chem. J. 89, 552.
Straub, F. B. (1943), *Studies Inst. Med. Chem. Univ. Szeged* (now *Studies on Muscle Inst. Med. Chem. Univ. Szeged*) *3*, 23.
Tsao, T. C., and Bailey, K. (1953), *Biochim. Biophys. Acta 11*, 102.
Ulbrecht, M., Grubhofer, N., Jaisle, F., and Walter, S. (1960), *Biochim. Biophys. Acta 45*, 443.

Distinct Subunits for the Regulation and Catalytic Activity of Aspartate Transcarbamylase*

John C. Gerhart and Howard K. Schachman

ABSTRACT: Kinetic investigations of regulatory enzymes have led to the current view that such enzymes bind their regulatory metabolites at sites distinct from the active sites, and that inhibition (or activation) requires the cooperative interaction of subunits within the enzyme molecules. This communication presents direct physicochemical studies concerning one such enzyme, aspartate transcarbamylase (ATCase), in terms of its subunit structure and binding of the specific inhibitory metabolite, cytidine triphosphate (CTP). As judged from sedimentation and viscosity studies, ATCase from *Escherichia coli* is a compact, globular protein with a sedimentation coefficient ($s_{20,w}$) of 11.7 S, an intrinsic viscosity of 0.045 dl/g, and a molecular weight of 3.1×10^5. Ultracentrifugal analysis of the binding of the CTP analog, 5-bromocytidine triphosphate (BrCTP), reveals the existence of eight receptor sites on the enzyme. Upon the addition of the mercurial, *p*-mercuribenzoate, native ATCase dissociates into two types of subunits which are easily separable by zone centrifugation or column chromatography. The larger, with $s_{20,w} = 5.8$ S and molecular weight of 9.6×10^4, possesses the entire catalytic activity of the native enzyme and is completely insensitive to the inhibitor, CTP. This protein is termed the *catalytic subunit*. The second protein, with $s_{20,w} = 2.8$ S and molecular weight of 3×10^4, is enzymically inactive and is unnecessary for the activity of the catalytic subunit. This smaller protein, termed the *regulatory subunit*, bears the receptor sites for the inhibitor, BrCTP, and is required for the control of enzymic activity. Since the regulatory subunits comprise 37% of the total weight of the native enzyme, it can be calculated that there are four such subunits in each ATCase molecule. Similarly it can be concluded that the native enzyme contains two catalytic subunits.

The native enzyme can be reconstituted from the unfractionated subunits upon removal of the *p*-mercuribenzoate through the addition of mercaptoethanol. Also, upon mixing of the separated subunits from which the mercurial had been removed, aggregation occurs spontaneously to produce a complex having the catalytic and regulatory properties of the native enzyme.

Although the numerous metabolic pathways of a normal cell are biochemically independent, they are closely interconnected and coordinated with regard to the rates of synthesis of their products. Much of the coordination derives from a small, important group of regulatory enzymes which operate at critical steps in the sequences of metabolic reactions. These enzymes are equipped to interact specifically with metabolites in addition to their substrates and products. Characteristically, a regulatory enzyme of one pathway is strongly activated or inhibited by a specific metabolite arising from another pathway, or from another step of the same pathway. Thus, through these enzymes, a mechanism exists for the close coordination of metabolism.

Some of the better-known regulatory enzymes have been found in the biosynthetic pathways of bacteria (Umbarger, 1961; Wilson and Pardee, 1964; Monod *et al.*, 1963). Generally the first enzyme of a sequence is inhibited by the end product of the entire pathway. This pattern of control, known as feedback inhibition, couples the production of low molecular weight metabolites to their consumption for the synthesis of macromolecules. One such example is observed in pyrimidine biosynthesis in *Escherichia coli*. The first enzyme in that pathway is aspartate transcarbamylase (ATCase)[1]

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[1] Abbreviations used in this work: ATCase, aspartate transcarbamylase; CTP, cytidine triphosphate; BrCTP, 5-bromocytidine triphosphate.

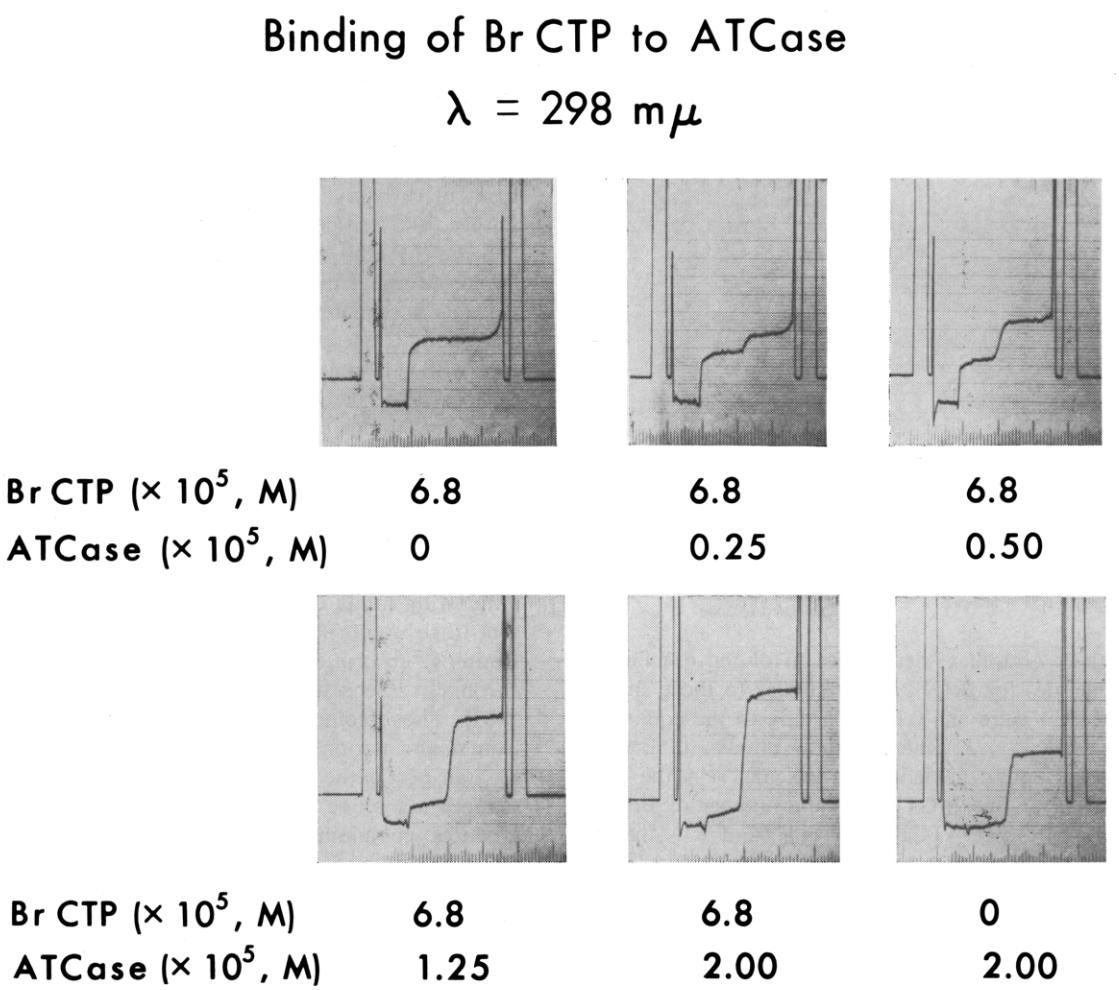

FIGURE 1: The binding of BrCTP to native ATCase. Samples contained 6.8 × 10^{-5} M BrCTP; ATCase at the concentrations indicated; 0.10 M Tris–Tris-HCl, *p*H 8.0; 0.02 M potassium phosphate, *p*H 7.0; 10^{-3} M mercaptoethanol; and 10^{-4} M EDTA. Samples were centrifuged at 50,740 rpm at temperatures from 3 to 5°. The representative scanner traces were made approximately 60 minutes after speed was reached. Optical density at 298 mμ (along the ordinate) is drawn as a function of distance from the center of the rotor (along the abscissa). The direction of sedimentation is to the right.

which is inhibited specifically by an end product, cytidine triphosphate (CTP) (Gerhart and Pardee, 1962). As long as the nucleoside phosphates produced via this pathway are converted into nucleic acids, ATCase continues to catalyze the formation of the first product, carbamyl aspartate. However, if CTP accumulates, ATCase action is inhibited and pyrimidines are not formed.

Metabolic control of this type is attributable to the structure of these enzymes. In particular, the protein structure must determine not only the strength and specificity of binding the regulatory metabolites (which often differ greatly from the substrates and products in size, shape, and charge), but also the effect of the regulatory metabolites on catalytic activity (i.e., activation or inhibition). Recent kinetic experiments with several enzymes (Gerhart and Pardee, 1962; Changeux, 1963; Martin, 1963; Cohen and Patte, 1963) have indicated that the regulatory metabolite binds to the enzyme at a site (called the regulatory, or allosteric, site) which is distinct from the active site. Moreover, with two regulatory enzymes in particular (Changeux, 1963; Gerhart and Pardee, 1963) inhibition or activation appears to require the participation of subunits within the enzyme molecules.

Since these conclusions were inferred from kinetic data, efforts were made to obtain direct physical and chemical information about the structure of a regulatory enzyme, in terms of its subunits and interactions with substrates and inhibitors. As shown in this communication, ATCase is composed of two types of subunits. The larger of these possesses the entire catalytic activity of the enzyme and is insensitive to the feedback inhibitor, CTP, whereas the second subunit is devoid of

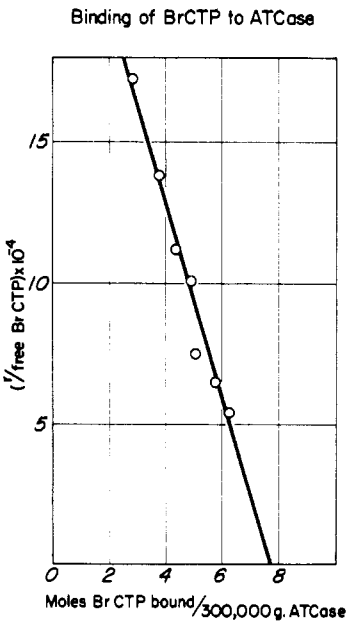

FIGURE 2: Graphical determination of the number of binding sites for BrCTP. Data similar to those shown in Figure 1 were analyzed according to the equation: $r/A = n/K - r/K$, where r is the number of BrCTP molecules bound per enzyme molecule, A is the concentration of free BrCTP, n is the number of BrCTP receptor sites per ATCase molecule, and K is the intrinsic dissociation constant of the BrCTP-ATCase complex.

catalytic activity and bears the regulatory sites required for the control of catalytic activity.

Materials and Methods

The procedure of Sheperdson and Pardee (1960) with modifications to be described elsewhere was employed for the purification of ATCase. The enzyme was obtained from a strain of *E. coli* K12, diploid for the chromosomal region bearing the ATCase cistrons. When this strain was derepressed for enzymes of the pyrimidine pathway, ATCase comprised approximately 12% of the total soluble protein. Purification involved ammonium sulfate precipitation, heating to 73° in concentrated salt solutions of high *p*H, and, finally, zone centrifugation in a sucrose gradient. This last step proved necessary for the removal of desensitized ATCase which sediments more slowly than the native enzyme and which on occasion constitutes as much as one-third of the total enzyme activity. ATCase purified by this procedure has a specific activity (Shepherdson and Pardee, 1960) of 6000–7000 units/mg. Protein concentrations were determined refractometrically from synthetic-boundary experiments with an ultracentrifuge equipped with a Rayleigh interference optical system (Richards and Schachman, 1959; Schachman, 1963a). The absorbance at 280 mμ of a 0.1% solution of ATCase is 0.59, and the ratio of absorbances at 280 and 260 mμ is 1.85. Fractionation of the subunits of the dissociated enzyme was achieved by zone centrifugation in a sucrose gradient (see legend of Figure 6) or by chromatography on a column of DEAE-Sephadex. Enzymic activity was assayed by measurement of carbamyl aspartate production (Gerhart and Pardee, 1962) (see legend of Figure 6).

Sedimentation experiments were performed in a Spinco Model E ultracentrituge equipped with a phase plate as a schlieren diaphragm and a rotatable light source for Rayleigh interference optics. Double-sector cells with a 12-mm optical path and sapphire windows were used routinely. For some experiments with dilute solutions 30-mm cells were employed. Sedimentation equilibrium experiments for measurement of molecular weights were performed with liquid columns of 2.7 mm, using interference optics (Richards and Schachman, 1959).

Studies of the interaction of the enzyme and its subunits with inhibitor were conducted with an ultracentrifuge equipped with a split-beam photoelectric scanning absorption optical system and a monochromator (Schachman *et al.*, 1962; Lamers *et al.*, 1963). The binding of the inhibitor was determined from sedimentation velocity patterns by measurement of the transport of the low molecular weight inhibitor when sedimenting alone as compared to its movement in mixtures containing the inhibitor and protein. To facilitate these determinations, 5-bromocytidine triphosphate (BrCTP) was substituted for the usual inhibitor, CTP. With BrCTP, light with a wavelength of 298 mμ was used, thereby reducing the interference caused by the light absorption of the protein.

Viscosity measurements were performed with an Ostwald viscometer having an average shear gradient of 265 sec^{-1}, and outflow times at 25.0 ± 0.005° were determined to ±0.03 seconds (Schachman, 1957). All chemicals were obtained commercially with the exception of BrCTP, which was synthesized according to the procedure of Bessman *et al.* (1958).

Results

Molecular Weight and Shape of ATCase. Native ATCase sediments as a single, sharp boundary (see top left-hand pattern of Figure 3). The sedimentation coefficient, s, in svedbergs, decreases slightly with increasing enzyme concentration according to the equation, $s_{20,w} = 11.75(1 - 0.09\,c)$ where c is concentration of enzyme in g/100 ml. This small concentration dependence is characteristic of compact globular macromolecules (Schachman, 1959), and the negative slope of s versus c, even at high dilution, indicates the absence of association-dissociation equilibria throughout the concentration range examined (0.05–0.8 g/100 ml). The intrinsic viscosity, $[\eta]$, was found to be 0.045 dl/g, a value similar to that found for many compact globular macromolecules, and substantially less than that observed with swollen or elongated particles (Schachman, 1963b).

Combination of s_0 and $[\eta]$ in the Scheraga-Mandelkern equation (Scheraga and Mandelkern, 1953), with an assumed partial specific volume of 0.74 ml/g and a value of β (the shape factor) of 2.16×10^6, gave a molecular weight estimate of 3.0×10^5. A more reliable value of the molecular weight was obtained from sedimentation equilibrium experiments on dilute solutions of ATCase. At concentrations of 0.25 g/100 ml the apparent weight-average molecular weight was found to be approximately 3.1×10^5. The dependence of log c on x^2, where x is the distance from the axis of rotation, was linear, indicating a high degree of homogeneity. This molecular weight is not thought to be accurate to more than 5% since the range of concentrations examined was not sufficient for an evaluation of the nonideality of the solutions (Schachman, 1959) and because of occasional trace contamination from the catalytic subunit of ATCase (see later).

Binding of BrCTP to ATCase. Figure 1 shows representative sedimentation velocity patterns from a series of experiments with a fixed concentration of BrCTP and increasing concentrations of ATCase. The pattern of BrCTP alone shows that all of the material absorbing light at 298 mμ migrates slowly with a sedimentation coefficient of about 0.4 S. Upon the addition of ATCase (0.25×10^{-5} M) about 22% of the BrCTP migrates rapidly with a sedimentation coefficient of 11.7 S. When the ATCase concentration is increased (0.50×10^{-5} M), as in the third pattern, about 36% of the BrCTP is bound in the form of rapidly sedimenting complexes and the remainder sediments slowly as free BrCTP. As seen in the remaining patterns, the bulk of

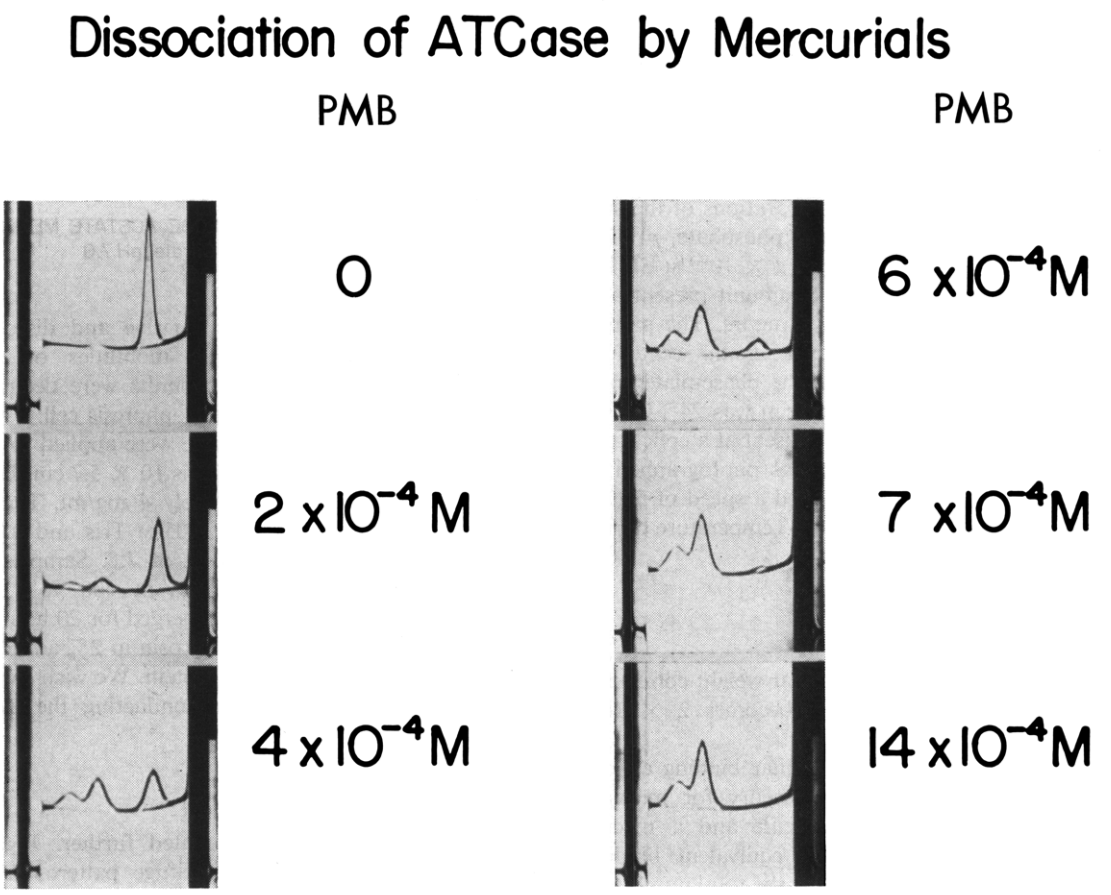

FIGURE 3: Dissociation of ATCase by mercurials. Samples were prepared at room temperature and contained 0.04 M potassium phosphate, *p*H 7.0; 8.7 mg/ml ATCase (2.9×10^{-5} M native enzyme); and *p*-mercuribenzoate (PMB) at the concentrations indicated. ATCase was dialyzed previously to remove mercaptoethanol and EDTA. Samples were centrifuged about 40 minutes after preparation. The Schlieren photographs reproduced above were taken 50 minutes after the centrifuge reached a speed of 60,000 rpm. Menisci are on the left. The angle of the schlieren diaphragm was 50°. Temperature was maintained at approximately 20° during centrifugation. The sedimentation rate of the fastest boundary was approximately 11.3 S, the intermediate boundary was 5.6 S, and the slowest boundary 2.8 S.

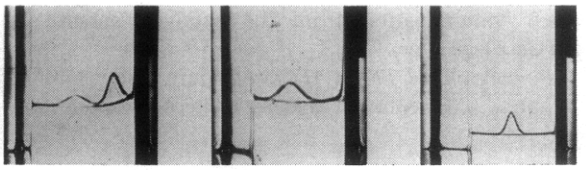

FIGURE 4: Separated subunits of ATCase. On the left is shown the schlieren pattern of a sample of ATCase dissociated in a solution containing 7.5 mg/ml of native enzyme, 0.04 M potassium phosphate, *p*H 7.0, and 1.4×10^{-3} M *p*-mercuribenzoate. The pattern was photographed 108 minutes after the centrifuge reached a speed of 59,780 rpm, at a temperature of 22° and a phase-plate angle of 50°. The preparations of the subunits contained 0.04 M potassium phosphate, *p*H 7.0, 2×10^{-3} M mercaptoethanol, and 2×10^{-4} M EDTA. In the center is shown the 2.8 S subunit present at a concentration of approximately 5 mg/ml. The pattern was photographed 104 minutes after the centrifuge reached a speed of 59,780 rpm. The phase-plate angle was 40°. Temperature during the run was 24°. On the right is shown the 5.8 S subunit present at a concentration of 6.3 mg/ml. The pattern was photographed 48 minutes after the centrifuge reached a speed of 59,780 rpm, at a phase-plate angle of 70°. Temperature during the centrifugation was 23°.

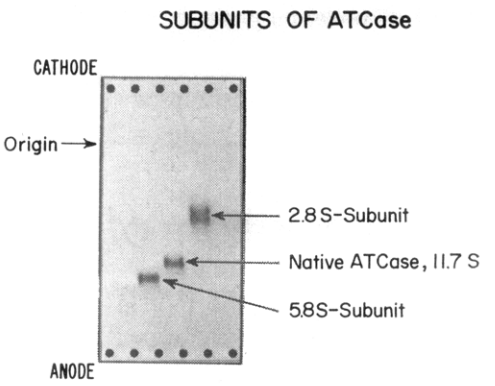

FIGURE 5: Electrophoresis of native and dissociated ATCase. The electrophoretic mobilities of native ATCase and the separated subunits were determined in a Beckman Microzone electrophoresis cell. Samples of approximately 0.5-μl volume were applied to a cellulose acetate strip of dimensions 10 × 5.7 cm. Protein concentration was approximately 4 mg/ml. The electrophoresis buffer contained 0.05 M Tris and 0.012 M citric acid, yielding a final *p*H of 7.8. Samples were electrophoresed for 20 minutes at 400 volts, after which time the strips were submerged for 20 hours in a staining solution of 0.002% nigrosin in 2% acetic acid, to detect the position of the protein. We wish to thank Mrs. Frances Hamburg for conducting the electrophoresis.

the inhibitor exists as high molecular weight complexes when the enzyme concentration becomes 2×10^{-5} M.[2]

Figure 2 shows results from similar binding experiments plotted according to the theory for multiple equilibria between a small molecule and a macromolecule possessing independent equivalent binding sites (Klotz, 1953). In this plot, *r* is the total number of moles of bound BrCTP divided by the total number of moles of ATCase. Extrapolation of the data to *r*/(free BrCTP) equal to zero, i.e., infinite concentration of unbound BrCTP, gives a value of 8 as the number of BrCTP binding sites per molecule of ATCase. The intrinsic dissociation constant, estimated from the intercept on the ordinate, is 3×10^{-5} moles/liter.[3]

Dissociation of ATCase into Subunits. Earlier studies (Gerhart and Pardee, 1962) showed that ATCase can be rendered insensitive to inhibition by CTP as the result of treatment with mercurials or mild heating. In the case of heating, "desensitization" seems to be accompanied by dissociation of ATCase into smaller units. Therefore the effect of these treatments on the structure of ATCase was investigated further. Figure 3 shows representative ultracentrifuge patterns from a series of experiments with a constant concentration of ATCase and increasing amounts of the mercurial, *p*-mercuribenzoate. Concomitant with the decrease in the amount of the 11.7 S component (native ATCase) is the formation of two other components with sedimentation coefficients 2.8 and 5.8 S. The relative amounts of these two components are independent of the amount of ATCase dissociated. Even when a large excess of *p*-mercuribenzoate is added, these two components are present in the same relative amounts: approximately 63% for the 5.8 S subunit and 37% of

[2] Since the enzyme contributed slightly to the optical density of the solution at 298 mμ (as seen in the right-hand pattern in the lower row), the change in the optical density across the boundary due to the inhibitor-enzyme complexes was not taken directly as a measure of the amount of bound BrCTP. Instead, the amount of slowly sedimenting (unbound) BrCTP, when compared to the control containing no enzyme, was used as a direct indication of the fraction of free BrCTP in the various mixtures.

[3] It should be noted that binding experiments of this type are not free of ambiguity because of possible complications from coupled flows (Gosting, 1956) and the reequilibration of the mixtures if the equilibrium is disturbed owing to differential sedimentation of the various molecular species (Schachman, 1959, 1960; Gilbert and Jenkins, 1959; Williams *et al.*, 1958). However, these complications are likely to be minor for systems involving the interaction of macromolecules with much smaller materials (Schachman, 1960). Since the sedimentation coefficients of ATCase in the presence and absence of inhibitor are almost equal, the disturbance of the equilibria during the sedimentation experiments is probably negligible. As a consequence the estimates of the number of binding sites in ATCase and the dissociation constant for BrCTP can be considered reliable.

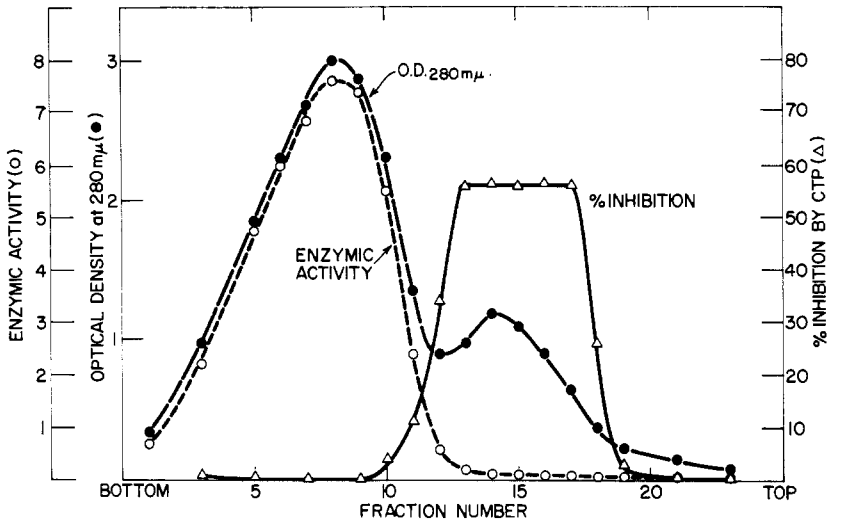

FIGURE 6: Function of 5.8 S and 2.8 S subunits of ATCase. The subunits of ATCase were separated by sucrose-gradient centrifugation as follows: A solution (0.4 ml) containing 7 mg purified ATCase, 0.04 M potassium phosphate, *p*H 7.0, and 3×10^{-3} M *p*-mercuribenzoate was layered onto a 4.4-ml gradient of 6–25% sucrose containing 0.04 M potassium phosphate, *p*H 7.0, and 10^{-4} M *p*-mercuribenzoate. Samples were then centrifuged for 20 hours at 10° and 38,000 rpm in the SW-39 rotor in the Spinco Model L centrifuge. Drops were collected, 12 per fraction, from the bottoms of the tubes. Fractions were diluted 10-fold for optical density readings at 280 mμ. The scale of the ordinate is corrected to the undiluted values. ATCase activity was determined at 28° in assay mixtures containing 5×10^{-3} M aspartate, 3.6×10^{-3} M carbamyl phosphate, 0.04 M potassium phosphate, *p*H 7.0, and 0.1 volume of a 1000-fold dilution of each fraction in dilution buffer (0.04 M potassium phosphate, *p*H 7.0, 2×10^{-3} M 2-mercaptoethanol, 2×10^{-4} M EDTA). The scale of the ordinate for enzymic activity is μmoles carbamyl aspartate produced per ml per 30 min, × 16. Inhibition by CTP was measured as follows: Fraction 6 (10 μl) (containing ATCase activity but no sensitivity to CTP) was mixed with 25 μl of other fractions, diluted to 1 ml with dilution buffer, allowed to stand at room temperature for 2 hours, diluted further 20-fold in dilution buffer, and assayed as described before, plus or minus 4×10^{-4} M CTP.

the 2.8 S subunit. No time dependence for the conversion of the enzyme into subunits has been observed. The ultracentrifuge pattern for the solution containing 14×10^{-4} M *p*-mercuribenzoate is unchanged whether the sample is examined immediately (i.e., after 30 minutes) or after 24 hours. This indicated that the 5.8 S component is not an intermediate in the dissociation of the enzyme into the 2.8 S component but that the two components are distinct subunits.

Direct evidence for the independent nature of the subunits came from fractionation experiments of the dissociation mixture by zone centrifugation in a sucrose-density gradient. (See legend of Figure 6 for a description of the separation procedure.) After separation, the subunits were freed of *p*-mercuribenzoate by dialysis against a solution of 0.04 M potassium phosphate, *p*H 7.0, and 2×10^{-3} M mercaptoethanol. Figure 4 shows the sedimentation velocity patterns of the separated subunits. The separated subunits do not tend to aggregate after the removal of the mercurial, since they still sediment at the characteristic rates of approximately 5.8 and 2.8 S. Preliminary molecular weight determinations by sedimentation equilibrium yielded values of 9.6×10^4 for the 5.8 S subunit, and 3×10^4 for the 2.8 S subunit.

Evidence for the chemical difference of the subunits was obtained from electrophoresis on cellulose acetate membranes. As seen in Figure 5 the two subunits differ from each other and from the native enzyme in their mobilities, presumably because of differences in their amino acid composition. In recent experiments it has been possible to separate the two subunits by chromatography on DEAE-Sephadex.

Functions of the Subunits of ATCase. With the recognition of distinct and separable subunits in ATCase, attempts were made to ascribe to them the two specific functions of regulation and catalysis. ATCase was dissociated by *p*-mercuribenzoate and the subunits were separated by zone centrifugation on a sucrose gradient and characterized as shown in Figure 6. The optical density profile of the samples from the centrifuge tubes showed two separate components. Material collected from tubes 4–10 had a sedimentation coefficient of 5.8 S whereas material from tubes 14–18 had a sedimentation coefficient of 2.8 S. Enzymic activity was assayed and found associated exclusively with the 5.8 S fraction, which exhibited a specific activity almost 2-fold greater than that of the native enzyme.

Although the 5.8 S subunit is catalytically active, it is not at all inhibited by CTP. In order to test whether the capacity for inhibition resides in the 2.8 S subunit, a recombination experiment was done. An aliquot of 5.8 S subunit from fraction 6 of the zone centrifugation experiment was added to aliquots of all other fractions.

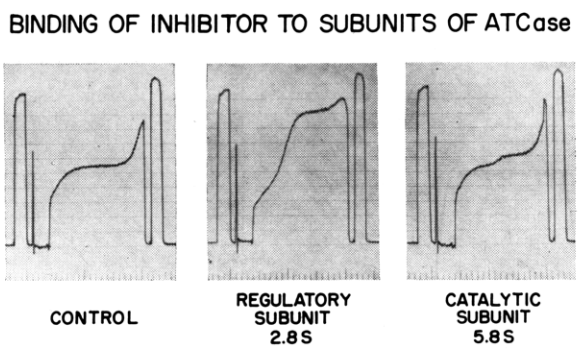

FIGURE 7: Binding of BrCTP to the separated subunits of ATCase. Samples were prepared as described in the legend of Figure 1, except that the concentration of BrCTP was 1.4×10^{-4} M. The subunits were present at a concentration of approximately 2 mg/ml. Temperature during the centrifugation was 22°. The traces of the sample of BrCTP alone and of BrCTP plus the 5.8 S subunit were made 56 minutes after the centrifuge reached a speed of 59,740 rpm. The trace of the sample of BrCTP plus 2.8 S subunit was made 104 minutes after the same speed was reached. Sedimentation is to the right.

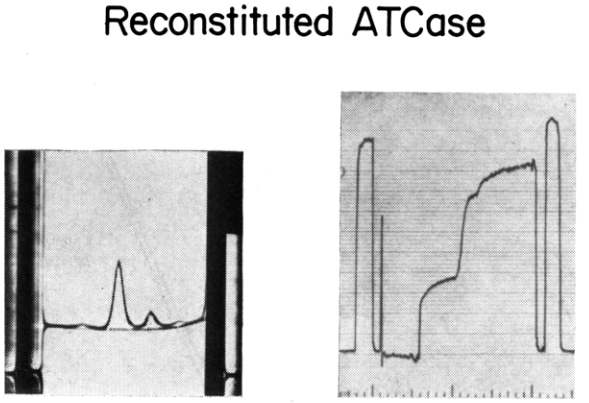

FIGURE 8: Reconstitution of ATCase after dissociation by mercurials. ATCase was dissociated in a solution containing 7.5 mg/ml of native enzyme, 0.04 M potassium phosphate, *p*H 7.0, and 10^{-3} M *p*-mercuribenzoate. After incubation at 25° for 60 minutes, the sample was dialyzed overnight at 4° against 100 volumes of a solution containing 0.04 M potassium phosphate, *p*H 7.0, and 10^{-4} M mercaptoethanol. After dialysis the sample was made 2×10^{-3} M in mercaptoethanol and then characterized in the ultracentrifuge. Above on the left is shown a schlieren pattern photographed 32 minutes after the centrifuge reached a speed of 60,000 rpm at a phase plate angle of 60°. Temperature during centrifugation was 20°. The principle component moved at 11.2 S, and the minor faster components at 15.8 and 19.4 S. The trace of slowly sedimenting material moved at 6.5 S. On the right is shown a scanner trace of the binding of BrCTP to reconstituted enzyme of the same sample. Conditions for binding are identical to those described in the legend of Figure 7. (See Figure 7 for the control centrifugation in the absence of enzyme.) The scanner trace was made 32 minutes after the centrifuge reached a speed of 59,780 rpm, at 26°.

After a 2-hour incubation period in the presence of mercaptoethanol, the production of carbamyl aspartate was measured both in the presence and absence of CTP in order to determine the per cent of inhibition. As seen in Figure 6, inhibition (by about 60%) was observed only when the 5.8 and 2.8 S subunits were both present. It can be concluded, therefore, that the 2.8 S subunit, though devoid of enzymic activity, is essential for the inhibition.

The role of the 2.8 S subunit in the inhibition by CTP was explored further through binding experiments similar to those described earlier for the native enzyme. When BrCTP was added directly to *p*-mercuribenzoate dissociated ATCase, a pattern was obtained which showed clearly that about 55% of the BrCTP is bound to the 2.8 S subunit, whereas no BrCTP is bound to the 5.8 S subunit, and the remainder of the BrCTP is free in solution. This binding experiment was repeated with the separated subunits from which *p*-mercuribenzoate was removed with excess mercaptoethanol, as shown in Figure 7. Equivalent concentrations of protein (by weight) were used for the two subunits. (The 2.8 S subunit absorbs more light at 298 mμ per mg protein than does the 5.8 S subunit.) Again it was found that the 2.8 S subunit binds BrCTP strongly, whereas the 5.8 S subunit does not appear to bind BrCTP at all. Thus one reason for the importance of the 2.8 S subunit in the inhibition by CTP is that the 2.8 S subunit bears the binding sites for CTP.

Reconstitution. The dissociation of ATCase by *p*-mercuribenzoate is successfully reversed by the addition of the sulfhydryl compound, mercaptoethanol. In a representative experiment, ATCase was dissociated with an excess of *p*-mercuribenzoate (see the legend of Figure 8) and an aliquot of the sample was examined in the ultracentrifuge to observe dissociation. Then the sample was dialyzed against a solution of 0.04 M potassium phosphate, *p*H 7.0, containing 10^{-4} M mercaptoethanol. Figure 8 shows the sedimentation characteristics of the ATCase in this dialyzed sample. As much as 80–90% of the material reconstitutes to form a complex which sediments at 11.2 S, similar to native ATCase. (The remaining material sediments faster, at approximately 15 and 19 S, perhaps owing to the formation of higher aggregates.) The reconstituted enzyme binds BrCTP (Figure 8) and is similar to native and dissociated ATCase in its catalytic activity and inhibition by CTP. The complex could also be reconstituted by mixing the separated subunits from which the *p*-mercuribenzoate had been removed. At *p*H 8.5 and 37°, the reconstitution of the separated subunits proceeded optimally. Thus, the formation of the native enzyme appears to proceed spontaneously from the subunits.

Discussion

ATCase is composed of two easily separable proteins which together confer on the enzyme the two functions of catalysis and metabolic control. One protein possesses the entire catalytic activity and must therefore bear the active sites. This protein will be referred to here as the *catalytic subunit*. It is stable and catalytically active, and by itself shows little tendency to aggregate in the presence or absence of mercurials. In electrophoresis, it moves as a particle slightly more negatively charged than the native enzyme. The catalytic subunit is estimated to have a molecular weight of 9.6×10^4 and may contain two or more polypeptide chains as judged from preliminary dissociation studies with 3.0 M guanidine hydrochloride.

The other protein of native ATCase has no catalytic activity and is not essential for the activity of the catalytic subunit. This protein does bind CTP and therefore must bear the regulatory sites found in the native enzyme. Since this protein is indispensable for the inhibition of the catalytic subunit by CTP, it will be referred to here as the *regulatory subunit*.[4] The regulatory subunit is stable and retains its affinity for CTP, and by itself shows only a slight tendency to aggregate after the removal of mercurials. In electrophoresis it migrates as a particle more positively charged than the native enzyme. It is considerably smaller than the catalytic subunit, having a molecular weight of approximately 3×10^4. Clearly the two proteins differ functionally and chemically.

The quantitative composition of the native enzyme in terms of catalytic and regulatory subunits may be calculated as follows: Native ATCase has a molecular weight of 3.1×10^5 of which 63% (i.e., 2.0×10^5 molecular weight units) is derived from catalytic subunits. Consequently there must be two catalytic subunits per native enzyme molecule. The remaining 37% (i.e., 1.1×10^5 molecular weight units) is made up of regulatory subunits. Therefore there must be four regulatory subunits per native enzyme molecule. It is noteworthy that the eight receptor sites found for CTP in the native enzyme must be on the four regulatory subunits, requiring the presence of two sites per subunit (and possibly indicating the presence of two polypeptide chains per subunit). Formation of the native structure of molecular weight 3.1×10^5 requires the presence of both kinds of subunits. Thus native ATCase appears to be a coaggregate in which unlike subunits are bonded together.

Sulfhydryl and other mercurial-reactive groups may play an important part in the aggregation and interaction of the two kinds of subunits. The disaggregation of the native enzyme by *p*-mercuribenzoate has not been characterized fully, but seems to resemble the "all-or-none" pattern found for phosphorylase A (Madsen and Cori, 1956), potato X virus capsid protein (Kaper and Houwing, 1962), and hemerythrin (Keresztes-Nagy and Klotz, 1963). In the case of ATCase approximately twenty-four to twenty-six groups are titrated per native enzyme molecule in the course of dissociation. Perhaps these reactive groups occupy critical positions between subunits and become exposed or buried *in unison* as the subunits rapidly and reversibly change their spatial arrangement (as has been suggested by Monod, Wyman, and Changeux for hemoglobin and other allosteric proteins [Changeux, 1964; Monod *et al.*, 1965]).

One of the striking changes accompanying the dissociation of ATCase by mercurials is the increase in catalytic activity (Gerhart and Pardee, 1964). This enhanced catalytic activity is also characteristic of the isolated catalytic subunit. For example, at low aspartate concentrations, where the effect is most pronounced, the catalytic subunit is more than four times as active as the native enzyme. Apparently at low substrate concentrations the catalytic subunit has a greater affinity for aspartate than does the native enzyme. (The turnover number may be higher also.) Clearly, each catalytic subunit loses activity when combined with another catalytic subunit and four regulatory subunits as in the native enzyme. A possible explanation for the loss of activity is that the catalytic subunit is inhibited by the regulatory subunit, perhaps due to distortion of its tertiary structure as it becomes part of the complex. At high substrate concentrations, however, the affinity of the complex for aspartate does become strong (probably even better than that of the free catalytic subunit). It appears that the proposed inhibition of the catalytic subunit by the regulatory subunit is reversible. The regulatory subunit is viewed then as a proteinaceous inhibitor which acts in a reversible fashion. When CTP binds to the regulatory subunit, the less-active conformation of the native enzyme is stabilized (an idea fully developed by Changeux [1964] and Monod *et al.* [1965]). CTP, the regulatory metabolite, could be viewed as a co-inhibitor acting on the regulatory subunit or apo-inhibitor.

The regulatory subunit represents a class of proteins produced by the cell not for catalytic activity but for the control of catalytic activity. In the case of ATCase, it appears that the control can be achieved by quite a small polypeptide chain (of only 15,000 molecular weight per CTP binding site), which is present in multiple copies in the native enzyme molecule. A rather large fraction of the total protein of ATCase, about 37%, is devoted exclusively to the control of activity. Other proteinaceous inhibitors of enzymes have long been known (e.g., trypsin inhibitors [Desnuelle, 1960], the heat-labile inhibitor of diphosphopyridine nucleotidase [Schwarz *et al.*, 1958], and pepsin inhibitor [Bovey and Yanari, 1960]). The regulatory protein described here differs from these in its presumed rapidly reversible inhibition and its specific binding of a low molecular weight metabolite.

At this time it is impossible to say whether the presence of two kinds of subunits in ATCase reflects a characteristic unique to regulatory enzymes, or whether any protein having stereospecifically different sites will

[4] The name *regulatory subunit* is not meant to imply that the catalytic subunit does not participate in the regulation. Probably both subunits together produce the regulatory effects.

necessarily have different kinds of polypeptide chains. The latter supposition is supported by the case of tryptophan synthetase from *E. coli* (Yanofsky *et al.*, 1961). Perhaps the two subunits of tryptophan synthetase are present because the enzyme binds two quite different substrates (serine and indoleglycerol phosphate) and no single polypeptide chain could contribute the precise structure for two such different binding sites.

The evolution of regulatory enzymes was apparently not constrained by the necessary formation of the regulatory and active sites from the same polypeptide chain. Instead, the regulatory site developed from a separate protein, one possibly descended from a primitive enzyme which bound CTP as a substrate or product. The mutations most important for the appearance of regulatory enzymes were those which allowed the association and specific interaction of the two proteins. Thus, irrespective of the biochemical details of the enzymic reaction (which are the province of the catalytic subunit alone), regulatory connections developed between and within pathways wherever they led to a more efficient metabolism.

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References

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

Bovey, F. A., and Yanari, S. S. (1960), *Enzymes 4*, 68, 79.

Changeux, J.-P. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 497.

Changeux, J.-P. (1964), *Brookhaven Symp. Biol. 17* (*BNL 869* (*C-40*)), 232.

Cohen, G. N., and Patte, J.-C. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 513.

Desnuelle, P. (1960), *Enzymes 4*, 128.

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

Gerhart, J. C., and Pardee, A. B. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 491.

Gerhart, J. C., and Pardee, A. B. (1964), *Federation Proc. 23*, 727.

Gilbert, G. A., and Jenkins, R. C. L. (1959), *Proc. Roy. Soc.* (*London*) *A253*, 420.

Gosting, L. J. (1956), *Advan. Protein Chem. 11*, 430.

Kaper, J. M., and Houwing, C. (1962), *Arch. Biochem. Biophys. 96*, 125.

Keresztes-Nagy, S., and Klotz, I. M. (1963), *Biochemistry 2*, 923.

Klotz, I. M. (1953), *Proteins 1*, 727.

Lamers, K., Putney, F., Steinberg, I. Z., and Schachman, H. K. (1963), *Arch. Biochem. Biophys. 103*, 379.

Madsen, N. B., and Cori, C. F. (1956), *J. Biol. Chem. 223*, 1055.

Martin, R. G. (1963), *J. Biol. Chem. 238*, 257.

Monod, J., Changeux, J.-P., and Jacob, F. (1963), *J. Mol. Biol. 6*, 306.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 11* (in press).

Richards, E. G., and Schachman, H. K. (1959), *J. Phys. Chem. 63*, 1578.

Schachman, H. K. (1957), *Methods Enzymol. 4*, 32.

Schachman, H. K. (1959), Ultracentrifugation in Biochemistry, New York, Academic.

Schachman, H. K. (1960), *Brookhaven Symp. Biol. 13* (*BNL 608* (*C 22*)), 49.

Schachman, H. K. (1963a), *Biochemistry 2*, 887.

Schachman, H. K. (1963b), *Cold Spring Harbor Symp. Quant. Biol. 28*, 409.

Schachman, H. K., Gropper, L., Hanlon, S., and Putney, F. (1962), *Arch. Biochem. Biophys. 99*, 175.

Scheraga, H. A., and Mandelkern, L. (1953), *J. Am. Chem. Soc. 75*, 179.

Schwarz, M. N., Kaplan, N. O., and Lambory, M. F. (1958), *J. Biol. Chem. 232*, 1051.

Shepherdson, M., and Pardee, A. B. (1960), *J. Biol. Chem. 235*, 3233.

Umbarger, H. E. (1961), *Cold Spring Harbor Symp. Quant. Biol. 26*, 301.

Williams, J. W., Van Holde, K. E., Baldwin, R. L., and Fujita, H. (1958), *Chem. Rev. 58*, 715.

Wilson, A., and Pardee, A. B. (1964), *Comp. Biochem. 6*, 73.

Yanofsky, C., Helinski, D. R., and Maling, B. D. (1961), *Cold Spring Harbor Symp. Quant. Biol. 26*, 11.