Subunit Interactions in Aspartate Transcarbamylase

THE INTERACTION BETWEEN CATALYTIC AND REGULATORY SUBUNITS AND THE EFFECT OF LIGANDS

WILLIAM W.-C. CHAN

From the Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada

SUMMARY

The interaction between the catalytic subunit \(c_3\) and the regulatory subunit \(r_2\) of aspartate transcarbamylase from Escherichia coli was studied by measuring the reversible formation of the \(c_3r_2\) complex as a function of \(r_2\) concentration. Conversion to the native enzyme was prevented by using a very low concentration of \(c_3\) (40 ng per ml) in the presence of bovine serum albumin. A simple hyperbolic \(r_2\) saturation curve was obtained suggesting the presence of only one kind of \(c:r\) domain. From the association constant for the formation of \(c_3r_2\), the free energy of \(c:r\) interaction can be estimated to be about \(-10 \) Cal per mole. Neither CTP nor ATP appears to affect the strength of \(c:r\) interaction in this complex. Succinate in the presence of carbamyl phosphate promotes tighter binding. At higher concentrations of \(c_3\) and nonsaturating levels of \(r_2\), conversion to the native enzyme \((c_6r_6)\) takes place. This renaturation process is second order with respect to the concentration of \(c_3\) and is virtually irreversible. Renaturation is inhibited by saturating levels of \(r_2\) and to some extent by both CTP and ATP. The effect of ligands on \(c:r\) interactions reported here may have significance in the allosteric mechanism of the native enzyme.

In the preceding paper (2), physicochemical evidence was reported indicating that the catalytic subunit \(c_3\) of aspartate transcarbamylase interacts reversibly with the regulatory subunits \(r_2\) to form a complex which most probably has an \(c:r\) structure. Although at nonsaturating levels of \(r_2\), the reversible formation of the complex is accompanied by conversion to the native enzyme \((c_6r_6)\), these two processes can be studied separately by selecting the appropriate \(c_3\) concentration. Thus, in the reversible reaction \(c_3 + 3r_2 \rightleftharpoons c_3r_2\), the ratio of \(c_3\) to \(c_6r_6\) should be independent of the total concentration of \(c_3\) and \(c_6r_6\) whereas the conversion of \(c_3\) and \(c_6r_6\) to \(c_6r_6\) is concentration-dependent. At extremely low concentrations of \(c_3\), conversion to \(c_6r_6\) therefore becomes insignificant and the formation and breakdown of the \(c_3r_2\) complex should then appear as a simple equilibrium. The association constant then provides a direct measure of the strength of subunit interactions. We report here results indicating that at low \(c_3\) concentrations, the interaction between \(c_3\) and \(r_2\) may indeed be described as an equilibrium involving only one type of binding site. An unexpected finding is that the strength of the \(c:r\) subunit interaction so determined is unaffected by CTP and ATP. Also presented here are the kinetics of conversion of the \(c_3r_2\) complex to the native enzyme at somewhat higher concentrations of \(c_3\) and nonsaturating levels of \(r_2\). This process is second order and is inhibited by high levels of \(r_2\) and by both CTP and ATP. The implications of these results on the allosteric mechanism of aspartate transcarbamylase will be discussed in the following paper (3).

MATERIALS AND METHODS

The preparation of aspartate transcarbamylase, the separation into subunits, and the enzyme activity assays were as described in the preceding paper (2). The chemicals used were also the same except that the l-aspartic acid used in the assays was \(U^{-14}\)C-labeled (208 mCi per mmol) rather than tritium labeled.

For studying the extent of \(c_3r_2\) formation as a function of \(r_2\), the regulatory subunit (0.25 to 5 \(\mu\)g) in 10 to 200 \(\mu\)l of potassium phosphate buffer (40 mM, pH 7.0) containing 2-mercaptoethanol (20 mM) and bovine serum albumin (50 \(\mu\)g per ml) was added to a solution of \(c_3\) at 25 (0.04 \(\mu\)g per ml) in Tris-acetate buffer (0.2 M, pH 8.5) containing 2-mercaptoethanol (20 mM), potassium \(\alpha\)-aspartate (0.5 or 1.0 \(\mu\)mol, 2 \(\times\) 10\(^6\) cpm per \(\mu\)mol) and bovine serum albumin (50 \(\mu\)g per ml). Volume was 0.49 ml at this stage. After incubation for 1 min, dialithium carbamyl phosphate (0.5 \(\mu\)mol in 10 \(\mu\)l) was added to start the enzymic reaction. Duplicate samples were assayed for and 10 min as described previously. Controls were performed without \(r_2\) to determine \(v_0\) (see elsewhere in text) and without \(c_3\) to determine the background activity in the \(r_2\) preparation (accounting for 0.5 to 5% of the counts in the assay). For the reciprocal plots, the data were analyzed by a computer procedure to obtain a least squares fit to a straight line without the weighting of points.

In the experiments on reassociation, the mixture (1 ml) containing \(c_3\) (0.1 to 0.4 \(\mu\)g) in Tris-acetate buffer (0.2 M, pH 8.5) containing 2-mercaptoethanol (20 mM) and the albumin (50 \(\mu\)g per ml) and reassociation was started by adding \(r_2\) (5 to 20 \(\mu\)g in 5 to 20 \(\mu\)l). At various time intervals, duplicate samples (normally 50 \(\mu\)l each) were withdrawn and added to 0.44 ml of Tris-acetate buffer (0.2 M, pH 8.5) containing potassium \(\alpha\)-aspartate (0.5 \(\mu\)mol, 2 \(\times\) 10\(^4\) cpm per ml).
cpm per μmol), r2 (5 μg), and the same amount of 2-mercaptoethanol and albumin. Enzymic reaction was then started by adding carbamyl phosphate (0.5 μmol in 10 μl of H2O) and duplicates were assayed at 25°C for 10 or 20 min. For reassociation mixtures containing the lowest concentration of r1 (0.1 μg), the size of the withdrawn samples was increased to 100 μl and the final volume of the assay mixture was maintained at 0.5 ml by appropriate adjustments. Assays with CTP (1 mM) or ATP (5 mM) were conducted similarly but with a higher aspartate concentration (20 mM). The renaturation rate was found to be dependent to some extent on the age of the cr preparation and for rate comparison under different conditions each set of experiments was conducted within the shortest practicable period (a few days). For studies of the effects of CTP and ATP on renaturation rate, each series was done in 1 day using the same diluted cr solution so that the greatest possible uniformity might be achieved. In these experiments, because of the large number of assays required in each series, only two sampling times (30 and 60 min) were used (with duplicates at each time point). Linearity was always found for the second order plots extending between the zero time value and the values at the two sampling times.

RESULTS

Extent of c:r6 formation as a function of r2 concentration—At low aspartate concentrations (1 or 2 mM), the postulated c:r6 complex is more active than the catalytic subunit (c3) because the $K_m$ of the complex is smaller (2, 4). Using the increase in enzymic activity to measure c:r6, we have studied the extent of c:r6 formation as a function of r2. In order to minimize the extent of conversion to the native enzyme, very low concentrations of c3 (40 ng per ml) were used in these experiments. Previous work has shown using the continuous titrimetric assay that the formation of c:r6 was so fast that the increase in activity upon the addition of r2 occurred with no apparent time lag (4). It was therefore possible to start the enzymic reaction soon (1 min) after the addition of r2 to cr.

As judged by the increase in activity upon adding r2, the formation of the c:r6 complex appears to be a simple saturation process and its dependence on r2 concentration follows a hyperbolic curve (Fig. 1a). These results suggest that only a single binding step is involved. The simplest, though not the only possible explanation (see “Discussion”), is that the following equilibria exist (Equation 1) and that the three c:r domains in c:r6 are independent of each other.

$$\begin{align*}
cr + r2 & \rightleftharpoons c2r2 \\
cr + r2 & \rightleftharpoons cr6
\end{align*}$$

(1)

This explanation further assumes that the formation of each c:r contact affects only the active site located in the particular catalytic polypeptide involved and not the neighboring active sites. If these assumptions are valid, we can simply consider that the catalytic polypeptides can exist in two states (c3 and c6) depending respectively on whether a regulatory subunit is bound to it or not. The above equilibria can then be represented as a single process, $c3 + r2 \rightleftharpoons c6$ for which Equation 2 holds.

$$\frac{[c6]}{[c3][r2]} = K_{assoc}$$

(2)

where the square bracket terms indicate the concentration of the species and $K_{assoc}$ is the association constant for c:r binding. Because c3 and c6 have different kinetic properties, the change in activity at 1 mM or 2 mM aspartate can be related to the concentration of these two states. Thus the increase in activity, ($v - v_0$) is given by

$$v - v_0 = [c3] \times \text{specific activity of c3}$$

(3)

At saturation levels of r2, all of the catalytic polypeptides will be in the form of c6 and the maximum increase in activity ($v_{sat} - v_0$) is given by

$$v_{sat} - v_0 = [c6] \times \text{specific activity of c6}$$

(4)

from which we derive the expression

$$\frac{v - v_0}{v_{sat} - v_0} = \frac{[c3]}{[c6]}$$

(5)

and from Equations 2 and 5 we can obtain

$$\frac{1}{v - v_0} = \frac{1}{[r2]K_{assoc}} \times \frac{1}{v_{sat} - v_0} + \frac{1}{v_{sat} - v_0}$$

(6)

A plot of $1/(v - v_0)$ versus $1/[r2]$ should therefore be a straight line and when $1/(v - v_0) = 0$, the intercept $1/[r2]$ is equal to $-K_{assoc}$. The double reciprocal plot (Fig. 1b) shows that the above relationship holds at both 1 mM and 2 mM aspartate con-
appears that CT1' and AT1 have little or no effect on the c:r interactions in the C3r6 complex. On the other hand, the sub-

of the data also do not differ substantially (Table I). It thus

interaction (AGf,) can be calculated from -1/\( K_{assoc} \) to

ligand. The values of \( K_{assoc} \) computed from a least squares fit

terest to examine the possible effects of the allosteric ligands CTP

and ATP on the strength of subunit interactions as measured by

data (Fig. 2) obtained in the presence of either 1 mM CTP or 5

rnM ATP are quite similar to those obtained in the absence of the

be = -10.2 Cal per mol.

These experiments could not be performed because it would then

make similar measurements at higher concentrations of aspartate,

in aspartate concentration. Although it would be desirable to

intercepts (Table I) are not significantly affected by the change

centrations. The association constants determined from the

intercepts (Table I) are not significantly affected by the change

in aspartate concentration. Although it would be desirable to

make similar measurements at higher concentrations of aspartate,

these experiments could not be performed because it would then

be impossible to distinguish between c3r6 and cQ on the basis of

activity. If the observed r2 saturation indeed represents the

equilibrium of binding at the c:r domain, then the free energy of

interaction (\( \Delta G_{c:r} \)) can be calculated from -\( RT \ln K_{assoc} \) to

be = -10.2 Cal per mol.

Effect of Ligands on r2 Saturation—it was of considerable in-

terest to examine the possible effects of the allosteric ligands CTP

and ATP on the strength of subunit interactions as measured by

the r2 saturation method described above. Surprisingly, the data

(Fig. 2) obtained in the presence of either 1 mM CTP or 5

mM ATP are quite similar to those obtained in the absence of the

ligand. The values of \( K_{assoc} \) computed from a least squares fit

of the data also do not differ substantially (Table I). It thus

appears that CTP and ATP have little or no effect on the c:r

interactions in the c3r6 complex. On the other hand, the sub-

strate analog succinate appears to promote tighter binding be-

tween c3 and r2 as reflected in the higher association constant.

To gain some insight into the nature of the forces involved in
c:r binding, we also determined the effect of urea, ionic strength,

and organic solvent on the subunit interactions. It was found

that urea at a concentration of 2 M greatly weakened the binding

whereas reduction in the ionic strength resulted in tighter binding

(Fig. 3 and Table I). Addition of 0.2 M propanol to the lower

ionic strength medium was shown to lead to weaker binding. It

thus appears that ionic charges, hydrophobic interactions, and

perhaps hydrogen bonds may all be involved in the association

between the catalytic and the regulatory subunits.

Conversion into Native Enzyme (c3r6)—It has been shown that

at higher concentrations of c2 and nonsaturating concentrations

of r2, conversion to the native enzyme takes place (2, 4). Al-

though it was possible previously to monitor this renaturation

process in a continuous manner by observing the decrease in ac-

tivity with time (4), this method was unreliable for quantitative

rate measurements because the depletion of substrates and the

accumulation of products might introduce serious errors.

Because the renaturation process is essentially irreversible (2), it

was decided to follow it by removing samples at intervals and
diluting into the assay mixture containing r2. As the conversion

c3r6 (see Footnote 2) to the native enzyme (c3r6) is known to

be dependent on c3r6 concentrations (4), the 5- or 10-fold dilution

into the assay mixture will essentially stop further renaturation.

The presence of a relatively high concentration of r2 in the assay

mixture ensures that practically all of the remaining c3r6 will

be converted to c3r6, and this also serves to prevent further renatra-

tion in the withdrawn samples. Previously, it was found that

in the presence of substrates, renaturation occurred over approxi-

mately 15 min at 0.75 \( \mu \)g of c3 and 2.5 \( \mu \)g of r2 per ml. However,

in the absence of substrates, the renaturation appeared to proceed

faster, and lower initial c3 concentrations were necessary to per-

mit convenient study of the process. The \( r2 \) concentration was

also increased to help further reduce the rate of renaturation.

The course of renaturation at 0.2 \( \mu \)g of c3 and 5 \( \mu \)g of r2 per ml is

shown in Fig. 4. Samples from the same renaturation mixture

TABLE I

Association constants for formation of c3r6 from c2 and r2

For conditions see text.

| Aspartate concentration | Additions                  | \( K_{assoc} \)     |
|-------------------------|----------------------------|--------------------|
| 1 mM                    | None                       | 2.93 ± 0.14        |
| 2 mM                    | None                       | 3.18 ± 0.14        |
| 1 mM                    | 1 mM CTP                   | 3.74 ± 0.14        |
| 1 mM                    | 5 mM ATP                   | 2.94 ± 0.12        |
| 2 mM                    | 20 mm succinate            | 7.56 ± 0.40        |
| 1 mM                    | 2 M urea                   | 0.74 ± 0.24        |
| 1 mM                    | 50 mm Tris-acectate*       | 6.73 ± 0.25        |
| 1 mM                    | 50 mm Tris-acectate* + 0.2 M propanol | 2.95 ± 0.10 |

* Tris-acectate (50 mm) was used in these experiments instead of Tris-acectate (0.2 M).

\( \Delta G_{c:r} \) is the same graph as in Fig. 16 for 1 mM aspartate obtained in

the absence of either CTP or ATP. However, for determining the association constants in the CTP and ATP experiments (Table I), a least squares procedure was used.

\( \Delta G_{c:r} \) is defined as the free energy of interaction between c3 and r2 as reflected in the higher association constant.

Although the mechanism of formation of c3r6 from c3 and r2 is unknown, evidence (2, 4) indicates that the process is inhibited when all of the initially added c3 is converted to c3r6. Thus, strictly speaking, it is not correct to refer to the process as the conversion of c3r6 to c3r6. We have therefore used the term c3r6 (\( n = 0, 1, 2, \) or 3) to represent the mixture of the species c3, c3r6, c3r6, and c3r6 from which c3r6 is formed via a bimolecular reaction.

Fig. 2. Effect of ligands on r2 saturation. (A), 20 mm succinate and 2 mm aspartate; (D), 5 mm ATP and 1 mm aspartate; (X), 1 mm CTP and 1 mm aspartate. For the data in the presence of succinate the line shown is obtained by least squares fit. For the ATP and CTP data, in order to provide comparison, the line shown here is the same graph as in Fig. 16 for 1 mm aspartate obtained in the absence of either CTP or ATP. However, for determining the association constants in the CTP and ATP experiments (Table I), a least squares procedure was used.

Fig. 3. Effect of the aqueous environment on r2 saturation. Aspartate (1 mM) was used in these experiments. (O), 2 M urea; (O), 50 mm Tris-acectate instead of 0.2 M Tris-acectate; (X), 50 mm Tris-acectate and 0.2 M propanol.

Downloaded from http://www.jbc.org/ by guest on March 23, 2020
were assayed under three different conditions in order to provide some information about the nature of the product. Initially no native enzyme was present and all of the c6rZn was converted in the assay mixture to c6r6 which had about the same activity at 20 mM aspartate in the presence of either CTP (1 mM) or ATP (5 mM). As renaturation proceeded, the activity in the CTP assay decreased by large amounts and approached a low final level whereas the activity in the ATP assay decreased to a much smaller extent and considerable final activity was maintained. At 1 mM aspartate, the relatively low initial activity due to c6r6 dropped further and finally reached only a barely detectable level. These results are consistent with the expected conversion of c6rZn (which is measured in the assay as c6r6 characterized by a low Ka and insensitivity to CTP and ATP) to the native enzyme which is known to have little activity at low aspartate concentrations and to be sensitive to CTP and ATP. The duplicate 10-min and 20-min assays agreed within experimental error indicating that no further renaturation took place once the samples were diluted into the assay mixture. Because the activity in the 1 mM aspartate assay decreased 20-fold during renaturation, this assay provided the most accurate means of monitoring the renaturation process and was used in all of the subsequent experiments. The 5-fold decrease in activity in the CTP-containing assay at 20 mM aspartate also gave similar (though always slightly higher) renaturation rates. The assay containing ATP was, however, less reliable for following renaturation because the maximum decrease in activity amounted to less than 40%.

The kinetic data on renaturation were analyzed by converting the activities to concentrations of c6rZn and c6r6. In the following analysis, r0 represents the activity at zero time; r1 the activity at time t; and r∞, the activity at the end of renaturation. If we consider the specific activity of c6r6 and c6r6 in terms of the amount of catalytic polypeptide contained in these species, then we obtain

$$v_{t} - v_{∞} = \frac{\text{amount of catalytic polypeptide}}{\text{specific activity of c6r6}}$$

$$v_{∞} = \frac{\text{amount of catalytic polypeptide}}{\text{specific activity of c6r6}}$$

and therefore $(v_{t} - v_{∞})/v_{∞}$ is equal to $(a)$ the fraction of the total catalytic polypeptides existing in the form of c6r6. When $1/a$ is plotted against renaturation time (t), a straight line is obtained (Fig. 5) indicating second order dependence on c6r6. This result is also confirmed by varying the initial concentration of c6 and the second order rate constants obtained (Table II) with 0.1, 0.2, or 0.3 μg of c6 per ml all agree within experimental error. The renaturation rate was found to depend also on the r2 concentration (Fig. 6). The rate constant decreased approximately linearly with the increase in r2 concentration within the range of r2 levels used. These results are in agreement with our earlier qualitative observation (2, 4) indicating that reassociation to the native enzyme is inhibited when all of the c6 present is converted to c6r6. The implication of this finding on the mechanism of reassociation is discussed in a later section.

The renaturation process was found to be inhibited to some extent by CTP and by ATP (Fig. 7). In both cases, as the concentration of the nucleotide was increased, the amount of inhibition reached a maximum and then declined. Maximal inhibition occurred at nucleotide concentrations (0.5 mM CTP and 1 mM ATP) which are known to alter significantly the activity of the native enzyme (5). These concentration levels are in the

![Graph](https://example.com/graph.png)

**Fig. 4.** Time course of renaturation of aspartate transcarbamylase from the catalytic and regulatory subunits. The mixture contained initially 0.2 μg of c6 per ml in 0.2 M Tris-acetate, pH 8.5, 20 mM 2-mercaptoethanol, and 50 μg per ml of the albumin at 25° and renaturation was started by adding 5 μg per ml of r2. Samples from the same renaturation mixture were assayed in three different assay systems for 10 and 20 min as described under "Materials and Methods." The substrates and ligands indicated on the graphs denote the conditions in the assay systems.

![Graph](https://example.com/graph.png)

**Fig. 5.** Dependence of renaturation rate on c6 concentration. a represents the fraction of the initially added c6 not yet converted to the native enzyme and therefore existing as c6r6. Initial c6 concentrations are indicated on the graph. Ten micrograms of r2 per ml were added to start renaturation in each case. Other conditions were the same as in Fig. 4. Duplicate samples were assayed for 10 and 20 min at 1 mM aspartate.

**TABLE II**

| Initial concentrations | $k_{renat}$ |
|------------------------|-------------|
| c6 | r2 | $10^{9}$ M⁻¹ min⁻¹ |
| 0.1 | 10 | 17.3 |
| 0.2 | 10 | 17.7 |
| 0.3 | 10 | 16.8 |
| 0.2 | 5 | 42.0 |
| 0.2 | 10 | 19.8 |
| 0.2 | 20 | 7.4 |
the same as in Fig. 5.

added per ml are indicated on the graph. Other conditions were equal to five times the Ki.

not obtained with the competitive

sites on r2 reported by various workers (8-M). The effect is not caused by the occupation of the carbamyl phosphate site in

ATP' binding to r2 (6, 7). The tendency to produce lower in-

taking duplicate samples at 30 and at 60 min. Other conditions were the same as in Fig. 5. The second order rate constants were then plotted as percentages against concentration of ligand. (O), with CTP; (●), with ATP; (X), 0.5 mM CTP with 0.5 mM pyrophosphate; and (△), 1 mM ATP with 0.5 mM pyrophosphate.

same range as the binding constants determined for CTP and ATP binding to r2 (6, 7). The tendency to produce lower

inhibitor pyrophosphate at a level equal to five times the Ki.

of varvina amounts of CTP and ATP and the rate was followed by

of aspartate concentrations. We must therefore consider the fol-

manner to that of c3 and c3r6. The observation that the curve

activity equal to two-thirds the activity of c3 plus one-third the

similarly c3r4) at low levels of aspartate: (a) that it has the same activity as c1; (b) that it has the same activity as c3r6; (c) that c3r3 has an activity equal to two-thirds the activity of c1 plus one-third the activity of c3r6; and (d) that its activity is not related in a simple manner to that of c3 and c3r6. The observation that the curve relating r2 concentration with the increase in activity is a simple hyperbola is difficult to reconcile with the complex situation postulated under (d) but is not inconsistent with the remaining alternatives. If the binding of one or two r2 subunits to c3 produces no change at the active sites (alternative a) and the characteristic properties of c3r4 are brought on only when the third r2 subunit binds, then a simple hyperbolic saturation curve would result.

However, from a mechanistic point of view, it is unlikely that such an all-or-none system operates. Similarly, it would be difficult to envisage a molecular mechanism whereby the binding of the 1st r2 subunit results in all of the properties of c3r4 whereas the binding of two subsequent r2 subunits produces no effect (alternative b). Thus the most reasonable explanation of the hyperbolic r2 saturation curve is to postulate that as each r2 subunit binds to a catalytic polypeptide in c3 it affects only the active site on that polypeptide giving therefore one-third the effect observed in c3r6 (alternative c). This mechanism, together with the assumption that the binding of each r2 subunit does not affect the binding of other r2 subunits, is able to account for the observed r2 saturation curve in a mechanistically reasonable manner. The hyperbolic saturation curve then simply reflects the single process of r2 binding to one of three independent domains on c3. This explanation of the r2 saturation results is in agreement with previous evidence indicating that the catalytic polypeptides in c3 behave independently (11).

If we assume that the above explanation is correct, then the r2 saturation provides a most convenient measure of the strength of the c:r interaction. Of the three types of subunit interactions present in native aspartate transcarbamylase, the c:r interaction appears to be the weakest. The c:r interaction must be stronger because no dissociation of c3 into an individual c subunit is detectable under non-denaturing conditions (11). In the case of r2, spontaneous dissociation into monomers has been observed (6); however, the tendency to dissociate is significantly less than in the case of c3r6. Therefore, the r:r interaction should be somewhat intermediate in strength between the c:c and the c:r interactions. The c:r interaction is however sufficiently strong to maintain the c6r6 structure of the native enzyme because even the most energetically favorable mode of dissociation (i.e. c3r6 = c6r4 + r2) would require the break-up of two c:r contacts. The standard free energy change for this process would therefore be about 20 Cal corresponding to a \( K_{\text{asso}} \) of \( 4.5 \times 10^{5} \) M\(^{-1}\). This estimate of the minimum energy required to dissociate the native enzyme is consistent with the observation that no dissociation can be detected at room temperature even when the aspartate transcarbamylase concentration is in the microgram per ml range (12). Our estimate of the strength of c:r interaction also implies that c3r6 should be relatively stable because its dissociation would also involve the breakup of two c:r contacts regardless of which of the following processes occurs: c3r6 \( \rightleftharpoons \) c3r5 + r2; c3r6 \( \rightleftharpoons \) c3 + c6r3; or c3r6 \( \rightleftharpoons \) 2 c6r3. On the other hand c3r6 should dissociate as readily as c3r6 because this process involves only one c:r contact. These calculations are supported by the recent reports of the isolation of c3r6 from renaturation mixtures deficient in r2 (13, 14).

![Fig. 6. Dependence of renaturation rate on r2 concentration. Initial c3 concentration was 0.2 μg per ml and the amounts of r2 added per ml are indicated on the graph. Other conditions were the same as in Fig. 5.](http://www.jbc.org/)
The results on the kinetics of reassociation clearly show that the formation of native enzyme is second order with respect to the concentration of \( c_3r_6 \). This indicates that a rate-determining bimolecular step is involved but does not provide further information regarding the reacting species. The observation that saturating levels of \( r_2 \) strongly inhibit the reassociation process shows that 2 molecules of \( c_3r_6 \) react poorly if at all. This result is to be expected because in this case no \( c:r \) contact can be made between the two reacting components. Apart from \( c_3r_6 \), the most prevalent \( c_3r_2 \) species under our experimental conditions is \( c_3r_4 \) because the \( r_2 \) concentrations used (5 to 20 \( \mu \)g per ml) are many times greater than the half-saturation level (1 \( \mu \)g per ml).

From a mechanistic point of view, the most favorable reaction would be the combination of \( c_3r_6 \) with \( c_3r_4 \) directly to give \( c_6r_6 \) as this simply requires a "snapping together" of two components. If this would be the main pathway for the formation of \( c_6r_6 \), then the reassociation constant would be proportional to \( [c_3r_6] \times [c_3r_4] \). We have shown in an earlier section that most probably the concentration of the different species are governed by the following relationship:

\[
\frac{[c_3r_6]}{[c_3r_6][r_2]} = K_{assoc} = \frac{[c_3r_4]}{[c_3r_4][r_2]}
\]

from which we can derive the expression

\[
[c_3r_4][c_3r_6] = \frac{[c_3r_6][r_2]}{[r_2]K_{assoc}}
\]

At the relatively high levels of \( r_2 \) in our experiments, the \( c_3r_6 \) concentration does not vary appreciably with the \( r_2 \) concentration and therefore \( [c_3r_6] \times [c_3r_2] \) should vary approximately as \( 1/[r_2] \). Although the observed \( K_{assoc} \) decreases with increasing \( [r_2] \), the relationship is closer to \( K_{assoc} = 1/[r_2] \) than to \( K_{assoc} = 1/[r_2]^2 \). This result would suggest that renaturation under the above conditions does not proceed predominantly via a combination of \( c_3r_6 \) with \( c_3r_4 \), but that the main pathway consists of a rate-determining reaction between \( c_3r_6 \) and \( c_3r_2 \) followed by kinetically fast rearrangement steps to give the native enzyme. Much more extensive studies regarding the effect of \( r_2 \) concentration on the renaturation rate would be required to substantiate this suggested mechanism.

The effects of ligands on \( r_2 \) saturation and on the renaturation kinetics are important for the elucidation of the allosteric mechanism because they provide insight regarding changes in subunit interactions. Ideally, it would be desirable to investigate the effect of aspartate over the whole concentration range in which sigmoidal kinetics is observed. However, this has not been possible because it is only at low aspartate concentrations that \( c_3r_4 \) and \( c_3r_6 \) can be distinguished on the basis of activity. If the effect of aspartate is similar to that of succinate, then the observed increase in strength of \( c:r \) interaction induced by succinate may be significant in the allosteric transition between the tight and the relaxed forms of the native enzyme. One must be cautious however, in extrapolating from the succinate experiments because we have shown by inhibition studies that in addition to being a substrate analog, succinate has other effects on the native enzyme.\(^1\)

The effects of CTP and ATP are less ambiguous than those suggested above for aspartate and indicate that the binding of the nucleotide ligands primarily does not affect the \( c:r \) interactions. Although this result might at first seem surprising, it is in line with the lack of influence of these nucleotides on the activity of \( c_3r_4 \) observed earlier (2, 4). It is highly unlikely that this absence of effects by CTP and ATP is due to the inability of these ligands to bind to \( c_3r_6 \). If this were the case, then one would observe a shift of the \( r_2 \) saturation curve by CTP or ATP because these ligands are known to bind to \( r_1 \) (7) and therefore their presence would favor the dissociation of \( c_3r_6 \). The identical \( r_2 \) saturation curve obtained in the presence or absence of the nucleotides therefore implies that they bind equally well to \( r_2 \) and to \( c_3r_6 \). The fact that CTP and ATP influence the conversion of \( c_3r_2 \) to \( c_3r_6 \) is also consistent with their ability to bind to \( c_3r_6 \).

Although the well known effects of CTP and ATP on the kinetics of the native enzyme might lead one to expect that the two ligands should have opposite effects on \( c_3r_6 \), our results show that both CTP and ATP inhibit the conversion to \( c_6r_6 \). A possible explanation is that in the absence of these ligands, \( c_3r_6 \) has the optimal orientation of subunits for incorporation into the native \( c_3r_6 \) structure (perhaps by having a similar subunit orientation to that in aspartate transcarbamylase). If this is the case, then CTP and ATP, by changing this orientation of subunits in opposite directions, both would inhibit the process of conversion to \( c_6r_6 \). It is probably significant in this regard that Colman and Markus (15) have observed that both CTP and ATP stabilize the native enzyme against sodium dodecyl sulfate-induced dissociation. Both their results and our data on renaturation are probably concerned with the kinetics of interactions at the \( c:r \) domain (breakdown in one case and formation in the other). These two sets of observations suggest that the activation energy for formation and breakdown of \( c:r \) interactions in native aspartate transcarbamylase is lowest in the absence of CTP or ATP and both are consistent with the above suggestion that the orientation of subunits in \( c_3r_6 \) is closest to that of \( c_3r_6 \) under these conditions. These considerations and the fact that CTP and ATP have no direct effect on \( c:r \) interactions lead us to the tentative conclusion that these ligands primarily affect \( c:r \) interactions probably resulting in a change in orientation. (In the native enzyme, this tendency to change in orientation is likely to be opposed by quaternary constraints.) These aspects of the action of CTP and ATP and their possible significance on the allosteric mechanism will be developed more fully in the following paper (3).

An interesting aspect of the above study which remains to be investigated is the question of how aspartate (or succinate) influences the renaturation rate. It was not possible to determine the effects of aspartate or succinate in this respect because in the case of aspartate the influence on \( r_2 \) saturation is unknown and in the case of succinate the \( r_2 \) saturation is changed. Because \( r_2 \) saturation itself has an effect on the renaturation rate, comparison of rates is only meaningful when conditions are such that \( r_2 \) saturation remains constant. The effects of aspartate and succinate can be studied only when the dependence of renaturation rate on \( r_2 \) concentration is more fully understood.

Acknowledgment—The skillful technical assistance of Mr. Duncan Chong is gratefully acknowledged.

REFERENCES
1. Chan, W. W.-C. (1974) Abstracts of the Ninth Meeting of the Federation of European Biochemical Societies, Budapest, August, 9, 388
2. Mort, J. S., and Chan, W. W.-C. (1974) J. Biol. Chem. 249, 653-660
3. Chan, W. W.-C. (1974) J. Biol. Chem. 249, 668-674

\(^1\) J. S. Mort, and W. W.-C. Chan, manuscript in preparation.
4. Chan, W. W.-C., and Mort, J. S. (1973) J. Biol. Chem. 248, 7014-7016
5. Gerhart, J. C. (1970) Curr. Top. Cell. Regul. 2, 275-325
6. Cohnberg, J. A., Piglet, V. P., and Schachman, H. K. (1972) Biochemistry 11, 3386-3411
7. Changeux, J. P., Gerhart, J. C., and Schachman, H. K. (1968) Biochemistry 7, 531-538
8. Winland, C. C., and Chamberlin, M. J. (1970) Biochem. Biophys. Res. Commun. 40, 43-49
9. Matsumoto, S., and Hamme, G. G. (1973) Biochemistry 12, 1388-1394
10. Cook, R. A. (1972) Biochemistry 11, 3792-3797
11. Schachman, H. K. (1973) in Protein-Protein Interactions (Jaenicke, R., and Helmerich, E., eds) pp. 17-56 Springer Verlag, New York
12. Schachman, H. K., and Edelstein, S. J. (1966) Biochemistry, 5, 2681-2705
13. Jacobson, G. R., and Stark, G. R. (1973) J. Biol. Chem. 248, 8003-8014
14. Yang, Y. R., Syvanen, J. M., and Schachman, H. K. (1974) Proc. Nat. Acad. Sci. U. S. A. 71, 918-922
15. Colman, P. D. and Markus, G. (1972) J. Biol. Chem. 247, 3829-3837
Subunit interactions in aspartate transcarbamylase. The interaction between catalytic and regulatory subunits and the effect of ligands.

W W Chan

*J. Biol. Chem.* 1975, 250:661-667.

Access the most updated version of this article at [http://www.jbc.org/content/250/2/661](http://www.jbc.org/content/250/2/661)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/250/2/661.full.html#ref-list-1](http://www.jbc.org/content/250/2/661.full.html#ref-list-1)
Additions and Corrections

Vol. 250 (1975) 653–660

In Mort, John S., and William W.-C. Chan. Subunit Interactions in Aspartate Transcarbamylase. Characterization of a Complex between the Catalytic and the Regulatory Subunits.

Page 655, left-hand column, Line 10 from end should read:

for $e_s$ and $c_4 e_s$ (Table I) . . .

Page 660, Reference 10 should read:

10. Chan, W. W.-C. (1975) J. Biol. Chem. 250, 661–667

Page 660, Reference 11 should read:

11. Chan, W. W.-C. (1975) J. Biol. Chem. 250, 668–674

Vol. 250 (1975) 661–667

In Chan, William W.-C. Subunit Interactions in Aspartate Transcarbamylase. The Interaction between Catalytic and Regulatory Subunits and the Effect of Ligands.

Page 666, Reference 2 should read:

2. Mort, J. S., and Chan, W. W.-C. (1975) J. Biol. Chem. 250, 653–660

Page 666, Reference 3 should read:

3. Chan, W. W.-C. (1975) J. Biol. Chem. 250, 668–674

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprint they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Additions and Corrections

Vol. 250 (1975) 653-660

In Mort, John S., and William W.-C. Chan. Subunit Interactions in Aspartate Transcarbamylase. Characterization of a Complex between the Catalytic and the Regulatory Subunits.

Page 655, left-hand column, Line 10 from end should read:

for $c$ and $c_{i}^{\ell}$ (Table I) . . .

Page 660, Reference 10 should read:

10. Chan, W. W.-C. (1975) J. Biol. Chem. 250, 661-667

Page 660, Reference 11 should read:

11. Chan, W. W.-C. (1975) J. Biol. Chem. 250, 668-674

Vol. 250 (1975) 661-667

In Chan, William W.-C. Subunit Interactions in Aspartate Transcarbamylase. The Interaction between Catalytic and Regulatory Subunits and the Effect of Ligands.

Page 666, Reference 2 should read:

2. Mort, J. S., and Chan, W. W.-C. (1975) J. Biol. Chem. 250, 653-660

Page 666, Reference 3 should read:

3. Chan, W. W.-C. (1975) J. Biol. Chem. 250, 668-674

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprint they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.