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# Allosteric Regulation of Aspartate Transcarbamoylase. Changes in the Sedimentation Coefficient Promoted by the Bisubstrate Analogue *N*-(Phosphonacetyl)-L-aspartate†

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ABSTRACT: Although it is known that aspartate transcarbamoylase from *Escherichia coli* undergoes a 3.6% *decrease* in sedimentation coefficient upon the addition of the substrate, carbamoyl phosphate, and succinate, an analogue of the second substrate, aspartate, it has been difficult to establish an exact relationship between the conformational change and the extent of ligand binding because of the low affinity of the enzyme for succinate. Hence studies were performed on the enzyme with the bisubstrate analogue, *N*-(phosphonacetyl)-L-aspartate (PALA) since its binding is determined readily. The maximal change in sedimentation coefficient, −3.1%, was attained when only four of the six active sites in the enzyme were saturated. Carbamoyl phosphate and ATP facilitated the conformational transition promoted by PALA while CTP retarded the transition. Moreover the effects of carbamoyl phosphate and the nucleotide effectors were additive; with both carbamoyl phosphate and ATP present only 2.5 mol of PALA per mol of enzyme was required to produce a maximal decrease in sedimentation coefficient. ATP or CTP alone caused a reduction in sedimentation coefficient of about −0.5%. In contrast, when carbamoyl phosphate was also present, the change promoted by ATP was −1.1% whereas that caused by CTP was −0.5%. The conformational changes in aspartate transcarbamoylase appear to be concerted and the results are consistent with the two-state model of allosteric proteins (Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12,* 88). Analogous studies with the purified catalytic subunit, which exhibits noncooperative enzyme kinetics, showed that the *increase* in the sedimentation coefficient, +1.4%, promoted by PALA was closely linked with binding.

All molecular models proposed to account for the cooperativity exhibited by allosteric enzymes implicate ligand-promoted conformational changes of the oligomeric protein from a constrained or low-affinity state to a relaxed form with catalytic sites having a high affinity for substrates (Monod et al., 1965; Koshland et al., 1966). In this series of papers on the regulatory enzyme, aspartate transcarbamoylase (ATCase)[1] (EC 2.1.3.2; carbamoylphosphate:L-aspartate carbamoyltransferase) from *Escherichia coli,* we present studies of the conformational changes of the enzyme as a function of its extent of saturation by ligands in order to test whether the experimental results can be explained by the simple hypothesis that the allosteric transition from one state to the other is concerted.

As shown by Gerhart and Schachman (1968) the addition of the substrate, carbamoyl phosphate, and the substrate analogue, succinate, to ATCase led to a 3.6% decrease in the sedimentation coefficient and to a six-fold increase in the reactivity of the sulfhydryl groups of the regulatory subunits of the enzyme. These changes appeared to attain their maximal values prior to the saturation of the enzyme by succinate (Changeux et al., 1968). However, the earlier binding data for succinate are not readily rationalized with the present structural evidence that ATCase contains six catalytic polypeptide chains distributed within two catalytic trimers (Weber, 1968; Wiley and Lipscomb, 1968; Meighen et al., 1970; Rosenbusch and Weber, 1971; Cohlberg et al., 1972). Hence it seemed

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[1] Abbreviations used are: ATCase, aspartate transcarbamoylase; PALA, *N*-(phosphonacetyl)-L-aspartate; DEAE, diethylaminoethyl.

desirable to reexamine the conformational changes at varying degrees of saturation of the enzyme with the bisubstrate analogue, *N*-(phosphonacetyl)-L-aspartate (PALA), which binds with high affinity (dissociation constant about $10^{-8}$ M) to the six catalytic sites of ATCase (Collins and Stark, 1971; Jacobson and Stark, 1973). PALA is a particularly useful ligand since it not only binds tightly to the enzyme in the absence of carbamoyl phosphate but it also binds competitively with carbamoyl phosphate (the affinity of the enzyme for PALA is about $10^3$ times that for carbamoyl phosphate) (Collins and Stark, 1971). Indeed at low levels of PALA where only a few of the active sites are occupied the enzyme is activated in a manner analogous to that observed at low concentrations of succinate (Gerhart and Pardee, 1963; Collins and Stark, 1971). Hence it is possible to titrate the change in the sedimentation coefficient of ATCase with PALA alone or in the presence of carbamoyl phosphate. In the latter case some of the sites would be occupied by PALA and others in the same molecule would have bound substrate. Thus it is possible to determine the effect of the substrate on the PALA-promoted conformational transition. Similarly it is possible to measure the effect of the inhibitor, CTP, and the activator, ATP, on the titration curve.

As shown below, the change in the sedimentation coefficient of ATCase attains its maximal value even though several sites are unliganded and the data are consistent with the view that the enzyme undergoes a concerted conformational change which is affected by the presence of carbamoyl phosphate, CTP and ATP. In the second paper in this series (Blackburn and Schachman, 1977), analogous studies are presented on the change in the reactivity of the sulfhydryl groups of the enzyme. Both sets of data are treated in the final paper (Howlett et al., 1977) according to the theoretical formulation of the model of Monod et al. (1965) and it is shown that this model accounts satisfactorily for the experimental findings.

## Experimental Section

### *Materials*

ATCase preparations purified according to Gerhart and Holoubek (1967) were found by polyacrylamide gel electrophoresis to contain small amounts of a species lacking one regulatory subunit (Yang et al., 1974) and small amounts of aggregated material. Accordingly purified regulatory subunit was added to these preparations and chromatography of the resulting solutions on Sephadex G-200 yielded much more homogeneous samples of ATCase which were used for the difference sedimentation experiments. Protein concentrations were determined spectrophotometrically at a wavelength of 280 nm with an extinction coefficient of 0.59 $cm^2$ $mg^{-1}$ or ultracentrifugally with a synthetic boundary cell and interference optics (Richards and Schachman, 1959; Babul and Stellwagen, 1969). A value of $1.83 \times 10^{-3}$ dL/g was used for the specific refractive increment at 546 nm. Catalytic subunit was prepared from ATCase using neohydrin and DEAE-cellulose chromatography (Kirschner, 1971), and the concentration was determined spectrophotometrically at a wavelength of 280 nm using an extinction coefficient of 0.72 $cm^2$ $mg^{-1}$ (Kirschner, 1971).

*N*-(Phosphonacetyl)-L-aspartate (PALA) was a generous gift from Dr. G. R. Stark. The concentration of PALA was determined by acid hydrolysis followed by aspartate analysis using a Beckman 120 B amino acid analyzer. Dilithium carbamoyl phosphate was obtained from Sigma Chemical Co. and purified further by precipitation from cold water and ethanol (Gerhart and Pardee, 1962). Aliquots of a stock solution were stored at −20 °C and thawed just prior to use. CTP and ATP were purchased from Sigma Chemical Co. Succinate was obtained from Eastman Organic Chemicals. Most of the experiments were performed with 40 mM potassium phosphate buffer at pH 7.0 ± 0.02, containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA.

### *Methods*

*Ultracentrifugation.* Sedimentation experiments were performed with a Beckman-Spinco Model E ultracentrifuge. Two ultracentrifuge cells were used in each experiment; both cells contained 12-mm, 2° single-sector charcoal-filled epon centerpieces. One cell had conventional plane quartz windows, while the other had a lower plane window and either a 1° positive or negative wedged upper window. In this way the schlieren pattern of the second cell was displaced relative to that of the other cell, permitting measurements to be made simultaneously on both solutions. The reference hole of the An-D rotor was modified as discussed by Gerhart and Schachman (1968). The rotor temperature during the course of each experiment was controlled and monitored by use of the RTIC unit. All experiments were performed at 20 ± 2 °C.

For measurements of the effect of PALA on the sedimentation coefficient of ATCase, solutions were prepared as follows. A small aliquot of PALA solution (4.5 mM) was added with a Hamilton syringe to solutions of enzyme in the appropriate buffer. In the same manner reference solutions were prepared by adding $H_2O$ instead of the PALA solution. Since the final concentration of PALA was generally less than 0.1 mM and most of it was bound to protein, the contribution of PALA to the viscosity and density of the solution was negligible. In order to test the effect of various ligands (carbamoyl phosphate, ATP, and CTP) on the PALA titration curve, the ligand was added to the stock solution of ATCase and solutions were prepared as above. In this way the effects on the sedimentation of ATCase due to the viscosity and density increments of the ligands in the reference and sample solutions cancelled. The effects of these ligands alone on the sedimentation coefficient of ATCase involved corrections for the density, viscosity, and buoyant density increments due to the ligands. Values for the partial specific volume of carbamoyl phosphate (0.48 mL/g), succinate (0.61 mL/g), and phosphate (0.35 mL/g) were those used by Kirschner and Schachman (1971a) which had been calculated according to the method of Traube (1899) and McMeekin et al. (1949). In a similar way a value of 0.52 mL/g was estimated for the partial specific volume of PALA. The partial specific volume used for CTP was 0.44 mL/g (Cohlberg, 1972) and the same value was assumed for ATP. Corrections for the viscosity of the ligands involved the use of the following reduced viscosities: ATP (3.33 mL/g), CTP (3.21 mL/g), and succinate (3.39 mL/g) (J. Fraser and H. K. Schachman, unpublished data). The reduced viscosity of carbamoyl phosphate was assumed to be 3.3 mL/g. For the buoyant density corrections made according to Kirschner and Schachman (1971a), it was assumed that the volume change on binding of ligands was zero and that the binding of one molecule of either carbamoyl phosphate, PALA, ATP, or CTP caused the displacement of one molecule of phosphate. The binding of each molecule of PALA to ATCase in the presence of carbamoyl phosphate was assumed to displace one molecule of carbamoyl phosphate. It was assumed further that, under the conditions used, carbamoyl phosphate bound to six sites on ATCase and that the binding constants obtained by Winlund and Chamberlin (1970) for the binding of ATP and CTP to ATCase in 10 mM phosphate buffer, pH

7, were the same for the 40 mM phosphate buffer used in this study.

*Computations.* A Gaertner two-dimensional microcomparator was used to measure the change in the $r$ position of the maximum ordinate of the schlieren peak as a function of time, $t$, and the sedimentation coefficient was calculated from the slope of the plot of ln $r$ versus $t$. Where necessary the measured sedimentation coefficients were corrected to values ($s_{20,w}$) corresponding to a solvent with the viscosity and density of water at 20 °C (Svedberg and Pedersen, 1940). Calculations were performed with the aid of a Hewlett-Packard 9820a calculator equipped with a 9862a calculator plotter. It was found that by using two cells in the same rotor the difference in the two sedimentation coefficients could be measured with a standard deviation of 0.005–0.01 S, which for ATCase, with a sedimentation coefficient, $s_{20,w}$, of 11.7 S, corresponds to an error less than 0.1%. The difference in sedimentation coefficients, $\Delta s$, was calculated as follows.

The differential equation relating boundary position, $r$, to the time, $t$, is written for each solution and that for the reference solution, 1, is subtracted from the equation for the sample containing the ligand, 2, to give

$$\frac{1}{\omega^2}\frac{d}{dt}(\ln r_2 - \ln r_1) = \Delta s \qquad (1)$$

where $\Delta s$ is the difference between the two sedimentation coefficients, $s_2$ and $s_1$. This equation can be rearranged to give

$$\frac{1}{\omega^2}\left(r_1\frac{dr_2}{dt} - r_2\frac{dr_1}{dt}\right) = r_1 r_2 \Delta s \qquad (2)$$

Introducing the terms, $\bar{r} = (r_1 + r_2)/2$, and $\Delta r = r_2 - r_1$, followed by collection of the appropriate terms gives

$$\frac{1}{\omega^2}\frac{d(\Delta r/\bar{r})}{dt} = \frac{r_1 r_2}{\bar{r}^2}\Delta s \qquad (3)$$

For small values of $\Delta r$, eq 3 reduces to

$$\frac{1}{\omega^2}\frac{d(\Delta r/\bar{r})}{dt} = \Delta s \qquad (4)$$

This treatment is similar to the procedure of Springer et al. (1974) for the analysis of difference sedimentation equilibrium experiments. The standard deviation in $\Delta s$ is computed directly from plots of $(\Delta r/\bar{r})$ versus $t$. Values of $\Delta s$ obtained from eq 4 are combined with the values of the sedimentation coefficient of the reference solution, $s_1$, to calculate the fractional change in sedimentation coefficient, $\Delta s/s$, caused by the addition of ligand. This procedure minimizes errors due to plate alignment and is similar to that proposed by Schumaker and Adams (1968), although it did not involve extrapolation of the values of $\Delta s/s$ to infinite dilution of protein. Since the small effects due to radial dilution in each sector effectively cancel, it was consjdered preferable to perform all difference sedimentation experiments at a fixed protein concentration rather than invoke the uncertainties inherent in such correction procedures.

## Results

*Effect of PALA on the Sedimentation Coefficient of ATCase.* As seen in Figure 1, the sedimentation coefficient of ATCase *decreased* progressively upon the addition of increasing amounts of PALA.[2] At a molar ratio of PALA to ATCase of about 4 this decrease attained its maximum value, −3.1% for $\Delta s/s$, and subsequent additions of PALA had no further effect. Figure 1 shows also the significant displacement of the PALA titration curve caused by the presence of the substrate, carbamoyl phosphate, at a concentration of 1 mM. In these solutions the plateau value of −3.1% for $\Delta s/s$ was obtained when there were only about 3 molecules of PALA per ATCase molecule. Since the same maximum value of −3.1% was observed in the presence or absence of carbamoyl phosphate, it seemed that this substrate did not cause any significant change in the sedimentation coefficient of the enzyme.[3] This conclusion is supported by the direct measurement of the effect of carbamoyl phosphate. As seen in Table I the measured value of $\Delta s/s$ due to 1 mM carbamoyl phosphate was only −0.08%. Correction of this value for the density and viscosity increments resulting from the carbamoyl phosphate and the contribution to the buoyancy from the bound ligand gave a value of −0.09% for $(\Delta s/s)_{corr}$. Table I also summarizes the results for the effect of the two ligands, carbamoyl phosphate and succinate. The measured value of $-3.2_5$% for $\Delta s/s$ upon correction is −3.3% which is in good agreement with the plateau value, −3.5% for $(\Delta s/s)_{corr}$, obtained with PALA.

Figure 1 also summarizes data for the spectral change in ATCase caused by the addition of PALA. In marked contrast to the sedimentation data, the spectral change (ΔOD) varies linearly with the amount of PALA and reaches a plateau at a molar ratio of 6 for PALA/ATCase. This result is in excellent agreement with the prior observations (Collins and Stark, 1971; Jacobson and Stark, 1973) showing that this bisubstrate analogue binds with high affinity to the 3 active sites of the isolated catalytic subunits and to the 6 sites of the intact enzyme.

*Effect of Nucleotides on the Conformational Transition Promoted by PALA.* Figure 2a shows the effect of PALA on the sedimentation coefficient of ATCase in the presence of the

[2] Previous studies on the ligand-promoted conformational changes in ATCase (Gerhart and Schachman, 1968) were performed with phosphate buffers. Imidazole acetate was considered as an alternative buffer because phosphate ions have been found to be competitive with the binding of carbamoyl phosphate (Porter et al., 1969). However, the concentration dependence of the sedimentation coefficient of ATCase in the imidazole buffer was indicative of an association-dissociation equilibrium which could be described by a monomer-dimer equilibrium with an association constant of $3 \times 10^3$ $M^{-1}$. Moreover the sedimenting boundaries were much broader than those obtained in phosphate buffers. Upon the addition of PALA to the enzyme in the imidazole buffer the boundaries became much sharper and the concentration dependence of the sedimentation coefficient corresponded to that expected for nonassociating globular proteins (Smith et al., 1973). Although the effects of the association-dissociation equilibrium could be eliminated by extrapolating all values of $\Delta s/s$ to infinite dilution, it seemed preferable to circumvent this complication by using phosphate buffer for most of the studies. In phosphate, both in the absence and presence of PALA, there was no evidence for association of ATCase molecules. Moreover similar values of $\Delta s/s$ were obtained in the two buffers when the observed values were extrapolated to infinite dilution.

[3] During the course of this work it became apparent that in $\Delta s/s$ studies with both ATCase and catalytic subunit the use of 2 mM carbamoyl phosphate yielded results slightly different from those with 1 mM carbamoyl phosphate. The effect was apparently specific for ATCase and catalytic subunit since 1 mM or 2 mM carbamoyl phosphate had no effect on the sedimentation coefficient of aldolase. Since the binding constant for carbamoyl phosphate binding to ATCase in phosphate buffer is less than 0.1 mM (Y. R. Yang and H. K. Schachman, unpublished data), it is unlikely that the effect was due to failure to saturate the enzyme. Carbamoylation of ATCase is known to occur at higher concentrations of carbamoyl phosphate (Nelbach, 1972) and it is possible that a specific carbamoylation may be the reason for the above discrepancy. It was found that 10 mM potassium cyanate caused a change in the sedimentation coefficient of ATCase of −0.35%. There seemed to be a time dependence in the $\Delta s/s$ measured for the effect of carbamoyl phosphate on ATCase, although these results were somewhat irreproducible. If carbamoylation was the source of variability in $\Delta s/s$ measurements, then this would emphasize the need for the use of freshly prepared carbamoyl phosphate solutions in difference sedimentation velocity measurements.

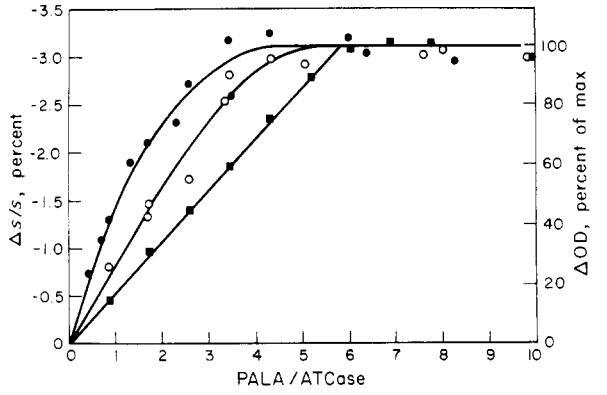


FIGURE 1: Effect of PALA on the sedimentation coefficient of ATCase. For all sedimentation velocity experiments, the protein concentration was 3 mg/mL and the buffer was 40 mM potassium phosphate, pH 7.0, containing 0.2 mM EDTA and 2 mM 2-mercaptoethanol. In each experiment the sedimentation coefficient of the sample solution containing PALA was determined relative to a reference solution of the enzyme which did not contain the bisubstrate analogue. Results, designated by ○, show the effect of PALA, in terms of molar ratio of PALA to ATCase, on $\Delta s/s$. Analogous results for solutions containing carbamoyl phosphate (1 mM) in both the sample and reference solutions are represented by ●. The temperature for all experiments was 20 ± 2 °C. The binding of PALA to ATCase, designated by ■, was measured by the change in the ultraviolet absorption spectrum (Collins and Stark, 1971; Jacobson and Stark, 1973). Data are plotted as percent of maximal change in optical density ($\Delta OD_{290nm} - \Delta OD_{286nm}$) versus molar ratio of PALA to ATCase. The maximum change was 0.031 unit for a protein concentration of 2.4 mg/mL in 40 mM potassium phosphate at pH 7.0.

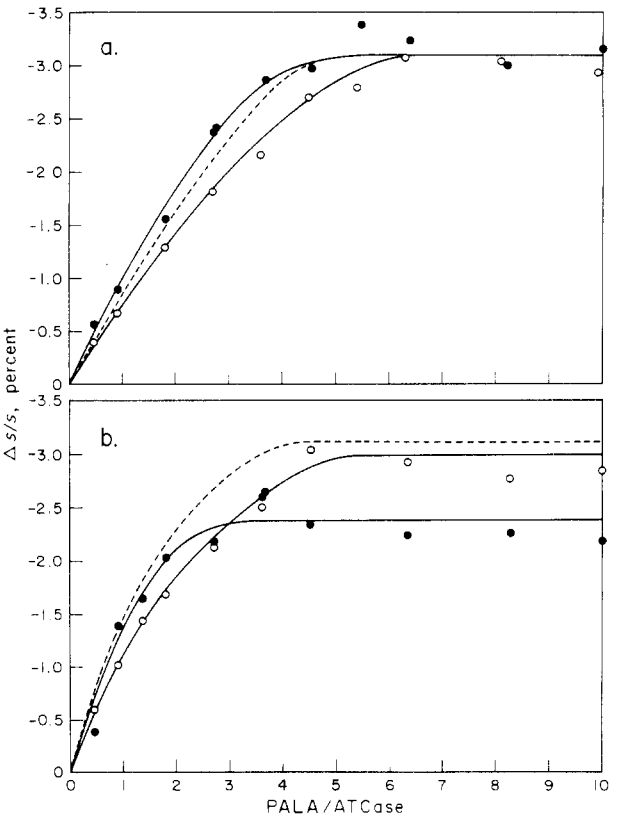


FIGURE 2: Effect of ATP and CTP on the PALA-promoted change in the sedimentation coefficient of ATCase in the absence (a) and presence (b) of carbamoyl phosphate (1 mM). Experiments were conducted as described in the legend of Figure 1. (a) Data represented by ● were obtained with 2 mM ATP in both the sample and reference solutions and data, designated by ○, were determined with solutions containing 0.5 mM CTP. The dashed curve (- - -) from Figure 1 represents the results for the PALA titration curve in the absence of other ligands. (b) Data represented by ● show the effect of PALA on $\Delta s/s$ in the presence of 2 mM ATP and 1 mM carbamoyl phosphate in both the sample and reference solutions and the data, designated by ○, were obtained with both 0.5 mM CTP and 1 mM carbamoyl phosphate in both solutions. The dashed curve (- - -) represents the data from Figure 1 for the effect of PALA in the presence of carbamoyl phosphate (1 mM).

TABLE I: Effect of Ligands on the Sedimentation Coefficient of ATCase and Catalytic Subunit.[a]

| Sample | Ligand[b] | $(\Delta s/s)$ (%) | $(\Delta s/s)_{corr}$[c] (%) |
|---|---|---|---|
| ATCase | None | −0.06 | −0.06 |
| ATCase | Carbamoyl phosphate | −0.08 | −0.09 |
| ATCase | Carbamoyl phosphate + succinate | $-3.2_5$ | −3.3 |
| ATCase | PALA | $-3.1_0$ | −3.5 |
| Catalytic subunit | Carbamoyl phosphate | +0.6 | +0.6 |
| Catalytic subunit | Carbamoyl phosphate + succinate | +1.6 | +1.3 |
| Catalytic subunit | PALA | +1.8 | +1.2 |

[a]Sedimentation velocity experiments were performed at 20 ± 2 °C. The buffer was 40 mM potassium phosphate, pH 7.0, containing 0.2 mM EDTA and 2 mM 2-mercaptoethanol. [b]The concentrations of carbamoyl phosphate, succinate, and PALA were 1, 3, and 0.08 mM, respectively. [c]The changes in sedimentation coefficient were corrected for the density and viscosity contributions of the unbound ligands and for the change in buoyant weight due to binding as described in Methods.

inhibitor, CTP (0.5 mM), and the activator, ATP (2 mM). Included in the figure for comparison is the curve (from Figure 1) for the effect of PALA in the absence of any other ligands. Several features merit comment. First, all three curves are similar in that there is a progressive *decrease* in the sedimentation coefficient of the enzyme upon the addition of PALA and the plateau value is about −3.1% for $\Delta s/s$. Second, although the effects of CTP and ATP on the conformational transition are small, there does seem to be a general tendency for CTP to inhibit the conformational change and for ATP to promote the transition. In the presence of CTP the maximum value of $\Delta s/s$ is observed only when the enzyme is almost completely saturated with PALA (a molar ratio of about 6). Since the effect of ATP was qualitatively similar to that observed with carbamoyl phosphate and CTP had the opposite effect, it seemed of interest to examine the influence of nucleotides on the PALA-promoted conformational transition in the presence of carbamoyl phosphate.

In Figure 2b results are presented for the change in the sedimentation coefficient of ATCase as a function of added PALA in the presence of both 1 mM carbamoyl phosphate and either 0.5 mM CTP or 2 mM ATP. Shown for comparison as a broken line is the PALA conformational titration curve (from Figure 1) obtained in the presence of 1 mM carbamoyl phosphate. Several features of these results are noteworthy. First, in comparison with Figure 2a both the CTP and ATP curves were shifted to the left by the addition of carbamoyl phosphate. In the presence of the substrate and CTP the plateau value of $\Delta s/s$ is attained at a PALA/ATCase ratio of about 5 as compared with 6 when carbamoyl phosphate was absent. Similarly this ratio was only about 2.5 when both ATP and carbamoyl phosphate were present as compared with a value of about 3 with only carbamoyl phosphate or about 4 when only ATP was

TABLE II: Effect of Nucleotides on the Sedimentation Coefficient of ATCase.[a]

| Ligands | | $(\Delta s/s)$ | $(\Delta s/s)_{corr}$[c] |
|---|---|---|---|
| Sample | Reference | (%) | (%) |
| ATP (2 mM) | CTP (2 mM) | −0.02 | −0.02 |
| ATP (2 mM) + carbamoyl phosphate[b] | CTP (2 mM) + carbamoyl phosphate[b] | −0.63 | −0.63 |
| CTP (0.5 mM) | None | +0.73 | −0.53 |
| CTP (2 mM) | None | +0.72 | −0.46 |
| CTP (0.5 mM) + carbamoyl phosphate[b] | Carbamoyl phosphate[b] | +0.72 | −0.54 |
| ATP (2 mM) | None | +0.74 | −0.38 |
| ATP (2 mM) + carbamoyl phosphate[b] | Carbamoyl phosphate[b] | +0.03 | −1.09 |

[a]Experiments were performed at 20 ± 2 °C as described in Methods. The buffer was 40 mM potassium phosphate, pH 7.0, containing 0.2 mM EDTA and 2 mM 2-mercaptoethanol. [b]Carbamoyl phosphate concentration was 1 mM. [c]The changes in sedimentation coefficient were corrected for the density and viscosity contributions of the unbound ligands and for the change in buoyant weight due to binding; the procedure is described by Kirschner and Schachman (1971a) and in Methods.

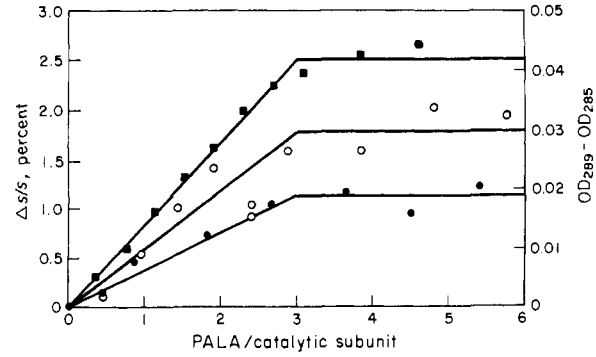


FIGURE 3: Effect of PALA on the sedimentation coefficient of the catalytic subunit of ATCase. The PALA-promoted change was measured as described in the legend to Figure 1 and in Methods. Experiments with solutions containing no additional ligands are designated by ○ and those with solutions containing 1 mM carbamoyl phosphate in both the sample and reference are represented by ●. Binding data measured from difference spectra ($\Delta OD_{289nm} - \Delta OD_{286nm}$) are represented by ■. The protein concentration was 2.6 mg/mL.

present. Second, the maximum value of $\Delta s/s$ in the presence of CTP and carbamoyl phosphate was −3%, a value which is approximately equal to that (−3.1%) observed in the absence of CTP. In contrast, the maximum value of $\Delta s/s$ was only −2.4% when both ATP and carbamoyl phosphate were present.

*Effect of Nucleotides on the Sedimentation Coefficient of ATCase in the Absence of PALA.* Since, in the presence of varying amounts of PALA, CTP inhibits or retards the conformational transition of ATCase and ATP promotes or favors the conformational change, it seemed of interest to examine the effect of the nucleotides directly on the sedimentation coefficient of the enzyme.

As seen in Table II, the value of $\Delta s/s$ for ATCase in the presence of ATP as compared with the enzyme with CTP was −0.02%. In this experiment the nucleotide concentrations were 2 mM which is sufficiently high to saturate the 6 binding sites on the regulatory chains of the enzyme (Gray et al., 1973). Hence buoyant corrections due to the bound ligand are very nearly equal. Also the corrections due to the viscosity and density increments from the free nucleotides are virtually the same. Thus ATP and CTP either have no effect on the sedimentation coefficient of the enzyme or they cause similar changes. When, however, this same experiment is performed with carbamoyl phosphate present in both solutions, the value of $\Delta s/s$ was −0.63%. This result indicates that ATCase is partially converted to the slower sedimenting conformational state by the addition of both carbamoyl phosphate and ATP.

Table II also presents additional data for the effects of CTP and ATP on the sedimentation coefficient of the enzyme without compensating ligands in the reference solution. For example, the addition of CTP at 0.5 mM and 2 mM led to values of $\Delta s/s$ of +0.73% and +0.72%, respectively, or −0.53% and −0.46% for $(\Delta s/s)_{corr}$. The addition of carbamoyl phosphate along with the CTP had no further effect on the value of $\Delta s/s$. With ATP alone the observed value of $\Delta s/s$ was +0.74% and the corrected value was −0.38%. But ATP in the presence of carbamoyl phosphate as compared with carbamoyl phosphate alone gave a measured $\Delta s/s$ of +0.03%, a value significantly lower than that caused by CTP in the presence of carbamoyl phosphate (+0.72%). This value of $\Delta s/s$ due to ATP in the presence of carbamoyl phosphate becomes −1.09% after correction for the molecular weight and density of the bound ATP and contributions to the viscosity and density of the solution from the ATP. Although there are some uncertainties in the parameters involved in the calculation of $(\Delta s/s)_{corr}$ from the experimental values of $\Delta s/s$, it is clear that the addition of carbamoyl phosphate to both solutions has no effect on the magnitude of the change in sedimentation coefficient of ATCase caused by CTP. In contrast, Table II shows that the presence of the substrate influences markedly the value of $\Delta s/s$ produced by ATP. Thus the two ligands together promote a conformational change in the enzyme from a fast-sedimenting species to a more slowly sedimenting form.

*Effect of Ligands on the Sedimentation Coefficient of the Catalytic Subunit of ATCase.* Figure 3 presents the results of a series of difference sedimentation velocity experiments on the catalytic subunit of ATCase at varying degrees of saturation with PALA. Spectral data are also presented which are in agreement with the findings of Collins and Stark (1971) showing that 3 molecules of PALA are tightly bound to the subunit. Unlike the intact enzyme, the catalytic subunit exhibits an *increase* in sedimentation coefficient upon the binding of PALA. Moreover, this change is closely linked with the binding of the ligand as seen by the approximately linear plots of $\Delta s/s$ versus the ratio of PALA to catalytic subunit and the attainment of plateau values of $\Delta s/s$ when this ratio was 3. This behavior was observed both in the presence and absence of carbamoyl phosphate where the values of $\Delta s/s$ were +1.1% and 1.8%, respectively. Since PALA is known to displace carbamoyl phosphate from the catalytic subunit (Collins and Stark, 1971), this difference between the values of $\Delta s/s$ indicates that carbamoyl phosphate causes an increase in the sedimentation coefficient amounting to 0.7%. This conclusion from the plateau values in Figure 3 is supported by the direct measurement (see Table I) of 0.6% for $\Delta s/s$ caused by the addition of carbamoyl phosphate to the catalytic subunit. Table I also shows that the addition of carbamoyl phosphate and succinate caused an increase in the sedimentation coefficient of 1.6%, which corresponds to 1.3% for $(\Delta s/s)_{corr}$. This value is in excellent agreement with that produced by adding PALA to the catalytic subunit.

## Discussion

Small changes in the sedimentation coefficient of a protein as a result of the addition of a specific ligand can be attributed to a number of factors which have been discussed in detail elsewhere (Richards and Schachman, 1959; Gerhart and Schachman, 1968; Schumaker, 1968; Kirschner and Schachman, 1971a,b). Distinguishing between a conformational change in the protein which affects the frictional coefficient and a perturbation in an association–dissociation equilibrium involving different oligomeric species requires additional information, such as difference sedimentation equilibrium measurements (Springer et al., 1974; Springer and Schachman, 1974) or the concentration dependence of the sedimentation coefficient (Smith et al., 1973).

The results on ATCase in imidazole buffer indicated clearly the hazards in interpreting the ligand-promoted change in the sedimentation coefficient. As shown by the dependence of the sedimentation coefficient on concentration there was significant association of the enzyme when no ligand was present. Upon the addition of PALA, however, the concentration dependence was altered to that of a globular, nonassociating species. In contrast the dependence of the sedimentation coefficient of the enzyme in phosphate buffer was virtually linear both in the absence and presence of PALA. The results could be described by the equation

$$s = s_0(1 - kc) \tag{5}$$

where $s_0$ is the sedimentation coefficient at infinite dilution, $c$ is the protein concentration, and the parameter, $k$, is related to the properties of the macromolecules (Schachman, 1959; Creeth and Knight, 1965). In the absence of PALA, $k$ was 0.007 L/g and it increased slightly to 0.0077 L/g upon the addition of PALA. This small change in the value of $k$ which may be ascribed to an increased hydrodynamic resistance experienced by the liganded ATCase molecules is similar to that observed earlier by Gerhart and Schachman (1968) who used carbamoyl phosphate and succinate (instead of PALA). Because of this difference in $k$, the values of $\Delta s/s$ at any particular ratio of PALA/ATCase depend on the protein concentration. Hence all difference sedimentation velocity experiments were conducted at a fixed concentration of ATCase (approximately 3 mg/mL). The values of $\Delta s/s$ obtained in this way were slightly greater than those evaluated for infinite dilution by extrapolation of the data at various protein concentrations.

Since the ligand-promoted *decrease* in the sedimentation coefficient of ATCase in phosphate buffer cannot be attributed to a change in the state of association of the enzyme, we must look elsewhere for an explanation. The experimental results could be interpreted in terms of an increase in either the partial specific volume or the frictional coefficient (or both). As pointed out by Gerhart and Schachman (1968), the binding of a ligand of higher density than the protein probably would not lead to an increase in partial specific volume. Moreover, it is unlikely that a change in partial specific volume upon binding of ligand to the active sites would account for both the *decrease* in sedimentation coefficient of ATCase and the *increase* observed for the catalytic subunit. Thus the ligand-promoted decrease in the sedimentation coefficient of ATCase was attributed to an increase in the frictional coefficient of the hydrodynamic unit (Gerhart and Schachman, 1968). This conclusion gained support recently from the observation (Dubin and Cannell, 1975) that the diffusion coefficient of ATCase decreased by about 4% upon the addition of carbamoyl phosphate and succinate. An increase in the frictional coefficient of ATCase would occur if the molecules became more elongated (or flattened) or their effective volume was increased (about 10%). For our present purposes a distinction between these alternatives is not necessary. It is sufficient to conclude that the conformation of the enzyme is altered upon binding PALA and that the liganded protein can be considered as existing in a more "swollen" state than the enzyme in the absence of ligands. Here we are concerned primarily with the manner in which the conformational change is achieved and, in particular, with the dependence of the conformational change on the extent of saturation of the enzyme by PALA.

As seen in Figures 1 and 2, the *decrease* in the sedimentation coefficient of ATCase associated with the binding of PALA is loosely linked to the degree of ligand saturation. These results with the intact enzyme are in marked contrast with those for the isolated catalytic subunit. With the subunit, there was an *increase* in the sedimentation coefficient upon binding PALA which can be interpreted as resulting from a conversion of the protein to a more compact (or symmetrical) structure. Moreover, as shown in Figure 3, the observed changes in the sedimentation coefficient were proportional to the extent of saturation of the active sites by PALA. The catalytic subunit is noncooperative in its kinetic behavior and the tightly linked conformational changes associated with ligand binding to it have been interpreted in terms of local effects (Collins and Stark, 1969; Kirschner and Schachman, 1973a,b).

The conversion of ATCase to the more slowly sedimenting conformation was complete when only a fraction of the PALA binding sites was saturated. In the presence of carbamoyl phosphate and ATP, the enzyme has undergone the complete transition to the "swollen" conformation even though only two of the active sites are liganded with PALA (the remaining four are occupied by carbamoyl phosphate). By themselves the activator, ATP, and the inhibitor, CTP (Gerhart and Pardee, 1962), have only a slight and similar effect on the sedimentation coefficient of the enzyme. However, ATP facilitates the transition promoted by PALA and CTP retards the conformational change (Figure 2a). Moreover, carbamoyl phosphate is an effector which acts in the same direction as ATP. As seen in Tables I and II, ATP and carbamoyl phosphate together cause a larger decrease in the sedimentation coefficient of ATCase than the sum of their separate effects. Hence it appears that, in the presence of these two ligands, one binding to the regulatory subunits and the other to the catalytic subunits, a significant number of the enzyme molecules are converted to the more "swollen" (or relaxed) conformation which kinetically is the more active form. As a consequence the PALA-promoted change in the sedimentation coefficient (Figure 2b) is only about −2.4%, whereas it is −3.1% where no other ligands are present. In contrast to these synergistic effects of carbamoyl phosphate and ATP, the effects of carbamoyl phosphate and CTP were antagonistic. These conformational changes can be interpreted satisfactorily in terms of a concerted transition according to the two-state model of Monod et al. (1965). The quantitative aspects of these changes together with a consideration of their relevance to the functional behavior of ATCase are discussed in the final paper in this series (Howlett et al., 1977).

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