Self-Association and Allosteric Properties of Glutamine-dependent Carbamyl Phosphate Synthetase

REVERSIBLE DISSOCIATION TO MONOMERIC SPECIES*

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SUMMARY

Glutamine-dependent carbamyl phosphate synthetase from Escherichia coli can exist in two different conformations which exhibit, respectively, monomer sedimentation coefficients ($s_{20,W}$) of 7.3 S and 8.7 S. Both conformations undergo rapid reversible self-association upon addition of potassium ions or ornithine. Formation of the 8.7 S conformation is promoted by the addition of phosphate ions. Low concentrations of urea (0.2 to 2.0 M) or guanidine hydrochloride (0.05 to 0.5 M), both of which inhibit the enzymatic activity, favor dissociation in potassium phosphate buffer, which is reversible. Ammonia has been found to be a potent allosteric activator of the enzyme. The presence of ammonia or ornithine (also an allosteric activator) promotes oligomer formation; the maximum sedimentation coefficient observed (in potassium phosphate buffer containing ornithine) is 14.8 S. Sedimentation equilibrium studies indicate the existence of tetramer or higher species in potassium phosphate buffer containing ornithine. Models are presented which explain the apparent relationship between allosteric regulation of the enzyme and its state of association.

Glutamine-dependent carbamyl phosphate synthetase catalyzes the following reaction (1):

$$2\text{ATP} + \text{HCO}_3^- + \text{H}_2\text{O} + \text{l-glutamine} \rightarrow \text{Mg}^{2+} \rightarrow \text{NH}_4\text{CO}_3\text{PO}_4^- + \text{l-glutamate} + 2\text{ADP} + P_i$$

The enzyme also catalyzes carbamyl phosphate synthesis when glutamine is replaced by ammonia. The glutamine-dependent synthetase activity of the enzyme is allosterically regulated by a number of purine and pyrimidine nucleotides. Thus, purine nucleotides (e.g. IMP) stimulate activity, and pyrimidine nucleotides are either inhibitory (e.g. UMP) or have no effect (cytidine nucleotides) on the activity (2); this indicates a mechanism by which a balance can be maintained between the rates of purine and pyrimidine biosynthesis. IMP is a more potent activator than are XMP, GMP, GDP, GTP, and AMP, while UMP inhibits more effectively than do UDP and UTP; in general, the degree of activation or inhibition decreases as the number of steps required to synthesize the various nucleotides from IMP and UMP, respectively, increases (2). The finding that ornithine is an effective allosteric activator arose from the observation by Piérard (3) that ornithine counteracts the inhibition produced by pyrimidine nucleotides. Anderson and Marvin (4) subsequently demonstrated that ornithine is a potent allosteric activator even in the absence of UMP. Since E. coli has only one carbamyl phosphate synthetase which catalyzes carbamyl phosphate formation for both the arginine and pyrimidine biosynthetic pathways (5), the ability of ornithine to stimulate the enzyme seems to offer a mechanism that can provide carbamyl phosphate for arginine biosynthesis even when UMP is present in excess. Studies on the several partial reactions catalyzed by carbamyl phosphate synthetase (6) suggest that the allosteric effectors influence the over-all activity by altering the affinity of the enzyme for the ATP utilized in phosphorylation of enzyme-bound carbamate, rather than for the ATP that is used for the activation of carbon dioxide (2).

Anderson and Marvin (4, 7) carried out sucrose gradient centrifugation studies of the enzyme in potassium phosphate and reported that the sedimentation coefficient varied with increasing enzyme concentration. They also found that IMP and ornithine increased the sedimentation coefficient while UMP decreased it, and suggested that a monomer-oligomer equilibrium accounted for the changes in the sedimentation behavior of the enzyme.

Independent studies in this laboratory (8), in which the sedimentation properties of the enzyme were examined by analytical ultracentrifugation, showed that the sedimentation coefficient of the enzyme can vary from about 7 S to 15 S depending upon the nature of the solvent. In an effort to understand these observations we undertook a systematic investigation of the effects of solvent perturbation on the sedimentation velocity behavior of the enzyme as observed in the analytical ultracentrifuge. The results obtained have led to the finding of appropriate solvent conditions for the reversible
production of alternate monomer conformations. We have conducted sedimentation equilibrium studies of the monomer-oligomer equilibrium produced in the presence of potassium phosphate and ornithine, and also of the monomer which can be obtained in the absence of potassium phosphate. These studies have shed light on the apparent parallelism between stimulation of enzymatic activity and self-association of the enzyme. In the course of this work we discovered that ammonia promotes self-association of the enzyme, and that ammonia is also a positive allosteric effector of glutamine-dependent carbamyl phosphate synthetase activity. The effect of ammonia, which may have metabolic significance, explains the relative insensitivity of the ammonia-dependent carbamyl phosphate synthetase activity to other allosteric effectors.

EXPERIMENTAL PROCEDURE

Materials—Glutamine-dependent carbamyl phosphate synthetase was purified from E. coli B (Grain Processing Corp.; grown on minimal medium three-fourths of the way through the log phase) by the procedure of Anderson et al. (9). This method of isolation yields a highly purified enzyme which, however, contains at least two minor impurities which migrate between the light and heavy subunits on sodium dodecyl sulfate acrylamide gel electrophoresis. The enzyme preparation used in sedimentation equilibrium studies was further processed as follows. The high molecular weight contaminant was removed in a 0 to 52% ammonium sulfate fractionation at 0°C; the enzyme was then precipitated by increasing the ammonium sulfate concentration to 65%. Low molecular weight contaminants were removed by Sephadex G-200 gel filtration in the presence of L-ornithine (150 mM potassium phosphate, 10 mM L-ornithine, 0.5 mM EDTA, pH 7.8).

L-Ornithine, L-glutamine, and the nucleotides were obtained from Sigma. Recrystallized urea and recrystallized guanidine hydrochloride were obtained from Mann. Sodium [14C]carbonate was obtained from New England Nuclear Corp.

Determination of Enzyme Activity—Carbamyl phosphate synthetase activity was determined by conversion of [14C]bicarbonate (30,000 to 50,000 cpm per pmole) to [14C]carbamyl phosphate, which was quantitated by conversion to [14C]urea. The [14C]urea was separated from unreacted [14C]bicarbonate by the use of Dowex 1-X8, as previously described (1, 6, 10). In determinations of the glutamine-dependent carbamyl phosphate synthetase activity carried out in the presence of ammonia, the amount of L-glutamate formed was quantitated. In these studies, the reaction was stopped by addition of 0.1 ml of 1 N hydrochloric acid; after standing at 0°C for 10 min, the solution was neutralized by addition of 0.1 ml of 1 M Tris, and the formation of L-glutamate was then determined by use of the glutamate dehydrogenase reaction (11).

Ultracentrifugal Analyses—Sedimentation velocity determinations were carried out on enzyme preparations which were dialyzed at 4°C against the appropriate buffer. The sedimentation studies were carried out in a Spinco model E analytical ultracentrifuge equipped with RTIC unit, electronic speed control, and photoelectric scanning system. Double sector cells of 3.5, 12, and 30 mm path lengths were used and scanning was carried out at 280 nm. Sedimentation coefficients were measured from the rate of movement of the 50% position of apparent boundaries with time, or, when required by the shape of the boundary for associating systems, by calculation of the weight average sedimentation coefficient.

High speed sedimentation equilibrium using interference optics

![Fig. 1. Concentration dependence of the sedimentation coefficient of the enzyme under various solvent conditions. Enzyme (15 to 18 mg per ml) was dialyzed at 4°C against the buffers indicated and was then diluted with the dialysate to various protein concentrations as indicated. Sedimentation coefficients were determined at 25°C at 52,000 to 60,000 rpm. A, 150 mM potassium phosphate (K-phosphate), 10 mM L-ornithine (ORN), pH 7.8; B, 150 mM potassium phosphate, 20 mM ammonium chloride, pH 7.8; C, 150 mM potassium phosphate, pH 7.8; D, 150 mM sodium phosphate, pH 7.8; E, 30 mM Veronal-HCl, 100 mM NaCl, 10 mM L-ornithine, pH 7.6; F, 30 mM Veronal-ICl, 100 mM potassium chloride, pH 7.6; G, 30 mM Veronal-ICl, 100 mM sodium chloride, pH 7.6. All buffers contained 0.5 mM EDTA.](http://www.jbc.org/)

was conducted essentially as described by Yphantis (12). Attainment of equilibrium was ascertained experimentally. All data were analyzed using the computer program of Roark and Yphantis (see Reference 13) to provide smoothed concentrations and various molecular weight moments throughout the cell.

RESULTS

Alternate Conformations of Carbamyl Phosphate Synthetase

Monomer—The sedimentation coefficient of the enzyme in Veronal buffer (Fig. 1, Curve G) is concentration independent at 7.3 S. The results of sedimentation equilibrium experiments reported below, taken in conjunction with previously reported estimates of the molecular weights of the two constituent polypeptide chains, confirm that this species is monomeric. In the presence of sodium phosphate (Fig. 1, Curve D), the sedimentation coefficient shows a slight increase with increasing protein concentration, indicating some association. A mathematical analysis of these data as described in the “Appendix” confirms the suspicion that the data do not represent an association of the 7.3 S monomer observed in Veronal. For this to be true, nearly complete association to a dimer with a sedimentation coefficient of 9.7 S would be required. Since the monomer appears to be nearly spherical upon examination under the electron microscope, no reasonable value for the frictional ratio

1 R. H. Haschemeyer and P. P. Trotta, unpublished observations.
for the dimer can explain such an extreme deviation from the expected sedimentation coefficient for similarly hydrated spheres that have a 2-fold difference in molecular weight (e.g. $S_2 = S_1(2^{2/3})$). The only reasonable interpretation of the gentle increase in sedimentation coefficient observed upon increasing protein concentration is that the enzyme has a grossly different conformation in sodium phosphate from that in Veronal. If the monomer in phosphate were greatly expanded, its sedimentation coefficient would be substantially below 7 S and the enzyme would represent a slightly dissociating dimer. If the enzyme in phosphate were more compact, then the curvature (Fig. 1, Curve D) would represent a slight tendency toward dimerization. Both alternatives were tested for reasonable least-square fit of the data by approaching the two local minima with bounded variables as discussed in the "Appendix," and, as expected for a curve that is barely rising, both fit the data adequately. Covalent cross-linking experiments in sodium phosphate show only minor amounts of bands expected for dimers (15, 16). In addition, the presence of significant monomer is required to fit the sedimentation equilibrium data in potassium phosphate and ornithine (see below); both results exclude the "mostly dimer" model. The least-square results show that the monomer has a sedimentation coefficient of 8.7 S and that values for $K_z$ (see "Appendix") and $S_2$ of 0.07 and 13.4 S, respectively, fit the data well.

Both the compact 8.7 S and expanded 7.3 S conformations exhibit self-association in the presence of certain effectors. The association of the 7.3 S conformation promoted by potassium ions (Fig. 1, Curve F) is well described by a monomer-dimer equilibrium. The concentration span covered is inadequate to evaluate unambiguously the three unknowns, $S_1$, $S_2$, and $K_2$, but if the relation $S_2 = S_1(2^{2/3})$ is used, $S_1$ is returned as 7.3 S with $K_2 = 0.2$ with a resultant excellent over-all least-square fit of the data. The effect of potassium ions on the 8.7 S conformation (Fig. 1, Curve C) is more pronounced, and analysis requires self-association to include polymers beyond dimer for least square agreement within experimental error of the data. Alternate models that fit the data are presented as examples in the "Appendix." Additional confirmation of the proposed change in monomer conformation is that no reasonable association scheme with a monomer sedimentation coefficient of 7.3 S fits these data, whereas values in the vicinity of 8.7 S are the least square choice.

Both the conformational change and the association are reversible. For example, the same sedimentation coefficient is obtained in potassium phosphate at a given concentration by dilution of a concentrated sample or by concentrating a dilute sample. Fig. 2 demonstrates both effects in that the velocity pattern in potassium phosphate (A, sedimenting at 10.5 S) is transformed to that of the 7.3 S monomer conformation by dialysis against Tris buffer (B). Subsequent addition of potassium phosphate shows a pattern (C) characteristic of the associated 8.7 S monomer and essentially identical to A. Only low concentrations of potassium phosphate are required to produce major changes in the sedimentation coefficient (Fig. 3).

**Effect of Ammonia and l-Ornithine on Enzyme Activity and State of Association**—The final step in isolation of the enzyme
Fig. 4. Effect of ammonia concentration on the sedimentation coefficient and on the glutamine-dependent carbamyl phosphate synthetase activity (CPSase).  

Involves its precipitation by addition of ammonium sulfate. It was observed that when the enzyme was not dialyzed at this stage in order to remove traces of ammonia, unusually high sedimentation coefficients were obtained. This discovery led to a study of the effect of ammonium ion concentration on the sedimentation coefficient. As shown in Fig. 4A, increasing the concentration of ammonium ions led to an increase in the sedimentation coefficient which reached a maximum at about 14 S at an ammonium ion concentration of 20 mM. Such an increase in sedimentation coefficient resembles the effect produced by l-ornithine, which is a positive allosteric effector (see Fig. 1, Curves A, B, and E). Anderson and Marvin (4, 7) have observed a similar associating effect of ornithine in a potassium phosphate buffer in studies in which sucrose gradient centrifugation was employed. As demonstrated in Fig. 1, Curve E, l-ornithine also promotes a self-association of the enzyme in the absence of potassium and phosphate ions. (Two boundaries were observed at a protein concentration of 4.4 mg per ml, whereas at lower protein concentrations, only a single boundary was found as in the other studies described in Fig. 1.) These observations led us to suspect that ammonium ion might also be a positive allosteric effector. The studies described in Fig. 4B indicate that the increase in sedimentation coefficient produced by increasing the concentration of ammonium ions is accompanied by stimulation of enzymatic activity. The concentration of ammonium ions required for half-maximal effect on the sedimentation coefficient and on the enzymatic activity is about the same. The activation by ammonium ions (Fig. 5) follows a relationship with ATP concentration that is typical of the other allosteric effectors of the enzyme (2), and it thus seems probable that ammonium ion acts by affecting the affinity of the enzyme for ATP.

Fig. 5. ATP dependence of the glutamine-dependent carbamyl phosphate synthetase activity in the presence and absence of ammonia. The enzyme (3.7 μg) was assayed by incubation for 10 min at 37° in a solution (final volume, 0.3 ml) containing 20 mM l-glutamine, 20 mM sodium bicarbonate, 20 mM L-glutamine, 100 mM potassium chloride, and 50 mM Tris-HCl (pH 7.8), and varying amounts of ATP and magnesium chloride, at equimolar concentrations. The upper curve was obtained under the same conditions but with 20 mM ammonium chloride added. Activity was determined by measurement of the glutamate formed.

It is of interest that the ammonia-dependent carbamyl phosphate synthetase activity is not appreciably sensitive to the allosteric activators IMP and ornithine (Fig. 6); in contrast, glutamine-dependent carbamyl phosphate synthetase activity is markedly activated by these effectors in the absence of phosphate (2, 4). On the other hand, UMP produces substantial inhibition of the ammonia-dependent synthetase activity, although this inhibition is significantly less than that exhibited toward the glutamine-dependent carbamyl phosphate synthetase activity (2). These findings seem to reflect the dual function of ammonia as both substrate and allosteric activator. Thus, we may explain the finding that ornithine and IMP fail to produce significant activation of the ammonia-dependent synthetase activity as indicating that ammonia has already interacted with the enzyme so as to promote the conformational state that exhibits increased affinity for ATP. However, UMP can apparently antagonize the activating effect of ammonia by favoring a conformational state of the enzyme which binds ATP less strongly.

Addition of ADP and Mg^{2+} leads to a considerable increase in the sedimentation coefficient, and addition of potassium ion (50 mM) in the presence of ADP and Mg^{2+} leads to a small but probably significant further increase in sedimentation coefficient (Fig. 7). Similar results were obtained when ADP...
The enzyme (3.7 μg) was assayed by incubation for 10 min at 37° in a solution (final volume, 0.3 ml) containing 300 mM ammonium chloride, 20 mM sodium bicarbonate, 50 mM Tris-HCl (pH 7.8), and varying amounts of ATP and magnesium chloride at equimolar concentrations. The following effectors were added as indicated: 3 mM UMP, 10 mM L-ornithine, and 5 mM IMP. The amount of ADP produced was determined by using the coupled DPN-lactate dehydrogenase-pyruvate kinase system (1).

was replaced by ATP, in agreement with the findings of Anderson and Marvin (7). Thus, it is notable that a number of compounds which cause an increase in sedimentation coefficient are also required for, or stimulate, enzymatic activity (potassium ion, ADP, ATP, L-ornithine, and ammonia). The only exception to this generalization so far noted is phosphate ion, which promotes a sizeable increase in sedimentation coefficient (Fig. 1), but actually somewhat inhibits glutamine-dependent synthetase activity (Fig. 8). (Magnesium ions do not reverse the inhibition found with phosphate.) These data are consistent with the observation noted under "Alternate Conformations of Carbamyl Phosphate Synthetase Monomer," i.e. that the increase in sedimentation coefficient promoted by the addition of phosphate is related to a large conformational alteration. In distinction, the increase in sedimentation coefficient promoted by those compounds which stimulate enzymatic activity is probably related mainly to a change in the state of association.

Sedimentation Equilibrium Studies—Sedimentation equilibrium experiments on carbamyl phosphate synthetase in the various buffer systems used for the sedimentation velocity studies reported above were generally not possible because the enzyme was not sufficiently stable for the several days required to reach equilibrium. Although some variation has been noted with different preparations, certain generalizations regarding stability may be made. The enzyme stability is enhanced by increasing the degree of association. Thus, enzymatic activity in potassium phosphate buffer is reasonably maintained only at high protein concentrations; positive allosteric effectors also stabilize the enzyme. In addition, the monomeric form of the enzyme in Tris or Veronal buffer loses its activity and ability to fully reassociate (e.g. when potassium phosphate is added) within a few days, despite the fact that sedimentation velocity behavior in this case is time-independent. These time-dependent conformational changes prevent sedimentation equilibrium studies except under the maximum associating conditions (in ornithine and potassium phosphate) or on the inactive monomer.

The enzyme exhibits behavior characteristic of a highly homogeneous monomer when examined by the criterion of the
sedimentation equilibrium carried out in Veronal-sodium chloride buffer (Fig. 9). The weight average molecular weight under these conditions is 163,000 ± 4,000 based on a partial specific volume of 0.734 as calculated from the amino acid composition. Addition of ornithine to the enzyme in potassium phosphate buffer shifts the equilibrium strongly towards oligomeric species. At high protein concentration (1.75 mg per ml), the weight average molecular weight ($M_w$) is essentially three times the monomer molecular weight, and the number average molecular weight is less than $M_w$, which in turn is less than $z$-average molecular weight. Thus, the association can only be interpreted by including at least one species with molecular weight corresponding to tetramer or higher.

The observation of a maximum sedimentation coefficient corresponding to 15 S (Fig. 1, Curve A) seems somewhat low considering the existence at the higher concentration levels of considerable quantities of trimer and higher polymers. One explanation of this apparently low value of the sedimentation coefficient may be that there are compensating effects of the association by a relatively strong sedimentation coefficient versus $c$ dependence of the individual sedimenting species. Analysis of the molecular weight data by a process identical with that described in the “Appendix” shows several ways in which the sedimentation equilibrium data can be fit. The simplest is a monomer-dimer-trimer-tetramer system with equilibrium constants of $K_2 = 7$, $K_3 = 520$, and $K_4 = 1750$. Using these equilibrium constants and calculating the sedimentation coefficients by the equation $S_j = S_i(2^n)/(f/f_0)$, where $(f/f_0)$ is calculated for linear polymers with axial ratios of 1, 2, 3, and 4 for monomer, dimer, trimer, and tetramer, respectively, the calculated curve fits the data well. We wish to emphasize that neither the evaluation of $K$ values from the limited sedimentation equilibrium data, nor the derivation of a model for association from the limited curvature present in the sedimentation coefficient versus $c$ data should be taken literally. The analysis serves only to present one model for which the two sets of data would be consistent.

Effect of Urea and Guanidine Hydrochloride on Sedimentation Velocity Behavior and on Enzymatic Activity—In the course of these studies, we examined the effects of urea and guanidine on the sedimentation behavior of the enzyme. As indicated in Fig. 10, relatively low concentrations of urea or guanidine hydrochloride produced a dramatic decrease in the sedimentation coefficient when the determinations were carried out in potassium phosphate buffer. When enzyme preparations containing 2 M urea or 0.5 M guanidine hydrochloride were allowed to stand at 26°C for 1 hour and then dialyzed against potassium phosphate buffer (150 mM; pH 7.8) containing 0.5 mM EDTA, the original sedimentation coefficients were restored. As indicated in Fig. 10, sedimentation in Veronal-HCl buffer led to a sedimentation coefficient of about 7.3 S over the same range of urea and guanidine hydrochloride concentrations. Thus, the association of the enzyme which occurs in the presence of potassium phosphate is weakened by urea or guanidine hydro-
for self-association can be shifted in either direction by various solvent perturbations. Thus, formation of the monomer is promoted by (a) removal of potassium ions, (b) addition of urea or guanidine hydrochloride, (c) addition of a negative allosteric effector (e.g. UMP (4)). Self-association of the monomer is favored by (a) addition of a positive allosteric effector (e.g. ammonia, ornithine, IMP), and (b) addition of ADP (or ATP) in the presence of magnesium ions. Some of these effects were noted by Anderson and Marvin (4, 7), who carried out studies in which sucrose gradient centrifugation was employed. The stoichiometry of self-association and the symmetry of various polymers is still uncertain. The results presented here show that species larger than dimer are present in potassium phosphate and that an even more complex association is promoted by the addition of ornithine.

Neither the present studies nor those of Anderson and Marvin (4, 7) can presently serve to define the state of association of the active form of the enzyme under assay conditions. The possible relationship between enzyme activation and association of the monomer may be considered in terms of two general hypotheses. According to one of these, the monomer is the principal active species, and activation (or inhibition) is associated with a shift in the equilibrium between an active conformation and a less active (or inactive) conformation. The active conformation has a more efficient catalytic center and also intermolecular contacts that are more thermodynamically favorable for association. Since marked association requires a substantial increase in protein concentration, the capacity to form a dimer or higher species is viewed as a coincidental phenomenon and has no direct relationship to stimulation of activity.

According to the second hypothesis, allosteric regulation is explained in terms of effects on the association in which the monomer is postulated to be much less active than the dimer. For example, at high concentrations of ATP and magnesium ions, a larger proportion of dimer would be formed, thus minimizing activation by ammonia, ornithine, or IMP. However, UMP (or urea or guanidine hydrochloride) would still inhibit under these conditions. Decrease in the concentrations of ATP and magnesium ions would shift the equilibrium toward monomer and thus increase the potential stimulatory effect of allosteric activators. This explanation implies that the sigmoidal nature of the ATP dependence of the glutamine-dependent synthetase activity can be correlated with the extent of dimerization.

The present work provides evidence that carbamyl phosphate synthetase is allosterically regulated by ammonia as well as by various metabolites of the pyrimidine, purine, and arginine biosynthetic pathways; a diagram summarizing the various metabolic and regulatory phenomena has been published (Fig. 2 of Reference 13). One may speculate that activation by ammonia may have the important function of maintaining the synthesis of carbamyl phosphate under conditions of low cellular energy supply, e.g. ATP deprivation. When the intracellular concentration of ATP falls, at least two mechanisms might function to increase ammonia levels: (a) decreased glutamine synthesis from ATP, glutamate, and ammonia catalyzed by glutamine synthetase; and (b) increased glutaminase activity. Concentrations of ATP greater than 1 mM have been reported to inhibit E. coli glutaminase B (10). The consequent decrease in glutamine concentrations would be expected to reduce product formation by carbamyl phosphate synthetase and by other glutamine amidotransferases. The activation of carbamyl phosphate synthetase by the increased concentration of ammonia produced under these conditions could then provide a mechanism...
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