Determination of the Number of Regulatory and Catalytic Sites on Aspartate Transcarbamylase*

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ABSTRACT: Difference spectroscopy and the method of continuous variation have been used to study the stoichiometry of the binding of 5-bromocytidine triphosphate (BrCTP) and of carbamyl phosphate to aspartate transcarbamylase. The regulatory subunit binds 1 equiv of BrCTP to a protein equivalent molecular weight of 17,000, and native aspartate transcarbamylase binds 6 equiv to a protein equivalent molecular weight of 310,000. The catalytic subunit binds three equivalents of the substrate, carbamyl phosphate, per catalytic subunit of mol wt 100,000.

Since native aspartate transcarbamylase has two catalytic subunits and a molecular weight of 310,000, these data suggest that the enzyme has six regulatory and six catalytic sites.

Earlier studies have suggested that the aspartate transcarbamylase of *Escherichia coli* can be dissociated into two types of subunits: four regulatory subunits, each of mol wt 27,000, and two catalytic subunits, each of mol wt 100,000 (Gerhart and Schachman, 1965; Changeux *et al.*, 1968). The catalytic subunits can be dissociated further to single polypeptide chains of mol wt 50,000 each (Weber, 1968a). These results have been supported by binding studies which indicate that native aspartate transcarbamylase binds 4 equiv of the allosteric inhibitor, cytidine triphosphate, or its analog, BrCTP,[1] and 4 equiv of succinate, an unreactive analog of the substrate aspartate. The regulatory subunit was reported to bind 1 equiv of CTP/subunit of mol wt 27,000, and the catalytic subunit to bind 2 equiv of succinate (Changeux *et al.*, 1968).

However, more recent studies have suggested that the molecular weight of the regulatory subunit is 17,000 and that of the catalytic subunit is 33,000 per polypeptide chain, indicating a structure of six regulatory and six catalytic chains per native enzyme molecule of mol wt 310,000 (Weber, 1968b). This is consistent with the observation that a native aspartate transcarbamylase crystal has been found with a threefold axis of symmetry, and another has been found with a twofold axis of symmetry (Wiley and Lipscomb, 1968).

We have used the method of continuous variation (Job, 1928) to redetermine the binding stoichiometry of BrCTP to native aspartate transcarbamylase and to the regulatory subunit, and to determine the binding stoichiometry of the substrate, carbamyl phosphate, to the catalytic subunit.

Experimental Section

Enzyme. Aspartate transcarbamylase and its dissociated subunits were prepared according to the method of Gerhart and Holoubek (1967). Protein concentrations were determined spectrophotometrically at 280 nm, using a specific absorbance of 0.59 cm^2 mg^{-1} for native aspartate transcarbamylase (Gerhart and Holoubek, 1967) and of 0.70 cm^2 mg^{-1} for the catalytic subunit (Collins and Stark, 1969). Protein concentration for the regulatory subunit was first determined by the Lowry procedure (Lowry *et al.*, 1951), using native aspartate transcarbamylase as the protein standard, and then confirmed by amino acid analysis. We are grateful to Mr. H. T. Tjan for performing the amino acid analyses.

Regulatory subunit was used immediately after preparation and dialysis *vs.* 0.04 M imidazole-HCl buffer (pH 7.0) containing 10^{-3} M dithiothreitol and 5×10^{-4} M EDTA. Catalytic subunit was used immediately after preparation and dialysis versus a buffer solution consisting of 0.2 M Tris, 0.1 M succinate, 5×10^{-4} M EDTA, and 2×10^{-3} M dithiothreitol (pH 7.5).

BrCTP. BrCTP was synthesized by a procedure modified from that of Bessman *et al.* (1958), as described by Eckfeldt *et al.* (1970). The concentration of BrCTP was determined spectrophotometrically, using an extinction coefficient of 9200 cm^{-1} M^{-1} at 299 nm in 0.1 N HCl (Bessman *et al.*, 1958).

Carbamyl Phosphate. Carbamyl phosphate, dilithium salt (Sigma Chemical Corp.), was purified by precipitation from aqueous solution by addition of cold ethanol (Gerhart and Pardee, 1962), washed with absolute ethanol, then dried thoroughly, and used immediately. Organic phosphate concentration was measured by determination of orthophosphate concentration before and after acid hydrolysis (Lowry and Lopez, 1946). Freshly prepared carbamyl phosphate contained four per cent of the total phosphate as inorganic phosphate and no bound water. Solutions of carbamyl phosphate were stored in ice and were never kept for more than 2 hr. Under these conditions, the decomposition is insignificant.

Difference Spectrophotometry. Difference absorbances were determined using a Zeiss PMQ II spectrophotometer. For different absorbance measurements with BrCTP, rectangular tandem cells with a path length of 0.44 cm/cell compartment (Pyrocell Manfacturing Co.) were employed. For difference absorbance measurements of the catalytic subunit with carbamyl phosphate, standard 1-cm path-length cells were em-

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[1] Abbreviation used is: BrCTP, 5-bromocytidine triphosphate.

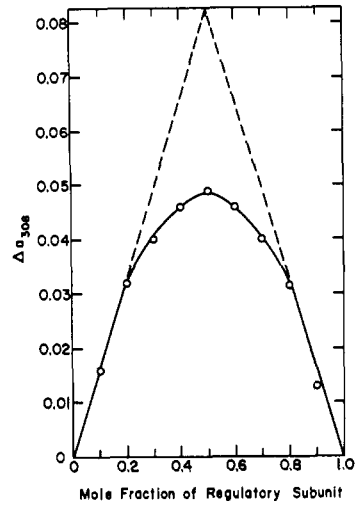

FIGURE 1: Binding of BrCTP to regulatory subunit. Difference absorbance at 308 nm *vs.* mole fraction of regulatory subunit. The constant sum of concentrations was 4×10^{-4} M, assuming an equivalent protein molecular weight of 17,000 per site. The buffer was 0.04 M imidazole-HCl (pH 7.0) with 10^{-3} M dithiothreitol and 5×10^{-4} M EDTA.

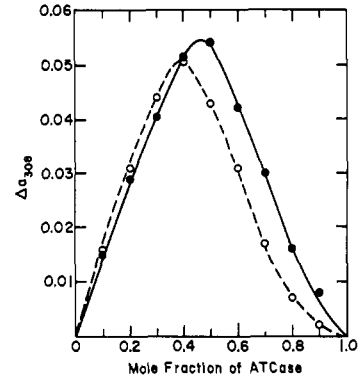

FIGURE 2: Binding of BrCTP to native ATCase, with and without carbamyl phosphate. Difference absorbance at 308 nm *vs.* mole fraction of ATCase: solid line and filled circles (●), with carbamyl phosphate (2×10^{-3} M); broken line and open circles (○), without carbamyl phosphate. The constant sum of concentrations was 4×10^{-4} M, assuming an equivalent protein molecular weight of 52,000 per site. The buffer was 0.04 M imidazole-HCl (pH 7.0) with 10^{-3} M dithiothreitol and 5×10^{-4} M EDTA.

ployed. The slit width was maintained at a constant value for all measurements at the same wavelength. All experiments were conducted at room temperature (~25°).

Method of Continuous Variation. Binding stoichiometries were determined by the method of continuous variation (Job, 1928; Asmus, 1961). The total concentration of protein and ligand was held constant, and their relative concentrations were varied. The difference absorbance was used as a measure of complex formation. The position of the maximum difference absorbance indicates the mole ratio of the complex. The extrapolated linear portions of the curve intersect at this same ratio.

Results

BrCTP Regulatory Subunit. The observed BrCTP regulatory subunit difference spectrum has a peak at 308 nm (Eckfeldt *et al.*, 1970). Figure 1 shows the difference absorbance at 308 nm as a function of the mole fraction of regulatory subunit. The molar concentration of regulatory subunit was calculated from the protein concentration, assuming a molecular weight of 17,000 (Weber, 1968b). A maximum difference absorbance was achieved at a mole fraction of 0.5, which corresponds to a 1:1 mole ratio of regulatory subunit to BrCTP. The intersection of the extrapolated linear portions of the experimental curve occurs at the same 1:1 ratio, and the plot is completely symmetrical. This indicates one BrCTP binding site per regulatory subunit of mol wt 17,000. If the equivalent molecular weight of the regulatory subunit were 27,000 instead of 17,000, then the intersection and maximum would occur at a mole fraction of 0.61.

BrCTP Native Aspartate Transcarbamylase. Figure 2 shows the results obtained with BrCTP and native ATCase. The molar concentration of ATCase was calculated from the protein concentration, assuming a molecular weight of 310,000 and six BrCTP sites per molecule, *i.e.*, an equivalent protein molecular weight of 52,000. In the presence of carbamyl phosphate (solid line in Figure 2), a maximum difference absorbance occurs at a 1:1 mole ratio, although the plot is slightly skewed, suggesting slightly more than six binding sites per molecule. If only four BrCTP sites were present, the maximum would occur at a mole fraction of 0.6.

In the absence of carbamyl phosphate (dashed line in Figure 2), the position of the maximum occurs at a mole fraction less than 0.5 and suggests 10–12 sites/molecule. Porter *et al.* (1969) have shown that CTP binds to the carbamyl phosphate site on the catalytic subunit, although less tightly than to the regulatory site of native ATCase. Therefore the large number of sites observed in the absence of carbamyl phosphate can be attributed to BrCTP binding at both the regulatory and catalytic sites. In the presence of carbamyl phosphate, binding is essentially restricted to the regulatory sites, and the data suggest six sites per molecule of native enzyme.

Carbamyl Phosphate Catalytic Subunit. Collins and Stark (1969) have shown that carbamyl phosphate induces tight binding of succinate and the production of a large difference spectrum. Carbamyl phosphate alone produces a smaller difference spectrum, and succinate alone binds weakly and produces a small difference spectrum at high concentrations. We have observed that in the presence of 0.1 M succinate, the addition of carbamyl phosphate induces tighter binding of succinate and a large difference spectrum. When measured with a slit width of 0.2 mm, this difference spectrum has a maximum at 289.5 nm. For 2×10^{-3} M carbamyl phosphate, this difference peak corresponds in magnitude to 3.6% of the total protein absorbance at the same wavelength.

Extremely small concentration differences in the two spectrophotometric cells can produce small but significant errors in the difference spectrum when the total absorbance is large. A correction term can be derived, based on the fact that the extinction coefficients for both forms of the enzyme, the enzyme–succinate complex and the enzyme–carbamyl phosphate–succinate complex, are the same at a crossover point (*e.g.*, 285.5 nm at a slit width of 0.5 mm) of the difference spectrum. The correction term is simply the observed difference absorbance at a crossover point, multiplied by the ratio

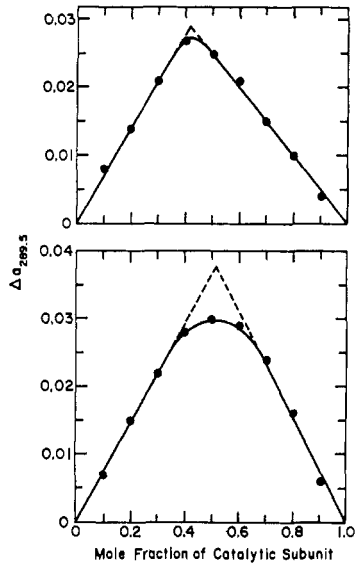

FIGURE 3: Binding of carbamyl phosphate to catalytic subunit. Corrected difference absorbance at 289.5 nm *vs.* mole fraction of catalytic subunit. Upper: constant sum of concentrations is 0.90×10^{-4} M, assuming an equivalent protein molecular weight of 50,000, *i.e.*, two carbamyl phosphate binding sites per subunit of mol wt 100,000. Lower: constant sum of concentrations is 1.35×10^{-4} M, assuming an equivalent protein molecular weight of 33,000, *i.e.*, three carbamyl phosphate binding sites per subunit of mol wt 100,000. The buffer was 0.2 M Tris (pH 7.5) with 0.1 M succinate, 2×10^{-3} M dithiothreitol, and 5×10^{-4} M EDTA.

of the extinction coefficients at the difference peak and the crossover point. The corrected difference absorbance is the observed difference absorbance at the peak, minus the correction term, which can be positive or negative. In all cases, the correction term was much smaller than the measured difference absorbance at the peak and was often less than 0.001. The largest correction term was 0.003, and the average magnitude of the correction terms was 0.001.

Figure 3 shows a plot of the corrected difference absorbance *vs.* the mole fraction of catalytic subunit. In the lower plot, the molar concentration of catalytic subunit was calculated from the protein concentration, assuming an equivalent molecular weight of 33,000, or three sites per catalytic subunit. The data show a maximum difference absorbance at 0.5 mole fraction and the intersection of the extrapolated linear portions at a mole fraction of approximately 0.52, which indicates 2.8 carbamyl phosphate binding sites per catalytic subunit of mol wt 100,000. This is within experimental error of three sites per subunit. If only two sites were available per subunit, then the maximum would occur at a mole fraction of 0.6.

The upper plot in Figure 3 shows the results of a similar experiment in which two carbamyl phosphate binding sites were assumed, instead of three, for an equivalent protein molecular weight of 50,000. In this case, the maximum should occur at a mole fraction of 0.5 for two sites and at 0.4 for three sites. The intersection of the extrapolated linear portions occurs at a mole fraction of 0.42, again indicating 2.8 carbamyl phosphate binding sites per catalytic subunit of molecular weight 100,000, or three sites per 107,000, for an equivalent molecular weight of 36,000 per carbamyl phosphate binding site.

Unfortunately, the binding of succinate to enzyme is too weak to permit direct determination of the binding stoichiometry of succinate to enzyme by the method of continuous variation.

Conclusion

In summary, these results support a structural model for ATCase with six regulatory sites and six catalytic sites per molecule of native enzyme.

References

Asmus, E. (1961), *Z. Anal. Chem. 183*, 321.

Bessman, M. J., Lehman, I. R., Adler, J., Zimmerman, S. B., Simms, E. S., and Kornberg, A. (1958), *Proc. Nat. Acad. Sci. U. S. 44*, 633.

Changeux, J.-P., Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 531.

Collins, K. D., and Stark, G. R. (1969), *J. Biol. Chem. 244*, 1869.

Eckfeldt, J. H., Hammes, G. G., Mohr, S. C., and Wu, C. W. (1970), *Biochemistry 9* (in press).

Gerhart, J. C., and Holoubek, H. (1967), *J. Biol. Chem. 242*, 2886.

Gerhart, J. C., and Pardee, A. B. (1962), *J. Biol. Chem. 237*, 891.

Gerhart, J. C., and Schachman, H. K. (1965), *Biochemistry 4*, 1054.

Job, P. (1928), *Ann. Chim. (Paris) 9*, 113.

Lowry, O. H., and Lopez, J. A. (1946), *J. Biol. Chem. 162*, 421.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Porter, R. W., Modebe, M. O., and Stark, G. R. (1969), *J. Biol. Chem. 244*, 1846.

Weber, K. (1968a), *J. Biol. Chem. 243*, 543.

Weber, K. (1968b), *Nature (London) 218*, 1116.

Wiley, D. C., and Lipscomb, W. N. (1968), *Nature (London) 218*, 1119.