Elimination of cooperativity in aspartate transcarbamylase
by nitrination of a single tyrosine residue

(aspaplate transcarbamylase/chemical modification/peptide sequence)

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ABSTRACT In a previous report [Landfear, S. M., Lipscomb, W. N. & Evans, D. R. (1978) J. Biol. Chem. 253, 3985-3996] we demonstrated that tetraniromethane can be employed to nitrate a limited number of tyrosine residues in aspartate transcarbamylase (carbamoylphosphate: L-aspartate car-

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bamylotransferase, EC 2.1.3.2); such modification eliminates cooperativity, feedback inhibition, and enzymatic activity, and reduces binding of the feedback inhibitor cytidine triphosphate. Cooperativity is lost more rapidly than other properties, and this loss correlates with the nitratit of a single tyrosine residue. In this paper, we describe the saturation kinetics of hybrid species constructed from nitrated subunits of one type (either catalytic or regulatory) and native subunits of the other type. We conclude that the modification responsible for loss of cooperativity is on the catalytic subunit. The tryptic peptide containing this modification has been isolated and identified.

The allosteric enzyme aspartate transcarbamylase (carba-

moylphosphate: L-aspartate carbamylotransferase, EC 2.1.3.2) from Escherichia coli functions at a control locus for the regula-

tion of the pyrimidine biosynthetic pathway (1, 2). This enzyme, which catalyzes the first step unique to that pathway, the condensation of L-aspartate and carbamyl (carbamoyl) phosphate to form carbamylaspartate (3, 4), exhibits a sigmoidal saturation curve (2) indicative of homotropic or cooperative subunit interactions. The rate of enzymatic catalysis is further modulated by the heterotropic effectors (2); cytidine triphosphate, the end product of the pyrimidine pathway, is a potent inhibitor, while adenosine triphosphate, the product of the parallel purine biosynthetic pathway, is an activator.

The protein is an oligomer of 12 polypeptide chains (5, 6) and has a total molecular weight of 310,000. Mercurials dissociate the intact molecule (7) into catalytic trimers (C6) containing three polypeptide chains, each of molecular weight 33,000, and regulatory dimers (R2) consisting of two polypeptide chains, each with molecular weight 17,000 (6-9). Crystallographic (5, 10-12) and biochemical (6, 8, 9) studies have revealed that the native molecule contains six copies of each chain (C6R6) arranged in D3 symmetry.

In an earlier paper (13) we demonstrated that progressive nitrination of aspartate transcarbamylase with the tyrosine-specific reagent tetraniromethane affects the loss of all regulatory properties: cooperativity is eliminated most rapidly, correlating with the nitration of one tyrosine per RC unit, followed by CTP inhibition, enzymatic activity, and the reduction of CTP binding, in that order. The transition state analogue N-(phospho-

nacetyl)-L-aspartate protects against the loss of activity. Hence, limited nitration in the presence of this analogue produces a mildly nitrated species (1.2 to 1.6 nitrotyrosines per catalytic chain, 1.4 nitrotyrosines per regulatory chain) that is completely devoid of homotropic and heterotropic interactions but that retains most of the original activity and CTP-binding potential. The hybrid enzymes (C6N)6R6 and C6(NR)6, constructed by reconstituting these nitrated subunits with native subunits, exhibited reduced but substantial CTP inhibition, indicating that the loss of heterotropic interactions is due to the combined effect of modifications on both the catalytic and regulatory subunits.

In the present study we have investigated more fully the regulatory properties of these hybrids. The (C6N)6R6 species exhibits no measurable homotropic interactions at pH 8.3, whereas the C6(NR)6 hybrid retains substantial cooperativity; we conclude that the modification responsible for elimination of cooperativity resides on the catalytic subunit. The position of the relevant tyrosine in the primary sequence of the catalytic chain has been determined by isolation and identification of a single nitrotyrosine-containing tryptic peptide.

MATERIALS AND METHODS

Aspartate Transcarbamylase and Subunits. The purification of aspartate transcarbamylase, nitration of the enzyme with tetraniromethane, isolation of native and nitrated subunits, and reconstitution of hybrid enzyme molecules have been reported previously (13).

Materials. Sodium dodecyl sulfate was purchased from Pierce Chemical Company; Sephadex G-50 superfine and dansyl chloride, from Sigma; diethylaminoethyl-cellulose (DE 52), from Whatman; and thin-layer polyamide sheets, coated on both sides, were obtained from the Cheng Chin Trading Company, Taiwan, and were cut into squares 5 cm on a side. Carboxypeptidases A and B were from Boehringer Mannheim and trypsin treated with tosylphenylalanyl chloromethyl ketone was from Worthington. Reagents for manual Edman degra-

dations were sequenation grade from either Pierce or Beck-

man; dimethylylalalmine (Eastman) was refluxed for 2 hr over phthalic anhydride and distilled twice (bp 61–62\(^\circ\)). Other chemicals were either analytical reagent grade or were those described in an earlier report (13).

Electrophoresis. Sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis was performed by the method of Laemmli (14).

High-voltage paper electrophoresis of peptides (10 nmol) was performed on a Savant apparatus using Whatman 3MM paper and pyridine/acetic acid/H\(_2\)O (400:12:3516, vol/vol) buffer.

Abbreviations: C, catalytic chain of aspartate transcarbamylase; R, regulatory chain of aspartate transcarbamylase; (C6N)6R6, hybrid constructed from nitrated catalytic chains and native regulatory chains; C6(NR)6 hybrid constructed from native catalytic chains and nitrated regulatory chains.

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pH 6.5. After a run at 2500 V for 4.5 hr, the paper was dried and sprayed with 0.3% ninhydrin (Sigma) in ethanol to stain the peptides.

**Isolation of the Nitrotyrosine-Containing Peptide.** Nitrated catalytic subunit, 57.2 mg in 4.4 ml of 0.10 M KH2PO4, pH 8.0, was mixed, at 3-min intervals, with five 100-μl aliquots of succinic anhydride, each containing a 2-fold molar excess of anhydride over the 14 lysines of the catalytic chain. Fifteen minutes after the last addition, a 1.2-fold molar excess (with respect to the 32 threonine and serine residues of the catalytic chain) of hydroxylamine hydrochloride was added as two 50-μl aliquots. Throughout the above procedure, the pH was maintained at 8.0 on a Radiometer TTT:pc pH-stat by addition of 1 M NaOH. The succinylated protein was transferred to 50 mM NaHCO3, pH 8.9, by elution from a column of Sephadex G-25 fine, concentrated to 1 ml, and incubated for 2 hr at room temperature with 0.57 mg of trypsin (treated with tosylphenylalanyl-chloromethyl ketone), added in 100 μl of 1 mM HCl. Another aliquot of trypsin was added, and the incubation was continued overnight. The resulting hydrolysate was applied to a column (1.5 × 55 cm) of DE 52 that had been thoroughly equilibrated with 50 mM NaHCO3, pH 8.9. The peptides were fractionated by eluting with a 600-ml linear gradient of 50 mM to 400 mM NaHCO3, pH 8.9. Fractions comprising the major peak absorbing at 428 nm were pooled, lyophilized, redissolved in 1.0 ml of 50 mM NaHCO3, pH 8.9, and eluted from a column (1.5 × 141 cm) of Sephadex G-50 superfine. The material in the A428 peak, separated from two smaller A280 peaks, was pooled, lyophilized, and dissolved in 750 μl of 50 mM NaHCO3, pH 8.9. The peptide was stored at −20°C.

**Amino Acid Analysis.** The purified nitrotyrosine peptide (4 nmol) was hydrolyzed under reduced pressure at 110°C for 22 hr in constant-boiling HCl (Fierce), and ½ of the sample was applied to a Beckman 121M amino acid analyzer.

**NH2-Terminal Studies.** Five nanomoles of peptide was lyophilized, dissolved in 25 μl of 0.10 M NaHCO3, mixed with 12.5 μl of dansyl chloride (concentration 2.5 mg/ml in dry acetonitrile), and incubated at 37°C for 50 min. The solution was dried under a stream of N2, hydrolyzed at 110°C overnight in 400 μl of constant-boiling HCl, dried under reduced pressure over NaOH pellets, and dissolved in 20 μl of 50% pyridine (refluxed over ninhydrin and then distilled). The dansyl amino acid was identified by chromatography (15) on polyamide sheets coated on both sides; the sample was spotted onto one side and a mixture of standards onto the opposite side. Initial identifications were confirmed by applying the sample and the corresponding dansyl amino acid on the same side.

**COOH-Terminal Studies.** Nine nanomoles of peptide was lyophilized and redissolved in 50 μl of 0.10 M N-ethylmorpholine, pH 8.5. Carboxypeptidase B or both carboxypeptidases A and B were added (1/3 of the peptide, by weight, in 10 μl) and the solution was incubated at 37°C for 2 hr, mixed with 37.5 μl of dansyl chloride (2.5 mg/ml in acetonitrile), and incubated at 37°C for another 30 min; this mixture was dried under a stream of N2 and redissolved in 20 μl of 50% pyridine. Dansyl amino acids were identified as above. A control containing enzyme but no peptide was treated identically to ensure that the amino acids identified were not released by autodigestion of carboxypeptidase.

**Sequencing Studies.** Manual Edman degradations were performed on 37 nmol of peptide by the method of Sauer et al. (16), and the resultant phenylthiohydrodantoin amino acids were identified by chromatographing against standards on a Beckman model 45 gas chromatograph or on thin-layer plates by the method of Summers et al. (17).

**RESULTS**

**Nitratred Subunits and Hybrids.** Aspartate transcarbamylase selectively nitrated in the presence of N-(phosphonacetyl)-L-aspartate until all homotropic and heterotropic properties

![Fig. 1. Aspartate saturation curves at pH 8.3, 5 mM carbamyl phosphate, for reconstituted aspartate transcarbamylase, C6R4 ( ), and for C6(RN)6R6 in the presence ( ) and absence ( ) of 0.5 mM CTP. Assays were performed by the radioactive method in 0.2 M Tris/acetate buffer. (Inset) Double reciprocal plots of the saturation curves.](image1)

**Enzyme Assays.** A modification of the radioactive assay of Davies et al. (18) was employed to measure enzyme activity, as detailed previously (13). For assays at pH 7.0, the buffer was 0.20 M imidazole/acetate, the enzyme concentration was above 1 μg/ml, and the reaction was terminated after 5 min.

![Fig. 2. Aspartate saturation curve for C6(RN)6R6 assayed radioactively at pH 8.3, 0.5 mM CTP, 5 mM carbamyl phosphate, 0.2 M Tris/acetate. (Inset) Double reciprocal plot of the data in the main body of the figure.](image2)
Buffers eliminated isolated the pH and 7.0. Polypeptide per (13). As retained 1.4 analysis proved these subunits. Although side reactions involving inter- and intramolecular crosslinking of tyrosines may occur upon nitration of some proteins (20, 21), electrophoresis on sodium dodecyl sulfate gels demonstrated that no crosslinked oligomers were present in these samples of isolated subunits. These nitrated subunits were reconstituted (13) with native subunits to form a hybrid containing nitrated catalytic chains and unmodified regulatory subunits, (CN)3R6, and another hybrid containing native catalytic subunits and nitrated regulatory chains, (CN)3(RN)6; these hybrids migrated with intact native enzyme on cellulose acetate electrophoresis.

Repeated attempts to obtain subunits with discrete modifications (i.e., 1.0 nitrotyrosine per polypeptide chain) were not successful; a mixture of nitrated and native catalytic subunits was not resolved by elution from DEAE-Sephadex with either salt or pH gradients of various steepness.

Regulatory properties of the hybrids. As previously reported (13), both these hybrids retained substantial sensitivity to CTP, revealing that the loss of heterotropic interactions in the intact nitrated enzyme results from modification of tyrosines on both the catalytic and the regulatory chains. Because the loss of cooperativity correlates with the nitration of one tyrosine (13), studies of the hybrids should resolve whether this tyrosine is on the catalytic or the regulatory chain.

Measurement of saturation kinetics for (CN)3R6 and for reconstituted aspartate transcarbamylase (CN)3R6 at pH 8.3 (Fig. 1) reveal the absence of cooperative interactions for the hybrid, as evidenced by the hyperbolic aspartate saturation curve; even in the presence of 0.5 mM CTP, which should amplify any re-

**FIG. 3.** The effect of the substrate analogue succinate on the specific activity of aspartate transcarbamylase (CN)3R6 and (CN)3R6 at pH 8.3 and 7.0. Radioactive assays were performed at 1 mM aspartate, 5 mM carbamyl phosphate, and increasing concentrations of succinate, using the buffers previously described. (A) Aspartate transcarbamylase (●, ○), (CN)3R6 (●, ○) and reduced (CN)3R6 ( ■) at pH 8.3 (solid symbols) and at pH 7.0 (open symbols). For ease of visualization, the scale of the ordinate has been changed at a relative velocity of 1.0. (B) (CN)3R6 at pH 8.3 ( ) and 7.0 ( ). Note the 10-fold difference in the scales of the abscissa for the data at pH 8.3 (bottom scale) and pH 7.0 (top scale).

**FIG. 4.** Aspartate saturation curves at pH 7.0, 5 mM carbamyl phosphate for (CN)3R6 in the absence of effectors (○) and in the presence of 0.5 mM CTP (●) or 2.0 mM ATP (■). Assays were performed by the radioactive method in 0.2 M imidazole/acetate. (Inset) Double reciprocal plot of assays in the presence of 0.5 mM CTP.

**FIG. 5.** Aspartate saturation curves of reduced (CN)3R6 assayed at pH 8.3 (●) and at pH 7.0 (○), 5 mM carbamyl phosphate, employing the radioactive assay and the buffers previously described. (Inset) Double reciprocal plots of the data in the main body of the figure.
Fig. 6. Ion exchange chromatography of the tryptic digest of the nitrated catalytic subunit. Nitrated subunit (57.2 mg) was digested with trypsin, and the peptides were resolved on a column (1.5 x 55 cm) of DEAE-cellulose, as described in the text. Fractions 58 through 62 (6 ml each) were pooled, lyophilized, and rechromatographed on a column of Sephadex G-50 superfine. (Inset) Amino acid sequence (William Konigsberg and coworkers, personal communication) of the nitrotyrosine-containing peptide. Our sequence information is indicated below the full sequence. NH₂-terminal residues were determined by Edman degradation and COOH-terminal residues by digestion with carboxypeptidases A and B. Table 1 gives a comparison of the amino acid composition of our peptide with the theoretical composition for residues Leu⁶⁰₆₋Lys²¹⁷.

Residual cooperativity, there is no detectable sigmoidicity in the (CN)₆R₆ data. The double reciprocal plots (Fig. 1) exhibit straight lines for the hybrid species (except for high values of 1/v where substrate inhibition occurs⁴), indicative of hyperbolic saturation kinetics. In contrast, the C₆(R₆)₆ hybrid displays reduced but observable cooperativity (Fig. 2) apparent in the sigmoidal aspartate curve and the nonlinear double reciprocal plot.

Another method for assessing the extent of cooperativity, which is not subject to complications from substrate inhibition, relies on enzymatic assays in the presence of low aspartate concentrations (1 mM) and increasing concentrations of substrate analogues (22) such as succinate. If substantial cooperative interactions exist, the activity is increased by addition of moderate concentrations of substrate analogues, which induce a cooperative conformational transition analogous to that elicited by aspartate binding; high concentrations of substrate analogues inhibit by saturating the active site. Fig. 3A reveals that, while native enzyme is activated 10-fold by 20 mM succinate, the (CN)₆R₆ hybrid is inhibited by all concentrations of succinate; i.e., it exhibits no evidence of homotropic effects. Together, these results demonstrate that the modification that disrupts homotropic subunit interactions is contained in the (CN)₆R₆ hybrid alone; i.e., it is on the catalytic chain.

Assays performed at pH 7.0 (Fig. 4) disclosed the unexpected result that residual cooperativity reappeared for the (CN)₆R₆ hybrid; the saturation curve in the presence of 0.5 mM CTP is visibly sigmoidal, and double reciprocal plots in the presence and absence of effectors are all curved, even though substrate inhibition is minimal at this pH.¹ This result is particularly surprising, because the native enzyme displays a substantial increase in cooperativity when the pH is raised from 7.0 to 8.3²; in the (CN)₆R₆ species, the pH dependence of cooperativity is the opposite of that for native enzyme. Fig. 3A and B confirms this conclusion: succinate does elicit some residual activation of (CN)₆R₆ at pH 7.0 but not at pH 8.3.⁶

Reduction of Nitrotyrosine with Sodium Hydrosulfite. One possible explanation for the reversed pH dependence of cooperativity in the (CN)₆R₆ hybrid could involve the ionization of the phenolic hydroxyl group on the modified tyrosine. The pK for the hydroxyl of an isolated tyrosine is about 10, whereas for nitrotyrosine is about 7 (23, 24). If the protonated form of the tyrosine is necessary to maintain cooperative interactions, nitration of the residue might reduce the pK to the point where the hydroxyl would be almost completely ionized at pH 8.3, eliminating homotropic properties. Lowering the pH to 7.0 should reionize a portion of the residues, partially restoring cooperativity.

This hypothesis was tested in the following way. For amino-tyrosine, the pK of the hydroxyl is about 10 (23, 24) and that of the amino group is about 5 (23). Reduction of the nitro-tyrosine with sodium hydrosulfite (23, 24) should then restore cooperative interactions in (CN)₆R₆ at pH 8.3 if the preceding explanation is correct. In fact, the (CN)₆R₆ hybrid that had been reduced with sodium hydrosulfite (23, 24) (no absorbance peak at 428 nm) did not regain cooperative interactions at pH 8.3 (Fig. 3A); the behavior of cooperativity as a function of pH was identical to that for the (CN)₆R₆ hybrid prior to reduction (Fig. 5). Hence, nitration of the tyrosine does not effect the loss of subunit interactions by promoting ionization of the phenolic hydroxyl; rather, some steric mechanism must be operative.

Isolation of the Nitrotyrosine-Containing Peptide. The

¹ S. C. Pastra-Landis, D. R. Evans, and W. N. Lipscomb, unpublished data.

⁶ Unpublished data of S. M. Landfear indicate that altered aspartate transcarbamylase molecules with a small degree of residual cooperativity may not be activated by succinate under these conditions. Hence, we cannot conclusively demonstrate that cooperativity has been completely eliminated in (CN)₆R₆ at pH 8.3; however, it has certainly been very substantially reduced at pH 8.3, while some cooperativity is clearly present at pH 7.0.
position of the relevant modification in the primary sequence of the catalytic chain was determined by isolating and identifying the nitrotyrosine-containing peptide. A 147-mg sample of aspartate transcarbamylase (at 7.0 mg/ml in 0.10 M Tris/acetate, pH 8.0), containing a 10-fold excess of N-(phosphonoacetyl)-L-aspartate per active site, was nitrated at 25° for 15 min with a 750-fold excess of tetranitromethane; these conditions suffice to eliminate cooperativity without substantially altering other functions (13). The modified catalytic subunit was isolated and a 57.2-mg sample was succinylated, digested with trypsin (treated with tosylphenylalanly chloromethyl ketone), and purified by ion exchange and gel filtration chromatography, as described above. The elution profile from the ion exchange column (Fig. 6) exhibits only one major absorbance peak at 428 nm, confirming that loss of cooperativity results from nitration of a single tyrosine residue (i.e., one tryptic peptide). High-voltage paper electrophoresis of the purified peptide revealed one very strong spot and two very weak contaminants; purity was estimated at 95% or better.

The results of amino acid analysis of this peptide are presented in Table 1. Dansylolation revealed that leucine is the NH₂-terminal residue, and sequential Edman degradation established leucine, aspartate, and proline as the first three amino acids. Digestion with carboxypeptidase B yielded lysine, and digestion with both carboxypeptidases A and B released lysine, valine, and asparagine. These results are entirely consistent with the sequence presented in Fig. 6, which is taken from the sequence data for the catalytic subunit, kindly communicated prior to publication by William Konigsberg; the numbers designating amino acids are his tentative assignments of sequence position. We conclude that the tyrosine we have modified is the one tentatively designated as number 213.1

**DISCUSSION**

From the results presented here, we conclude that nitration of a single tyrosine on the catalytic chain of aspartate transcarbamylase abolishes cooperativity at pH 8.3. We have located the position of this tyrosine within the preliminary amino acid sequence. It is remarkable that the introduction of one nitro group on the ring of a single tyrosine suffices to greatly reduce or completely eliminate cooperativity at pH 8.3; this fact argues in favor of a direct role for the residue in transmitting the allosteric signal. However, a full understanding of the function of this tyrosine will require the interpretation of the high-resolution x-ray map for aspartate transcarbamylase.

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1. The presence of lysine as the carboxyl terminal residue indicates that succinic anhydride did not modify this residue.

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The existence of a hybrid species, (C₇H₅NO₄)₆, that is virtually devoid of homotropic interactions but still retains substantial heterotropic functions is consistent with the notion, promulgated by several authors (25–30), that these two types of allosteric interaction are separated (i.e., occur by different mechanisms) in aspartate transcarbamylase.

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