# Ligand-Induced Dimer-to-Tetramer Transformation in Cytosine Triphosphate Synthetase*

Alexander Levitzki† and D. E. Koshland, Jr.‡

ABSTRACT: CTP synthetase is a dimer of mol wt 108,000. The dimer associates to form a tetramer (mol wt 216,000) in the presence of either of the substrates ATP or UTP. ATP and UTP are synergistic in the sense that the combined effect of the two ligands is higher than the sum of their effects. Binding curves, conformational changes, and the degree of aggregation as a function of ATP and/or UTP concentration have been measured. The correlation between the three titration curves leads to the conclusion that the dimer-to-tetramer conversion is ligand induced.

A wide variety of enzymes exhibit an association–dissociation behavior. Some of these polymerizations occur outside physiological ranges (Constantinides and Deal, 1969) and some at concentrations which indicate they may play a role in cellular processes (Katsunuma *et al.*, 1968; Brown and Reichard, 1969; Kvamme *et al.*, 1970). A number of enzymes which possess characteristics of allosteric enzymes are found to associate and dissociate in the presence of ligands. In Table I are listed a number of examples which fall in this category.

Association–dissociation phenomena are also of interest in relation to protein design. Even those cases which fall outside the physiological range are interesting in the sense that their pattern of dissociation can be important in understanding the subunit interactions of the protein (Klotz *et al.*, 1970; Cornish-Bowden and Koshland, 1970). CTP synthetase falls in the category of such enzymes and provides an excellent opportunity for examining such a behavior.

## Experimental Section

*Materials.* 6-Diazo-5-oxonorleucine[1] was obtained from Parke-Davis. The concentration of DON was measured spectrophotometrically (Hartman, 1968). $^{3}H$- and $^{14}C$-labeled ATP and UTP were obtained either from New England Nuclear Corp. or Schwarz BioResearch, Inc. All nonradioactive nucelotides were obtained from Schwarz BioResearch, Inc.

*CTP Synthetase.* The enzyme was purified and assayed as described previously (Long *et al.*, 1970; Levitzki and Koshland, 1970). By comparison of the $A^{280}$ of the enzyme with the protein value found by the method of Lowry *et al.* (1951) the $A^{280}_{0.1\%}$ value was found to be 0.89. Either $A^{280}$ or Lowry assay values were used to determine protein concentration. The purity of the enzyme was established by gel electrophoresis and electrofocusing (Levitzki *et al.*, 1971). The enzyme was stored in 20% glycerol containing: 4 mM glutamine, 70 mM $\beta$-mercaptoethanol, 2 mM EDTA, and 0.02 M sodium phosphate buffer (pH 7.2) either at 4° or at −20°.

*Binding Measurements.* Equilibrium dialysis was performed as described previously (Levitzki and Koshland, 1972). The filtration method of Paulus (1969) as modified (Levitzki and Koshland, 1972) was also used for binding measurements.

*Fluorescence Measurements.* Fluorescence measurements were carried out on the Hitachi spectrofluorimeter. Experimental details are given in the legends to the figures.

*Polymerization Studies.* The aggregation study was carried out using G-200 columns so that the change in molecular weight could be studied at low enzyme concentrations. Since Sephadex gives weight-average molecular weights, the fraction of tetramer is calculated from eq 1 where $M_w$, the measured molecular weight, is obtained from the position of the

$$M_w = \frac{(210,000)^2X + (105,000)^2(1 - X)}{210,000X + 105,000(1 - X)} \quad (1)$$

peak in the Sephadex column, and $X$ is the fraction of the enzyme in the tetramer form. The values used for the molecular weights of the dimer and the tetramer were 105,000 and 210,000, respectively (Long *et al.*, 1970). These values have been reconfirmed in this study.

## Results

It was shown earlier that the enzyme is a dimer in solution as concentrated as $10^{-4}$ M and that it aggregates to a tetramer in the presence of ATP and UTP when the enzyme is $10^{-7}$ M and the ATP and UTP concentrations are 0.75 mM (Long *et al.*, 1970). The aggregation studies were done at 4° and at 4° it was also found that the enzyme remains as a dimer in the presence of either 0.75 mM ATP or UTP. It has also been shown to exist as a dimer at very low concentrations of enzyme ($10^{-7}$ M) unless the substrates ATP and UTP are added. In that case, it undergoes polymerization to form a tetramer of 216,000 molecular weight. Furthermore, these changes are related to cooperativity as it can be shown that the quantitative aspects of the GTP activation and the glutamine and ammonia reactions are related to the state of polymerization of the enzyme (Levitzki and Koshland, 1972). It therefore seemed of value to study in detail the properties of the two forms of the protein and the changes in conditions which affect this polymerization. Fluorescence, binding, and molecular weight studies were conducted and were shown to clarify some aspects of the ligand induced dimer–tetramer equilibrium in CTP synthetase.

* From the Department of Biochemistry, University of California, Berkeley, California 94270. *Received June 14, 1971.* This work was supported in part by research grants from the National Science Foundation (GB-7057) and the U. S. Public Health Service (AM-GM-09765).

† Present address: Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel.

‡ To whom to address correspondence.

[1] Abbreviation used is: DON, 6-diazo-5-oxonorleucine.

TABLE I: Ligand-Induced Dissociation or Association in Regulatory Enzymes.

| Enzyme | Ligand Affecting Mol Wt: Substrate | Ligand Affecting Mol Wt: Allosteric Effector | Ref |
|---|---|---|---|
| Association | | | |
| Homoserine dehydrogenase (*Rhodospirillum rubrum*) | Homoserine | Isoleucine, methionine | Datta *et al.* (1964) |
| Acetyl-CoA carboxylase (rat adipose tissue) | Homoserine | Citric acid, other TCC[a] intermediates | Vagelos *et al.* (1963) |
| Glutaminase (rat liver) | Glutamine | L-Malic acid, *N*-acetylglutamine | Katsunuma *et al.* (1968) |
| Threonine deaminase (*E. coli*) | Glutamine | 5′-AMP | Kvamme *et al.* (1970) |
| dCMP deaminase (chick embryo) | Glutamine | dCTP + $Mg^{2+}$ | Maley and Maley (1968) |
| CTP synthetase (*E. coli*) | ATP, UTP | | |
| Dissociation | | | |
| Homoserine dehydrogenase (*Rhodospirillum rubrum*) | ATP, UTP | L-threonine | Datta *et al.* (1964) |
| Aspartokinase (*E. coli*) | ATP, UTP | L-Threonine | Wampler and Westhead (1968) |
| Phosphorylase (rabbit muscle) | Glycogen | | Metzger *et al.* (1967) |
| Glutamic dehydrogenase (liver) | DPNH | GTP | Frieden (1963) |

[a] TCC, tricarboxylic acid cycle.

TABLE II: Binding, Fluorescence, and Tetramerization Parameters for CTP Synthetase.[a]

| Ligand | Property Measured | Enzyme Concn (M)[b] | Midpoint Ligand Satn, $S_{0.5}$, M | Hill Coef, $n_H$ | UTP (M) | ATP (M) | $GluNH_2$ (2 mM) |
|---|---|---|---|---|---|---|---|
| ATP | Binding | $4.17 \times 10^{-7}$ | $7.0 \times 10^{-4}$ | 1.63 | | | Yes and no[d] |
| ATP | Binding | $1.49 \times 10^{-6}$ | $3.0 \times 10^{-4}$ | 1.0 | $1.5 \times 10^{-3}$ | | No |
| ATP | Fluorescence | $1.2 \times 10^{-7}$ | $4.8 \times 10^{-4}$ | 1.56 | | | Yes |
| ATP | Aggregation | $1.67 \times 10^{-6}$–$1.2 \times 10^{-7}$ [c] | $1.3 \times 10^{-3}$ | | | | Yes |
| UTP | Binding | $2.45 \times 10^{-6}$ | $7 \times 10^{-4}$ | 1.95 | | | Yes and no[d] |
| UTP | Binding | $8.88 \times 10^{-7}$ | $5.7 \times 10^{-5}$ | 1.0 | | $1.5 \times 10^{-3}$ | No |
| UTP | Fluorescence | $1.5 \times 10^{-7}$ | $3.85 \times 10^{-4}$ | 1.0 | | | Yes |
| UTP | Aggregation | $1.67 \times 10^{-6}$–$1.2 \times 10^{-7}$ | $1.2 \times 10^{-3}$ | | | | Yes |

[a] Conditions: in all experiments the concentration of the following was: 0.01 M $MgCl_2$–0.02 M imidazole acetate (pH 7.2)–70 mM β-mercaptoethanol. [b] Calculated as dimer. [c] Enzyme concentration drops during the gel filtration experiment. [d] Makes no difference.

In Figure 1 is shown the effects of ATP concentration on the dimerization of CTP synthetase. In the same figure is shown the effect of UTP and of the two combined. It is quite apparent that there is a synergistic effect and that ATP and UTP in concert are more effective than an equilvalent concentration of either substrate alone. In Figure 2 the binding of [$^{14}$C]ATP to CTP synthetase under conditions similar to those of the aggregation studies were made. In Figure 3, the binding of UTP to the enzyme is recorded. In the absence of ATP the binding of UTP is cooperative and in the absence of UTP the binding of ATP is cooperative. In the presence of saturating amounts of the alternate ligand the binding of each of these substrates fits the Michaelis–Menten hyperbola. To examine some conformational changes, the fluorescence quenching was examined and the change in this property was measured as a function of ATP (Figure 4) and UTP (Figure 5) concentrations. A quantity which we shall label $\bar{F}$ is designated as a fractional conformation change and will be represented by the quantity $(F_0 - F_x)/(F_0 - F_\infty)$, where $F_\infty$, $F_x$, and $F_0$ represent the fluorescence at saturating, intermediate, and zero concentration of ligand, respectively.

The results of these various studies are summarized in Table II. Comparisons of the half-saturation values are immediately indicative. In the first place it is noted that the half-saturation point for ATP-induced fluorescence quenching is less than the half-saturation point for the binding. This automat-

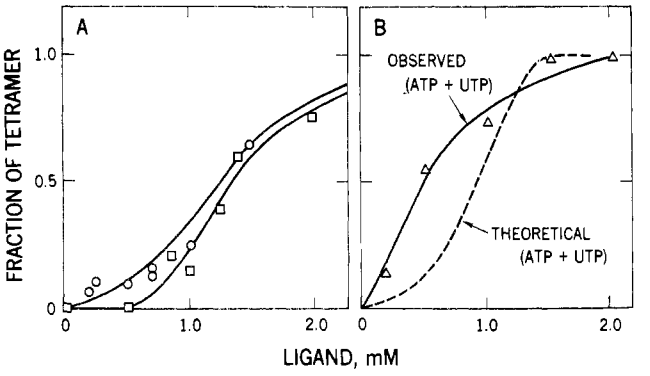
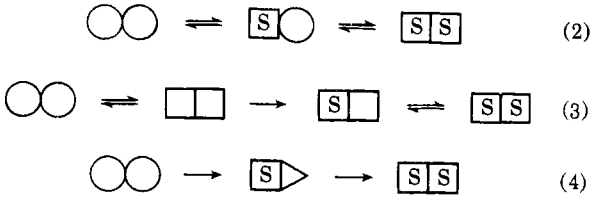



FIGURE 1: The ATP- and UTP-induced dimerization of CTP synthetase. Sephadex G-200 columns (2.5 × 100 cm) with 2-mm layer of G-25 coarse at the bottom were used. The columns were run at low pressure with a head of 10–15 cm. The buffer was 0.02 M sodium phosphate (pH 7.4) containing 1 mM EDTA, 70 mM β-mercaptoethanol, and 10 mM $MgCl_2$, with or without 4 mM L-glutamine. The column was equilibrated with the desired concentration of ATP or UTP or both in the above buffer prior to the addition of the sample. All runs in the presence of both UTP and ATP were done in the absence of $GluNH_2$. Glutamine does not affect either the ATP or the UTP induced dimerization. A sample containing 4 to 6 CTP synthetase units, 15 mg of γ-globulin, 15 mg of bovine serum albumin, 15 mg of chymotrypsinogen, 5 mg of cytochrome *c*, and Blue Dextran in a final volume of 2.5 ml of buffer was applied to the column. The column was run at 25° at a flow rate of 6–12 ml/hr. The fractions were assayed on a Zeiss PMQ-II at 620 (Blue Dextran), 280, and 415 mμ (cytochrome *c*). CTP synthetase was assayed using the standard assay. The molecular weight of CTP synthetase was measured from the standard curve using specific markers in each experiment. The fraction of the enzyme in the tetramer form was computed as described in the text (eq 1). The concentration of the enzyme in terms of dimers when applied was $1.67 \times 10^{-6}$ and $1.2–1.7 \times 10^{-7}$ M in the peak fraction where eluted. (A) The fraction of tetramer as a function of ATP (□) or UTP (○) concentrations. (B) Summation of the ATP and UTP effects from A (— — —) and the observed (Δ) aggregation curve against [ATP + UTP].

ically excludes a simplest sequential model (Koshland *et al.*, 1966) of the type shown in eq 2 and indicates either a concerted model (Monod *et al.*, 1965) of the type shown in eq 3 or a more general sequential model (Koshland, 1970) of the type shown in eq 4.




(2)

(3)

(4)

In the absence of added UTP the binding of ATP is cooperative with a Hill coefficient of 1.6. In the presence of UTP at saturating levels the binding of ATP becomes hyperbolic with a Hill coefficient of 1. Thus, if ATP induces a conformation change on binding to the UTP saturated protein, it must be a change which is not communicated to the neighboring subunits. The fact that the midpoint of the binding curve is approximately half the value of the midpoint of the aggregation curve indicates that there is appreciable binding to the dimeric species before aggregation to a tetramer occurs.

When the ATP influence on binding of UTP is studied, similar phenomena are found. Fluorescence studies indicate that either a general sequential model or a concerted model must apply. Again, the presence of ATP converts a cooperative curve with a Hill coefficient of $n_H$ equal 2.0 to a hyperbolic one with a Hill coefficient of 1.0.

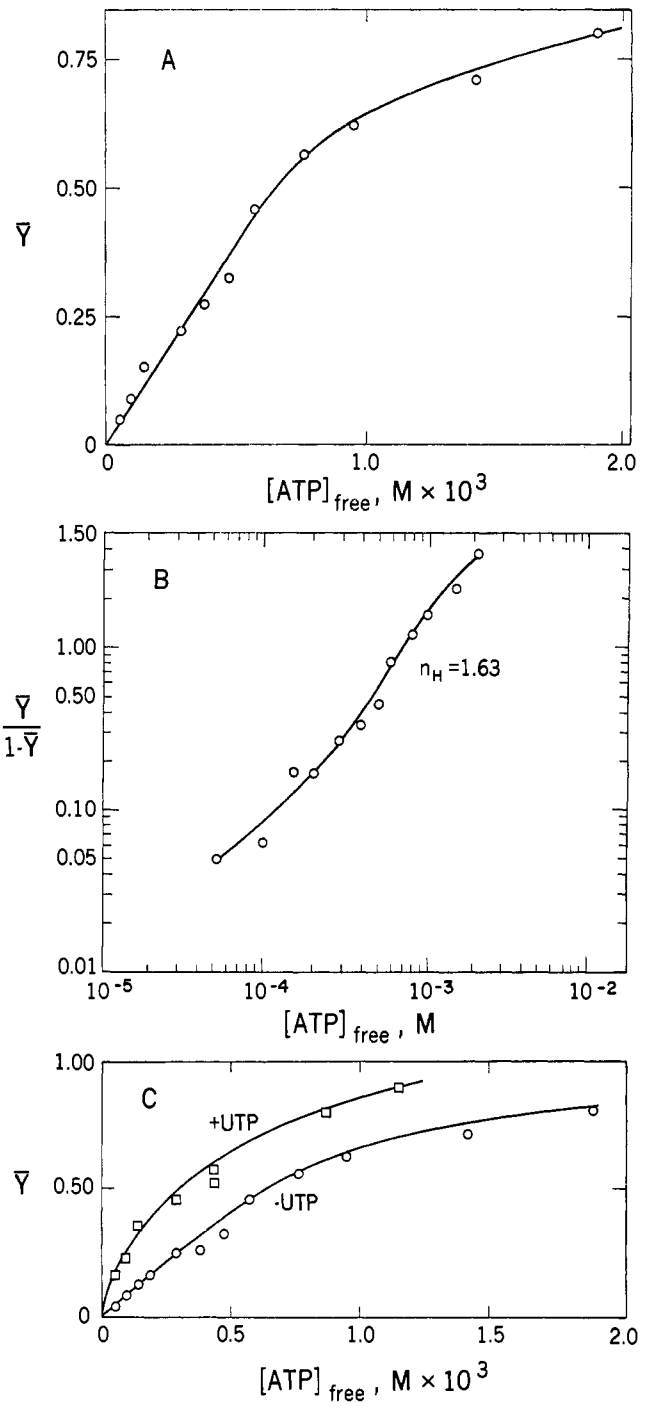



FIGURE 2: Binding of [$^{14}$C]ATP to CTP synthetase. The Paulus binding method was used as described in the Experimental Section. The enzyme–ligand mixtures contained: 10 mM $MgCl_2$, 20 mM imidazole acetate (pH 7.2), 8 mM sodium phosphate (pH 7.4), 2 mM glutamine, 35 mM β-mercaptoethanol, 0.5 mM EDTA, 0.208 nmole of pure CTP synthetase (final concentration $4.16 \times 10^{-7}$ M dimer), and [$^{14}$C]ATP ($2.03 \times 10^5$ cpm/μmole) at different concentrations. The protein–ligand mixtures were filtered for 3.5 hr at 25°. (A) The binding curve for ATP in the absence of UTP. (B) Hill plot of data from part A. (C) ATP binding in the presence of 2 mM UTP as compared to the binding in the absence of UTP.

It is apparent from considering these values that there is not an exactly symmetrical relationship between ATP and UTP. Thus, the presence of excess UTP reduces the midpoint of the

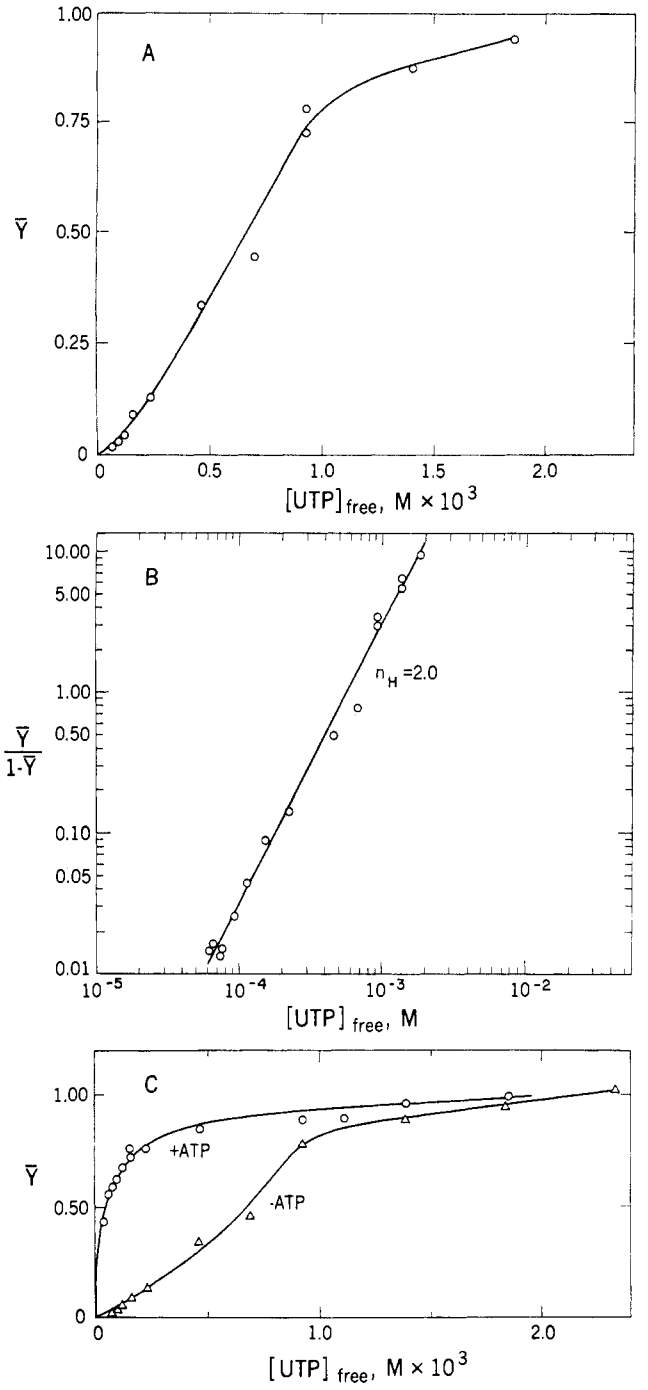



FIGURE 3: Binding of [$^{14}$C]UTP. The conditions were identical with those described for [$^{14}$C]ATP binding. The enzyme concentration in these experiments was $2.45 \times 10^{-6}$ M in terms of dimer. The specific activity of the [$^{14}$C]UTP was $1.245 \times 10^{5}$ cpm/$\mu$mole. (A) The binding curve for UTP. (B) Hill plot of data from A. (C) UTP binding in the presence of 2 mM ATP as compared to the binding in the absence of UTP.

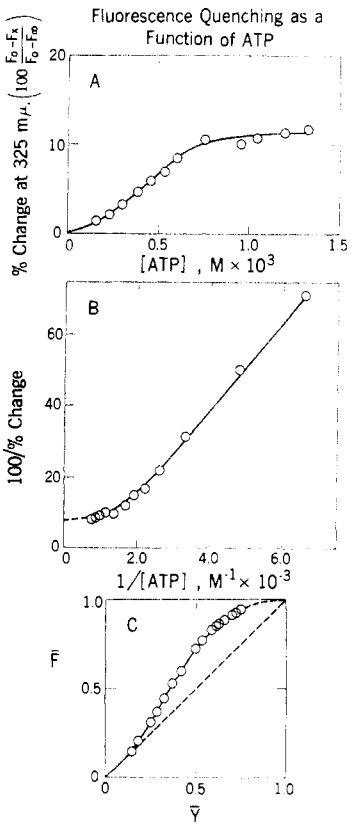



FIGURE 4: Fluorescence change as a function of ATP binding to CTP synthetase. The enzyme concentration was $1.2 \times 10^{-7}$ M in 0.06 M sodium phosphate (pH 7.4), $1.3 \times 10^{-2}$ M $MgCl_2$, 2 mM glutamine at a final volume of 3.0 ml, excited at 295 m$\mu$. The emission spectra was recorded five times at each ATP concentration. Increments of 10 or 15 $\mu$l of ATP ($4.64 \times 10^{-2}$ M, pH 7.4) were added. The change at the emission peak (325 m$\mu$) was used to plot the data. The reduction in emission intensity due to absorption by ATP was measured by the change in the emission spectra of *N*-acetyltryptophan. This reduction in *N*-Ac-Trp fluorescence was used to compute the real net fluorescence quenching of the protein induced by the ligand. (A) Fluorescence change *vs.* [ATP]. $F_0$ is the fluorescence value of the enzyme, $F_x$ is the corrected value at any ligand concentration, and $F_\infty$ is the corrected fluorescence value of the completely liganded protein. (B) Double-reciprocal plot of data in part A. (C) Fraction of fluorescence change ($\bar{F}$) *vs.* the fractional saturation ($\bar{Y}$).

binding curve for ATP to half whereas the presence of excess ATP reduces the midpoint of the binding curve of UTP by a factor of 10. That these two conformational changes are not equal and the second ligand is not simply adding to a single form of the protein is further indicated by the complex relationship between fluorescence, binding, and aggregation. A simple two-state model based on preexisting equilibria could not possibly explain these data.

## Discussion

The detailed evaluation of the association–dissociation phenomena of CTP synthetase indicates a number of significant observations. These are: (a) that there is essentially no formation of tetramer from dimer in the absence of added ligands, (b) that either ATP or UTP alone added at high enough concentrations can induce the tetramerization of the enzyme, (c) that the combination of ATP and UTP causes a greatly increased tetramerization compared to each ligand by itself, (d) that the midpoint of the binding curve occurs at a lower concentration than the midpoint of the aggregation curve, (e) that the midpoint of the fluorescence curve occurs at a lower concentration than the midpoint of the binding curve, (f) that the binding of ATP or of UTP occurs in a positively cooperative manner in the absence of the other ligand, and (g) that the binding of ATP or UTP occurs in a Michaelis–Menten manner in the presence of saturating amounts of the other ligand.

As discussed above, the binding cannot follow the simplest sequential model because the midpoint of the conformational

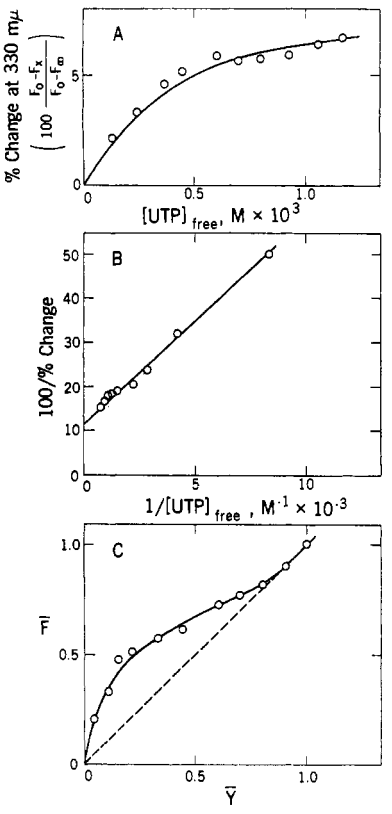



FIGURE 5: Fluorescence change as a function of UTP binding to CTP synthetase. The conditions were identical with those described above for the ATP experiments. The enzyme concentration was 1.5 $\times 10^{-7}$ M dimer. The excitation band was 300 mμ and the emssion band was at 330 mμ. The correction for UTP absorption was performed as described in Figure 4. (A) Fluorescence changes *vs.* [UTP]. (B) Double-reciprocal plot of data in part A. (C) Fraction of fluorescence change ($\bar{F}$) *vs.* the fractional saturation ($\bar{Y}$).

change precedes the binding midpoint of the ligands, and this means that the binding of the first ligand (either ATP or UTP) must induce a significant conformational change in the adjacent subunit of the dimer. Either a partial (as shown in eq 4) or a concerted change (as shown in eq 3) would agree with the fluorescence data. However, the association data exclude the concerted situation because the midoint in the conformational curve is so far below the midpoint of the tetramerization curve. This therefore requires an intermediate species in which the ligand-induced change in the neighboring subunit is not complete. The combination of binding, tetramerization, and conformational data therefore shows that the binding of ATP or UTP is sequential in nature as indicated in eq 4.

The next question which arises is whether ATP and UTP induce the same conformational change. Such a hypothesis would be attractive since it is noted that they have approximately similar Hill coefficients and the same midpoints and that the saturation with either ligand makes the binding of the alternate ligand follow a hyperbolic curve. In fact, however, this mechanism can also be excluded. The limitations imposed on the binding affinities by the similarities in $S_{0.5}$ and $n_H$ would require that the binding constant for ATP in the presence of saturating UTP be essentially the same as the binding constant for UTP in the presence of saturating ATP. Since this is not the case (as shown in Table I and Figures 2 and 3), the binding of ATP must induce a different conformation than the binding of UTP. From the synergism between ATP and UTP (Figure 1) it follows that a further conformational

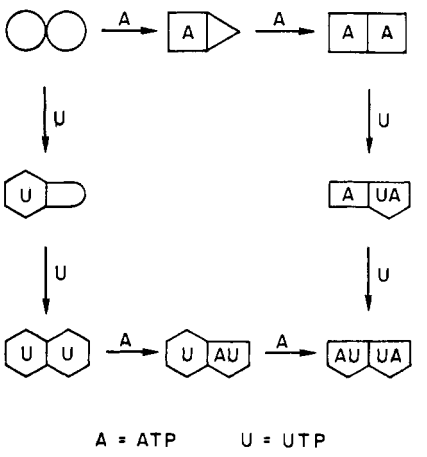



FIGURE 6: Conformation changes induced in the CTP synthetase dimer by ATP and UTP.

change must occur during the binding of one ligand in the presence of saturation concentrations of the other. If both were inducing the same conformational change, a combination of the two would not lead to a greater effect than equivalent concentrations of a single ligand. Since the binding of one ligand in the presence of the other ligand at saturation is found to be hyperbolic, this conformational change is not transmitted to the neighboring subunits.

A combination of all of these facts can be rationalized by the scheme of Figure 6. Here the various species are delineated for the various conformations and states of the dimer. The information which was used to derive this mechanism can also be used to indicate the approximate extent of tetramerization of the various dimer species and this is summarized in Figure 7. Figures 6 and 7 show schematically that dimer species with both UTP and ATP bound associate more strongly than those with only one ligand. This behavior is the basis for the observed synergism between ATP and UTP. The failure to observe any tetramer even at $10^{-4}$ M in the absence of ligands and the quantitative conversion of the dimer to tetramer even at $5 \times 10^{-8}$ M in the presence of ligands strongly suggest that the aggregation is ligand induced. It could very well be that the tetramer form of the enzyme does not dissociate even at concentrations below $5 \times 10^{-8}$ M; however they were not accessible experimentally.

It is perhaps worthy of emphasis that the midpoint of the saturation curve for conformational change is at a lower concentration than the midpoint for binding. This has occasionally been used as an argument that the allosteric mechanism must be concerted but as discussed elsewhere the reasoning is not correct (Koshland, 1970). The mechanism of eq 4 in which the change in one subunit distorts a neighboring subunit will cause the conformation change to "lead" the binding curve. Furthermore, other observations in the CTP synthetase system such as the negative cooperativity with respect to GTP (Levitzki and Koshland, 1972) and the half-of-the-sites reactivity (Levitzki *et al.*, 1971) are incompatible with a concerted mechanism.

It is of interest that the association–dissociation behavior of this enzyme occurs in a range of enzyme concentration and of substrate concentration which are approximately those of the physiological system. From the level of CTP synthetase isolated from *Escherichia coli* the enzyme in the total cell is approximately $6 \times 10^{-7}$ M. The estimated concentration of UTP and ATP are in the millimolar range. Of course, these

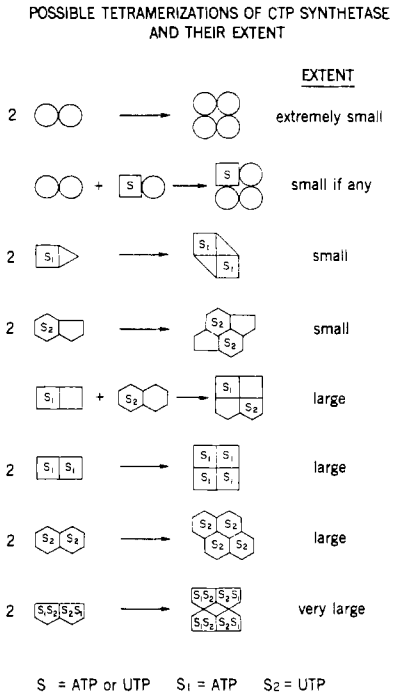



FIGURE 7: Extent of tetramerization of CTP synthetase as a function of the bound ligands.


FIGURE 8: The structure of CTP synthetase.

represent averages over a total cell and might be in error due to compartmentalization but they seem to be reasonable estimates. Thus, the association–dissociation phenomena described here probably occur *in vivo*. Association–dissociation of multisubunit enzymes coupled to ligand binding as a means of control have been observed in other regulatory systems, some of which are listed in Table I. One of the most elegant studies (Brown and Reichard, 1969) demonstrated that ribonucleoside diphosphate reductase dimerizes to an inactive form in the presence of its negative effectors. This dimerization was shown to be inhibited by the positive effectors. This type of control is in essence another manifestation of the change in subunit interactions.

The structure of the CTP synthetase must involve isologous binding as indicated in Figure 8. The absolute value of the pp binding domains must be so strong that no dissociation is observed under normal experimental or physiological conditions. On the other hand, the absolute value of the forces across the qq domain must be considerably weaker than the pp domain. Therefore, dissociation breaking the qq bonds does occur in physiological ranges. As a result the ligand induced changes in structure can be seen at the qq interface in terms of association–dissociation behavior. The ligand-induced distortions are similar in kind in either case. If the distortions do not weaken the subunit interactions to the point of dissociation, the energy is transferred between subunits in a manner similar to the intrachain distortions. This is true in CTP synthetase at the pp interface. If the interactions between subunits are marginal, the induced change in conformation may be just sufficient to cause dissociation. Conversely, a dimer which has little tendency to associate could undergo a conformation change aligning its surface residues so that association is engendered. This is true at the qq domain of CTP synthetase.

In the induced fit model the significant feature of cooperativity is the *change* in subunit interactions, not the absolute magnitude of these interactions. Either an associating–dissociating system or a system which stays in the same polymeric form can have large changes in subunit interactions. The absolute magnitude of the forces between subunits will determine whether these changes lead to dissociation. Therefore it cannot be said that the main cooperative effects occur in the qq domains simply because dissociation–association appears more dramatic than changes of quaternary structure at the same state of polymerization. Conformational changes transmitted across the pp domain will contribute significantly as in the case of the DON-induced conformational changes reported earlier (Levitzki *et al.*, 1971). The fact that the association–dissociation phenomena occurs across the qq plane only emphasizes the importance of the entire quaternary structure of the protein and the importance of both the absolute magnitude and the changes in the subunit interactions.

## References

Brown, N. C., and Reichard, P. (1969), *J. Mol. Biol. 46*, 39.
Constantinides, S. M., and Deal, W. C., Jr. (1969), *J. Biol. Chem. 244*, 5695.
Cornish-Bowden, A. J., and Koshland, D. E., Jr. (1970), *J. Biol. Chem. 245*, 6241.
Datta, P., Gest, H., and Segal, H. L. (1964), *Proc. Nat. Acad. Sci. U. S. 51*, 125.
Frieden, C. (1963), *Enzymes 7*, 3.
Hartman, S. C. (1968), *J. Biol. Chem. 243*, 853.
Katsunuma, T., Temma, M., and Katunuma, N. (1968), *Biochem. Biophys. Res. Commun. 32*, 433.
Klotz, I. M., Langerman, N. R., and Darnall, D. W. (1970), *Annu. Rev. Biochem. 39*, 25.
Koshland, D. E., Jr. (1970), *in* The Enzymes, Vol. 1, Boyer, P. D., Ed., 3rd ed, New York, N. Y., Academic Press, p 341.
Koshland, D. E., Jr., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5*, 365.
Kvamme, E., Tveit, B., and Svenneby, G. (1970), *J. Biol. Chem. 245*, 1871.
Levitzki, A., and Koshland, D. E., Jr. (1970), *Biochim. Biophys. Acta 206*, 473.
Levitzki, A., and Koshland, D. E., Jr. (1972), *Biochemistry 11*, 241.
Levitzki, A., Stallcup, W. B., and Koshland, D. E., Jr. (1971), *Biochemistry 10*, 3371.
Long, C. W., Levitzki, A., and Koshland, D. E., Jr. (1970), *J. Biol. Chem. 245*, 80.
Long, C. W., and Pardee, A. B. (1967), *J. Biol. Chem. 242*, 4715.
Lowry, O. H. Rosebrough, N. J., Farr, A. L., and Randall, R. T. (1951), *J. Biol. Chem. 193*, 265.
Maley, G. F., and Maley, F. (1968), *J. Biol. Chem. 239*, 1168.
Metzger, B., Helmreich, E., and Glaser, L. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 994.
Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.
Paulus, H. (1969), *Anal. Biochem. 32*, 91.

Vagelos, P. R., Alberts, A. W., and Martin, D. B. (1963), *J. Biol. Chem. 238*, 533.

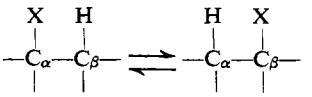
Wampler, D. E., and Westhead, E. W. (1968), *Biochemistry 7*, 1661.

# Mechanism of Action of Coenzyme $B_{12}$. Hydrogen Transfer in the Isomerization of β-Methylaspartate to Glutamate*

R. G. Eagar, Jr., B. G. Baltimore, M. M. Herbst, H. A. Barker, and J. H. Richards†

ABSTRACT: Use of a mixture of unlabeled and tetradeuterio-β-methylaspartate with coenzyme $B_{12}$ dependent β-methylaspartate–glutamate mutase has shown that the hydrogen that migrates becomes one of three equivalent hydrogens during the isomerization. Kinetic isotope effects suggest that cleavage of the bond in the substrate from carbon to that hydrogen which migrates is an important component of the rate-determining step. The evidence also supports the existence of an intermediate which can partition with similar probabilities to β-methylaspartate or to glutamate. Mechanistic implications of these findings are discussed.

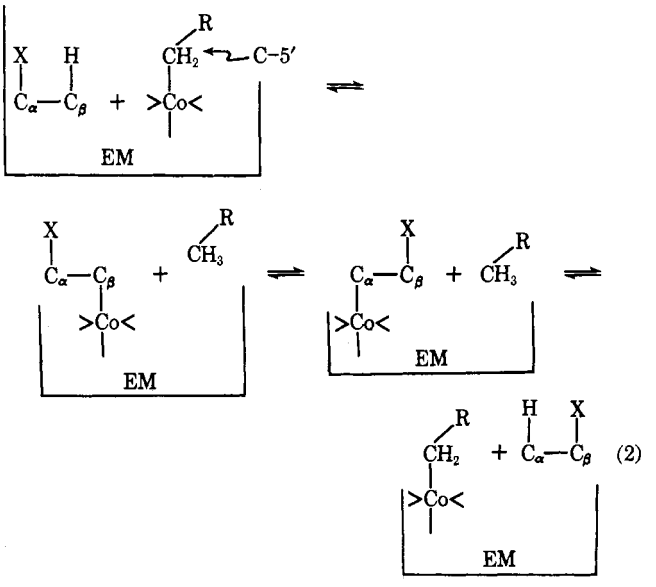
The cobalt-containing coenzyme $B_{12}$ (α-dimethylbenzimidazolyl Co-5′-deoxyadenosylcobamide) has been shown to be an obligatory cofactor in several processes in which hydrogen atoms and other groups undergo 1,2 migrations. Some of these are: dehydration of 1,2-propanediol to propionaldehyde (Brownstein and Abeles, 1961; Lee and Abeles, 1963), deamination of ethanolamine to acetaldehyde (Bradbeer, 1965; Babior 1969), isomerization of methylmalonyl coenzyme A to succinyl coenzyme A (Smith and Monty, 1959; Wood *et al.*, 1964), isomerization of β-methylaspartate to glutamate (Barker *et al.*, 1958; Iodice and Barker, 1963; Barker *et al.*, 1964a), the isomerization of β-lysine to 3,5-diaminohexanoic acid (Stadtman and Renz, 1968), and the isomerization of α-methyleneglutarate to α-methylene-β-methylsuccinate (Kung *et al.*, 1970). These six reactions have several features in common which can be summarized (eq 1).




(1)

In the isomerizations of both β-methylaspartate (Barker *et al.*, 1964a,b) and methylmalonyl coenzyme A (Kellermeyer and Wood, 1962; Wood *et al.*, 1964; Phares *et al.*, 1964), the X group ($CHNH_2COOH$ and COSCoA, respectively) is known to be transferred intramolecularly. The hydrogen which is transferred does not exchange with the hydrogen of water during the isomerization of β-methylaspartate (Barker *et al.*, 1964a; Iodice and Barker, 1963; Suzuki and Barker, 1966), the isomerization of methylmalonyl coenzyme A (Overath *et al.*, 1962; Erfle *et al.*, 1964a,b), the dehydration of propanediol (Brownstein and Abeles, 1961), or the deamination of ethanolamine (Babior and Gould, 1969). Exchange of hydrogen between substrate and C-5′ of coenzyme $B_{12}$ is, however, observed for propanediol dehydrase (Abeles and Zagalak, 1966; Riley and Arigoni, 1966; Frey and Abeles, 1966; Abeles and Frey, 1966), methylmalonyl coenzyme A mutase (Riley and Arigoni, 1966), ethanolamine deaminase (Babior, 1969), and glutamate mutase (Switzer *et al.*, 1969). Stereochemical studies of the rearrangments have shown that inversion of configuration is observed at the carbon to which hydrogen migrates in the isomerization of β-methylaspartate to glutamate (Sprecher and Sprinson, 1964) and for dehydration of propanediol (Retey *et al.*, 1966; Zagalak *et al.*, 1966). However, configurational inversion is not observed in the rearrangement of methylmalonyl coenzyme A to succinyl coenzyme A (Sprecher *et al.*, 1964).

The hydrogen which migrates has been shown to become one of three equivalent hydrogens in the case of methylmalonyl coenzyme A mutase suggesting that transfer of hydrogen from substrate to C-5′ of the deoxyadenosyl residue to the coenzyme cleaves the cobalt C-5′ bond and transforms C-5′




(2)

* Contribution No. 4228 from the Gates and Crellin Laboratories of Chemistry and Church Laboratory of Chemical Biology, California Institute of Technology, Pasadena, California 91109, and from the Department of Biochemistry, University of California, Berkeley, California 94720. *Received July 12, 1971.* Supported by National Institutes of Health Grants GMS-10218 and AI-00563.

† To whom to address correspondence.