Functionally Important Arginine Residues of Aspartate Transcarbamylase

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The reaction of phenylglyoxal with aspartate transcarbamylase and its isolated catalytic subunit results in complete loss of enzymatic activity (Kantrowitz, E. R., and Lipscomb, W. N. (1967) J. Biol. Chem. 251, 2888-2895). II-(phosphonacetyl)-L-aspartate is used to protect the active site, we find that phenylglyoxal causes destruction of the enzyme's susceptibility to activation by ATP and inhibition by CTP. Furthermore, CTP only minimally protects the regulatory site from reaction with this reagent. The modified enzyme still binds CTP although with reduced affinity. After reaction with phenylglyoxal, the native enzyme shows reduced cooperativity. The hybrid with modified regulatory subunits and native catalytic subunits exhibits slight heterotrophic or homotropic properties, while the reverse hybrid, with modified catalytic subunits and native regulatory subunits, shows much reduced homotropic properties but practically normal heterotrophic interactions. The decrease in the ability of CTP to inhibit the enzyme correlates with the loss of 2 arginine residues/regulatory chain (M, = 17,000). Under these reaction conditions, 1 arginine residue is also modified on each catalytic chain (M, = 33,000). Reaction rate studies of p-hydroxymercuribenzoate, L-aspartate, and N-ethylmorpholine were used to prepare [14C]phenylglyoxal, with a specific activity of 1.75 x 106 cpm/mol, by the method of Riley and Gray (31).

Each 300,000-dalton aspartate transcarbamylase molecule is composed of two catalytic and three regulatory subunits (6-8). The molecular structure of the enzyme and its complex with CTP have been determined at low resolution (9-11), while several reviews extensively summarize the chemistry of the enzyme (12, 13).

A number of studies have been performed to determine the nature of the interactions which occur between the enzyme and its substrates (14-18). Chemical modification studies have indicated that the active site contains 1 lysine (19), 2 histidine (19), 1 tyrosine (20), and 1 arginine (21) residue, along with a cysteine residue located near the active site (22, 23). London and Schmidt (24) have proposed a model which successfully predicts the interactions of the allosteric nucleotides with the enzyme. However, little information is available about the specific interactions between the nucleotides and the amino acid residues at the regulatory site.

In the course of our previous study (21), we found that phenylglyoxal, a reagent fairly specific for arginine residues, caused a loss in the ability of the enzyme to be activated by ATP or inhibited by CTP when the active site of the enzyme was protected by the transition state analog N-(phosphonacetyl)-L-aspartate. Since arginine residues have been implicated in the binding of ATP to glutamine synthetase (25), carbamyl phosphate synthetase (26), creatine kinase (26), and adenosine triphosphatase (27); and in the binding of NAD to malate dehydrogenase (28), lactate dehydrogenase (29), and alcohol dehydrogenase (30), we thought that arginine residues might also be involved in binding at the regulatory site of aspartate transcarbamylase. Therefore, we decided to investigate further the reaction of phenylglyoxal with aspartate transcarbamylase in order to probe the nature of the regulatory site of the enzyme.

EXPERIMENTAL PROCEDURES

Materials - The CTP, ATP, carbamylphosphate, succinate, p-hydroxymercuribenzoate, L-aspartate, and N-ethylmorpholine were obtained from Sigma Chemical Co. Sephadex G-25, G-100, and A-50 were obtained from Pharmacia. The carbamylphosphate was purified by precipitation from 50% ethanol and stored desiccated at -20° (5). Phenylglyoxal monohydrate was purchased from Aldrich and was used without purification. [14C]aetophenone and [3H]CTP were obtained from New England Nuclear. The [14C]acetophenone was used to prepare [14C]phenylglyoxal, with a specific activity of 1.75 x 106 cpm/mol, by the method of Riley and Gray (31).

Aspartate Transcarbamylase and Subunits - Aspartate transcar-

Aspartate transcarbamylase from Escherichia coli (car bamylphosphate: L-aspartate carbamoyltransferase EC 2.1.3.2) catalyzes the reaction of carbamylphosphate with aspartate to form carbamylaspartate and inorganic phosphate (1, 2). The enzyme exhibits a sigmoidal saturation curve for substrates (3), indicative of the enzyme's allosteric nature. The enzyme shows feedback inhibition by CTP (4) and is activated by ATP (3).

The 300,000-dalton aspartate transcarbamylase molecule is composed of two types of subunits (5). The larger subunit, with a molecular weight of 100,000, is composed of three identical polypeptide chains. This catalytic subunit exhibits enzymatic activity which is unmethylated by moderate concentrations of the nucleotide effectors. The smaller subunit, with a molecular weight of 33,000, is composed of two identical polypeptide chains. This regulatory subunit binds the allosteric effectors and shows no catalytic activity.
bamyase was isolated from *Escherichia coli* by the procedure of Gerhart and Holoubek (32). Large quantities of cells were grown at the New England Enzyme Center from an 8.4-liter culture of the kind strain of *E. coli* ATCC 32902, Berkeley, Calif. The enzyme was stored under sterile conditions in 0.04 M phosphate buffer, pH 7.0, 2 mM *β*-mercaptoethanol, and 0.2 mM EDTA in sealed vials under nitrogen at 5°C. Before use, the enzyme was exhaustively dialyzed against either 0.125 M potassium bicarbonate, pH 8.3, or 0.1 M N-ethylmorpholine, pH 8.3.

The catalytic and regulatory subunits were isolated by chromatography on DEAE-Sephadex A-50 after dissociation of the native enzyme with *p*-hydroxymercuribenzoate. For this purpose, the usual procedure (32) was modified. The catalytic and regulatory subunits were prepared by dialysis against 0.1 M potassium phosphate buffer, pH 7.0, 2 mM *β*-mercaptoethanol and 0.2 mM EDTA in order to produce the regulatory subunit (33). After elution of the catalytic subunit from the A-50 column, the fractions containing protein were pooled, and *β*-mercaptoethanol was added to a final concentration of 0.2 mM in order to produce the catalytically active enzyme subunit (32). For the regulatory subunit and for enzyme modified with phenylglyoxal, the method of Lowry et al. (34) was used exclusively.

Cellulose acetate electrophoresis was used both to detect dissociation of the enzyme into its subunits, and to check the purity of the subunits after preparation. A Shandon Electrophoretic System was employed with cellulose acetate sheets (78 × 150 mm). After electrophoresis for 18 to 20 min in 0.05 M Tris/citrate buffer, pH 7.8, at a constant current of 10 mA, the strips were stained with Coomassie brilliant blue using the procedure described by Fazekas de St. Groth et al. (35).

**Enzyme Activity**—The transcarbamylase activity was assayed by continuously monitoring the enzyme-catalyzed release of protons at pH 8.3, on a Radiometer FT1c pH-stat equipped with an SFU1a syringe burette unit. Routinely, assays in the absence of ATP or CTP were performed with 4.8 mM carbamylphosphate and 30 mM aspartate for assays in the presence of the nucleotide effectors CTP or ATP, the aspartate concentration was reduced to 9 to 15 mM since at saturating aspartate (30 mM), little or no activation by ATP or inhibition by CTP is observed. The CTP and ATP concentrations, 0.5 mM and 4 mM, respectively, in these assays are sufficient to exert the nucleotide's full effect.

Activity in the figures is reported as specific activity in units of millimoles of carbamylaspartate formed/h/mg of enzyme concentration and radioactivity. The second part was concentrated by gel filtration on Sephadex G-25 produced the modified regulatory subunit in very poor yield. The subunits were used for reconstitution experiments immediately after preparation in order to minimize the loss of modification.

**Preparation of Hybrids with Phenylglyoxal-modified Subunits—** Hybrid aspartate transcarbamylase molecules were prepared with either the catalytic or regulatory subunits modified with phenylglyoxal, along with a control hybrid in which both subunits were unmodified. Reassociation of the isolated subunits was accomplished by mixing the catalytic subunit with an excess of zinc regulatory subunit in 0.01 M Tris/acetate buffer, pH 8.3, 10 mM *β*-mercaptoethanol, 0.2 mM zinc acetate at 37°C for 30 min followed by centrifugation to check for complete reassociation. Excess regulatory subunit was removed by sucrose gradient centrifugation at 4°C for 15 h in a Beckman SW 25.1 rotor. The peak fractions from each of the subunits were then used for analysis of protein concentration and radioactivity.

Radioactivity was determined by mixing 100 to 200 μl of sample with 10 ml of Aquasol (New England Nuclear) and counting in a Packard Tri-Carb liquid scintillation counter. The number of arginine residues modified was calculated using a stoichiometry of 2 phenylglyoxal molecules/arginine residue modified (36).

**Binding Measurements—** Measurements of CTP binding were carried out by the technique of equilibrium dialysis using Union Carbide cellulose dialysis tubing No. 27. Before use, the membrane underwent four 2-h treatments at 60°C; two with 2% sodium carbonate, one with distilled water, and one with 1.0 M EDTA. After each step, the tubing was thoroughly washed with distilled water. The treated dialysis tubing was routinely stored at 4°C in 1.0 mM EDTA.

Each microdialysis chamber had a capacity of 60 μl on each side of the membrane. The mean to obtain a distribution of *K* values (moles of CTP bound per mole of enzyme), the concentration of enzyme was varied from 2 mg/ml to 10 mg/ml and the concentration of CTP was varied from 10^−12 M to 3 × 10^−6 M. Carbamylphosphate was present during the dialysis at an equilibrium concentration of 2 mM to prevent CTP binding at the active site (37). Dialyses were carried out at 4°C for 24 h.
Native enzyme used for the binding experiments was dialyzed against 0.1 M imidazole/acetate buffer, pH 7.0, 5 mM β-mercaptoethanol, and 0.2 mM EDTA before use. Modified enzyme was prepared by the standard procedure employing the above buffer as eluent in the gel filtration step.

Aspartate Transcarbamylase Reaction with p-Hydroxymercuribenzoate - The reaction of p-hydroxymercuribenzoate with aspartate transcarbamylase and phenylglyoxal-reacted enzyme was followed spectrophotometrically at 250 nm (38). At this wavelength, an increase in absorbance is observed due to the formation of the mercapto complex with the enzyme’s sulfhydryl groups (39). The sample and reference cells of the spectrophotometer were filled with 2.5 ml of a 1.6 × 10⁻⁴ M solution of p-hydroxymercuribenzoate in 0.04 M potassium phosphate buffer, pH 7.0. For reactions in the presence of ligands, the above solution of p-hydroxymercuribenzoate also contained 10 mM carbamylphosphate and 10 mM succinate.

Since the reactions were relatively fast, initiation was accomplished by adding an aliquot of enzyme (i.e., 0.3 mg in 20 to 50 μl) in 0.04 M potassium phosphate buffer, pH 7.0, to the spectrophotometer cuvette by means of an apparatus designed to allow addition and mixing simultaneously. This apparatus, a modified version of the continuous ligand titration apparatus previously described (40), could be used to obtain data about 2 to 3 s after initiation of the reaction.

Spectrophotometric Measurements - All spectrophotometric measurements were made on a Zeiss PMQ II or a Beckman DB-GT spectrophotometer, in quartz cells having a 1-cm path length.

Data Analysis - Fitting the experimental data to kinetic equations was accomplished by a fitting and plotting program written in Fortran IV and implemented on a PDP 11/45 computer. The program incorporated the MIT nonlinear least squares subroutines LSQR and LSMERR (Applications Program 84). Preliminary versions of the plots with theoretical fits were drawn on a Calcomp 565 plotter.

**RESULTS**

Loss of Heterotropic Interactions - We have previously shown that phenylglyoxal rapidly reacts with aspartate transcarbamylase to eliminate all catalytic activity (21). This reaction can be almost totally prevented by using the transition state analog N-[(phosphonacetyl)-l-aspartate (41) to protect the active site during the phenylglyoxal reaction.

With the active sites protected, we have found that the heterotropic properties of the enzyme are abolished by reaction with phenylglyoxal (see Fig. 1). The rate of elimination of regulatory properties is much slower than is the rate of elimination of enzyme activity in the absence of N-(phosphonacetyl)-l-aspartate. The second order rate constant for loss of the regulatory effect is 6.0 M⁻¹ min⁻¹ in the presence of N-(phosphonacetyl)-l-aspartate, which protects catalytic activity, as compared to 96.2 M⁻¹ min⁻¹ for the loss of enzymatic activity at pH 8.3, in 0.1 M N-ethylmorpholine buffer without N-(phosphonacetyl)-l-aspartate.

Protection experiments were performed in order to locate the arginine residues involved in the loss of CTP inhibition. The phenylglyoxal reaction was carried out in the presence of 10 mM CTP, at which concentration all the CTP sites should be saturated with ligand. As seen in Fig. 1, only a slight degree of protection is afforded the enzyme by CTP. This effect is much smaller than that afforded the active site arginine residue by carbamylphosphate (21). No dissociation of the enzyme into its constituent subunits could be detected under these reaction conditions by cellulose acetate electrophoresis. Therefore, one can rule out dissociation of the enzyme as a possible cause for the loss of the regulatory properties upon reaction with phenylglyoxal.

**Binding of CTP to Phenylglyoxal-modified Aspartate Transcarbamylase** - The elimination of nucleotide binding at the regulatory site alone would explain the loss of the regulatory effect. Therefore, CTP binding to phenylglyoxal-modified aspartate transcarbamylase was determined by equilibrium dialysis. The enzyme was reacted with phenylglyoxal for 60 min under experimental conditions identical to those shown in Fig. 1. N-(Phosphonacetyl)-l-aspartate was also present during the reaction to prevent phenylglyoxal from reacting with the catalytic site arginine residue. Fig. 2 shows the binding of CTP to reacted and unreacted enzyme. The binding experiments were performed in the presence of 2 mM carbamylphosphate to prevent CTP from binding to the active site (37). The data show that CTP still binds to the phenylglyoxal-modified enzyme. The dissociation constant for the strong set of CTP sites is increased from 5.6 × 10⁻⁷ M to 1.2 × 10⁻⁶ M. This small change in the binding constant cannot explain the loss of the CTP inhibition observed upon reaction with phenylglyoxal.

Since reliable data for the low affinity CTP sites are extremely difficult to obtain by equilibrium dialysis, any conclusions about the loss of any of these binding sites is not really valid.

**Elimination of Homotropic Interactions** - As Fig. 3 shows, the phenylglyoxal-modified aspartate transcarbamylase has a substantially altered aspartate saturation curve. The sigmoidal shape indicative of the cooperative interactions between the active sites in the native enzyme is completely eliminated upon reaction with phenylglyoxal. That the modified enzyme shows no detectable sigmoidicity is substantiated by the standard double reciprocal plot. All the homotropic interactions of the normal enzyme are lost upon reaction with this reagent. The most straightforward explanation would be that two classes of arginine residues are reacting; one class involved with the loss of the enzyme's homotropic properties and a second class directly involved with the loss of the enzyme's heterotropic properties. In order to evaluate such a possibility, the exact number of arginine residues actually reacting with phenylglyoxal under these conditions was determined.

**[14C]Phenylglyoxal Incorporation** - We have previously em-


**Fig. 2.** The binding of CTP to native and phenylglyoxal-reacted aspartate transcarbamylase. Equilibrium dialysis experiments were performed at 4° for 24 h in 0.1 M imidazole/acetate buffer, pH 7.0, 2 mM β-mercaptoethanol, 0.2 mM EDTA. The binding data are represented as a Scatchard plot, $R/\left[S\right]$ versus $R$, where $R$ is the number of moles of CTP bound per mole of aspartate transcarbamylase ($M = 300,000$) and $\left[S\right]$ is the concentration of free CTP. The binding experiments were carried out in the presence of an equilibrium concentration of 2 mM carbamylphosphate to prevent CTP from binding to the active site (37). The binding data were analyzed in terms of two sets of three independent binding sites (37). For the native enzyme (○), the curve shown corresponds to dissociation constants of $5.6 \times 10^{-6}$ M and $1.4 \times 10^{-4}$ M, for the high and low affinity sites, respectively. For the modified enzyme (●), the corresponding dissociation constants were $1.2 \times 10^{-4}$ M and $5 \times 10^{-4}$ M.

**Fig. 3.** The loss of homotropic interactions upon reaction with phenylglyoxal. The aspartate saturation curve for native aspartate transcarbamylase (○) and enzyme modified with phenylglyoxal (●). The modification reaction was performed in 0.125 M potassium bicarbonate buffer, pH 8.3, at 25° employing an aspartate transcarbamylase concentration of 1.38 mg/ml and a phenylglyoxal concentration of $4.1 \times 10^{-3}$ M. After 60 min, the reaction was stopped by gel filtration on Sephadex G-25. N-(Phosphonacetyl)-L-aspartate was present during the reaction at a concentration of 0.049 mM to prevent reaction with the arginine residue located at the active site. The enzymatic activity was determined by the pH-stat assay at 25°.

**Fig. 4.** The incorporation of phenylglyoxal into aspartate transcarbamylase. The number of arginine residues modified was determined by incorporation of [14C]phenylglyoxal. The modification reaction was carried out at 25° in 0.125 M potassium bicarbonate buffer, pH 8.3, employing an aspartate transcarbamylase concentration of 1.52 mg/ml and a phenylglyoxal concentration of $9.7 \times 10^{-4}$ M. The enzymatic activity was determined by the pH-stat assay performed at 15 mM aspartate in the presence and absence of 0.5 mM CTP. The loss of CTP inhibition was expressed as a relative CTP effect which is defined under “Experimental Procedures.” The left ordinate defines the decrease in the relative CTP effect as a function of reaction time (○), and the right ordinate defines the number of arginine residues modified per 50,000 daltons (one catalytic plus one regulatory chain) as a function of reaction time (●). For further details see “Experimental Procedures.”
In order to prevent arginine residues in the intersubunit contacts from reacting with phenylglyoxal, the modified subunits were prepared by reaction of phenylglyoxal with native aspartate transcarbamylase. The modified subunits were then isolated by gel filtration after dissociation of the intact enzyme with p-hydroxymercuribenzoate.

Hybrids were prepared combining modified catalytic subunits and native regulatory subunits \((\text{C}_\text{PG},\text{R},_\text{PG})\), native catalytic subunits and modified regulatory subunits \((\text{C},_\text{PG},\text{R})\), and native regulatory and catalytic subunits \((\text{R},_\text{PG},\text{C})\) as a control. As an additional safeguard, the procedure for isolation of the modified subunits, reconstitution, and purification of the hybrids was carried out once with \(^{14}\text{C}\text{phenylglyoxal. In this way, the exact extent to which the phenylglyoxal modification was lost (21, 36) during the procedure could be determined. Not more than 30% of the original phenylglyoxal was lost during the entire procedure.}

As seen in Table I, the regulatory nucleotides had practically no effect on the hybrid which had only modified regulatory subunits. By comparison, the hybrid with only modified catalytic subunits exhibited almost normal activation by ATP and inhibition by CTP. Reduced cooperativity was observed with both hybrids containing modified subunits (Fig. 6). The relative cooperativity of the various hybrids as expressed by the Hill coefficient is given in Table I.

**Number of Arginine Residues Modified on Individual Subunits**—The reconstitution experiments have indicated that a site of modification which causes the loss of heterotropic and homotropic properties is on the regulatory subunit of the enzyme. The determination of the distribution of modified arginine residues between the two subunits would act a maximum to the number of arginine residues involved in the loss of the enzyme’s heterotropic and homotropic properties.

The modified subunits were isolated from native enzyme previously reacted with phenylglyoxal. The phenylglyoxal-reacted native enzyme was dissociated with p-hydroxymercuribenzoate and the subunits separated by sucrose gradient centrifugation. Table II summarizes the results of this experiment in which arginine modification was determined as a function of reaction time with phenylglyoxal. About one-third of the phenylglyoxal reacts with arginine residues on the catalytic chains, while the other two-thirds reacts with arginine residues on the regulatory chains. The loss of the phenylglyoxal modification, which averaged approximately 20%, was determined for each time point.

The regulatory subunit data are plotted in Fig. 7 as relative CTP effect versus the number of arginine residues modified per regulatory chain \((M_r = 17,000)\). The curvature of this plot suggests that some arginine residues not related to the loss of CTP inhibition are also reacting. Extrapolation of the initial portion of these data to zero CTP inhibition indicates that 1.65 arginine residues/17,000 daltons correlates with the loss of CTP inhibition. Taking into account that approximately 20% of the phenylglyoxal label was lost during the time necessary to separate the subunits, the actual number of residues causing this effect would be very close to 2.

**Table I**

<table>
<thead>
<tr>
<th>Species</th>
<th>CTP inhibition</th>
<th>ATP activation</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>((\text{C},<em>\text{PG},\text{R},</em>\text{PG}))</td>
<td>38</td>
<td>51</td>
<td>2.2</td>
</tr>
<tr>
<td>((\text{C},_\text{PG},\text{R}))</td>
<td>4</td>
<td>3</td>
<td>1.4</td>
</tr>
<tr>
<td>((\text{C},\text{PG})/\text{R},_\text{PG})</td>
<td>39</td>
<td>30</td>
<td>1.1</td>
</tr>
</tbody>
</table>

* For preparation of the hybrids see "Experimental Procedures."

* CTP inhibition is defined as \(100 \times (A - A^{\text{CTP}})/A\), where \(A\) is the enzymatic activity in the absence of CTP, and \(A^{\text{CTP}}\) is the enzymatic activity in the presence of 0.5 mM CTP.

* ATP activation is defined as \(100 \times (A^{\text{ATP}} - A)/A\), where \(A\) is the enzymatic activity in the absence of ATP and \(A^{\text{ATP}}\) is the enzymatic activity in the presence of 4 mM ATP.

* The Hill coefficient was calculated by fitting the experimental data to the Hill equation by nonlinear least squares.

* \((\text{C},_\text{PG},\text{R},_\text{PG})\), native aspartate transcarbamylase reconstituted from isolated subunits; \((\text{C},_\text{PG},\text{R})\), reconstituted aspartate transcarbamylase with only the regulatory subunits modified with phenylglyoxal; \((\text{C},\text{PG})/\text{R},_\text{PG}\), reconstituted aspartate transcarbamylase with only the catalytic subunits modified with phenylglyoxal.

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**Fig. 5.** The correlation of the loss of CTP inhibition of native aspartate transcarbamylase with the number of arginine residues modified. The number of arginine residues modified is expressed per 50,000 daltons (one catalytic plus one regulatory chain). The dashed line is an extrapolation of the initial linear portion of the curve. Relative CTP effect is defined under "Experimental Procedures." The reaction conditions are the same as those reported in the legend to Fig. 4.

**Fig. 6.** The kinetic properties of aspartate transcarbamylase molecules containing subunits modified with phenylglyoxal. The enzymatic activity was determined by the pH-stat assay at pH 8.3, immediately after preparation and purification of the hybrids. A reconstituted enzyme with both subunits unmodified \((\text{C},_\text{PG},\text{R},_\text{PG})\) served as the control (O), along with the hybrid with only the regulatory subunits modified \((\text{C},_\text{PG},\text{R})\) (X), and the hybrid with only the catalytic subunits modified \((\text{C},\text{PG})/\text{R},_\text{PG}\) (C). Table I summarizes the heterotropic properties of the hybrids along with Hill coefficients.
Important Arginine Residues of Aspartate Transcarbamylase

The reaction with phenylglyoxal was carried out at 25° in 0.12 M potassium bicarbonate buffer, pH 8.3, for various lengths of time employing an aspartate transcarbamylase concentration of 1.38 mg/ml and a \(^{14}C\)phenylglyoxal concentration of 2.0 mM.

### Table II

<table>
<thead>
<tr>
<th>Reaction time (min)</th>
<th>Number of arginine residues modified per catalytic chain</th>
<th>Number of arginine residues modified per regulatory chain</th>
<th>Number of arginine residues modified per catalytic plus regulatory chain</th>
<th>Number of arginine residues modified per label recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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<td>30</td>
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<td>240</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
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</tbody>
</table>

**a** Relative CTP effect is defined under "Experimental Procedures."

**b** Number of arginine residues as determined immediately after the reaction with phenylglyoxal.

**c** Number of arginine residues as determined after the subunits were separated by sucrose gradient centrifugation.

**d** As calculated from the sum of the catalytic plus regulatory chain arginine residues modified after sucrose gradient separation of the modified subunits to the number of arginine residues modified as determined immediately after the modification of the native enzyme.

**Fig. 7.** The correlation of loss of CTP inhibition of native aspartate transcarbamylase with the number of arginine residues modified on the regulatory chain. The number of arginine residues modified is expressed per regulatory chain (17,000 daltons). The dashed line is an extrapolation of the initial linear portion of the curve. Relative CTP effect is defined under "Experimental Procedures" and was determined on the native enzyme after reaction with phenylglyoxal before the enzyme's subunits were dissociated by p-hydroxymercuribenzoate and separated by sucrose gradient centrifugation.

**Amino Acid Analysis**—Amino acid analyses were performed on samples of native aspartate transcarbamylase and isolated regulatory subunit which had been reacted with phenylglyoxal. For the native enzyme and the regulatory subunit the amino acid analyses showed that, within the experimental error of about 3%, no amino acid residues other than arginine were affected. The sample of regulatory subunit used for the amino acid analysis was the 240-min reacted sample in Table III.

### Table III

<table>
<thead>
<tr>
<th>Species</th>
<th>Carbamylphosphate</th>
<th>Succinate</th>
<th>Rate Constant (k_s)</th>
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<tr>
<td>C(_R_1)</td>
<td>0.0</td>
<td>0.0</td>
<td>68.8</td>
</tr>
<tr>
<td>C(_R_2)</td>
<td>10.0</td>
<td>10.0</td>
<td>318.8</td>
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<tr>
<td>(C(_R_1))-PG</td>
<td>0.0</td>
<td>0.0</td>
<td>243.8</td>
</tr>
<tr>
<td>(C(_R_2))-PG</td>
<td>10.0</td>
<td>10.0</td>
<td>249.3</td>
</tr>
<tr>
<td>(C(_R_1)(RPG))$_i$</td>
<td>0.0</td>
<td>0.0</td>
<td>225.0</td>
</tr>
<tr>
<td>(C(_R_2)(RPG))$_i$</td>
<td>10.0</td>
<td>10.0</td>
<td>368.8</td>
</tr>
</tbody>
</table>

**a** Second order rate constant.

**b** C\(_R_1\), native aspartate transcarbamylase; C\(_R_2\)-PG, native aspartate transcarbamylase which has been reacted with phenylglyoxal; (C\(_R_1\))(RPG)$_i$, reconstituted aspartate transcarbamylase with only the regulatory subunits modified with phenylglyoxal.

II. Of the 8 arginine residues in the regulatory chain, 1.3 were lost (as determined by the amino acid analysis). A result which is in reasonable agreement with the value determined by \(^{14}C\)phenylglyoxal incorporation.

**Reaction with p-Hydroxymercuribenzoate**—Gerhart and Schachman (38) have found that the rate of p-hydroxymercuribenzoate reaction with aspartate transcarbamylase is affected by the presence of the enzyme's substrates. This difference in reactivity of the liganded and unliganded forms of the enzyme is attributed to different conformational states. Native aspartate transcarbamylase reacts with p-hydroxymercuribenzoate about 5 times faster in the presence of saturating concentrations of carbamylphosphate and succinate.

The rate of reaction of phenylglyoxal modified enzyme with p-hydroxymercuribenzoate could yield information about the conformational state of the modified enzyme. Table III summarizes the kinetic data obtained from the p-hydroxymercuribenzoate reaction with native aspartate transcarbamylase and the phenylglyoxal-reacted enzyme. The modified enzyme does not show the increase in rate observed for the native enzyme in the presence of substrates. The observed rate is much closer to the rate observed for the liganded form of the native enzyme.

Although the phenylglyoxal-modified enzyme used for these experiments was protected by the transition state analog N-(phosphonacetyl)-L-aspartate during the actual phenylglyoxal reaction so that no modification would occur at the active site, the possibility still exists that other modified arginine residues on the catalytic subunit might influence the conformational changes normally induced by the binding of carbamylphosphate and succinate. In order to eliminate this problem, the p-hydroxymercuribenzoate reaction was also carried out on a hybrid prepared from unmodified catalytic subunits and phenylglyoxal-modified regulatory subunits. The results of these experiments (Table III) are substantially identical to the results obtained with phenylglyoxal-modified native enzyme. In this case, carbamylphosphate and succinate cause a small increase in the reaction rate, but the observed rate with and without substrates is significantly higher than that observed with the unliganded form of the native enzyme. The observed rate of reaction with the hybrid is comparable to that observed for the native enzyme with ligands.

**DISCUSSION**

During our previous investigation (21) of the phenylglyoxal reaction with aspartate transcarbamylase, we found this re-
agent to eliminate catalytic activity, and also influence the heterotropic properties of the enzyme. The ability of the nucleotide effectors to influence activity was destroyed 16 times more slowly than was the activity of the enzyme. Since the enzyme activity is necessary to monitor the effect of the nucleotides, the transition state analog $N$-(phosphonlactyl)-l-aspartate was used in all the present experiments to prevent the active site arginine residue from reacting with phenylglyoxal. Under the reaction conditions necessary to eliminate heterotropic properties, up to a 10% reduction in enzymatic activity was observed.

Arginine residues have been implicated in the binding of ATP to such proteins as glutamine synthetase (25), carbamylphosphate synthetase (25), creatine kinase (26), and adenosine triphosphatase (27). In all these cases, ATP greatly reduced the rate at which the arginine residues reacted. Similarly, the arginine residue critical for activity in aspartate transcarbamylase could be protected by either substrates or by $N$-(phosphonlactyl)-l-aspartate. We have used CTP, the nucleotide which binds most tightly to the regulatory site (37), to determine whether or not the reaction which causes elimination of nucleotide effects is located at the regulatory site (Fig. 1). Surprisingly, CTP slows the rate of the phenylglyoxal reaction by only 27%, a small amount compared to the protection afforded the active site arginine by the enzyme’s substrates. Unless the reaction with phenylglyoxal caused a conformational change which precluded the binding of the regulatory nucleotides, CTP should have markedly reduced the reaction rate.

Direct measurements of CTP binding to the phenylglyoxal-modified enzyme showed that CTP still was able to bind to the altered enzyme, although with a reduced affinity. The weaker binding constant of CTP to the modified enzyme could not fully account for the observed loss of CTP inhibition. It is still possible that the modification is at the regulatory site even though CTP does not prevent the reaction from taking place. If CTP binds to the modified enzyme which is in an altered orientation because of steric interactions between it and the modified arginine residue site, then the conformational change necessary to cause inhibition might be precluded. London and Schmidt (24) have determined that only small changes in the nucleotide can change its effectiveness. Similarly, a small change in the way binding occurs could substantially affect the heterotropic properties of the enzyme. Alternatively, the elimination of heterotropic properties could result from a more indirect change distant from the effector binding site.

Since many arginine residues might be reacting on one or both of the subunits, the allosteric properties of the modified enzyme were tested and the number of residues modified was determined by incorporation of $^{14}$C phenyl glyoxal. In order to check the allosteric properties of the enzyme, the aspartate saturation curves of normal and phenylglyoxal-modified enzyme were determined (Fig. 3). These data show that a great deal of the cooperativity of the native enzyme was lost upon reaction with phenyl glyoxal. Not only the heterotropic properties but also the homotropic properties of the enzyme were abolished. Incorporation experiments employing $^{14}$C phenyl glyoxal indicated reaction by fewer than 3 of the 24 possible arginine residues/50,000 daltons (one catalytic plus one regulatory chain). We found that 2 of these residues were on the regulatory chain while the remaining 1 was on the catalytic chain.

The loss of homotropic properties could be the result of modification on the catalytic chain and the loss of heterotropic properties the result of modification on the regulatory chain. Hybrids were used to determine the site of modification which caused the specific effects. The hybrid enzyme with only modified regulatory subunits showed practically no cooperativity or heterotropic properties. The extensive reduction of both the homotropic and heterotropic properties is due to the modifications of not more than 2 arginine residues on the regulatory chain. We were very surprised to find that the hybrid with modification only in the catalytic chain showed almost full nucleotide effects but little cooperativity. Since the control hybrid had nearly full cooperative effects, it seems that the 1 arginine residue modified/catalytic chain can also influence the homotropic cooperativity of the enzyme.

The rate of reaction of p-hydroxymercurobenzoate with aspartate transcarbamylase depends on the conformational state of the enzyme (38). The conformational change associated with the binding of substrates increases the reaction rate by about a factor of 5. Therefore, we have used this reaction to investigate the conformational state of the aspartate transcarbamylase modified with phenylglyoxal. These experiments indicated that the modified enzyme exists in a conformation normally associated with the liganded form of the enzyme, i.e. the enzyme saturated with substrates. Since the native enzyme fully saturated with substrates does not show any heterotropic properties, the substrate-induced conformational change must be involved in the mechanism of heterotropic properties. Therefore, it is not surprising that the modified enzyme, locked in the liganded conformation, also shows no heterotropic properties. In addition, the inability of the modified enzyme to undergo the transition between the liganded and unliganded conformations would also explain the elimination of homotropic interactions in the modified enzyme.

The mechanism by which the modification of these particular arginine residues can cause the loss of heterotropic or homotropic properties (or both) in the enzyme is a more subtle question. The most direct explanation would involve the modified residues in the transmission of allosteric effects among the subunits of the enzyme. Alternatively, the modification itself might cause a conformational change, or prevent a conformational change from occurring, and thereby eliminate the normal function of the enzyme. Until the data obtained here can be interpreted in terms of the three-dimensional structure of the enzyme, the exact mechanism by which phenylglyoxal causes the observed effects cannot be determined. However, some conclusions can be drawn from the data obtained with the hybrids in which only one type of subunit is modified with phenylglyoxal.

The hybrid with the catalytic subunits modified by phenyl glyoxal exhibits no homotropic cooperativity. The ability of this hybrid to retain heterotropic properties clearly indicates that the two functions can be separated. This behavior is very similar to that observed with a mutant aspartate transcarbamylase that is produced by growing the cells in the presence of thiouracil (42). On the other hand, the conformational change that is induced by the reaction of phenylglyoxal with the regulatory subunit alone is sufficient to alter the conformation of the molecule in the same way that the enzyme’s substrates do. This indicates that the regulatory subunits serve the major role in mediating the conformational changes associated with both the homotropic and heterotropic interactions of the enzyme.

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REFERENCES

Functionally important arginine residues of aspartate transcarbamylase.
E R Kantrowitz and W N Lipscomb