidin is preincubated with biotin.

Precipitation of the crude enzyme by addition of ammonium sulfate is associated with loss of enzymatic activity. Although the supernatant solution thus obtained is inactive, when the precipitated enzyme is resuspended in the supernatant solution, there is a considerable increase in activity. Heated supernatant solution also activated the enzyme. Dialyzed supernatant had no effect, but a concentrate of the dialysate activated the enzyme, as did biotin. Determination of the biotin content of the purified enzyme (after hydrolysis by Pronase or hydrochloric acid) by the 4′-hydroxyazobenzene-2-carboxylic acid method and by assay with *Saccharomyces cerivisiae* indicated the presence of about 6 mμmoles of biotin/mg of enzyme.

Previous studies have indicated that the initial step in the reaction catalyzed by *Escherichia coli* carbamyl phosphate synthetase is activation of carbon dioxide associated with cleavage of ATP[1] to ADP (Anderson and Meister, 1965). Since it is known that a number of enzymes that activate carbon dioxide are biotin-containing enzymes, it became of interest to determine whether carbamyl phosphate synthetase is a biotin enzyme. In an attempt to answer this question the effect of avidin on the purified enzyme was determined. It was found that avidin inhibits activity and that such inhibition is prevented by prior incubation of avidin with biotin. In the course of its purification the enzyme was precipitated from a relatively crude extract by addition of ammonium sulfate; the precipitate exhibited an appreciable loss of enzymatic activity, while the supernatant solution was inactive. When the crude precipitated enzyme was resuspended in the supernatant solution a considerable increase of activity was observed. It was subsequently found that biotin could substitute for the supernatant solution. Analysis of the purified enzyme for biotin indicated its presence. The data presented here therefore provide strong evidence that carbamyl phosphate synthetase is a biotin enzyme.

Experimental Section

Materials. 4′-Hydroxyazobenzene-2-carboxylic acid was obtained from Dajac Laboratories of the Borden Chemical Co., Philadelphia, Pa. Pronase was obtained from Calbiochem and *d*-biotin was purchased from Sigma Chemical Co. Avidin was obtained from Nutritional Biochemicals Corp. and Worthington Biochemical Corp. A preparation of avidin (Cornell, lot 24), kindly made available by Dr. Vincent du Vigneaud, was also used.[2]

Highly purified carbamyl phosphate synthetase and the other compounds used were obtained as previously reported (Anderson and Meister, 1965). In this paper, the most highly purified enzyme (Anderson and Meister, 1965) was used in all studies, except for expt 1–3 described in Table I; in these studies the ammonium sulfate fraction obtained in the course of purification of the enzyme (step 4 of the procedure of Anderson and Meister, 1965) was used.

Methods. Carbamyl phosphate synthetase activity was determined in reaction mixtures containing [^{14}C]HCO_3, L-glutamine, KCl, $MgCl_2$, ATP, and Tris-HCl buffer (pH 7.9); the carbamyl phosphate formed was determined after conversion into urea, and the ADP formed was measured by coupling with pyruvate kinase and lactate dehydrogenase as previously described (Anderson and Meister, 1965). Enzyme protein concentration was determined from its absorbancy at 260 and 280 mμ; the calculations were based on the values of absorbancy and dry weight for the most highly purified enzyme.

Biotin was determined by the 4′-hydroxyazobenzene-2-carboxylic acid method of Green (1965). We are indebted to Dr. Harland G. Wood for additional details concerning the application of this procedure to the determination of biotin in enzyme digests. Biotin was also determined by bioassay using *Saccharomyces cerivisiae* (ATCC 9896) as the test organism (Snell *et al.*, 1940).

Results

Inhibition of Purified Carbamyl Phosphate Synthetase by Avidin. As indicated in Figure 1, when the purified enzyme was incubated with avidin there was a progressive decrease in activity such that more than 80% of the

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[1] Abbreviations are listed in *Biochemistry 5*, 1445 (1966).

[2] A total of six avidin preparations was examined; all but one (from the first-mentioned source) inhibited the enzyme.

TABLE I: Activation of the Enzyme by Supernatant Solution and by Biotin.

Expt	Fraction and Additions	Carbamyl Phosphate (or ADP/2) Formed[a] (mμmoles)
1	$(NH_4)_2SO_4$ precipitate (A)	49
	$(NH_4)_2SO_4$ supernatant (B)	0
	A + B	82
	A + B (dialyzed)	49
	A + biotin	69
2	A	50
	B	0
	A + B	77
	A + biotin	69
3	A	43
	B	0
	A + B	66
	A + B (heated at 100°; 10 min)	66
	A + B (dialyzed)	41
	A + dialysate[b]	65
4	Purified enzyme	126
	Purified enzyme + B	122
	Purified enzyme + biotin	126

[a] Carbamyl phosphate synthesis was determined in a reaction mixture containing Tris-HCl buffer (pH 7.9, 50 μmoles), KCl (100 μmoles), ATP (20 μmoles), $MgCl_2$ (20 μmoles), [^{14}C]$NaHCO_3$ (20 μmoles, 740,000 cpm), L-glutamine (20 μmoles), 0.34 mg of $(NH_4)_2SO_4$-precipitated crude (step 4) enzyme (dialyzed for 2 hr against 0.15 M potassium phosphate buffer (pH 6.8)) and 0.05 ml of $(NH_4)_2SO_4$ supernatant solution (containing approximately 0.1 mg of protein) in a final volume of 1.0 ml. Biotin (10 μg) was added as indicated in certain experiments. The reaction mixtures were incubated at 37° for 15 min at which time they were deproteinized and analyzed for carbamyl phosphate as described in the text. In expt 4, highly purified enzyme (0.02 mg) was used and the reaction was followed by determinations of ADP. [b] The dialysate obtained in the experiment given on the line above was concentrated under reduced pressure and a proportionate amount was used in this experiment.

initial activity was lost within 40 min. In control experiments, in which avidin was omitted (Figure 1, curve 1) or in which avidin was incubated with biotin for 10 min (Figure 1, curve 2) prior to addition of enzyme, there was no appreciable loss of enzymatic activity.

Activation of the Enzyme by Biotin. When carbamyl phosphate synthetase is purified from *E. coli* according to the procedure of Anderson and Meister (1965), the first appreciable loss of activity occurs after the enzyme

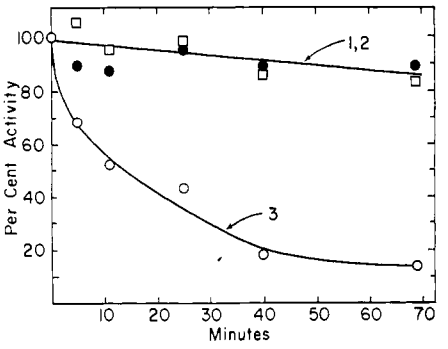

FIGURE 1: Inhibition of purified carbamyl phosphate synthetase by avidin. The reaction mixtures contained initially: curve 1 (closed circles), 0.45 ml of potassium phosphate buffer (pH 7.8, 20 μmoles); curve 2 (squares), 0.45 ml containing potassium phosphate buffer (pH 7.8, 20 μmoles), avidin (0.5 mg), and *d*-biotin (2 mg); curve 3 (open circles), 0.45 ml containing potassium phosphate buffer (pH 7.8, 20 μmoles) and avidin (0.5 mg). After incubation at 37° for 10 min, 0.25 mg of purified enzyme (0.05 ml) was added to each vessel, and incubation was continued at 37° for the indicated time intervals. Enzyme assays were then carried out on 0.02-ml samples; the uninhibited value (100%) was equivalent to 0.125 μmole of carbamyl phosphate and 0.25 μmole of ADP.

is precipitated from a relatively crude preparation by addition of ammonium sulfate (step 4). The observation that this loss of activity was substantial in a number of preparations led to experiments in which the activity of the precipitated protein was tested in the presence and absence of the supernatant solution (Table I). Although the supernatant solution (B) exhibited no enzymatic activity, it produced considerable activation when added to a solution of the precipitate containing the enzyme (A). As indicated in Table I, heating the supernatant at 100° for 10 min did not destroy its ability to activate. However, after the supernatant solution was dialyzed it failed to activate; on the other hand, the concentrated dialysate activated the enzyme. When biotin was substituted for the supernatant solution, appreciable activation was observed. Such activation was observed only with unpurified preparations of the enzyme; thus, incubation of highly purified preparations of the enzyme with biotin did not lead to an increase in activity.

Analysis of the Purified Enzyme for Biotin. As indicated in Table II the determination of the biotin content of the enzyme by the 4′-hydroxyazobenzene-2-carboxylic acid method indicated the presence of 6–7 mμmoles/mg of enzyme. In these studies, the most highly purified enzyme was digested with Pronase for periods ranging from 6 to 72 hr. Under these conditions, the maximal amount of biotin was found after 48-hr Pronase digestion. This preparation of the enzyme was also analyzed after it was treated with 6 N HCl at 110° for 1 hr; the result was similar to those found after 48- and 72-hr Pronase digestion.

Bioassay using *S. cerivisiae* after treatment of the purified enzyme with 6 N HCl at 110° for 1 hr gave a value of 6 mμmoles of biotin/mg of enzyme.

TABLE II: Biotin Content of the Purified Enzyme Determined by the 4′-Hydroxyazobenzene-2-carboxylic Acid Method.

Time of Pronase Digestion[a] (hr)	Biotin/mg of Enzyme (mμmoles)
0	0
6	0.25
12	3.3
24	3.6
48	6.6
72	6.6
1 (6 N HCl, 110°)[b]	6.0

[a] The purified enzyme (1.5 mg) was incubated with Pronase (2 mg) in 0.1 ml of 0.15 M potassium phosphate buffer (pH 6.8) containing 5×10^{-4} M EDTA at 37° for the indicated time intervals. The mixtures were then placed in an autoclave at 121° for 15 min; after cooling the precipitated protein was removed by centrifugation, and the biotin content of the supernatant solutions was determined as described in the text. [b] The purified enzyme (1.5 mg) in 0.1 ml of 0.15 M potassium phosphate buffer (pH 6.8) containing 5×10^{-4} M EDTA was mixed with an equal volume of 12 N HCl and placed at 110° for 1 hr. After cooling, the solution was evaporated to dryness under reduced pressure and the residue was dissolved in 0.1 ml of water and analyzed for biotin as described in the text.

Discussion

The several experimental approaches described here indicate that *E. coli* carbamyl phosphate synthetase is a biotin enzyme. Thus, we have observed characteristic inhibition of the purified enzyme by avidin (and its prevention by biotin). The analytical data on the purified enzyme and the observation that biotin reactivates the crude (step 4) enzyme in the presence of ATP and Mg^{2+} provide strong confirmatory evidence. Presumably the fraction precipitated by ammonium sulfate contains biotin-activating enzyme which converts biotin into biotinyl adenylate, which reacts with a specific ε-amino group of the enzyme to yield the holoenzyme (Lane *et al.*, 1964a, b; Christner *et al.*, 1964). The activation of carbon dioxide involved in the synthesis of carbamyl phosphate thus seems to be analogous to that which occurs in the activation of carbon dioxide catalyzed by such enzymes as acetyl coenzyme A carboxylase (Wakil *et al.*, 1958; Wakil and Gibson, 1960), β-methylcrotonyl coenzyme A carboxylase (Knappe *et al.*, 1961), propionyl coenzyme A carboxylase (Kaziro and Ochoa, 1962), and oxaloacetic transcarboxylase (Swick and Wood, 1960; Wood *et al.*, 1963) (reaction 1). It is possible as suggested previously (Anderson and Meister, 1965, 1966) that the initial step in this reaction involves formation of an enzyme-bound carbonic acid–phosphoric acid anhydride, which reacts with enzyme-bound biotin to yield the enzyme-bound biotin–carbon dioxide complex. It is of interest in this connection that ^{18}O is transferred from carbon dioxide to inorganic phosphate in the course of the reaction catalyzed by carbamyl phosphate synthetase of liver (Jones and Spector, 1960); similar experiments with the enzyme from *E. coli* have not yet been carried out. Identification of biotin as a prosthetic group of *E. coli* carbamyl phosphate synthetase has significant implications in relation to further studies on the mechanism of action of this enzyme.

$$\text{enzyme–biotin} + \text{ATP} + HCO_3^- \xrightarrow{Mg^{2+}} \text{enzyme–biotin–}CO_2 + \text{ADP} + P_i \quad (1)$$

It would be of interest to learn whether the carbamyl phosphate synthetases of animal tissues are also biotin enzymes. Indeed, a number of observations that have been made in connection with biotin deficiency in animals are entirely consistent with this possibility. For example, MacLeod and Lardy (1949) observed that biotin deficiency in the rat was accompanied by substantially decreased incorporation of [^{14}C]bicarbonate into arginine. Feldott and Lardy (1951) found that the synthesis of citrulline from ornithine, with L-glutamate as a specific adjuvant, was greatly depressed in washed residue of liver homogenate from biotin-deficient rats as compared with similar preparations from pair-fed control animals. They also noted that when glutamate was replaced by carbamyl L-glutamate the biotin-deficient and control preparations catalyzed equal rates of citrulline synthesis. The present observations on *E. coli* carbamyl phosphate synthetase, when considered in the light of earlier work, seem to offer ample justification for investigation of the biotin content of both the ammonia-utilizing and the glutamine-dependent carbamyl phosphate synthetases of animal tissues.

References

Anderson, P. M., and Meister, A. (1965), *Biochemistry 4*, 2803.

Anderson, P. M., and Meister, A. (1966), *Biochemistry 5*, 3157.

Christner, J. E., Schlesinger, M. J., and Coon, M. J. (1964), *J. Biol. Chem. 239*, 3997.

Feldott, G., and Lardy, H. A. (1951), *J. Biol. Chem. 192*, 447.

Green, N. M. (1965), *Biochem. J. 94*, 23C.

Jones, M. E., and Spector, L. (1960), *J. Biol. Chem. 235*, 2897.

Kaziro, Y., and Ochoa, S. (1962), *J. Biol. Chem. 236*, 3131.

Knappe, J., Ringelmann, E., and Lynen, F. (1961), *Biochem. Z. 335*, 168.

Lane, M. D., Rominger, K. L., Young, D. L., and Lynen, F. (1964a), *J. Biol. Chem. 239*, 2865.

Lane, M. D., Young, D. L., and Lynen, F. (1964b), *J. Biol. Chem. 239*, 2858.

MacLeod, P. R., and Lardy, H. A. (1949), *J. Biol. Chem. 179*, 733.

Snell, E. E., Eakin, R. E., and Williams, R. J. (1940), *J. Am. Chem. Soc. 62*, 175.

Swick, R. W., and Wood, H. G. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 28.
Wakil, S. J., and Gibson, D. M. (1960), *Biochim. Biophys. Acta 41*, 122.
Wakil, S. J., Titchener, E. B., and Gibson, D. M. (1958), *Biochim. Biophys. Acta 29*, 225.
Wood, H. G., Allen, S. H. G., Stjernholm, R., and Jacobson, B. E. (1963), *J. Biol. Chem. 238*, 547.

Isolation of the Monomeric Subunit of Immunoglobulin M with Its Interchain Disulfide Bonds Intact*

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ABSTRACT: Immunoglobulin M (IgM) consists of five monomeric subunits, IgM_s, each apparently comprising two μ chains and light chains. The distribution of disulfide bonds between μ chains within IgM_s and those joining the μ chains of one IgM_s to another is not known with certainty. Our goal was to obtain IgM_s with its original inter-μ-chain and μ-light-chain disulfide bonds intact, and to determine whether one or two disulfide bonds join each IgM_s to another subunit. The amount of IgM_s released from IgM by reduction could be controlled by adjusting the concentration of mercaptoethylamine. In our hands, reduction of IgM with 0.015 M mercaptoethylamine resulted in a mixture containing about 50% IgM_s. The subunit, isolated by filtration of the mixture through Bio-Gel P-200, had a sedimentation coefficient of 6.17 S at a concentration of 6.21 mg/ml, electrophoretic mobility similar to its parent IgM, maintained the specific antigenic sites on the μ chain, and comprised both μ and light chains. Regardless of the time of reduction from 5 to 40 min with 0.015 M mercaptoethylamine, IgM_s contained about two carboxymethylcysteine residues per molecule. In addition, IgM_s isolated after 30-min reduction failed to dissociate in propionic acid into μ and light chain until it subsequently was reduced with 2-mercaptoethanol. In conclusion, the data indicated the IgM_s has maintained its original interchain disulfide bond integrity, and each IgM_s is bound to another by a single disulfide bond.

Considerable progress has been made in the last several years toward elucidation of the structures of the immunoglobulins. IgG,[1] which normally occurs in the largest quantity in serum, comprises two pairs of polypeptide chains. Two γ chains in rabbit IgG are joined covalently by one highly labile disulfide bond (Palmer and Nisonoff, 1964; Hong and Nisonoff, 1965) and by noncovalent forces in the Fc region (Marler *et al.*, 1964; Inman and Nisonoff, 1966). A single light chain is attached by one disulfide bond and noncovalent forces (Fleischman *et al.*, 1963) to each γ chain.

IgM occurs in small quantities in normal serum. It appears to be composed of five monomeric subunits held to one another by disulfide bonds (Miller and Metzger, 1965a,b; Lamm and Small, 1966). Miller and Metzger, (1965a,b) have proposed that the structural architecture of IgM subunits (IgM_s) resembles that of intact IgG, *i.e.*, IgM_s also comprises two pairs of polypeptide chains. The light chains apparently are very similar or identical with those of IgG (Miller and Metzger, 1965a; Lamm and Small, 1966), but the other pair of chains, μ, differs from the γ chains in several respects such as a higher molecular weight (Lamm and Small, 1966), and different amino acid composition (Chaplin *et al.*, 1965), antigenic analysis (Cohen, 1963; Miller and Metzger, 1965a), and carbohydrate content (Chaplin *et al.*, 1965; Lamm and Small, 1966). In addition, each μ chain appears to be involved with four interchain disulfide bonds (Miller and Metzger, 1965b). One of these bonds covalently links a μ and a light chain (Chaplin *et al.*, 1965; Miller and Metzger, 1965b). The remaining three bonds are intrasubunit inter-μ-chain and intersubunit inter-μ-chain. Since it was not possible under the conditions used to reduce selectively these bonds, it is not known whether there are one or two disulfide bonds between subunits.

Investigators have used several different chemical

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: Tris–NaCl, 0.05 M Tris–0.5 M NaCl (pH 8.2); 0.32 SB, 0.32 M NaCl buffered with 10^{-3} M sodium borate (pH 8); MEA, mercaptoethylamine; 2-ME, 2-mercaptoethanol, redistilled. The nomenclature used is that recommended by the World Health Organization Committee on Nomenclature for Human Immunoglobulins (*Bull. World Health Organ. 30*, 447 (1964)).