Conformational States of Aspartate Transcarbamoylase Stabilized with a Cross-linking Reagent*

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Modification of native aspartate transcarbamoylase from Escherichia coli with the bifunctional reagent tartryl diazide was performed in the absence of ligands. The treatment resulted in the complete loss of cooperativity and a low substrate affinity. The Michaelis constant for aspartate ranged from 66 to 130 mM for different preparations. These values approach that previously estimated for the taut (T) state of the enzyme. The alteration in functional properties was not accompanied by either dissociation or aggregation. The modified enzyme is presumed to be a stabilized T state in which the allosteric transition is prevented by cross-linking.

The structural modifications associated with the above changes in function were examined and compared to those of the stabilized relaxed (R) state previously described. The latter derivative was obtained under identical conditions except that substrate analogs were present during the reaction with the bifunctional reagent. Both derivatives contained on the average about 10 cross-links per molecule. The cross-links were predominantly intrachain in nature. The absence of cross-linkages between catalytic and regulatory polypeptides permitted the separation of the catalytic and regulatory subunits of these derivatives by usual procedures. The catalytic subunits obtained from both derivatives showed substrate affinities similar to that of the unmodified catalytic subunit. The results indicate the importance of subunit interactions in the expression of the T and R states.

The allosteric properties of aspartate transcarbamoylase (aspartate carbamoyltransferase EC 2.1.3.2) from Escherichia coli (1) can be accounted for by the existence of two or more conformational states which differ in substrate affinities (2). We have previously reported (3) on the stabilization of a conformation which resembles the relaxed R1 state. This derivative was obtained by modifying the native enzyme with a bifunctional reagent in the presence of substrate analogs. Unlike the native enzyme, the modified derivative showed Michaelis-Menten kinetics with a relatively low $K_m$ (7 mM) for aspartate. We have now found that modification with this same reagent in the absence of ligands will also abolish cooperativity, but the product has a much lower substrate affinity. Under appropriate conditions, a derivative can be obtained with a $K_m$ for aspartate approaching the value (130 mM) previously estimated (4) for the T state. The characterization of this derivative is reported here. In addition, we present evidence concerning the nature of the structural modifications responsible for the distinct functional behavior of both derivatives.

MATERIALS AND METHODS

Aspartate transcarbamoylase from E. coli was isolated according to the procedure of Gerhart and Holoubek (5). The subunits were separated by treatment of the native enzyme with neohydrin as described by Schachman (6). Preparations of the enzyme and subunits were stored as described previously (7).

Diethyl tartrate was obtained from Aldrich Chemical Co. and converted to tartryl dihydrazide as described by Lutter et al. (8). 2,4,6-trinitrobenzene sulfonic acid was purchased from Sigma Chemical Co. Sephacryl S-200 was a product of Pharmacia Fine Chemicals. Acrylamide for gel electrophoresis was a special grade material from Eastman Kodak Co. Dye reagent for protein determination was purchased from Bio-Rad Chemical Co. Neohydrin was obtained from K and K Laboratories Inc. and was purified as described by Yang et al. (9).

Analytical Procedures—Aspartate transcarbamoylase activity was measured by the radiometric assay of Porter et al. (10), modified as described before (7). The carbamoyl phosphate concentration was 4 mM in all activity assays. This concentration should be sufficient to saturate the enzyme with respect to this substrate since the half-saturation points for the native enzyme (11) and for the catalytic subunit (10) are less than $2 \times 10^{-4}$ M. As explained previously (10), the very large difference (up to 1000 fold) in the $K_m$ of the enzyme for aspartate and for carbamoyl phosphate makes the measurement of the latter very difficult. Thus, only the $K_m$ for aspartate is studied in this work. In control experiments, we have shown that the $K_m$ of the modified derivatives for aspartate are not altered when the concentration of carbamoyl phosphate is reduced from 4 to 2 mM. This result confirms that the level of carbamoyl phosphate used is saturating.

Protein concentrations were determined from the absorbance at 280 nm by the method of Lowry et al. (12). In addition, the Bio-Rad protein assay was used on dilute samples. This method is based on spectral change that occurs when protein interacts with Coomassie blue G-250 (13). The protein dye reagent concentrate was diluted 4-fold with water and 0.8 ml of diluted reagent was added to 0.2 ml of sample (0 to 20 $\mu$g of protein). After 5 min, the absorbance was read at 595 nm. The amount of protein was calculated from a standardization curve calibrated using native enzyme. Polycrylamide gels were stained quantitatively using the method of Kahn and Rubin (14). The relative concentration of proteins (0 to 10 $\mu$g) in the gel electrophoresis was determined by scanning with a Joyce-Loebel microdensitometer.

Modification of the enzyme in the absence of ligands was conducted at a protein concentration of 1 mg/ml for 2 h at 25°C in 0.3 M triethanolamine/HCl (pH 8.5). Owing to the variability between experiments, the minimum concentration of reagent required to abolish cooperativity completely ranged from 10 to 15 mM. Samples were modified at both of these concentrations and the products were evaluated on the basis of double reciprocal plots. The preparation used for detailed characterization was cross-linked with 15 mM reagent.
since the lower concentration did not completely abolish cooperativity in this case. In order to facilitate comparison, modification in the presence of substrate analogs (succinate 20 mM and carbamoyl phosphate 4 mM) was also conducted using the same reagent concentration and incubation time. The properties of this derivative (substrate affinity and effector sensitivity) were not significantly different from those of the derivative reported earlier (3) in spite of the differences in cross-linking conditions. After modification, ethylenediamine/HCl (pH 7.7) was added to a final concentration of 0.2 M and the mixture was incubated for 30 min at 25°C. The modified enzyme was stored at 4°C without purification.

The molecular size of modified derivatives was estimated by sucrose density gradient centrifugation. The sample (40 μg in 0.1 ml) was loaded on a 5-ml sucrose gradient (5 to 20%) in 0.2 M Tris/acetate (pH 7.5) containing 20 mM 2-mercaptoethanol and bovine serum albumin (50 μg/ml). Centrifugation was conducted at 4°C for 16 h at 34,000 rpm using a Beckman SW 50.1 rotor. Fractures were assayed for activity at 6 mM aspartate. The positions of native enzyme and catalytic subunit were determined using separate tubes but in the same run. An internal marker (rabbit muscle aldolase) was used for the alignment of individual gradients.

For amino group determination, the enzyme was dialyzed extensively against 40 mM potassium phosphate (pH 7.0) and duplicate samples at five concentrations (20 to 120 μg) were treated with 2,4,6-trinitrobenzene sulfonic acid essentially as described by Habeeb (15). After incubation, the usual addition of sodium dodecyl sulfate and HCl was omitted. Instead, the equivalent amount of water was added and the samples were placed in ice bath before the absorbance was measured. The method was standardized using aldolase and bovine serum albumin as well as native aspartate transcarbamoylase.

One-dimensional electrophoresis was carried out in 15% polyacrylamide slab gels in sodium dodecyl sulfate as described by Laemmli (16) and modified by Anderson et al. (17). In two-dimensional separations where cleavage of cross-links was desired it was necessary to omit Tris from the electrophoresis system in the first dimension because it would react with periodate. Thus, the samples were first run in cylindrical gels (10% acrylamide) using the phosphate buffer system of Weber and Osborn (18). The gels were then incubated with shaking for 2 h in 50 mM triethanolamine/HCl (pH 7.5) containing 20 mM sodium periodate and 0.1% sodium dodecyl sulfate. In order to achieve a higher resolution, the second dimension employed the buffer system of Laemmli (16).

Dissociation of subunits was carried out on samples of cross-linked enzymes which had been dialyzed extensively against 40 mM potassium phosphate (pH 7.0). The modified enzyme (1.2 to 1.7 mg/ml) was treated with purified neohydrin (0.7 to 2.1 mg/ml) for 10 min at 25°C.

RESULTS

Choice of Cross-linking Conditions—The selection of a diazide reagent instead of the more common diimidates was initially prompted by the consideration that the product would differ in charge from the starting material (Fig. 1, a and b). This difference could facilitate separation of species modified to various extents. In practice, separation was not found to be necessary as the various derivatives did not appear to be markedly heterogeneous in properties. Another factor in selecting tartryl diazide was the prospect of cleaving the vicinal diol group with periodate. This cleavage reaction should in theory permit the distinction between effects which are due to conformational rigidity imposed by the cross-links and effects of side chain modification alone. However, the usual conditions for cleavage were found in control experiments to affect the allosteric properties of the native enzyme. Thus, it has not been feasible to prove unequivocally that the distinct functional properties of the derivatives are specifically due to cross-linking per se. Although the theoretical advantages of tartryl diazide could not be fully exploited, its choice was fortunate since we later found that suberimidate severely inactivated the enzyme and did not generate states with well defined kinetic properties. The reasons for the large difference between these two reagents remain obscure.

Excess ethylenediamine was added at the end of the modification to react with any remaining azide groups. This treatment should restore a amino group to any position where the reagent has reacted with only one of its functional groups (Fig. 1c). Assuming that all such groups react with ethylenediamine, it is possible to measure the extent of cross-linking (as distinct from the total extent of modification) by determining free amino group content.

For modification in the presence of substrate analogs, we used the distinctly high substrate affinity of the R state as a criterion in establishing optimal conditions. The ratio of activity of the modified enzyme to that of the native enzyme at a low aspartate concentration rises to a maximum at a reagent concentration of about 12 mM (Fig. 2). The modification is accompanied by loss of sensitivity to ATP which is essentially complete at this point. The optimal concentration determined here is the same as that used previously to obtain a derivative resembling the R state.

Modification in the absence of ligands was expected to stabilize the T state and this process was followed by the loss of substrate cooperativity. As the concentration of the bifunctional reagent was increased, the nonlinearity of double reciprocal plots disappeared (Fig. 3). At the minimum reagent concentration (in this case 10 mM) required to abolish cooperativity, the Kₐ is about the same as that previously estimated for the T state (130 mM). However, at higher reagent concentration, the Kₐ decreased and approached the value for the catalytic subunit (24 mM). Thus, apart from eliminating cooperativity, modification also affects substrate affinity. This side effect is not due to dissociation of the enzyme since even...
the derivative modified at the highest concentration of reagent sediments in sucrose gradients to the same position as native enzyme. As discussed in a later section, preliminary evidence suggests that loss of cooperativity is due to modification at the catalytic subunit, whereas the decrease in $K_m$ is caused by modification at the regulatory subunit. Since we were primarily interested in the stabilization of the T state, we chose to study the derivative modified with the minimum amount of reagent required to abolish cooperativity.

Characterization of the Derivative Modified in the Absence of Ligands—We have studied the substrate saturation behavior of several preparations of the enzyme modified in the absence of ligands. The Michaelis constants for aspartate of these samples ranged from 66 to 125 mM. The variability in $K_m$ values stems from the difficulty in controlling the actual concentration of active reagent and in extrapolating from relatively low substrate concentrations. Activity at higher concentrations of aspartate could not be used to estimate $K_m$ because of substrate inhibition. However, this derivative is clearly distinct from the enzyme modified in the presence of substrate analogs because of the 10- to 18-fold difference in $K_m$. The results suggest that we have obtained a stabilized conformation resembling the T state.

In sucrose density gradients, the derivative modified in the absence of ligands sediments as a single peak in the same position as the native enzyme. Thus, the strikingly low substrate concentration, activity at higher concentration of active reagent and in extrapolating from relatively low substrate concentrations. Activity at higher concentrations of aspartate could not be used to estimate $K_m$ because of substrate inhibition. However, this derivative is clearly distinct from the enzyme modified in the presence of substrate analogs because of the 10- to 18-fold difference in $K_m$. The results suggest that we have obtained a stabilized conformation resembling the T state.

Modification—The superscript X represents modification (cross-linking) with tartryl diazide, whereas M represents modification with the monofunctional reagent acetyl azide. This is followed by either n or s depending on whether the modification was conducted with the native (n) enzyme or with the isolated subunit(s). The last symbol in the superscript indicates whether substrate analog was present (+) or absent (−) during modification. Absence of a superscript indicates the unmodified subunit. Thus, (c&$\gamma$)X+(c$\gamma$)$\delta$X− signifies native enzyme cross-linked in the presence of succinate and carbamoyl phosphate. As shown in this paper, this derivative can be dissociated into the two types of subunit $c_3^{X+}$ and $r_2^{X-}$.
The two derivatives (c,r)X+ and (c,r)X− were found to have essentially the same cross-linking pattern when examined by gel electrophoresis in sodium dodecyl sulfate. In simple one-dimensional gels (Fig. 4), the most prominent bands for both derivatives were at the same position as the unmodified c (Mr = 33,000) and r polypeptides (Mr = 17,000). No protein could be detected with a mobility expected of a c–r dimer (Mr = 50,000). The only band which clearly represented interchain cross-linked products appeared in the Mr = 66,000 region. Due to possible ambiguities resulting from the coincidence that the molecular weight of c is about twice that of r, the nature of the material in various bands was clarified by two-dimensional electrophoresis. This method involved cleavage of the cross-linking moiety by periodate after electrophoresis in the first dimension. Cleavage patterns (Fig. 5) indicated that the band at 33,000 contained not only catalytic polypeptide but also r–r dimers. On the other hand, the band at 66,000 consisted entirely of c–c dimers with no detectable amounts of c–r–r trimer. The cleavage reaction appeared to be complete under the experimental conditions since the entire band at 66,000 moved from the diagonal. However, some side reaction with periodate apparently took place which produced multiple bands of the catalytic polypeptide.

The relative amounts of protein with molecular weights of 17,000, 33,000, and 66,000 in sodium dodecyl sulfate gels (Fig. 4) were found by densitometry to be approximately in the ratio of 4:6:1 for both derivatives. In the two-dimensional electrophoretic pattern (Fig. 5), the amount of r–r dimer can be judged by eye to constitute no more than 10% of the material with Mr = 33,000. Although these values are far from precise, we can estimate that only 15% or less or either c or r chains are involved in interchain (c–c or r–r) cross-links. Thus, on the average there is substantially less than one c–c and one r–r cross-link/molecule of either derivative. Since amino group determinations have indicated the presence of about 10 cross-links/molecule, most of the cross-links are presumably located within individual polypeptides.

Separation of the Cross-linked Derivatives into Catalytic and Regulatory Subunits—In order to determine the effects of quaternary structure on the functional properties of the cross-linked derivatives, we dissociated the subunits by treatment with the organic mercurial reagent neohydrin (6). In the case of (c,r)X− the subunits dissociated readily and could be separated easily by gel filtration (Fig. 6b). The positions of the two peaks do not differ appreciably from those of unmodified c and r in calibration runs. The areas of the peaks are also in the expected 2:1 ratio. These results are consistent with the above evidence from gel electrophoresis indicating a lack of cross-linking between c and r polypeptides.

The other derivative (c,r)X+ was more resistant to dissociation and required a 2-fold increase in neohydrin concentration for the complete disappearance of higher molecular weight species. Although the separation of subunits by gel filtration was not as distinct as in the case of (c,r)X− (Fig. 6b), fractions could be obtained which were free from contamination by the other subunit.

When the substrate saturation characteristics of the subunits cX+ and cX− isolated above were examined (Fig. 7), they were found to be closely similar to those of the unmodified catalytic subunit (Km = 24 mM). Thus, in the case of (c,r)X+, the Km for aspartate increases upon dissociation from 5.3 to 18.2 mM, whereas for (c,r)X− there is a corresponding decrease from 66 to 22 mM. These results suggest the importance of the quaternary structure for the expression of high and low substrate affinities. A simple explanation for the above results might be that the crucial modifications are located on the regulatory subunits. However, our preliminary hybridization experiments have shown that the substrate affinities of the T and R states return when the modified catalytic subunits cX+ and cX− reassociate with unmodified regulatory subunits. Thus, the potential for high or low affinity is inherent in the modification at the catalytic subunit but is not expressed unless the quaternary structure is present.

These preliminary results also provide an explanation for the decrease in Km which occurs when modification is conducted at higher concentrations of the reagent. The hybrid obtained when the modified catalytic subunit cX+ reassociates with unmodified r has a Km of about 120 mM which is much higher than that (66 mM) of the original derivative (c,r)X−. Thus, modification of the regulatory subunit is probably responsible for the side effect of decrease on Km at higher concentration of the modifying reagent.
As judged by its substrate affinity, the derivative modified in the absence of ligands appears to represent a distinct state of the enzyme. The loss of cooperativity and the high $K_m$ for aspartate can be explained most simply by postulating that under the conditions of modification the enzyme exists in the T state and this conformation has been stabilized by cross-linking. The stabilization could be due to the rigidity imposed by the cross-links or alternatively to the modification of groups which are essential for the allosteric transition between T and R states. These possibilities could in principle be distinguished by cleavage of the cross-links with periodate. Unfortunately under various conditions tested, this reagent shows significant side effects on the functional properties of the enzyme.

Properties of the T State—Owing to the variability among samples of (c(xa))X− and the decrease in $K_m$ at higher concentrations of cross-linking reagent, it has not been possible to establish a precise value for the $K_m$ of the T state. From analysis of the sigmoidal substrate saturation curve of the native enzyme, Howlett et al. (4) have estimated a value of 130 mM. Our most accurate determination (based on one preparation of the modified enzyme) is 123 ± 25 mM. The only other derivative of this enzyme that has substrate affinity in this range and intact quaternary structure is a hybrid containing wild-type and mutant subunits reported by Gibbon et al. (21). We have previously described a chymotrypsin-modified catalytic subunit with a $K_m$ for aspartate of 100 mM (22). Whether the similarity in substrate affinity among these derivatives is a coincidence or whether they are the result of similar structural alterations remains unclear.

Comparison between T and R States—It is remarkable that under otherwise identical modification conditions the products obtained in the presence or absence of substrate analogs should differ in substrate affinity by as much as 18-fold. These results provide independent evidence for the existence of at least two conformational states. Although the modification with bifunctional reagents is often considered to “freeze” a protein in a particular conformation (e.g. Ref. 23), the cross-linked derivative should not be regarded as completely rigid. The cross-linkage consists of a series of saturated carbon bonds which have considerable freedom of rotation. The lysine side chains which are modified also have a certain amount of flexibility. Thus, the enzyme derivatives are unlikely to be so rigid that conformational motility of 1 or 2 Å would be completely prevented. It is therefore noteworthy that the two derivatives studied here show no trace of cooperativity in

Fig. 6. Separation of catalytic and regulatory subunits of the cross-linked enzyme derivatives. Samples in a and b were 0.5 mg (c(xa))X− (O) and 0.3 mg (c(xa))X− (□), respectively, and had been treated with neohydron. The column (1 × 52 cm) consisted of Sephacyl S-200 in 40 mM potassium phosphate buffer and was run at 4°C at 1.5 mg/hour. Sample and fraction volumes were both 0.5 ml.

Fig. 7. Double reciprocal plots of modified derivatives and their corresponding catalytic subunits. a, (c(xa))X− (■) and c(xa)X− (□); b, (c(xa))X+ (○); and c(xa)X+ (□). Lines were based on least square fit of the data.
substrate binding. This observation suggests that the transition between the T and R states is accompanied by a much larger conformational change than that permitted by the limited flexibility of the cross-links. The above interpretation is consistent with various previous studies (24, 25) which indicate that a major structural change occurs upon substrate binding.

For preparation of $(c,t)_{X_{n+1}}$, saturating concentrations of substrate analogs have been used in this study. Under non-saturating conditions, stabilization of both conformations might be expected. Alternatively, cross-linked species possessing intermediate conformational changes might be obtained. The manner in which conformational changes take place represents an interesting aspect of the allosteric mechanism. Relevant information might be obtained by examining the enzyme cross-linked in the presence of nonsaturating concentrations of substrate analogs if mixtures of different derivatives could be separated on the basis of substrate affinity. This type of approach is being actively pursued.

In contrast to the striking difference in substrate affinity, the two derivatives are surprisingly similar in many structural aspects. In the first place, both derivatives apparently have the same molecular weight. Secondly, there is no difference in the number of cross-links/molecule. Finally, in both cases, there are no detectable c-r cross-links and only a relatively small number of other interchain (i.e. c-c and r-r) cross-links. The crucial modifications are presumably intrachain cross-links and at least some of these sites are expected to differ in the two derivatives. Detailed peptide mapping would be necessary to determine their precise location.

If we accept the number of cross-links determined by the indirect method in this paper, then there is on the average slightly less than one cross-link per polypeptide chain (10 for a dodecameric structure). The finding that the functional properties of each derivative are not significantly heterogeneous would suggest that functionally important conformational changes are to some extent concerted. Thus, the absence of cross-links on one or two subunits would not affect the properties of the molecule if the remaining subunits contain the specific modifications.

The I State—The absence of c-r cross-links in these derivatives is a valuable asset in studying the role of the quaternary structure in enzyme function. We have shown in this paper that upon dissociation the substrate affinities of both derivatives revert to that of the unmodified catalytic subunit. These results indicate that the establishment of cr contact has an important effect on substrate affinity. Consistent with this idea is the 4-fold decrease in $K_m$ which accompanies the conversion of $c_t$ to $c_tX_n$ (7). In this connection, it is noteworthy that with higher cross-linking reagent concentration and no ligands, the derivative obtained (Fig. 3) has a $K_m$ similar to that of $c_t$. Recently Landfear et al. (26) have described a derivative of this enzyme modified with tetranitromethane which again has a $K_m$ of about 20 mM and an intact subunit structure. Finally, the enzyme obtained from growth of $E. coli$ in 2-thiouracil has a $K_m$ of 14.4 mM rising to 28.8 mM in the presence of CTP (27). Thus, apart from the T and R states which have $K_m$ for aspartate of about 130 and 6.5 mM, respectively (Refs. 3 and 4 and this work), we must consider a state of the active site with an intermediate substrate affinity. We have previously (28) designated this conformation as the I state since it is characteristic of the isolated catalytic subunit.

The Structural Basis for the T, I, and R States—In a model previously proposed by one of us (28), the low substrate affinity of the T state was regarded as the result of the expenditure of energy which was required to convert the enzyme into the R state. The observation that the T state can be stabilized signifies that low substrate affinity is independent of the allosteric transition. This aspect of the previous model must therefore be modified.

We now postulate that the interaction at the cr domain can occur in at least three different ways, each of which has a distinct effect on substrate binding at the active site. In the absence of quaternary constraints, the preferred interaction at the cr domain gives rise to the R state at the active site. This effect is most clearly seen in the $c_tX_n$ derivative studied previously in this laboratory (29). In this structure, the catalytic and regulatory subunits can align themselves freely to establish the most favorable cr contact. In the intact enzyme $c_tX_n$, however, the type of cr contact formed is determined further by the necessity of having contacts at both c and r moieties. We believe that the resulting stereochemical constraints make it energetically preferable to form a different cr contact. The effect of this second type of contact is to decrease the substrate affinity to that of the T state. It is assumed that the difference in free energy between the T and R states is sufficiently small so that the energy of substrate binding is sufficient to shift the balance in favor of the R state.

As mentioned above, there are several derivatives of the enzyme with an intact subunit structure whose $K_m$ for aspartate (about 20 mM) is intermediate between those of the T and R states. We therefore postulate that substrate affinity of this magnitude (the I state) is associated with a third type of cr contact. The effect of this kind of cr interaction on the active site conformation is minimal since the $K_m$ is the same as in the isolated catalytic subunit (where the corresponding domains are in contact with water).

An important aspect of the allosteric mechanism concerns the action of the effectors ATP and CTP. The sensitivity to these effectors is essentially abolished in the stabilized T and R state derivatives (Refs. 3 and 7 and this work). A crucial question is whether the loss of sensitivity is an intrinsic property of these conformational states. Since the catalytic and regulatory subunits of the modified enzyme can be separated, hybridization might provide an answer to this question. Preliminary experiments indicate that the hybrids $c_tX_n$ and $c_rX_{n+1}$ have substrate affinities characteristic of the T and R states, respectively, and are both significantly sensitive to ATP and CTP. Thus, the modifications responsible for the changes in substrate binding are located in $c_t$, whereas those responsible for the loss of sensitivity to effectors are located in $r$. Detailed studies of the various hybrids are now in progress and should provide further insight into the allosteric mechanism.

REFERENCES
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Conformational states of aspartate transcarbamoylase stabilized with a cross-linking reagent.
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