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Conformational Changes in Proteins as Measured by Difference Sedimentation Studies. II. Effect of Stereospecific Ligands on the Catalytic Subunit of Aspartate Transcarbamylase*

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ABSTRACT: The technique of difference sedimentation has been employed to measure small changes in the sedimentation coefficient of the catalytic subunit of aspartate transcarbamylase (ATCase) in the presence of specific ligands. Succinate or carbamyl phosphate alone produced small increases in the sedimentation coefficient of the catalytic subunit which stemmed principally from the added weight and density of the bound ligands. Both ligands together at concentrations of 2 mM, however, produced an increase in s of 1.05%, which is three times larger than the expected increase due merely to binding the ligands. Differential sedimentation experiments using a synthetic boundary cell showed that the concentration dependence of the sedimentation coefficient was nearly the same in the presence or absence of ligands. This result indicated that the increase in sedimentation coefficient could not be due to a small shift in the association–dissociation equilibrium promoted by the addition of ligands. The difference in sedimentation coefficient plotted as a function of succinate concentration in the presence of carbamyl phosphate gave a titration curve with a midpoint of 2×10^{-4} M. Comparison of these results to those for the native enzyme (Gerhart, J. C., and Schachman, H. K. (1968), *Biochemistry 7*, 538) showed that the conformational changes for the subunit and the native enzyme occurred at the same succinate concentration though they were opposite in direction. It appears that carbamyl phosphate and succinate cause changes in the catalytic subunit which result in a more contracted or isometric conformation, but that these are linked to changes in the conformation in the whole enzyme complex which result in a more swollen or anisometric form for ATCase.

Much functional significance has been attributed to conformational changes in enzyme molecules. Alterations in the three-dimensional structure of an enzyme on binding substrates have been considered to be important as part of the mechanism of enzyme catalysis (Koshland, 1958; Lumry, 1959; Jencks, 1969) and in particular in the regulation of enzyme activity (Monod *et al.*, 1965; Koshland *et al.*, 1966). Some experimental techniques used to study conformational changes in proteins suffer from ambiguity in that they cannot distinguish local effects from bulk changes in the three-dimensional structure of the protein. These local effects stemming from the interaction between the ligands and protein may produce a marked change in some physical–chemical parameter (such as absorption or optical rotation) without a gross change in the protein conformation. Hydrodynamic methods, since they are a reflection of the volume and shape of the protein molecules, can make important contributions in removing this ambiguity. Since difference sedimentation has been shown to be an extremely sensitive and accurate hydrodynamic method (Richards and Schachman, 1959; Kirschner and Schachman, 1971), we have applied it to the study of conformational changes in the catalytic subunit of aspartate transcarbamylase (ATCase).[1]

ATCase catalyzes the first step specific to pyrimidine biosynthesis in *Escherichia coli*. It shows two types of binding site interactions, cooperative kinetics with the substrates aspartate and carbamyl phosphate and feedback inhibition with the end product of the pathway, cytidine triphosphate (CTP) (Gerhart and Pardee, 1962; Bethell *et al.*, 1968). It has been shown that the native enzyme can be dissociated by *p*-hydroxymercuribenzoate into two types of subunits (Gerhart and Schachman, 1965) which are readily separated and obtained in pure form. The catalytic subunits catalyze the reactions leading to the formation of carbamyl aspartate

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[1] Abbreviation used is: ATCase, aspartate transcarbamylase.

but show neither cooperative kinetics nor inhibition by CTP. The regulatory subunits bind CTP but have no enzymatic activity.

Gerhart and Schachman (1968) have shown that ATCase undergoes a conformational change on binding the substrate analog succinate in the presence of the substrate carbamyl phosphate. They found an increase in reactivity of the sulfhydryl groups and a decrease of 3.6% in sedimentation coefficient. CTP opposed both effects. In addition, the concentration of succinate which produces half the observed change in conformation was approximately 2×10^{-4} M for both enhancing the reactivity of the sulfhydryl groups and decreasing the sedimentation coefficient, while the binding curve was sigmoidal and had a half-maximal saturation of 7×10^{-4} M succinate. The data were interpreted as indicating that ATCase existed in two conformational states which could be interconverted by binding of ligands. Changeux and Rubin (1968) showed that the data were consistent with the allosteric model of Monod *et al.* (1965).

Whatever model is chosen to represent the experimental data, it seems unequivocal that succinate in the presence of carbamyl phosphate causes a change in the conformation of the native enzyme. Since succinate binds only to the catalytic subunit, it seemed reasonable to investigate the effect of succinate on the isolated catalytic subunit and to attempt to relate this to the effects observed in the native enzyme.

Gerhart and Schachman (1968) found that whereas the substrate analog succinate (2×10^{-3} M) in the presence of carbamyl phosphate (1.8×10^{-3} M) caused a 3.6% decrease in the sedimentation coefficient of ATCase, the same ligands caused a 1.8% increase in the sedimentation coefficient of catalytic subunit. Since the accuracy of measuring differences in sedimentation coefficient by conventional schlieren techniques employing simultaneous experiments with wedged window and plane window cells was about ±0.5%, their result was significant. However, limitations of the method made it impossible to titrate the change and determine the concentration of succinate at which the conformational change reached half its total value. Subsequently Collins and Stark (1969) have found that succinate in the presence of carbamyl phosphate produces a large perturbation of the ultraviolet spectrum.

In view of the fact that the catalytic subunit seems to undergo a conformational change opposite in direction to the native enzyme on binding the substrate analog succinate, it is clear that the whole enzyme is not simply eliciting the sum of the effects of two of the isolated catalytic subunits. It seemed appropriate, therefore, to study the relationship of the conformational changes of the isolated catalytic subunit to those of the native enzyme. In particular, it was necessary to determine whether the increase in the sedimentation coefficient represents a conformational change and to compare a titration of the conformational change as a function of succinate concentration with the binding of succinate.

General Considerations

Since the sedimentation coefficient is a function of both the molecular weight and the shape (or volume) of macromolecules, it is sensitive to the state of aggregation as well as changes in conformation of individual molecules. Thus information must be obtained about possible association–dissociation equilibria before a decision can be made as to whether an alteration in the sedimentation coefficient represents a change in the molecular weight or in the shape (or volume). Very small changes in sedimentation coefficient, such as can be measured by the difference sedimentation method, could be due to a shift in the association–dissociation equilibria which are too small to be detected by other methods. Hence systems which exhibit a ligand-promoted change in sedimentation coefficient must be examined to ascertain whether there is a shift in monomer–oligomer equilibria.

If the change in sedimentation coefficient is due to a reversible dissociation (or association) of the macromolecules, there should be a corresponding change in the dependence of the sedimentation coefficient on concentration. This latter effect could be detected from difference sedimentation experiments over a range of concentrations which would measure the concentration dependence of the change in sedimentation coefficient promoted by the ligands. A conformational change involving only the volume or shape of the sedimenting species would show very little concentration dependence, while a shift in the association–dissociation equilibria would be revealed by a substantial concentration dependence. Unfortunately difference sedimentation experiments can be performed accurately over only a limited range of concentrations. As a consequence the data from separate experiments at different concentrations would vary in precision. Hence an alternative method for studying the concentration dependence of the sedimentation coefficient was employed in conjunction with the difference sedimentation measurements on the effect of ligands at a single protein concentration.

The concentration dependence of the sedimentation coefficient can be measured accurately in a single experiment by examining the rate of movement of a "concentration boundary" formed by layering a protein solution at some concentration over a solution of the same protein at a higher concentration (Hersh and Schachman, 1955). Such differential sedimentation experiments produce two boundaries: one represents a conventional integral boundary between the protein at the lower concentration and the supernatant, and the other is the differential boundary between solutions at two different concentrations of the same protein. The difference in the rates of movement of these two boundaries yields directly the concentration dependence of the sedimentation coefficient as $\Delta s/\Delta c$, where Δs is the difference in the sedimentation coefficients for the two concentrations and Δc represents the concentration difference (Hersh and Schachman, 1955). Such layering experiments are performed readily through the use of a synthetic boundary cell such as that described by Pickels *et al.* (1952).

Since we are interested in measuring the difference in the concentration dependencies of the sedimentation coefficient of proteins in the presence and absence of specific ligands, we employed two synthetic boundary cells simultaneously in a single experiment. One cell contained conventional windows and the other had a wedged upper window so that the schlieren pattern was displaced vertically on the photographic plate. In this way one cell yielded the concentration dependence of the sedimentation coefficient in the absence of ligands and data from the other cell gave the effect of ligands. Comparison of the two values of $\Delta s/\Delta c$ gives $\Delta(\Delta s/\Delta c)$ in the same manner that measuring the concentration dependence of the change in sedimentation coefficient promoted by ligands would give $(\Delta/\Delta c)(\Delta s)$. From measurements of the integral boundaries the effect of ligands on the sedimentation coefficient can be detected as well for comparison to the results of the difference sedimentation experiments with interference optics.

In the case of ATCase the decrease of 3.6% in the sedimentation coefficient upon the addition of specific ligands has been interpreted in terms of a volume (or shape) change, since there was no evidence indicating a ligand-promoted dissociation of the enzyme molecules (Gerhart and Schachman, 1968). For this system studies were also made at varying protein concentrations (in separate experiments) and it was found that the enzyme in the presence of ligands had a slightly greater dependence of s upon c than in the absence. These data lend support to the interpretation that the enzyme molecules are more "swollen" in the presence of ligands and therefore experience greater hydrodynamic resistance during their movement in a centrifugal field. Comparable data are not available for the catalytic subunit of ATCase; moreover the changes in s upon the addition of ligands are so small that the method employed for ATCase is not applicable. Hence difference sedimentation experiments were conducted by the method described in the preceding paper (Kirschner and Schachman, 1971) to determine the effect of ligands. In addition, differential sedimentation measurements were made in order to study the concentration dependence of the sedimentation coefficient both in the absence and presence of ligands.

Materials and Methods

Succinic acid was purchased from Eastman and recrystallized three times from benzene. Glutaric acid from Mann Biochemicals was treated with decolorizing charcoal and recrystallized three times from 80% benzene and 20% ethyl ether. Both compounds were judged pure by paper chromatography in ethanol–water–ammonium hydroxide (8:1:1, v/v) and ether–acetic acid–water (15:3:1, v/v) according to the method of Jones *et al.* (1953). D-Aspartate obtained from Sigma was recrystallized three times from water and judged to be free of L-aspartate by enzymatic reaction with carbamyl phosphate using the colorimetric assay for ATCase of Gerhart and Pardee (1962). Dilithium carbamyl phosphate obtained from Sigma was reprecipitated from cold ethanol–water (Gerhart and Pardee, 1962).

ATCase and its catalytic subunit were prepared as described by Gerhart and Holoubek (1967). The catalytic subunit gave a single boundary when measured at 280 nm in the ultracentrifuge equipped with a photoelectric scanner (Schachman and Edelstein, 1966). The extinction coefficient at 280 nm was 0.72 $cm^2\ mg^{-1}$ when the protein concentration was determined interferometrically with a synthetic boundary cell. The specific enzyme activity of the catalytic subunit determined at 20 mM aspartate was 22–28 units/mg at 28°, pH 7.0, in 0.04 M phosphate buffer. Assays were performed according to the procedure of Porter *et al.* (1969).

All ultracentrifuge experiments were performed on a Beckman-Spinco Model E ultracentrifuge equipped with a Rayleigh optical system. The optical system was aligned according to Richards *et al.* (1971).

Difference sedimentation experiments were performed as described by Kirschner and Schachman (1971). In a typical experiment 0.4 ml of catalytic subunit at a concentration of 9 mg/ml in phosphate buffer was added to each of two tubes by means of a Hamilton syringe fitted with a Cheney adapter. A 0.1-ml portion of 0.01 M succinic acid and 0.01 M carbamyl phosphate was added to one tube and exactly the same volume of 0.01 M glutaric acid and 0.01 M phosphate in the same buffer as the protein were added to the other tube with a Hamilton syringe. The cells were filled by means of a Hamilton syringe with identical volumes of about 0.41 ml. A small volume of solution was then withdrawn from one sector so that the contents of the sectors differed in their meniscus positions by about 0.1 mm. The cells were double-sector centrifuge cells with unfilled epoxy centerpieces and sapphire windows. All calculations were performed as described previously (Kirschner and Schachman, 1971).

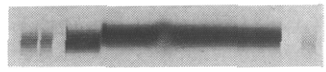

FIGURE 1: Difference sedimentation pattern for catalytic subunit. Both compartments were filled with catalytic subunit as a concentration of 8 mg/ml in 0.04 M potassium phosphate buffer containing 2 mM mercaptoethanol and 0.2 mM Na_2EDTA at pH 7.0. One compartment contained 2 mM carbamyl phosphate and succinate; the other, 2 mM phosphate and glutarate. The pattern was obtained 40 min after the rotor attained a speed of 60,000 rpm.

Simultaneous differential sedimentation experiments were performed with two cup-type synthetic boundary cells (Pickels *et al.*, 1952) according to the method of Hersh and Schachman (1955). One cell was assembled with plane quartz windows and the other had a 1° negative wedged upper window. In this way the schlieren patterns were displaced from each other vertically on the photographic plates and the position of all four boundaries could be measured readily on a Gaertner microcomparator. For both cells about 0.40 ml of the concentrated protein solution (10 mg/ml) was added to the sectoral cavity and the cups were filled with 0.24 ml of the dilute protein solution (5 mg/ml). The protein solutions were dialyzed against the same buffer. One cell contained succinate and carbamyl phosphate and the other had glutarate and phosphate in equivalent amounts. The rotor was accelerated at a constant current of 8 A until layering began at about 8000 rpm; then the drive voltage was reduced so that the current was 4 A. Boundary formation was complete at about 11,000 rpm and the rotor was then accelerated to a speed of 60,000 rpm in the usual manner.

Results

Change in Sedimentation Coefficient Promoted by Ligands. Figure 1 shows a typical difference sedimentation pattern for catalytic subunit at a concentration of 8 mg/ml. One sector contained succinate and carbamyl phosphate and the other sector contained glutarate and phosphate, each at a concentration of 2 mM. The values for $\Delta s/\bar{s}$ for a series of such experiments with the specific ligands, carbamyl phosphate and succinate, are listed in Table I.

The control experiment with identical solutions of catalytic subunit gave −0.005% (−0.0003 S) for $\Delta s/\bar{s}$. Succinate, which is known to bind weakly in the absence of carbamyl phosphate (Changeux *et al.*, 1968), produced a small increase in s as compared to glutarate which interacts very weakly. The observed value of 0.03% for $\Delta s/\bar{s}$ can be attributed to the increase in molecular weight. Carbamyl phosphate alone (*vs.* phosphate) produced a significant increase in s of 0.19% (0.018 S). When this value is corrected for the binding of carbamyl phosphate *vs.* phosphate (3 moles/mole of protein), a value of $(\Delta s/\bar{s})_{cor}$ of +0.03% is obtained.

Upon the addition of both ligands, succinate and carbamyl phosphate (as compared to glutarate and phosphate in the reference solution) there was an increase in s of 1.05% (0.061 S), a value about three times greater than would be expected on the basis of the increment in molecular weight.

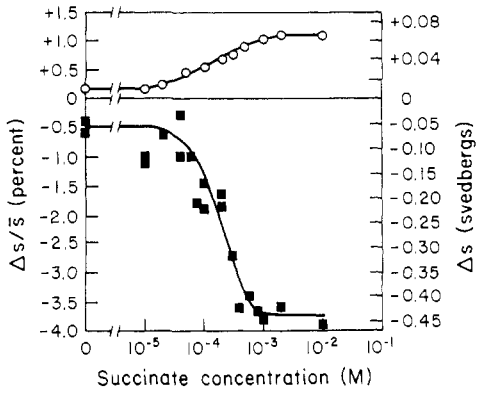

FIGURE 2: Conformational titration of ATCase and its catalytic subunit. The difference in sedimentation coefficient, $\Delta s/\bar{s}$, in per cent is plotted *vs.* succinate concentration for ATCase and its catalytic subunit. The scale for Δs in Svedbergs is different for the two proteins. Data on ATCase, replotted from Gerhart and Schachman (1968), who employed simultaneous experiments with wedged and plane window cells, are shown as ■. Each point represents the difference in sedimentation rate between a sample and reference solution. Both solutions contained ATCase at 4.2 mg/ml in 0.04 M phosphate buffer (pH 7.0). In the sample were 1.8×10^{-3} M dilithium carbamyl phosphate and a given concentration of succinate. In the reference was an additional 1.8×10^{-3} M phosphate and an equivalent amount of glutarate. Data for catalytic subunit obtained by difference sedimentation with Rayleigh optics are represented by o. For each experiment both sectors of a double-sector ultracentrifuge cell contained catalytic sununit at a concentration of 8 mg/ml in 0.04 M potassium phosphate buffer (pH 7.0), 2 mM mercaptoethanol, and 0.2 mM Na_2EDTA. One sector contained 2×10^{-3} M dilithium carbamyl phosphate and a given concentration of succinate. The other sector contained 2×10^{-3} M additional phosphate and an equivalent concentration of glutarate. All experiments were performed at 20° and the rotor speed was 60,000 rpm.

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

Expt	Ligand	$\Delta s/\bar{s}$ (%)	Δs (S)	$(\Delta s/\bar{s})_{cor}$[b] (%)
1	None *vs.* none	−0.005	−0.0003	−0.005
2	Succinate *vs.* glutarate	+0.03	+0.0018	0.00[c]
3	Carbamyl phosphate *vs.* phosphate	+0.19	+0.0110	+0.03
4	Succinate–carbamyl phosphate *vs.* glutarate–phosphate	+1.05	+0.0610	+0.62[c]
5	Succinate–carbamyl phosphate *vs.* none	+1.18	+0.0680	+0.75
6	Succinate–carbamyl phosphate *vs.* succinate–phosphate	+0.98	+0.0570	+0.58[d]
7	Succinate–carbamyl phosphate *vs.* D-aspartate–phosphate	+0.90	+0.0520	+0.47[c]
8	Succinate–carbamyl phosphate *vs.* glutarate–carbamyl phosphate	+0.48	+0.0278	*e*

[a] All experiments were performed at a temperature of 20 ± 0.5° in 0.04 M potassium phosphate buffer, 2 mM mercaptoethanol, and 0.2 mM EDTA (pH 7.0). The concentration of all ligands was 2×10^{-3} M. [b] $(\Delta s/\bar{s})_{cor}$ represents the values of $(\Delta s/\bar{s})$ after correcting for the binding (per mole of subunit) of 3 moles of carbamyl phosphate (Hammes *et al.*, 1970) and 1.8 moles of succinate (obtained from the titration curve, Figure 2 and the stoichiometry from Changeux *et al.*, 1968). It was assumed that in the absence of carbamyl phosphate, phosphate was bound with the same stoichiometry as carbamyl phosphate (Porter *et al.*, 1969). The corrections to $\Delta s/\bar{s}$ were made according to Kirschner and Schachman (1971). The partial specific volume of the ligands was calculated by the method of Traube (1899) and McMeekin *et al.* (1949): phosphate, 0.35 ml/g; carbamyl phosphate, 0.48 ml/g; and succinate, 0.61 ml/g. [c] It was assumed that glutarate and D-aspartate in the presence of phosphate did not bind at all. [d] For the binding of succinate in the presence of phosphate the value used for the dissociation constant was 0.02 M (Collins and Stark, 1969). [e] No estimate could be made for the binding of glutarate in the presence of carbamyl phosphate.

Omitting the glutarate and phosphate from the reference solution gave +1.18% for the effect of succinate and carbamyl phosphate. Similar results were obtained when the reference solution contained succinate and phosphate (+0.98%), or D-aspartate and phosphate (+0.90%). These latter ligand pairs have been shown to interact weakly with the catalytic subunit (Collins and Stark, 1969; Porter *et al.*, 1969; Davies *et al.*, 1970). The slight differences among these values of $\Delta s/\bar{s}$ are significant and probably reflect small variations in the interactions among these various combinations of ligands. Some indication of specific interactions is seen by comparison of the results of expt 4 and 5. The slightly higher value of 1.18% with no ligands in the reference solution (as contrasted to 1.05% in expt 4), coupled with the fact that succinate should cause a decrease in s of about 0.1%, due to its contribution to the viscosity and density of the solvent, indicate that there may be some slight positive effect on $\Delta s/\bar{s}$ caused by glutarate and phosphate. Support for this new hypothesis can be seen from experiment 8 where the value of $\Delta s/\bar{s}$ was only +0.48% when the catalytic subunit in succinate and carbamyl phosphate was compared to the same protein in glutarate and carbamyl phosphate. Thus glutarate does affect the conformation of the catalytic subunit especially when carbamyl phosphate is present. Collins and Stark (1969) have shown previously that glutarate and carbamyl phosphate cause a moderate change in the ultraviolet region of the spectrum even though the inhibition constant for glutarate at pH 7.9 is more than 70 times larger than that of succinate (Porter *et al.*, 1969). As yet, however, there have been no direct measurements of the binding of glutarate to the catalytic subunit. Hence a quantitative interpretation of the slight differences shown in Table I cannot be made at this time. Nonetheless the general trend of the results indicates that certain ligand pairs produce an increase in s of about 1%.

Effect of Ligand Concentration on the Sedimentation Coefficient. Figure 2 shows the change in sedimentation coefficient for both the catalytic subunit and the native enzyme as a function of succinate concentration in the presence of saturating amounts of carbamyl phosphate. The results for the catalytic subunit were obtained by the difference sedimentation method based on interference optics (Kirschner and Schachman, 1971), and those for the ATCase were replotted from Gerhart and Schachman (1968) who used schlieren optics with plane and wedged-window cells.

TABLE II: Dependence of Sedimentation Coefficient on Concentration.

Material	s_1[a] (S)	s_D[a] (S)	k[b] (ml/mg)	s_0[b] (S)
ATCase[c]			0.009	11.7
Catalytic subunit[c]			0.0086	5.8
Catalytic subunit[d] (glutarate, phosphate)	5.73	5.20	0.0089	5.84
Catalytic subunit[d] (succinate, carbamyl phosphate)	5.83	5.30	0.0086	5.92

[a] The coefficients s_1 and s_D are obtained as described in the text for the integral and differential boundaries in a differential sedimentation experiment using the synthetic boundary cell. [b] Data fit to $s = s_0(1 - kc)$, where s_0 is the sedimentation coefficient at zero protein concentration and k is a parameter representing the fractional decrease in the sedimentation coefficient with concentration of the protein. [c] Obtained from a series of velocity experiments at various protein concentrations. [d] Obtained from differential sedimentation experiments using the synthetic boundary cell. All samples were in 0.04 M potassium phosphate buffer (pH 7.0) containing 2 mM mercaptoethanol and 0.2 mM Na_2EDTA. The concentration of all ligands was 2 mM.

Although the total change in s (from zero succinate to high succinate) for the catalytic subunit was only about 0.05 S (0.9% for $\Delta s/s$), considerable accuracy was obtained with the average deviation of the points from the titration curve corresponding to 0.001–0.003 S. In contrast, the deviations of the data for the native enzyme were substantially larger. For both curves the half-titration values were about 2×10^{-4} M succinate. In both cases $\Delta s/s$ approached constant values at high succinate concentration (10^{-2} M) as would be expected in a titration of a conformational change from one form to another.

Concentration Dependence of Sedimentation Coefficient in Absence and Presence of Ligands. Although the titration curve for the catalytic subunit indicated that the ligands promoted a conformational change and there was no evidence suggesting an association–dissociation equilibrium, it seemed important, nonetheless, to obtain further data which would bear on these two possible interpretations. Accordingly studies were conducted on the concentration dependence of the sedimentation coefficient of the catalytic subunit both in the absence and presence of specific ligands.

Figure 3 shows representative patterns from a pair of differential sedimentation experiments in which protein solutions at a concentration of 5 mg/ml were layered over similar solutions at 10 mg/ml. The solutions producing the upper patterns contained succinate and carbamyl phosphate at a concentration of 2 mM; in the control these ligands were replaced by equivalent amounts of glutarate and phosphate. Measurements of the positions of the differential (or concentration) boundaries were made from a series of patterns at different times and corresponding data were obtained for the conventional boundaries formed by movement of the protein molecules from the menisci. Plots of the logarithms of the radial distance *vs.* time for each of the four

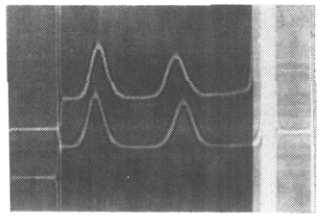

FIGURE 3: Patterns from simultaneous differential sedimentation experiments to determine the effect of ligands on the concentration dependence of the sedimentation coefficient. Catalytic subunit at a concentration of 5 mg/ml was layered over catalytic subunit at a concentration of 10 mg/ml in a cup-type synthetic boundary cell. The negative wedged window cell contained 0.04 M phosphate buffer (pH 7.0), 2 mM mercaptoethanol, 0.2 mM Na_2EDTA, and 2 mM glutarate, and 2 mM additional phosphate. The cell with the plane window contained the same buffer plus 2 mM succinate and 2 mM carbamyl phosphate. The temperature was 20° and the rotor speed 60,000 rpm. The patterns were obtained 20 min after reaching speed.

boundaries were linear. From the slopes of these curves, corrected for the angular velocity of the rotor, sedimentation coefficients, s_1, were calculated for the protein in the upper solutions at concentration, c_1. Similar calculations gave values, s_D, for the rate of movement of the differential (or concentration) boundaries formed between solutions at concentrations c_1 and c_2, where c_2 is the concentration of the lower solution. The results from various experiments are given in Table II.

As shown by Hersh and Schachman (1955) the rate of movement of a differential boundary (corrected for the magnitude of the centrifugal field), s_D, is related to the change in sedimentation coefficient with concentration, $\Delta s/\Delta c$. For experiments with a finite change in concentration across the differential boundary the relationship takes the form

$$s_D = s_1 + c_2(\Delta s/\Delta c) \quad (1)$$

The sedimentation coefficient, s, for globular macromolecules which exhibit only a slight concentration dependence can be expressed by

$$s = s_0(1 - kc) \quad (2)$$

where s_0 is the sedimentation coefficient at infinite dilution and the constant, k, which is equal to $-(1/s_0)(\Delta s/\Delta c)$, is a function of the shape and volume of the macromolecules (Schachman, 1959). The various values of s_1 and s_D were used in eq 1 and 2 for the calculation of s_0 and k and the results are given in Table II. For comparison results are also presented for the unliganded catalytic subunit and ATCase. These data were obtained from conventional sedimentation velocity experiments at a series of concentrations where the boundaries were observed with schlieren optics (Gerhart and Schachman, 1965).

The differential sedimentation experiments, as summarized in Table II, show clearly that essentially the same concentration dependence is obtained for the catalytic subunit in the absence of ligands, in the presence of weakly interacting ligands (glutarate and phosphate) and in the presence of strongly interacting ligands (succinate and carbamyl phosphate). A similar concentration dependence, $k = 0.009$ ml/mg, characteristic of nonassociating globular protein molecules, was obtained for ATCase.

Table II also shows, from the values of s_1 and s_0, that the catalytic subunit sediments more rapidly (about 1.5%)

in the presence of succinate and carbamyl phosphate than with glutarate and phosphate. This observation is in agreement with the results obtained by the difference sedimentation method with interference optics where the strongly interacting ligands promoted an increase of 1.05%.

Discussion

Strongly associating protein systems involving a rapidly reversible monomer–dimer equilibrium are readily recognized by sedimentation velocity studies since a single boundary is observed at all concentrations and the sedimentation coefficient increases with concentration in dilute solutions (Schwert, 1949; Gilbert, 1955; Rao and Kegeles, 1958; Schachman, 1959; Fujita, 1962). If the tendency toward dimerization is not large, this positive concentration dependence of the sedimentation coefficient will not be observed since the normal hydrodynamic effects lead to a slight negative concentration dependence (Gilbert, 1960; Schachman, 1959). For such systems the observed concentration dependence will not be as large, in a negative sense, as that produced by nonassociating systems (Gilbert, 1960). Thus, as Gilbert (1960) pointed out, the existence of a rapidly reversible monomer dimer equilibrium may be revealed by the magnitude of the concentration dependence of the sedimentation coefficient. Accordingly we must inquire whether the change in k for the catalytic subunit from 0.0089 to 0.0086 ml per mg upon the addition of the strongly interacting ligands can be attributed to reversible dimerization.

For a weakly associating system involving monomers and dimers the concentration dependence of the sedimentation coefficient can still be described by an expression of the same form as eq 2 (Gilbert, 1960). In this case, however, the constant, k_{app}, must contain a term accounting for the increase in sedimentation coefficient due to dimer formation as well as the normal term, k, which describes the decrease in sedimentation coefficient stemming from hydrodynamic effects. If the concentration range and the equilibrium are such that only a small amount of dimer is formed, then a simple treatment for a monomer–dimer equilibrium with an association constant, K in milliliters per milligram, suffices to give k_{app}. This treatment yields

$$k_{app} = k - \left[\frac{s_D}{s_M} - 1\right]K \qquad (3)$$

where s_D and s_M are the sedimentation coefficients of the dimer and monomer, respectively. In this derivation we assumed that the values for k for monomer and dimer were equal and that terms containing the square of the concentration were negligible. If both monomer and dimer are assumed to be spherical, s_D/s_M is about 1.59; hence the association constant, K, can be evaluated from the values of k_{app} and k.

If we now assume that the change in k from 0.0089 ml/mg in the absence of the strongly interacting ligands to k_{app}, 0.0086 ml/mg, upon the addition of succinate and carbamyl phosphate is due to dimer formation, a value of K equal to 5×10^{-4} l./g is obtained from eq 3. Thus a solution at 8 mg/ml would contain 0.4% dimers and the remainder as monomers. For such a solution, as compared to one composed of 100% monomer, the value of $\Delta s/\bar{s}$ would be only 0.24%. As seen in Table I, the observed value of $\Delta s/\bar{s}$ (1.05%) upon the addition of succinate and carbamyl phosphate is more than four times larger. Thus we can conclude that the small amount of dimer inferred from the change in the concentration dependence of the sedimentation coefficient cannot account for the increase in the sedimentation coefficient observed at 8 mg/ml. A similar conclusion is obtained if we attribute the value of $\Delta s/\bar{s}$ at 8 mg/ml to dimer formation and then calculate k_{app} for comparison with the observed value. If the increase in sedimentation coefficient at a fixed protein concentration (expressed as $\Delta s/\bar{s}$) is attributed solely to a shift from a nonassociating system to a monomer–dimer equilibrium, the association constant would be related to $\Delta s/\bar{s}$ for low values of c relative to $1/K$ by

$$K = \frac{\frac{\Delta s}{\bar{s}}}{\left[\left(\frac{s_D}{s_M} - 1\right) - \frac{(s_D/s_M - 1)}{2}\left(\frac{\Delta s}{\bar{s}}\right)\right]c} \qquad (4)$$

For a $\Delta s/\bar{s}$ of 1.05%, the association constant calculated from eq 4 is 1.7×10^{-3} l./g which corresponds to the presence of 1.4% dimer at a total protein concentration of 8 mg/ml. The value of k_{app} calculated from eq 3 would be 0.0079 whereas the observed value in the presence of succinate and carbamyl phosphate is 0.0086. Thus these two complementary sets of calculations show that the observed values of the effect of succinate and carbamyl phosphate on the sedimentation coefficient and its concentration dependence cannot be accounted for quantitatively by the assumption of a rapidly reversible monomer–dimer equilibrium.

Since the effects caused by succinate and carbamyl phosphate cannot be attributed to dimer formation, we conclude instead that there must be a conformational change in the catalytic subunit which results in a 1% decrease in the frictional coefficient. Such a change would lead to a corresponding increase in $\Delta s/\bar{s}$. In addition, this alteration in the frictional coefficient would produce a small decrease in k since more compact (or less anisometric) protein molecules would be expected to exhibit a smaller concentration dependence for their sedimentation coefficients (Schachman, 1959). In this respect the change in the catalytic subunit is the opposite of that observed with ATCase where the ligands promoted a decrease in the the sedimentation coefficient and an increase in k, both of which reflect a more swollen form for the hydrodynamic unit (Gerhart and Schachman, 1968).

The conformational titration curves shown in Figure 2 clearly demonstrate that both the binding of carbamyl phosphate alone and the binding of succinate and carbamyl phosphate have opposite effects on the size (or shape) of ATCase and the catalytic subunit. In each case a plateau was obtained in $\Delta s/\bar{s}$ at high succinate and the half-titration value was about 2×10^{-4} M succinate. With ATCase the titration of the change in the sedimentation coefficient was similar to that observed by other techniques which are sensitive to the conformation of proteins. For example, the change in the reactivity of sulfhydryl groups (Gerhart and Schachman, 1968), the alteration in the absorption spectrum as measured by difference spectroscopy, and the change in the optical rotatory dispersion (V. P. Pigiet and H. K. Schachman, unpublished results) all showed a half-titration level at 2×10^{-4} M succinate. In contrast, the binding of succinate, as measured by equilibrium dialysis (Changeux *et al.*, 1968) gave a distinctly different titration curve with a half saturation value of 7×10^{-4} M succinate. For the catalytic subunit the binding curve and the difference sedimentation titration curve are similar with the half-saturation value

for succinate, 5.5×10^{-4} M, being slightly greater than the level, 2×10^{-4} M, required to produce a half-maximal change in the sedimentation coefficient. The latter value was also obtained from measurements of the change in the optical rotatory dispersion (V. P. Pigiet and H. K. Schachman, unpublished results) and a slightly higher value, 3.7×10^{-4} M succinate, produced a half-maximal change in the absorbance of an environmentally sensitive chromophore attached covalently to the catalytic subunit (M. W. Kirschner and H. K. Schachman, unpublished results). These values for the catalytic subunit are less than those, 7.4×10^{-4} M, obtained by ultraviolet difference spectroscopy (Collins and Stark, 1969) and 8.3×10^{-4} M for the inhibition constant at pH 6.9 (Porter *et al.*, 1969). Since the inhibition constant was evaluated from assays of the catalytic subunit in imidazole acetate buffer at 28° and the difference spectra were performed with glycylglycine buffer at 25°, it is possible that the discrepancies can be attributed to variations in experimental conditions.

The conformation change in the catalytic subunit as measured by difference sedimentation thus seems to differ from the conformation change in the native enzyme not only in the direction and magnitude of the effect but in its relationship to the saturation curve for binding of the ligands. There are at least two possible interpretations of these differences. On the one hand it is possible that the changes observed in the isolated catalytic subunits are different from those which the subunits undergo when they are constrained in the native ATCase molecules. On the other hand, the catalytic subunits, free and in the complexes, may suffer the same changes in tertiary and quaternary structures but in ATCase complexes these changes could be linked to additional alterations in quaternary structure which are larger and opposite in direction.

Methods which are sensitive to short-range properties of molecules, such as spectral methods, could be used to compare the conformational changes of isolated catalytic subunits with those for catalytic subunits incorporated into ATCase. Preliminary evidence based on the similarity between the difference spectrum for ATCase (V. P. Pigiet and H. K. Schachman, unpublished results) with that for catalytic subunits (Collins and Stark, 1969) and experiments with catalytic subunit modified to produce an environmentally sensitive chromophore (M. W. Kirschner and H. K. Schachman, unpublished results) suggest that the conformational changes undergone by the isolated catalytic subunits are similar to those for catalytic subunits incorporated into ATCase. Since the hydrodynamic effects for the two species shown in Figure 2 are strikingly different, ATCase cannot be reflecting simply the sum of the changes in the catalytic subunits but probably a different type of transition involving changes in the packing or architecture of the whole complex. Recently a model has been proposed for ATCase which envisages a linkage between local structural changes in the catalytic subunits and a change in the quarternary structure of the aggregate (Gerhart, 1970).

Examination of only the spectral data would not have revealed the striking qualitative difference between conformational changes in the catalytic subunit and those in ATCase. The hydrodynamic data alone provide little information as to the relationship between the conformational changes in the isolated subunit with those for the subunit in the ATCase complex. The results from methods sensitive to bulk structure complement the data obtained by techniques sensitive to short-range effects. Together they can be used effectively to distinguish different levels of conformational transitions and to examine the linkage between changes in the tertiary structure in individual subunits with alterations in the quaternary structure of the native complexes.

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Development of Ordered Structures in Sequential Copolypeptides Containing L-Proline and γ-Hydroxy-L-proline*

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ABSTRACT: The conformational properties of poly(Pro-Gly), poly(Hyp-Gly), poly(Gly-Gly-Pro-Gly), poly(Gly-Gly-Hyp-Gly), and poly(Pro-Ala) have been investigated by measuring the circular dichroism in water, ethylene glycol–water (2:1, v/v), and trifluoroethanol as a function of temperature and by determining the intrinsic viscosity in water over the range 5–70°.

It has been possible to demonstrate the formation of ordered structures for poly(Hyp-Gly), poly(Gly-Gly-Hyp-Gly), and poly(Pro-Ala) under suitable conditions. Poly(Pro-Gly) and poly(Gly-Gly-Pro-Gly) are not ordered under the conditions studied. The ordered structures for poly(Pro-Ala) and poly(Hyp-Gly) are suggested to be similar to poly-L-proline form II. In poly(Pro-Ala) this conformation results from severe steric restraints to rotation about the dihedral angles ϕ and ψ. Puckering of the pyrrolidine ring allows intrachain hydrogen bonding between the hydroxyl group and the carbonyl oxygen on the second preceding residue to stabilize a similar conformation in poly(Hyp-Gly). A model for the ordered state of poly(Gly-Gly-Hyp-Gly) is not as clear but could involve an associated structure with some similarities to the known ordered state of poly(Pro-Gly-Gly).

The pyrrolidine ring in L-proline and γ-hydroxy-L-proline limits the nitrogen–α-carbon rotational angle, ϕ (Edsall *et al.*, 1966a–c), to about 102–122° (Donohue and Trueblood, 1952; Mathieson and Welsh, 1952; Cowan and McGavin, 1955; Leung and Marsh, 1958; Sasisekharan, 1959a,b), restricts the rotational freedom of the preceding residue in the chain (DeSantis *et al.*, 1965; Schimmel and Flory, 1967, 1968; Hopfinger and Walton, 1969; Holzwarth and Chandrasekaran, 1969; Madison and Schellman, 1970a,b), and does not allow the peptide bond to function as a proton donor in hydrogen-bond formation. In the solid state poly-L-proline form I exists as a right-handed helix with cis peptide bonds (Traub and Shmueli, 1963), and poly-L-proline form II forms a left-handed helix with trans peptide bonds (Cowan and McGavin, 1955; Sasisekharan, 1959a). In poly(γ-hydroxy-L-proline) A the chain conformation is similar to that of poly-L-proline form II, but in addition all possible intermolecular hydrogen bonds are formed between the hydroxyl groups and carbonyl oxygen atoms (Sasisekharan, 1959b). Polyglycine can form an ordered structure closely related to poly-L-proline form II and poly(γ-hydroxy-L-proline) A, but in polyglycine form II right- and left-handed helices are equally probable and all possible intermolecular hydrogen bonds are formed between the peptide units.

The conformational properties of a large number of sequential copolypeptides containing glycine and either L-proline or γ-hydroxy-L-proline have been reviewed (Harrington *et al.*, 1966; Andreeva *et al.*, 1967; Carver and Blout, 1967; Ramachandran, 1967; Venkatachalam and Ramachandran, 1969). The major emphasis has been on sequential copolypeptides containing glycine at every third residue since this periodicity occurs throughout large portions of the collagen chain (Schroeder *et al.*, 1954; Kang *et al.*, 1967; Bensusan, 1969; Butler, 1970; Kang and Gross, 1970). The proposed structures for the ordered portion of collagen also require that every third residue be glycine (Ramachandran and Kartha, 1955; Rich and Crick, 1955, 1961; Ramachandran and Sasisekharan, 1965; Ramachandran *et al.*, 1968). It has been shown that in the solid state poly(Pro-Gly-Pro) forms a collagen II type structure (Traub and Yonath, 1966; Yonath and Traub, 1969) and that several copolyhexapeptides with glycine at every third residue have a similar conformation (Segal *et al.*, 1969). Poly(Pro-Gly-Gly), however, forms a double-layered sheet in which each chain has a conformation similar to that of poly-L-proline form II and where there are two interchain hydrogen bonds per tripeptide unit (Traub, 1969). In solution it has been possible to demonstrate a heat-induced cooperative structural transition in poly(Pro-Gly-Pro) (Engel *et al.*, 1966). Subsequently it was found that the formation of the disordered form in monodisperse poly(Pro-Pro-Gly) is accompanied by a threefold reduction in molecular weight (Kobayashi *et al.*, 1970). Ordered conformations in solution at or near room temperature have also been detected in poly(Gly-Pro-Gly) (Oriel and Blout, 1966) and in several polyhexapeptides containing glycine at every third residue (Segal, 1969). Although poly(Gly-Pro-Ala) adopts a statistical conformation at room temperature, an ordered conformation is developed at low temperatures in ethylene glycol–water (2:1, v/v) (Brown *et al.*, 1969).

In the present work the conformational properties, in dilute solution, of five sequential copolypeptides containing L-proline or γ-hydroxy-L-proline are reported. Although four of

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