Essential Arginine Residues in Tryptophanase from Escherichia coli*

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Tryptophanase from Escherichia coli B/117-A is inactivated by the arginine-specific reagent, phenylglyoxal, in potassium phosphate buffer at pH 7.8 and 25°C. Apo- and holoenzyme are inactivated at the same rate, and inactivation of both is correlated with modification of 2 arginine residues/tryptophanase monomer. Substrate analogs having a carboxyl group protect the holoenzyme against both inactivation and arginine modification but have no effect on the inactivation or modification of the apoenzyme. Phenylglyoxal-modified apotryptophanase retains the capacity to bind the coenzyme, pyridoxal-P, but the spectrum of this reconstituted species differs from that of native holo tryptophanase. Neither this reconstituted species nor the phenylglyoxal-modified holoenzyme shows the 500 nm absorption characteristic of the native enzyme when substrates are added. These results demonstrate a requirement for specific arginine residues for substrate binding and are discussed in the context of the known conformational and spectral forms of tryptophanase with regard to a possible role for arginine residues in formation of a catalytically effective enzyme-pyridoxal-P complex.

Crystalline tryptophanase (EC 4.1.99.1) from Escherichia coli B/117-A (1) is a tetrameric enzyme, M∞ = 220,000, composed of identical, single polypeptide subunits, each with a pyridoxal-P binding site (2). A monovalent cation is required for tryptophanase activity (3), NH₄⁺ and K⁺ being by far the most effective. Holotryptophanase undergoes a pH-dependent shift (pK = 7.2) in its spectrum from an inactive, low pH form (λmax 405 nm) to an active, high pH form (λmax 337 nm) (4). The formation of the 337 nm form is also dependent on the presence of K⁺ (4, 5).

At present, the following information is available concerning the roles of specific amino acid residues in the catalytic functioning of tryptophanase. Structural studies in this laboratory established the sequence of the pyridoxyl decapptide obtained from borohydride-reduced holotryptophanase (6). One or more sulfhydryl groups are required for enzyme activity (7), but a detailed study of this point has not been carried out. From a study of the pH dependence of the hydrogen exchange reaction catalyzed by tryptophanase (4) it was deduced that a group on the enzyme with pK 6.65, perhaps a histidine residue, was responsible for the abstraction of the α proton from substrates during catalysis. Coenzyme specificity studies (8, 9) showed that the 5'-phosphate of pyridoxal-P plays a significant role in coenzyme binding but is not required for catalytic function, and that minor alterations at positions 2 and 6 of the 3-hydroxy pyridine ring system produce only minor alterations in coenzyme binding. A detailed discussion of the structure, catalytic activities, and mechanism of action of tryptophanase can be found in a recent review (10).

Phenylglyoxal (11, 12), cyclohexanedione (13, 14), and butanedione (15-17) have been used to show that a large number of enzymes contain arginine residues essential for substrate or cofactor binding. Among the enzymes so far selectively modified are carboxypeptidase (16), ribonuclease (17), several kinases (12, 19), several dehydrogenases (15, 20-22), aspartate transcarbamylase (23), mitochondrial ATPase (24), fructose-1,6-diphosphate phosphatase (25) and at least two pyridoxal-P dependent enzymes, aspartate aminotransferase (14, 26) and β-mercaptotetrahydrodase (27). In each case substrates or cofactors, or both, containing carboxyl or phosphoryl groups have assumed binding sites on the enzyme in which arginine residues subject to modification play a role. Crystallographic model studies have demonstrated the propensity of guanidinium groups to form complexes with both carboxyl (28) and phosphoryl groups (29-31) and examples of such complexes are found in enzyme structure (32, 33).

The present study establishes that arginine residues play a critical role in the function of tryptophanase.

MATERIALS AND METHODS

Enzyme Preparation and Assay—Tryptophanase from Escherichia coli B/117-A (34) was prepared as the apoenzyme by a modification (35) of previous procedures. Holotryptophanase was prepared by incubation of the purified apoenzyme with pyridoxal-P for 1 h at 37°C in 0.1 M potassium phosphate, pH 7.8, containing 1 mM EDTA and 10 mM dithioerythritol followed by gel filtration or dialysis to remove excess reagents. Enzyme activity was measured as follows. Protein samples (about 10 μg of protein) were incubated for 20 min...
at 60°C in 0.5 ml of the activation buffer (Buffer A) (10% (v/v) glycerol, 0.1 M potassium phosphate (pH 7.8), 1 mM EDTA, 10 mM dithioerythritol, 20 μM pyridoxal-P, and 100 μg of acetylated bovine serum albumin (30/mL) to ensure full activation. The samples were then cooled to 25°C, 0.5 ml of 1.2 mM S-o-nitrophenylcysteine was added, and the change with time in absorbance at 370 nm was recorded (97). A change in the molar extinction coefficient of 1050 M⁻¹ cm⁻¹ at 370 nm occurs upon decomposition of S-o-nitrophenylcysteine to pyruvate, NH₃, and o-nitrothiophenol (37) and was used to calculate enzyme activity. Protein concentrations were determined from the absorbance at 278 nm (2) or by the method of Lowry et al. (38) using bovine serum albumin as a standard. Tryptophanase preparations used had specific activities of 49 to 55 μmol of S-o-nitrophenylcysteine decomposed min⁻¹ mg⁻¹.

Inactivation and Modification—Phenylglyoxal monohydrate was obtained from Aldrich Chemical Co. [7-¹⁴C]Phenylglyoxal prepared from [7-¹⁴C]acetophenone (ICN Corp.) by selenium oxidation (39, 40) had a specific activity of 0.2 μCi/μmol. Inactivation experiments were carried out at 25°C in 0.1 M potassium phosphate, 0.2 mM dithioerythritol, at protein concentrations near 1 mg/mL. Samples (10 μL) removed at various times were added to 0.5 ml of Buffer A to stop the modification reaction and then were assayed for activity. To determine the amount of phenylglyoxal bound, the enzyme was treated with [7-¹⁴C]phenylglyoxal and assayed for activity and extent of incorporation of radioactivity. A sample of the modified enzyme (0.1 ml of an inactivation mixture) was separated from excess radioactive phenylglyoxal by gel filtration through a Sephadex G-25 column (0.5 × 17 cm) using 10 mM potassium phosphate, pH 7.8, for elution. Radioactivity was determined by liquid scintillation counting using a Triton/toluene mixture (41). The activity of the treated enzyme is expressed as the per cent of the activity of the untreated control enzyme.

Quantities of phenylglyoxal-treated tryptophanase sufficient for spectral analyses were isolated as follows. Apo- or holotryptophanase, 1 mg/mL in 0.1 M potassium phosphate, 2 mM dithioerythritol, pH 7.8, was treated with 10 mM phenylglyoxal for 1 h at 25°C. The modification reaction was terminated by adding 40 μM of L-alanine per milligram of starting protein and the reaction mixture was dialyzed for 3 h against two 500-ml changes of 0.1 M potassium phosphate, 0.2 mM dithioerythritol. Data points are shown in Fig. 1 as the per cent inactivation 25 and 60 min after phenylglyoxal addition. Both apo- and holotryptophanase were incubated with 2.8 mM CO, 4.5 mM (●), or 6.8 mM (●) phenylglyoxal in 0.1 M potassium phosphate (pH 7.8), 0.2 mM dithioerythritol. Data points are shown for holotryptophanase only. Data for apotryptophanase fell on the same curve.

Fig. 2 (right). Number of arginine residues modified during inactivation of apo- (●) and holotryptophanase (○) with [7-¹⁴C]phenylglyoxal. Enzyme samples (1 mg/mL) in 0.1 M potassium phosphate (pH 7.8), 0.2 mM dithioerythritol were treated with 5 mM [7-¹⁴C]phenylglyoxal; enzyme activity and phenylglyoxal incorporation were determined as described under "Materials and Methods." The number of arginine residues modified was calculated using the stoichiometry of 2 mol of phenylglyoxal/mol of arginine (11).

**TABLE I**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Kᵢ value*</th>
<th>Inactivation at:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>mM</td>
<td>25 min %</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
<td>70</td>
</tr>
<tr>
<td>Indoleacrylate</td>
<td></td>
<td>0.19</td>
<td>34</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td>16</td>
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<tr>
<td>L-Ethionine</td>
<td>5</td>
<td>0.52</td>
<td>32</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>30</td>
<td>3.0</td>
<td>31</td>
</tr>
<tr>
<td>Indole</td>
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<td>0.05-0.25</td>
<td>72</td>
</tr>
</tbody>
</table>

* Measured with tryptophan as substrate (10).

Results

Both apo- and holotryptophanase are inactivated at about the same rate and to the same extent by treatment with phenylglyoxal (Fig. 1). Approximately 4 mol of [7-¹⁴C]phenylglyoxal are incorporated/mol of either apo- or holotryptophanase monomer with full loss of activity (Fig. 2). Using the stoichiometry of 2 mol of phenylglyoxal/mol of arginine obtained in model systems by Takashashi (11), this corresponds to the modification of 2 arginine residues/enzyme monomer.

Protection by Substrate Analogs—Competitive inhibitors which are substrate analogs protect holotryptophanase against inactivation by phenylglyoxal only if they have a carboxyl group (Table I). All the analogs of this type tested protect to approximately the same extent when added at concentrations that are equal multiples of their Kᵢ values in the tryptophanase reaction. This result rules out a nonspecific cause for the protection effect, such as reaction of the analogs with phenylglyoxal and, since L-alanine protects as well as either indolepropionate or indolepropionate but indole does not protect, it also rules out inactivation directed solely by hydrophobic interaction of phenylglyoxal with the indole binding site on the enzyme.

Indolepropionate not only protects holotryptophanase against inactivation, but also decreases incorporation of [7-¹⁴C]phenylglyoxal (Fig. 3). From the extent of the decrease it appears that both of the arginine residues modified (with loss of activity) in the absence of indolepropionate are protected from reaction by this inhibitor. This conclusion is confirmed by replotting the data obtained in the presence of indolepropionate (Fig. 3) as the activity remaining versus the number of arginines modified. Such a plot shows that no modification...
takes place without accompanying inactivation, and extrapolation of this plot to zero remaining activity shows 2 arginine residues are modified/tryptophanase monomer. Indole has no effect on the extent of arginine modification in the holoenzyme.

None of the substrate analogs listed in Table I protect apotryptophanase from inactivation by phenylglyoxal. Indolepropionate does not reduce the extent of arginine modification in the apoenzyme and, as shown in Fig. 4, the observed loss of activity remains correlated with modification of 2 arginine residues of apotryptophanase in the presence of indolepropionate. Thus the simultaneous presence of substrate (or inhibitory analogue) with an α-carboxyl group and the coenzyme is necessary to protect tryptophanase from modification and inactivation by phenylglyoxal.

No change in the $K_m$ of S-o-nitrophenylcysteine or in the $K_i$ for alanine was observed during inactivation of either apo- or holotryptophanase.

Spectral Properties of Modified Enzyme—Apo- and holotryptophanase modified with phenylglyoxal until less than 20% of the original activity remained were prepared for spectral observation as described under "Materials and Methods." Phenylglyoxal modification of the apoenzyme markedly increases its absorbance above 350 nm (Fig. 5) and makes interpretation of the spectrum of the pyridoxal-P-reconstituted species difficult. From the difference spectra (Fig. 5, inset) it is clear that the spectrum of pyridoxal-P bound to the modified apoenzyme differs from both that of free pyridoxal-P and that of pyridoxal-P bound to the native enzyme. The spectrum of the modified holoenzyme appears to differ little from that of the native enzyme when the phenylglyoxal contribution is taken into account. Furthermore, unlike those of the native enzyme (4) the spectra of the modified species are essentially invariant between pH 6 and pH 8.5. Neither modified apoenzyme reconstituted with pyridoxal-P nor holotryptophanase modified with phenylglyoxal forms the 500 nm band which normally appears on the addition of α-amino acid substrates to the enzyme (4).

**Fig. 4 (right).** Number of arginine residues of apotryptophanase modified in the presence of indolepropionate during inactivation with [7-14C]phenylglyoxal. The experimental conditions are described in the legend to Fig. 3; activities and incorporation of phenylglyoxal were determined as specified in the legend to Fig. 2.

Pyridoxal-P Binding to Modified Enzyme—An apparent dissociation constant ($K_p$) for pyridoxal-P binding to both the native and phenylglyoxal-modified apoenzyme was determined by following the change in absorbance at 430 nm upon addition of pyridoxal-P. From the data shown in Fig. 6, values of $K_p = 0.1 \mu M$ for the native enzyme and $3.2 \mu M$ for the modified enzyme were obtained. The value for the native enzyme (42) of the titration data. The lines shown were determined by linear regression and the $K_p$ values calculated from the slopes. The $x$-intercept corresponds to 0.3 pyridoxal-P sites/native monomer and 1.1 pyridoxal-P sites/modified-monomer. $r$, concentration of bound pyridoxal-P; $F_0$, concentration of unbound pyridoxal-P; and $K_p$, dissociation constant for the protein-pyridoxal-P interaction.
enzyme obtained by this method is only slightly larger than the value obtained from equilibrium dialysis (43) and agrees closely with the concentration of pyridoxal-P required for half-maximal activity under comparable conditions (9). It was not possible to resolve pyridoxal-P from the phenylglyoxal-modified holoenzyme in order to determine the $K_m$ for this species. Treatment of the native enzyme with penicillamine and (NH$_4$)$_2$SO$_4$ normally removes about 90% of the bound cofactor (1). No change in the spectrum of the phenylglyoxal-modified holoenzyme was observed after repeated treatment of this sort. Recently, partial resolution of holotryptophanase during gel filtration chromatography (44) has been used to determine the effects of monovalent cations on pyridoxal-P binding to tryptophanase. In similar experiments at 25°C we have found 40% resolution of the native enzyme and 90% resolution of the phenylglyoxal-modified apoenzyme reconstituted with pyridoxal-P, but no resolution of the phenylglyoxal-modified holoenzyme.

**DISCUSSION**

The results reported here clearly demonstrate a critical role for modifiable arginine residues in the function of tryptophanase. That the rate and extent of modification by phenylglyoxal is the same for apo- and holoenzyme shows that the accessibility and reactivity of the modifiable arginine residues remains unchanged during the transition from apo- to holoenzyme.

Together with the results of the coenzyme specificity studies (8, 9), which demonstrate a major role for the 5'-phosphate group in the binding of pyridoxal-P, the spectral evidence that this coenzyme binds to phenylglyoxal-modified apotryptophanase nearly as well as to the native apoenzyme argues against a direct role for modifiable arginine residues in binding the 5'-phosphate moiety of the coenzyme. Small steric alterations, *e.g.* addition of a methyl group to the 5'-carbon, change values of $K_m$ (the concentration of pyridoxal-P required for half-maximal activity under the assay conditions), by 1 order of magnitude or more, while changes in charge, *e.g.* methylation of the 5'-phosphate, result in much smaller changes in $K_m$. Phenylglyoxal modification of an arginine residue involved in binding the 5'-phosphate of the coenzyme should thus result in a large (2 to 3 orders of magnitude) change in $K_m$ while the observed change is less than 1 order of magnitude, 0.7 to 3.2 µM.

The most facile conclusion, and one that is supported by the results of the substrate protection experiments, is that the modifiable arginine residues are involved in binding the substrate carboxyl group. Although substrate analogs protect tryptophanase against modification by phenylglyoxal, they do so only in the case of the holoenzyme, *i.e.* when bound coenzyme also is present. This is the expected result if substrate can bind to the holoenzyme but not to the apoenzyme, and would indicate that the known conformational differences between apo- and holotryptophanase (2) result, among other changes, in the generation of the substrate binding site of the holoenzyme. However, it seems unreasonable that both of the arginine residues that are modified in the holoenzyme (and protected from reaction by substrate analogs having a carboxyl group) are required for binding of a substrate containing a single negative charge.

Substrate specificity studies (1) (45) and investigations of the reversibility of the tryptophanase reaction (5) have demonstrated separate binding domains for the hydrophobic (indolyl, nitrophenyl) and carboxyl portions of substrates. If the only role for both of the arginine residues was in binding substrate at the carboxyl binding domain, their modification should cause a change in substrate $K_m$. This change should be greatest for those substrates such as serine or alanine, for which the carboxyl group provides the principal binding interaction, but should be less pronounced for substrates such as tryptophan or S-o-nitrophenylcysteine, where the principal binding force is the interaction of the hydrophobic portion of the substrate with the protein. No change in $K_m$ for either type of substrate was observed, but this observation might reflect the fact that in a mixture of modified and native tryptophanase, only the $K_m$ for the residual native species is observed. However, the data are consistent with the view that, although at least one of the two modifiable arginines must have a role in substrate binding, the second may play an indirect role in tryptophanase function. This contention is supported by the difference in the spectra of the native and modified enzyme and by the invariance with pH of the spectra of the modified enzyme. This invariance suggests that the pK of the functional group involved in the spectrally observed pH-dependent transition of the native enzyme is altered. Modification of an arginine residue near this functional group could have such an effect.

Based on previous knowledge and results obtained from the present work, the active site of tryptophanase appears to have the following features. Tryptophanase undergoes a conformational change upon pyridoxal-P binding (2), and the change is "felt" by adjacent subunits (46). In both the apo- and holoenzyme the domain ultimately responsible for binding the substrate carboxyl group is accessible and reactive to phenylglyoxal, but is effective in binding substrate only in the holoenzyme. Pyridoxal-P is bound to the phenylglyoxal-modified apoenzyme but some further transition required for formation of a catalytically active complex is blocked. If pyridoxal-P is bound prior to the phenylglyoxal modification, it is locked into the enzyme, either through steric blockage or some other effect, and is not resolvable by the usual procedures.

In a previous report we presented evidence indicating that one of 3 modifiable arginine residues of β-serine dehydratase was essential for pyridoxal-P binding (27). In tryptophanase, by contrast, arginine residues are required for substrate binding and appear to be involved only indirectly with interactions concerned in binding of pyridoxal-P. Thus, despite the formal similarity in the pyridoxal-P-dependent α,β-elimination reactions catalyzed by β-serine dehydratase (47) and by tryptophanase (10), it appears that, at least with respect to the amino acid residues involved in binding and catalysis, the detailed mechanisms of these two enzymes will turn out to be different.

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Essential Arginine Residues in E. coli Tryptophanase

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