Allosteric Regulation of Aspartate Transcarbamoylase. Effect of Active Site Ligands on the Reactivity of Sulfhydryl Groups of the Regulatory Subunits†

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ABSTRACT: Earlier studies on ligand-promoted conformational changes in aspartate transcarbamoylase (Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7,* 538) showed that the sulfhydryl groups of the enzyme reacted with excess *p*-mercuribenzoate according to pseudo-first-order kinetics and that the rate constant for the reaction of the 24 thiols of the three regulatory subunits was increased sixfold by the addition of the aspartate analogue, succinate, in the presence of the substrate, carbamoyl phosphate. Since succinate binding was so weak and difficult to measure accurately, the relationship between the enhancement in reactivity and ligand binding was uncertain. This limitation has been circumvented by using as a ligand the bisubstrate analogue, *N*-(phosphonacetyl)-L-aspartate (PALA), which binds extremely tightly to the 6 active sites in the enzyme (Jacobson, G. R., and Stark, G. R. (1973), *J. Biol. Chem. 248,* 8003). Like the other active site ligands, PALA in saturating amounts caused a marked enhancement (sixfold) in the reactivity of the thiols. However, with PALA at subsaturating levels biphasic reactions were observed whereas with succinate pseudo-first-order kinetics were always obtained. The biphasic kinetics were accounted for in terms of two concurrent pseudo-first-order reactions; the rate constant for the slower reaction was equal to that of unliganded enzyme and the rate constant for the faster reaction corresponded to that of the fully liganded enzyme. The fraction of sulfhydryl groups in the "fast" class increased with PALA concentration until, upon the addition of 4 to 5 equiv of this active site ligand per enzyme molecule, all of the thiols of the regulatory chains were fast reacting. Both carbamoyl phosphate and CTP affected the biphasic kinetics observed in the presence of subsaturating amounts of PALA with the former facilitating the isomerization and the latter antagonizing it. These results indicate that the enzyme exists in different conformations in the absence and presence of PALA and that both forms coexist in populations of partially liganded molecules. Moreover, the isomerization of the enzyme from a state with slowly reacting sulfhydryl groups to one with rapidly reacting thiols was complete prior to saturation of all six active sites. This gross change in the quaternary structure of aspartate transcarbamoylase is readily interpreted in terms of a concerted transition involving two states (Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12,* 88).

Aspartate transcarbamoylase (ATCase)[1] (EC 2.1.3.2; carbamoylphosphate:L-aspartate carbamoyltransferase) from *Escherichia coli* dissociates into separate catalytic (C) and regulatory (R) subunits upon the addition of the mercurial, *p*-mercuribenzoate (PMB) (Gerhart and Schachman, 1965). The sulfhydryl groups of the enzyme react with excess mercurial according to pseudo-first-order kinetics and the reactivity of the 24 groups on the R subunits is enhanced about sixfold upon the addition of both carbamoyl phosphate and succinate (Gerhart and Schachman, 1968). This large increase in the rate of reaction of these sulfhydryl groups and the ligand-promoted decrease (3.5%) in the sedimentation coefficient of intact ATCase were interpreted by Gerhart and Schachman (1968) in terms of a conversion of the enzyme molecules from a constrained state with low affinity for substrates into a relaxed conformation having a high affinity for substrates. The analysis of these results (Gerhart and Schachman, 1968; Changeux and Rubin, 1968) according to the two-state model of Monod et al. (1965) was based in part on data describing the binding of succinate to the enzyme (Changeux et al., 1968).

Since the binding of succinate to the enzyme is so weak and therefore difficult to measure accurately and the earlier data are not rationalized readily with the currently accepted structure of ATCase, we have reexamined the kinetics of the reaction of the enzyme with PMB at varying degrees of saturation of the active sites with the bisubstrate analogue, *N*-(phosphonacetyl)-L-aspartate (PALA). This ligand binds very tightly to the catalytic chains (with a dissociation constant about 10^{-8} M) and the binding is readily measured spectrophotometrically (Collins and Stark, 1971; Jacobson and Stark, 1973).

As in solutions containing saturating amounts of carbamoyl phosphate and succinate, all 24 sulfhydryl groups of the R subunits were found to react with excess PMB as a single class in the presence of 6 equiv of PALA (per mol of ATCase), and the pseudo-first-order rate constant was about sixfold higher than that observed in the absence of ligands. The partially liganded enzyme, in contrast, exhibited different kinetic behavior depending on the nature of the ligands. Whereas with saturating carbamoyl phosphate and varying concentrations of succinate pseudo-first-order kinetics were always observed, in solutions containing less than 4 mol of PALA per mol of ATCase biphasic kinetics were obtained. The biphasic reactions could be described readily in terms of two concurrent pseudo-first-order processes with the slower rate constant corresponding to that for the unliganded (constrained) form

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[1] Abbreviations used are: ATCase, aspartate transcarbamoylase; PMB, *p*-mercuribenzoate; PALA, *N*-(phosphonacetyl)-L-aspartate; R, regulatory subunit; C, catalytic subunit; N (subscript), native subunit; P (subscript), pyridoxylated subunit; $C_NC_PR_3$, hybrid ATCase molecule containing one native and one pyridoxylated catalytic subunit and three regulatory subunits.

of the enzyme and the faster rate constant equal to that for the fully liganded (relaxed) molecules. The fraction of sulfhydryl groups reacting in the fast class increased progressively upon the addition of PALA. Moreover at subsaturating levels of PALA the number of fast-reacting thiols depended upon the presence or absence of other ligands such as the substrate, carbamoyl phosphate, or the inhibitor, CTP. These results, which indicate that both the constrained and relaxed forms of the enzyme coexist in the presence of subsaturating concentrations of PALA, can be interpreted in terms of the two-state model of Monod et al. (1965).

Experimental Section

Materials. ATCase was prepared according to the method of Gerhart and Holoubek (1967). Dilithium carbamoyl phosphate, CTP, ATP, and PMB were obtained from Sigma Chemical Co. Succinate was obtained from Eastman Organic Chemicals. PALA was kindly provided by Dr. G. R. Stark.

Measurement of Rates of Reaction of ATCase with PMB. The experimental procedure was similar to that of Gerhart and Schachman (1968). ATCase, which was stored in 3.6 M ammonium sulfate, 10 mM 2-mercaptoethanol, 0.2 mM EDTA, was diluted to a protein concentration of approximately 0.6 mg/mL and dialyzed against several changes of 40 mM potassium phosphate buffer at pH 7.0 ± 0.02. All enzyme solutions were used within 1 day after removal of 2-mercaptoethanol and EDTA because solutions stored for longer periods seemed to undergo air oxidation with the loss of some sulfhydryl groups. PMB stock solutions (0.40 mM) containing 40 mM phosphate and 50 mM Tris-HCl at pH 7.0 were stored at −20 °C. The enzyme solution, 1 mL at a concentration of about 0.6 mg/mL containing the appropriate ligand at the desired concentration, was placed in one of the compartments of a rectangular mixing cell (Hellma) and 1 mL of the PMB stock solution was added to the other compartment. A corresponding reference cell was filled with a solution of 0.2 mM PMB and the instrument was adjusted to zero optical density after the solutions attained a temperature of 20 ± 0.2 °C. All measurements were made at 250 nm in a Cary 16 spectrophotometer equipped with a Model 16053 recorder and a thermostated cell holder. For studies on the effect of ATP or CTP on the kinetics an equivalent concentration of the ligand was added to the reference cell in order to compensate for the optical density contributed by the ligand. After mixing the enzyme and PMB solutions the increase in absorbance due to mercaptide formation was recorded for a period at least ten times the half-time of the reaction. The total change in optical density was approximately 0.15. A value of 7.6×10^3 M^{-1} cm^{-1} was used for the extinction increment at 250 nm corresponding to the formation of the mercaptide complex (Boyer, 1954).

Analysis of the Reaction of ATCase with PMB in Terms of Pseudo-First-Order Kinetics. All computations were performed with a Hewlett-Packard 9820a calculator equipped with a 9862 calculator plotter. In all experiments a slow linear increase in absorbance with time was observed (corresponding to about 5×10^{-6} absorbance units per s) and this change was not affected by the addition of any of the ligands. Accordingly the data were corrected for this effect and then plotted in terms of a pseudo-first-order reaction according to

$$\ln \frac{A_\infty - A_t}{A_\infty - A_0} = -kt \qquad (1)$$

where A_0 and A_∞ represent the initial and final absorbance and A_t represents the absorbance at time, t. The pseudo-first-order rate constant, k, was calculated from the least-squares slope for the data over 90% of the reaction. Since the consumption of PMB was only a small fraction of the initial amount, changes in the PMB concentration during the reaction were neglected.

In some experiments (see Results) biphasic curves were obtained and the data were analyzed according to a scheme involving two concurrent first-order reactions leading to the same products (Amdur and Hammes, 1966). For this analysis it was assumed that there were fast-reacting molecules (F) as well as those which reacted with PMB more slowly (S). The concentrations of these two classes at any time, t, were described by

$$F = F_0 e^{-k_f t} \qquad (2a)$$

and

$$S = S_0 e^{-k_s t} \qquad (2b)$$

where F_0 and S_0 were the initial concentrations of those species and k_f and k_s were the rate constants for the reaction with the mercurial. Since the change in absorbance provided a direct measure of the overall extent of the reaction, the equations were combined to yield

$$A_\infty - A_t = F_0 e^{-k_f t} + S_0 e^{-k_s t} \qquad (3)$$

The data for these experiments were analyzed by iteratively fitting the final and initial portions of the overall reaction until the calculated values for k_s, k_f, S_0, and F_0 converged. As described in Results it was found that the values for the two rate constants, k_s and k_f, could be assigned on the basis of independent experiments with solutions containing no ligand and saturating amounts of ligand, respectively. It was possible, therefore, to calculate the initial concentrations, S_0 and F_0, from a least-squares solution of eq 3.

Results

Extent and Rate of Reaction of ATCase with PMB. Both the number of sulfhydryl groups in the enzyme which reacted with PMB and the rate of the reaction under different conditions are summarized in Table I. A total of 29 SH groups per ATCase molecule reacted when no other ligands were present. In the presence of CTP, which binds to the R subunits, the number of reactive thiols (30) was unchanged. The addition of saturating amounts of carbamoyl phosphate carbamoyl phosphate and succinate, or PALA (all of which bind to the C subunits) led to a decrease in the number of sulfhydryl groups which reacted with PMB. In the presence of these ligands only 24–26 thiols per ATCase molecule reacted and the addition of CTP did not affect the number of reactive groups. As shown by Gerhart and Schachman (1968) the single SH group on each catalytic chain (in isolated C subunits) was less reactive in the presence of ligands which bind to the active sites; thus only the 24 thiols on the three R dimers in ATCase react under these conditions.

Although the reaction between ATCase and PMB involves the formation of 30 mercurial–cysteinyl complexes, the kinetics of this process can be described in terms of a pseudo-first-order reaction (Gerhart and Schachman, 1968). As shown in Figure 1, a straight line relationship was obtained when the data were plotted according to eq 1 and a pseudo-first-order rate constant, k, of $3.0 \pm 0.2 \times 10^{-3}$ s^{-1} was obtained. This rate constant was only slightly affected by the presence of either CTP or ATP (Table I).

In addition to decreasing the reactivity of the SH groups on the C subunits, the active site ligands enhance the reactivity of the thiols on the R subunits in ATCase. The value of k was

TABLE I: Reaction of Sulfhydryl Groups of ATCase with PMB.[a]

Ligand[b]	No. of SH groups reacted	$k \times 10^3$ (s^{-1})
None	29	3.0 ± 0.2
CTP	30	2.6 ± 0.2
ATP		3.5
Carbamoyl phosphate	25	9.0 ± 0.5
Carbamoyl phosphate + CTP	26	7.5 ± 0.5
Carbamoyl phosphate + ATP		10
Carbamoyl phosphate + succinate	25	25 ± 0.5
PALA	25	18 ± 0.5
PALA + CTP	25	18 ± 0.5
PALA + ATP		20
PALA + carbamoyl phosphate	25	21 ± 0.5
PALA + carbamoyl phosphate + CTP	24	19 ± 0.5
PALA + carbamoyl phosphate + ATP		22

[a] Experiments were performed as described in Experimental Section. The total number of sulfhydryl groups reacted was calculated from the measured change in absorbance at 250 nm; the increment in extinction coefficient was 7.6×10^3 M^{-1} cm^{-1} (Boyer, 1954). The pseudo-first-order rate constants, k, were the averages of at least five independent determinations for each ligand except ATP. Only single measurements were conducted on these solutions. [b] Concentrations of ligands were: CTP, 0.21 mM; ATP, 0.1 mM; carbamoyl phosphate, 2.1 mM; succinate, 2.1 mM. The concentration of PALA was approximately 0.01 mM corresponding to about 8 mol of PALA per mol of ATCase.

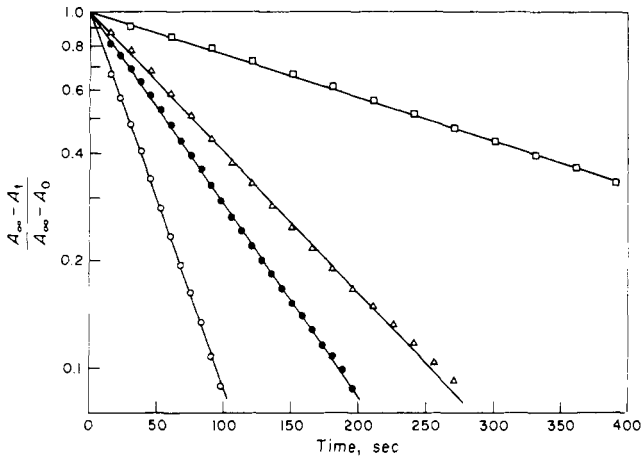

FIGURE 1: Analysis of the reaction between ATCase and PMB in terms of pseudo-first-order kinetics and the effect of ligands on the rate constant. Data obtained as described in Experimental Section were analyzed in terms of pseudo-first-order kinetics and plotted according to eq 1 where A_0 is the absorbance at zero time, A_t is the absorbance at various times during the reaction, and A_∞ is the absorbance after the reaction was essentially complete. The straight lines for the various experiments were drawn with the calculated values of the pseudo-first-order rate constant, k, evaluated from a least-squares fit of the data through 90% of the reaction. Different experiments are represented by the various symbols as follows: (□) no added ligand; (△) 2.1 mM carbamoyl phosphate; (● and ○) 2.1 mM carbamoyl phosphate plus 0.08 mM and 2.1 mM succinate, respectively.

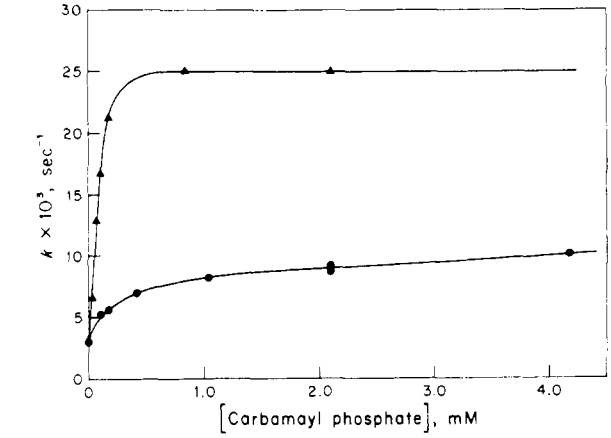

FIGURE 2: Effect of carbamoyl phosphate on the pseudo-first-order rate constant for the reaction between ATCase and PMB in the presence and absence of succinate. Pseudo-first-order rate constants, k, were determined as described in the legend to Figure 1 and in the Experimental Section. The symbol ▲ designates experiments with solutions containing 1.5 mM succinate, and the experiments represented by ● were performed on solutions containing no ligand other than the carbamoyl phosphate.

increased from 3×10^{-3} to $9.0 \pm 0.5 \times 10^{-3}$ s^{-1} by the addition of 2.1 mM carbamoyl phosphate (Figure 1 and Table I). Increasing the carbamoyl phosphate concentration to 4.2 mM led to only a slight additional increase in k to 10×10^{-3} s^{-1}. The dependence of the pseudo-first-order rate constant on carbamoyl phosphate concentration is shown in Figure 2. At a concentration of only 0.4 mM, the increase in k was about one-half the maximal enhancement. Succinate alone had no effect on the reactivity of the sulfhydryl groups ($k = 3.2 \times 10^{-3}$ s^{-1}). However, the presence of succinate had a marked effect on k at saturating levels of carbamoyl phosphate (Figure 1) and on the concentration of carbamoyl phosphate required for the maximal enhancement of the rate (Figure 2). In the presence of 1.5 mM succinate the addition of 1 mM carbamoyl phosphate caused an eightfold increase in the pseudo-first-order rate constant ($k = 25 \times 10^{-3}$ s^{-1}) and a half-maximal increase (to 14×10^{-3} s^{-1}) was attained at only 0.1 mM carbamoyl phosphate.

Representative pseudo-first-order-kinetic plots for the reaction of ATCase with excess PMB in the presence of saturating levels of carbamoyl phosphate and different amounts of succinate (0.08 mM and 2.1 mM) are presented in Figure 1. As seen in Table I the rate constant was increased eightfold when both carbamoyl phosphate and succinate were present at saturating concentrations. Table I shows also that at a saturating level of the bisubstrate analogue, PALA, there was a marked enhancement (sixfold) in k to a value about $18 \pm 0.5 \times 10^{-3}$ s^{-1}.

Biphasic Reaction of ATCase with PMB in the Presence of Subsaturating Levels of PALA. As seen in Figure 3a the reaction of ATCase with PMB was biphasic when less than four of the six catalytic sites were occupied by PALA. This observation is in striking contrast to those observed for the reaction in the presence of subsaturating concentrations of either carbamoyl phosphate or succinate (Figures 1 and 2). Since the reaction of ATCase with PMB in the presence of limiting amounts of PALA could not be accounted for by a single pseudo-first-order rate constant and since it was known that PALA promoted a conformational change in the enzyme molecules (Howlett and Schachman, 1977), attempts were made to analyze the biphasic kinetics in terms of two concurrent first-order processes (eq 2a, 2b, and 3).

In the iterative fitting procedure used for analyzing the biphasic kinetics the reaction of the purported "slow" class was first calculated over a range of the experiment corresponding to the final 20% of the change in absorbance. The "curve" for this class of molecules was then subtracted from the experimental data so as to permit calculation of the reaction rate for the "fast" class of molecules. Then the slow and fast rates were analyzed alternately until the data converged for the two pu-

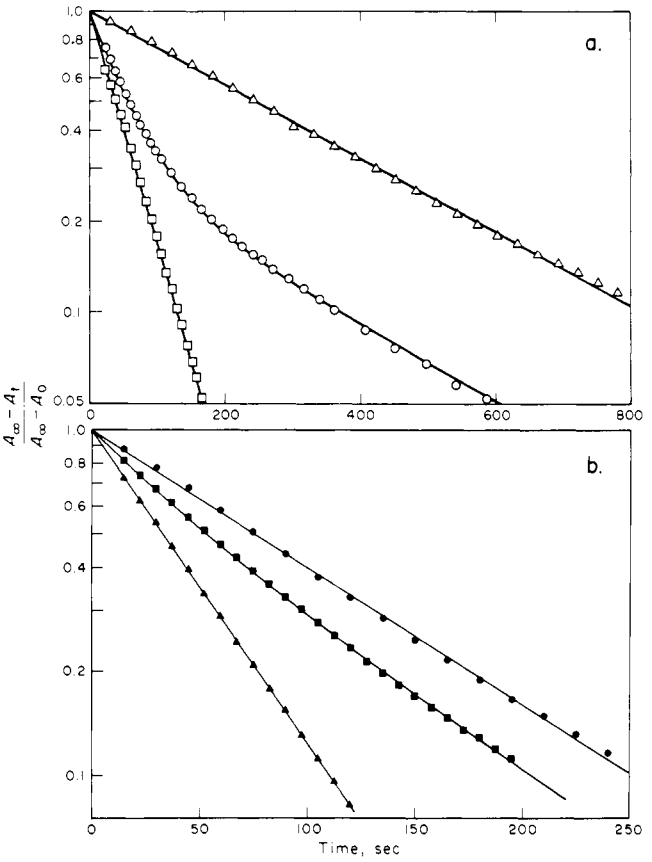

FIGURE 3: Biphasic reaction between ATCase and PMB in presence of subsaturating amounts of PALA. Data were obtained by procedure described in Experimental Section and plotted in terms of pseudo-first-order kinetics. (a) Experiments with no other ligands. Data designated by △ were obtained with ATCase in the absence of PALA and results represented by □ were obtained with solutions containing 7.2 equiv of PALA per ATCase molecule. The experiments designated by ○ were performed with solutions containing 3.2 equiv of PALA per molecule of ATCase and the least-squares curve through the data was drawn according to eq 3 with $k_f = 18 \times 10^{-3}$ s^{-1} and $k_s = 3 \times 10^{-3}$ s^{-1} which were the values of the rate constants for the fully liganded, □; and unliganded, △, enzyme. With this theoretical curve the values of F_0 and S_0 (see eq 3) for the fraction of sulfhydryl groups in the fast- and slow-reacting classes were determined. (b) Effect of carbamoyl phosphate on the biphasic reaction between ATCase and PMB in the presence of limiting amounts of PALA. All solutions contained 2.1 mM carbamoyl phosphate. Data represented by ● were obtained in the absence of PALA and experiments designated by ▲ were performed with solutions containing 7.2 equiv of PALA per ATCase molecule. The symbol ■ represents experiments with solutions containing 0.8 equiv of PALA per ATCase molecule and the least-squares curve was drawn with the rate constants, $k_f = 21 \times 10^{-3}$ s^{-1} and $k_s = 9 \times 10^{-3}$ s^{-1}, evaluated from the experiments designated by ▲ and ●, respectively.

tative species. In this way it was found that the fast rate calculated from the biphasic kinetics corresponded to the rate measured for the fully liganded enzyme, $k_f = 18 \times 10^{-3}$ s^{-1}, and the calculated slow rate was equal to that obtained for the unliganded enzyme, $k_s = 3 \times 10^{-3}$ s^{-1}. A variety of kinetic experiments at different degrees of saturation of the enzyme with PALA were analyzed by this procedure and the calculated values for k_s and k_f were found to be essentially constant. The calculated initial concentrations for the two species varied, however, with the fraction of molecules in the fast class increasing with the degree of saturation of the enzyme with PALA. In the light of this consistency in the two rate constants all the experimental data were analyzed by a least-squares solution of eq 3 so as to obtain the initial concentrations of the two species. The rate constants were assigned the values determined experimentally (Figure 3a) for the unliganded and fully liganded ATCase molecules. As seen in Figure 3a the curve calculated in this manner accounts for the experimental data over 95% of the course of a reaction in which there were 3.2 mol of PALA per mol of ATCase (a fit of comparable quality was obtained for other experiments). Even though only 53% of the active sites of ATCase were saturated with PALA, about 72% of the sulfhydryl groups were found to react with a rate constant corresponding to fully liganded enzyme.

A similar procedure was used for analyzing the effect of other ligands on the reaction between ATCase and PMB in the presence of limiting amounts of PALA. With saturating amounts of carbamoyl phosphate, for example, biphasic kinetics were observed but the curvature of the plots was not as pronounced as that observed when only PALA was present. Figure 3b shows one such experiment with 0.8 equiv of PALA per molecule of ATCase along with the kinetics for the reactions in the absence of any PALA and with 7.2 mol of PALA per mol of ATCase. When carbamoyl phosphate is present alone, k was 9×10^{-3} s^{-1} (compared with 3×10^{-3} s^{-1} in the absence of all ligands), and the enzyme in saturating amounts of both carbamoyl phosphate and PALA exhibited a rate constant of 21×10^{-3} s^{-1}. These values for the rate constants for the "slow" and "fast" classes, respectively, gave good fits for the biphasic kinetics and were used for calculations of the fraction of sulfhydryl groups in the two classes. Even though only 13% of the active sites in ATCase were occupied by PALA (the remaining sites contain bound carbamoyl phosphate), about 39% of the sulfhydryl groups were found to be in the "fast" class.

Figure 4a summarizes a series of experiments showing the effect of PALA on the fraction of sulfhydryl groups in the "fast" class. In the absence of carbamoyl phosphate all of the sulfhydryl groups appeared as fast reacting when there were only 4–5 equiv of PALA per ATCase molecule. When carbamoyl phosphate was present, only 3 mol of PALA per mol of ATCase was required to convert all of the sulfhydryl groups to the rapidly reacting class. The binding of PALA to ATCase is shown in Figure 4 by the linear change in optical density with the ratio of PALA to ATCase. Saturation was attained at 6 mol of PALA per mol of enzyme as found previously by Jacobson and Stark (1973).

Effect of CTP and ATP on Reactivity of Sulfhydryl Groups of ATCase. The results in Table I show that CTP had only a slight effect on the reactivity (rate constant) of the sulfhydryl groups of ATCase when PALA was either absent or present in saturating amounts. However, as is evident from a comparison of Figures 4a and 4b, the distribution of sulfhydryl groups in the "slow" and "fast" classes was markedly affected by CTP when the PALA concentration was insufficient to saturate all the ATCase molecules. The analyses of the biphasic kinetics of the experiments with CTP were slightly more complex since the best fits in terms of two concurrent reactions required slight changes in the value of k_s (from 2.7×10^{-3} to 3.5×10^{-3} s^{-1}) as the amount of PALA increased. Within the precision of the analysis, the fraction of sulfhydryl groups in the "fast" class varied linearly with the molar ratio of PALA to ATCase. Significantly more PALA was required to convert all the groups to the "fast" class in the presence of CTP (Figure 4b) than was required when CTP was absent (Figure 4a).

Addition of carbamoyl phosphate to solutions containing CTP significantly increased the rate of mercaptide formation ($k = 7.5 \times 10^{-3}$ s^{-1}) as compared with solutions containing CTP alone ($k = 2.6 \times 10^{-3}$ s^{-1}). However, as shown in Table I this enhancement by carbamoyl phosphate was not as great as that caused by the substrate when no CTP was present ($k = 9 \times 10^{-3}$ s^{-1}). In solutions containing CTP, carbamoyl

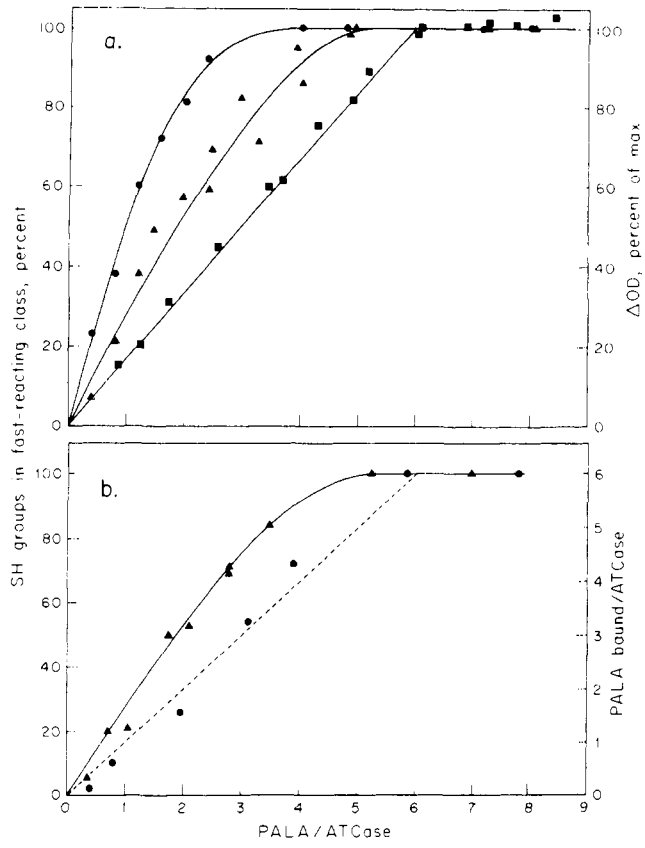

FIGURE 4: Effect of PALA on fraction of sulfhydryl groups in ATCase in fast-reacting class. The fraction of sulfhydryl groups in the fast-reacting class was determined by the procedure described in the legend to Figure 3 and in the Experimental Section. (a) Experiments with and without carbamoyl phosphate. Data indicated by ▲ and ● were obtained with solutions containing no other ligand and 2.1 mM carbamoyl phosphate, respectively (for those symbols the ordinate on the left gives the percent of sulfhydryl groups in the fast-reacting class vs. the number of equivalents of PALA per molecule of ATCase). The symbol ■ represents the change in the ultraviolet difference spectrum as measured by ($\Delta OD_{290} - \Delta OD_{286}$) as a function of the ratio of PALA to ATCase. These measurements were performed with a Cary 14 spectrophotometer equipped with a 0-0.1 expanded scale slide-wire as described by Blackburn and Schachman (1976). The results are plotted as percent of maximum (right ordinate); the maximum change was 0.031 unit for a protein concentration of 2.43 mg/mL in 40 mM potassium phosphate at pH 7. (b) Effect of CTP on the fraction of sulfhydryl groups in the fast-reacting class in solutions containing subsaturating amounts of PALA. All solutions contained 0.21 mM CTP. The symbols ● and ▲ represent experiments in the absence and presence of 2.1 mM carbamoyl phosphate, respectively, and the results are indicated on the left ordinate. The dashed line (- - - -) represents the binding of PALA to ATCase (moles/mole) as determined by spectral titration and indicated on right ordinate.

phosphate and limiting amounts of PALA biphasic kinetics were observed and the curves could be analyzed satisfactorily in terms of two concurrent reactions with $k_s = 7.5 \times 10^{-3}\ s^{-1}$ and $k_f = 19 \times 10^{-3}\ s^{-1}$. The results of these analyses are presented in Figure 4b and it is seen again that binding of carbamoyl phosphate to the sites not occupied by PALA facilitated the conversion of the sulfhydryl groups to the "fast" class (the PALA titration curve is displaced toward the left as compared with the data for solutions containing no carbamoyl phosphate).

Only a few experiments were performed on the effect of ATP on the kinetics of mercaptide formation because of the relatively weak binding of ATP to ATCase and the technical difficulties due to the large absorbance of ATP at 250 nm. As shown in Table I, the pseudo-first-order rate constant was increased slightly by the addition of ATP to solutions containing no ligand, carbamoyl phosphate, PALA, or mixtures of these two ligands.

Reactivity of Sulfhydryl Groups in Hybrid ATCase-Like Molecules Containing Three Active Sites. Pseudo-first-order kinetics were observed for the reaction between excess PMB and a hybrid containing one native C subunit (C_N) and one inactive, pyridoxylated C subunit (C_P) along with three native R subunits (Gibbons et al., 1974). In the absence of any ligands the rate constant for the thiols of $C_NC_PR_3$ was $4.2 \times 10^{-3}\ s^{-1}$ and the reactivity increased to $10 \times 10^{-3}\ s^{-1}$ upon the addition of carbamoyl phosphate (these rates though slightly greater than those for the native enzyme (Table I) were equal to those observed with enzyme molecules reconstituted from isolated C and R subunits). When both carbamoyl phosphate and succinate were present in saturating amounts, the value of k increased to $29 \times 10^{-3}\ s^{-1}$.

Discussion

Resolution of Two Coexisting Conformational States in Solutions of Partially Liganded ATCase. The dissociation of ATCase into two catalytic and three regulatory subunits upon the addition of excess PMB involves a sequence of reactions in which six noncovalent bonding domains between the catalytic and regulatory polypeptide chains must be disrupted (Cohlberg et al., 1972). Despite the complexity of the overall process, the rate of mercaptide formation can be accounted for satisfactorily as a single pseudo-first-order process (Gerhart and Schachman, 1968).[2] In the presence of saturating amounts of both carbamoyl phosphate and succinate or the bisubstrate analogue, PALA, mercaptide formation was still pseudo-first-order and, moreover, the rate of reaction of the 24 sulfhydryl groups on the R subunits was enhanced six- to eightfold. However, when their concentration was too low to saturate the enzyme, these ligands had markedly different effects on the reaction of the enzyme with PMB. With any combination of carbamoyl phosphate and succinate, pseudo-first-order kinetics were observed for the 24 sulfhydryl groups and, depending on the concentration of ligands, the rate constant varied between that for the unliganded enzyme and that found for fully liganded molecules (Figures 1 and 2). In contrast, when fewer than 4 equiv of PALA per ATCase molecule were present, the reaction of ATCase with PMB could not be described by a pseudo-first-order process (Figure 3a). Instead the process of mercaptide formation appeared to be biphasic.

[2] In the experiments of Gerhart and Schachman (1968) the dissociation of ATCase by PMB was shown to be an "all-or-none" process since no bound mercurial could be detected on the intact enzyme molecules and all the cysteinyl residues of the released R subunits were converted to mercaptide complexes regardless of the extent of the reaction. More recently it has been found that the process involves the formation of at least one intermediate which has been shown to be a relatively stable ATCase-like molecule lacking one R subunit (Yang et al., 1974; Evans et al., 1974, 1975; Bothwell and Schachman, 1974). This intermediate reacts further with PMB via a series of steps leading finally to free C and R subunits whose sulfhydryl groups are converted to mercaptide complexes. The observed rate of reaction of the intermediate with PMB is very similar to that of ATCase (S. Subramani and H. K. Schachman, unpublished). Hence the observed pseudo-first-order kinetics for the overall process, as measured by the increase in absorbance at 250 nm (Boyer, 1954), is not unexpected despite the formation and subsequent dissociation of a reactive intermediate. Isolated R subunits react very rapidly with PMB (as yet no rate constant for this reaction is available) and the rate of reaction of the free C subunits with PMB is comparable to that for unliganded ATCase (Gerhart and Schachman, 1968). Thus the reaction between ATCase and excess PMB involving 24 sulfhydryl groups on the three R dimers and 6 on the two C trimers appears to follow pseudo-first-order kinetics. In some of the plots representing the pseudo-first-order kinetics, e.g., Figures 1, 3a, and 3b, slight curvature is observed and it is not yet clear whether the departure from linearity is significant or due to experimental error.

As shown in Figures 3a and 3b the biphasic reaction at subsaturating concentrations of PALA could be accounted for satisfactorily by assuming that there were two concurrent pseudo-first-order reactions with the slow reaction having a rate constant corresponding to that measured for the unliganded enzyme and the last reaction with a rate equal to that for the fully liganded molecules. With these two measured rate constants it was possible to analyze a series of experiments at different ratios of PALA to ATCase. This analysis showed that the fraction of sulfhydryl groups in the "fast" class increased with the amount of PALA added to the enzyme. Moreover, as seen in Figure 4a, all of the sulfhydryl groups appeared in the "fast" class even though there was insufficient PALA to saturate all six active sites of the enzyme. A maximum enhancement in reactivity was attained at a molar ratio of 4 to 5 for PALA to ATCase. This result was virtually identical with that found for the PALA-promoted decrease in the sedimentation coefficient of ATCase (Howlett and Schachman, 1977). Moreover other ligands like carbamoyl phosphate and CTP had similar effects on the PALA-promoted changes in reactivity of the sulfhydryl groups and the sedimentation coefficient of the intact enzyme (Howlett and Schachman, 1977).

It thus appears that the ligand-promoted enhancement in the reactivity of the sulfhydryl groups and the decrease in sedimentation coefficient are both manifestations of the same change in the quaternary structure of the enzyme molecules. This gross conformational change appears to affect the entire enzyme molecule and cannot be attributed to local, direct effects of ligands since the observed increase in reactivity of the sulfhydryl groups occurred on different polypeptide chains from those to which the ligands were bound.[3] In addition the enhancement in reactivity (and decrease in sedimentation coefficient) was clearly not proportional to the fractional occupancy of the active sites by the ligand.

Although the biphasic kinetics (Figure 3) for mercaptide formation could be accounted for in terms of two classes of sulfhydryl groups with reactivities corresponding to either those of unliganded enzyme molecules (i.e., the constrained state) or those of fully liganded ATCase (i.e., the relaxed state), this observation alone does not exclude the possibility of a larger number of reactive species being present. Would a partially liganded enzyme molecule react with a rate corresponding to the unliganded enzyme or the fully liganded species, or would the rate be intermediate between these two extremes? This question was answered by examining the effect of ligands on the rate of reaction of hybrid ATCase-like molecules with excess PMB. The hybrid enzyme, $C_NC_PR_3$, which binds the substrate, carbamoyl phosphate, at all six catalytic chains and the analogue, succinate, at only the three native catalytic chains (Blackburn and Schachman, 1976), exhibited the homotropic and heterotropic effects characteristic of the native enzyme (Gibbons et al., 1974). As seen by the results presented above the hybrid molecules which could bind only 3 equiv of succinate per mol of enzyme exhibited a sevenfold enhancement in the reactivity of its sulfhydryl groups. Moreover, the hybrid showed the same ligand-promoted decrease in sedimentation coefficient as the native enzyme (Gibbons et al., 1974). In the light of these results showing that partially liganded hybrid molecules undergo the complete conformational change characteristic of the native enzyme, it seems reasonable to treat the biphasic kinetics for ATCase in terms of a mixture of molecules some of which are in the constrained state with low reactivity toward PMB and others in the relaxed state with a higher reactivity. Whether a particular molecule is in one or the other state would depend presumably on the extent to which it is liganded.

If both succinate and PALA enhance the reactivity of the sulfhydryl groups of the native enzyme through a similar change in the quaternary structure, why are the pseudo-first-order plots for partially liganded enzyme molecules in Figures 1 and 3 so different? It should be noted that the experimental conditions with succinate and PALA differ markedly. With succinate, which binds only weakly to ATCase (dissociation constant about 10^{-4} M (Changeux et al., 1968)), the ligand-promoted conformational changes are obtained only with a large excess of succinate to binding sites (about 100-fold). When the enzyme is partially saturated with succinate (in the presence of carbamoyl phosphate) there would be an equilibrium mixture of constrained and relaxed ATCase molecules and free succinate (Monod et al., 1965). If the interconversions between these two forms as well as the binding and dissociation reactions involving succinate were rapid compared with the rate of degradation of the enzyme and mercaptide formation, the various equilibria for the unreacted enzyme would be maintained throughout the reaction with PMB and pseudo-first-order kinetics would be obtained. The observed rate constant would be a measure of the fraction of enzyme molecules in the fast-reacting (or relaxed) conformation. In contrast, with PALA which binds very tightly to ATCase (dissociation constant about 10^{-8} M (Collins and stark, 1971)), the changes in the quaternary structure are obtained under conditions where there is virtually no free PALA in the solution (Howlett and Schachman, 1977). Hence in a partially liganded population of enzyme molecules those in the relaxed conformation would react with PMB rapidly (with a rate constant characteristic of that species) and those in the constrained state would react more slowly. Reequilibration of the unreacted enzyme molecules to reestablish the initial distribution of relaxed and constrained enzyme molecules could not occur because of the lack of sufficient free PALA in the solution. Thus biphasic kinetics would be observed. The experimental data can be accounted for satisfactorily in a treatment based on the two-state model of Monod et al. (1965) with parameters given in the following paper (Howlett et al., 1977). However, a test of this treatment and possible modifications of it must await further studies on the detailed mechanism for the dissociation of ATCase into subunits and measurement of the appropriate rate constants.

Effect of Carbamoyl Phosphate on the Reactivity of the Sulfhydryl Groups of ATCase. As shown by Gerhart and Schachman (1968) and extended in this study, the addition of saturating amounts of carbamoyl phosphate to ATCase leads to a threefold enhancement in the reactivity of the sulfhydryl groups of the R subunits. One-half the maximal enhancement was attained in the presence of approximately 0.4 mM carbamoyl phosphate. Moreover, as seen in Figure 2, the same enhancement in reactivity was observed at only 0.1 mM carbamoyl phosphate when succinate was also present (1.5 mM). Succinate, which by itself had no effect on the rate of mercaptide formation, thus caused a significant shift in the concentration dependence of the carbamoyl phosphate promoted

[3] There may be a local effect of carbamoyl phosphate on the reactivity of the sulfhydryl groups of the regulatory subunits. As seen in Table I the rate for ATCase fully liganded with PALA (18×10^{-3} s^{-1}) is less than that for the enzyme in the presence of saturating amounts of carbamoyl phosphate and succinate (25×10^{-3} s^{-1}). Similarly the addition of carbamoyl phosphate to enzyme saturated with PALA led to an increase in the rate constant (from 18×10^{-3} to 21×10^{-3} s^{-1}). It is possible, therefore, that the change in the pseudo-first-order rate constant for ATCase from 3×10^{-3} to 9×10^{-3} s^{-1} upon the addition of carbamoyl phosphate may represent more than merely an alteration in the quaternary structure as represented by the transition from the T to R conformation.

increase in reactivity of the sulfhydryl groups. This effect of succinate in lowering the carbamoyl phosphate requirement can be ascribed to the requirement for ordered binding of ligands to ATCase (Changeux et al., 1968; Porter et al., 1969; Wedler and Gasser, 1974; Jacobson and Stark, 1975).

Since carbamoyl phosphate alone promotes a significant shift in the equilibrium between the constrained and relaxed conformations of ATCase, it is of interest to consider its effect on the PALA-promoted conformational transition of the enzyme. As seen in Figure 3b, ATCase molecules partially saturated with PALA and in the presence of excess carbamoyl phosphate exhibited biphasic kinetics in the reaction with PMB. The curvature in these plots was slight (compared with those in which PALA was the only ligand), but this is to be expected since the pseudo-first-order rate constants, k_f and k_s, differed by only a factor of two. Nevertheless the coexistence of two classes of reacting molecules was evident, and carbamoyl phosphate clearly facilitated the PALA-promoted shift of sulfhydryl groups to the "fast" class. This conclusion is evident from the PALA titration curves in Figure 4a which show the displacement of the curve toward the left for solutions containing carbamoyl phosphate. The combined effect of carbamoyl phosphate and PALA is sufficient to promote the full conformational transition when only 3 equiv of PALA are bound per ATCase molecule (as compared with 4 to 5 equiv in the absence of carbamoyl phosphate). This can be expressed in an alternative manner by noting in Figure 4a that at approximately 1 equiv of PALA per molecule of ATCase the addition of carbamoyl phosphate essentially doubled the fraction of sulfhydryl groups in the fast-reacting class.

Effect of CTP and ATP on Reactivity of Sulfhydryl Groups of ATCase. The effects of CTP and ATP on the reactivity of the sulfhydryl groups are in general agreement with the view that CTP inhibits the enzyme by stabilizing the constrained conformation, whereas ATP is an activator because it binds preferentially to and thereby stabilizes the relaxed state (Gerhart and Pardee, 1962; Bethell et al., 1968; Gerhart and Schachman, 1968; Changeux and Rubin, 1968). As shown in Table I, CTP caused a slight decrease in the pseudo-first-order rate constant whereas the addition of ATP led to an increase. In the presence of carbamoyl phosphate, the effects of the nucleotides were again in opposite directions. However, CTP had virtually no effect when the enzyme was saturated with PALA. These results with CTP are analogous to the observation of Gerhart and Schachman (1968) that 5-bromocytidine triphosphate had no effect on the rate constant when succinate was present at high concentration but did cause a decrease in the reactivity when the concentration of succinate was low. The increase in the rate of reaction of ATCase with PMB caused by the addition of ATP is similar to that observed by Markus et al. (1971) who used a different technique.

In contrast to the active site ligands which bind to the C subunits and alter, by substantial amounts, the reactivity of the sulfhydryl groups on the R subunits, both CTP and ATP bind directly to the R subunits and have much smaller effects on the reactivity of those sulfhydryl groups. The changes in the rate of mercaptide formation promoted by CTP and ATP could arise from local, direct effects in addition to the indirect effects stemming from the gross conformational change generally identified as the allosteric transition affecting the kinetic behavior of the enzyme. Indeed the stabilization of ATCase against denaturation by sodium dodecyl sulfate and proteolytic digestion (Colman and Markus, 1972; McClintock and Markus, 1968) may be attributable to such local effects. That there is an influence of CTP on the equilibrium between the constrained and relaxed conformations is demonstrated by the effect of CTP on the distribution of enzyme molecules between the slow- and fast-reacting classes. The substantial effect of CTP in stabilizing the constrained state is evident from the antagonism of the PALA-promoted isomerization shown in Figures 4a and 4b. Part of this antagonism can be overcome by carbamoyl phosphate (cf. Figures 4a and 4b) which favors the relaxed conformation.

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Allosteric Regulation of Aspartate Transcarbamoylase. Analysis of the Structural and Functional Behavior in Terms of a Two-State Model†

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ABSTRACT: The kinetic and physical properties of the allosteric enzyme, aspartate transcarbamoylase from *Escherichia coli,* have been analyzed according to the two-state model (Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12,* 88). An internally consistent set of calculated parameters accounted quantitatively for the results from diverse experiments including: (a) enzyme kinetics as a function of the concentration of aspartate in the presence of saturating carbamoyl phosphate; (b) gross conformational changes of the enzyme, revealed by the decrease in the sedimentation coefficient and the increase in the reactivity of the sulfhydryl groups of the regulatory subunits, as a function of the extent of saturation of the active sites by the bisubstrate analogue, *N*-(phosphonacetyl)-L-aspartate; (c) stimulation of enzymic activity at low concentrations of the substrate analogue, succinate; and (d) effects of the inhibitor, CTP, and the activator, ATP, on the kinetic and physical properties of the enzyme. The data were interpreted in terms of an equilibrium between a constrained or low-affinity (T) state and a relaxed or high-affinity (R) form of the enzyme and the perturbation of the equilibrium by the addition of various ligands. In the absence of any ligand the equilibrium constant, [T]/[R], was 250 indicating that the T state was 3.3 kcal/mol more stable than the R state. The affinity of the T state for aspartate, succinate, and *N*-(phosphonacetyl)-L-aspartate was at least 20 times weaker than that of the R state. A slight preferential binding of carbamoyl phosphate to the R state caused a shift of the allosteric equilibrium constant to 7. When ATP which also binds slightly better to the R state was present along with carbamoyl phosphate, the equilibrium constant was reduced to 2. In contrast, the slight preferential binding of CTP to the T state led to a shift of the equilibrium constant to 35 when carbamoyl phosphate was present and to 1250 in the absence of that substrate (with ATP and no carbamoyl phosphate the constant is 70). Although the nucleotides and the substrate, carbamoyl phosphate, bind to different polypeptide chains in the enzyme molecule, their effects on the kinetic and physical properties are additive in terms of influencing the allosteric transition. The results cited are consistent with the view that the entire enzyme molecule undergoes a concerted transition which is influenced by binding of different ligands to the various polypeptide chains in the protein.

The demonstration that aspartate transcarbamoylase (ATCase)[1] (EC 2.1.3.2; carbamoylphosphate:L-aspartate carbamoyltransferase) from *Escherichia coli* exhibits a sigmoidal dependence of activity on substrate concentration and is subject to feedback inhibition by CTP (Gerhart and Pardee, 1962) represents one of the earliest examples of the regulation of enzyme activity. When it was shown subsequently (Gerhart and Schachman, 1965) that ATCase contained distinct catalytic and regulatory subunits, these two phenomena characteristic of allosteric enzymes, termed homotropic and heterotropic effects (Monod et al., 1965), became the subject of investigations relating structure to function (Gerhart, 1970; Jacobson and Stark, 1973a; Schachman, 1974). Both allosteric effects have been postulated to be the consequence of ligand-promoted conformational changes in the enzyme whereby the binding of ligands to the oligomeric protein affects the subsequent binding to other sites on the same molecule (Monod et al., 1965; Koshland et al., 1966).

Ligands which bind to the active sites on the catalytic subunits of ATCase lead not only to an increase in the effective hydrodynamic volume of the molecules but also to a sixfold enhancement of the chemical reactivity of the 24 sulfhydryl groups on the regulatory subunits (Gerhart and Schachman, 1968). These alterations in the properties of ATCase upon the binding of active-site ligands are indicative of a gross conformational change in the enzyme molecules and have been interpreted (Changeux and Rubin, 1968) in terms of the model proposed by Monod et al. (1965). However, at that time knowledge of the structure of ATCase was insufficient and binding data for substrate analogues were inadequate. Hence a renewed attempt was made to determine whether the two-state model could account for additional enzyme kinetic data and new results relating conformational changes to the extent of binding of the bisubstrate analogue, *N*-(phosphonacetyl)-L-aspartate (PALA).

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[1] Abbreviations used are: ATCase, aspartate transcarbamoylase; PALA, *N*-(phosphonacetyl)-L-aspartate; CbmP, carbamoyl phosphate; Asp, aspartate; PMB, *p*-mercuribenzoate; n_H, Hill coefficient.