A Spin Label Substrate Analogue as Active Site-directed Modifying Agent

TRYPTOPHAN 140 OF ASPARTATE AMINOTRANSFERASE*

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The interaction of cytoplasmic aspartate aminotransferase with the spin probe substrate analogue potassium nitrosodisulfonate (NDS) has been investigated using the α-subform of the pig heart enzyme. Electron paramagnetic resonance studies show that NDS binds to the enzyme. Scatchard plots with different concentrations of NDS are linear and show the presence of two binding sites/dimer (Kd = 0.7 mM). Substrates or substrate analogues displace enzyme-bound NDS. NDS is also a competitive substrate inhibitor (Kd = 0.72 mM) when tested in assays for transaminase activity. Treatment of the enzyme with 0.2 mM NDS at pH 7.5 results in an inactivation that follows pseudo-first order and saturation kinetics with a maximum inactivation rate of 0.04 min⁻¹ and Kmax of 1.2 mM. The NDS rate of inactivation is pH-independent, and substrates and substrate analogues protect the enzyme. These results are consistent with a role for NDS acting as an active site-directed reagent.

The absorption spectrum of NDS-treated apo- or holoenzyme shows an increase in absorbance in the 320-nm region that parallels the loss in activity and a decrease in the 280/260-nm absorbance ratio. The amino acid composition of NDS-modified enzyme shows only a loss of tryptophyl residues (1/subunit). Peptide fractionation of tryptic digests of the NDS-modified enzyme revealed only one extra peptide with absorption at both 220 and 320 nm. Both the amino acid composition and partial sequence of this peptide indicate that it corresponds to residues 130-153 in the cytoplasmic aspartate aminotransferase sequence. This protein fragment includes Trp 140 residue which, in the 2.8-Å x-ray maps of the enzyme's active site region, appears located in front of the pyridine ring of pyridoxal phosphate (Metzler, D. E., Jansonius, J. N., Arnone, A., Martinez-Carrion, M., and Manning, J. M. (1982) Fed. Proc. 41, 2432-2438).

The reaction mechanism and protein structure of the pyridoxal-P-dependent enzyme aspartate aminotransferase (EC 2.6.1.1) from pig heart have been extensively studied. Amino acid groups participating in the catalytic process or in coenzyme and substrate interactions have been investigated using reagents with specificity toward a given type of functional group, affinity labels of substrates, or of derivatives of the coenzyme. The first of such residues to be located was the lysyl (Lys 258) residue whose ε-amino group forms a Schiff base linkage to the coenzyme pyridoxal-P (1-3).

Chemical modification with tetranitromethane also affects cysteinyl residues (7-9) and the destruction of some histidyl residues by photooxidation of the enzyme in the presence of methylene blue (10, 11) have been reported. The properties of the enzyme modified in this way suggested the possibility that these groups might be binding sites for the carbonyl groups (7-9, 11) of the substrates or participate in proton removal from the substrate (12). Another guanidinium group forms an ion pair with the phosphate group of the coenzyme (13) but has not been chemically modified.

The arrangement of these above mentioned groups in the active site region are now being detected by x-ray diffraction methods (13, 14). Several other residues which appear in the active center region have not yet been tagged by chemical modification procedures, and their role in the enzyme's functions remains undefined. Of particular interest is the presence of a tryptophan residue, Trp 140, which appears to be located in front of the pyridine ring with its indole side chain within van der Waals contact with the coenzyme (14). In solution, this proximity appears to reflect on a fluorescence quenching for the tryptophan (15-17) and circular dichroism perturbations of the aromatic amino acid region (18) upon binding of coenzyme by apoenzyme. Identification of this residue in solution through chemical modification procedures has not been fruitful, and it appears desirable to selectively alter Trp 140 and investigate its potential role in enzyme function. To this end, the chemical reagent of choice is known from studies with model compounds (19, 20) and with different types of proteins (21, 22) to be a specific oxidizing agent mainly for tyrosine and tryptophan groups. Another important consideration which led us to the selection of NDS² as the modifier agent is its reported behavior as an analogue of oxalacetate when used as an active site probe for citrate synthase (23). This suggested the possibility that it could act as a more specific active site probe in enzymes employing oxalacetate as...
Transaminase Active Site Tryptophan

EXPERIMENTAL PROCEDURES

The α-form of cytoplasmic aspartate aminotransferase was isolated from pig hearts as previously described (24). Potassium nitrosodisulfonate (Fremy’s salt) was obtained from Aldrich. Protein concentrations were calculated from the absorbance at 280 nm (ε = 140,000 M⁻¹ cm⁻¹) for a molecular weight of 94,000. All of the absorption spectra and absorbance measurements were carried out with a Cary model 210 recording spectrophotometer.

Aspartate Aminotransferase with NDS—Enzyme modifications were carried out at 25 °C in 0.05 M potassium phosphate buffer, pH 7.5. The concentration of enzyme was 1 mg/ml and the concentration of NDS ranged from 0.2-4 mM. Modification reactions were initiated by adding a suitable volume of freshly prepared solution to the enzyme in the phosphate buffer along with any other reagent used. Aliquots of the sample containing the enzyme, since the amplitude of the absorbance of the spectra decreases with time under these conditions.

Treatment of Aspartate Aminotransferase with NDS—Enzyme modifications were carried out at 25 °C in 0.05 M potassium phosphate buffer, pH 7.5. The concentration of enzyme was 1 mg/ml and the concentration of NDS ranged from 0.2-4 mM. Modification reactions were initiated by adding a suitable volume of freshly prepared solution of reagent to the enzyme in the phosphate buffer along with any other compound being studied. All reagents were neutralized before they were added to the enzyme solution. Aliquots were withdrawn from the reaction mixtures at various time intervals and the activity monitored by spectrophotometric assay as previously described (26). When 80% inactivation had occurred, the modifier was removed from the sample by passing the modified enzyme through a Sephadex G-25 column (2 x 25 cm) equilibrated and eluted with 0.05 M potassium phosphate buffer, pH 7.5.

Protein concentrations were calculated from the absorbance at 280 nm (ε = 140,000 M⁻¹ cm⁻¹) for a molecular weight of 94,000. All of the absorption spectra and absorbance measurements were carried out with a Cary model 210 recording spectrophotometer.

Tryptic Digest—Protein samples (5 mg/ml) were dialyzed against 0.1 M Tris/HCl bufer, pH 8.0, and digested with TPCK-trypsin. The n-hexane solution of TPCK-trypsin was applied in 100 μl of this buffer and eluted with a gradient of this buffer.

Substrates and Analogues—The addition of different lipids such as substrates and analogues to the NDS-enzyme complex and the number of binding sites/substrate molecules. A Scatchard plot of the data (Fig. 2) was linear, indicating two specific binding sites/substrate dimer. A value of 0.7 mM is obtained for the dissociation constant (Kd) of NDS from these two sites.

As it has been reported (33), treatment of the enzyme with N-ethylmaleimide in the presence of the substrate pair L-glutamate (70 mM) and α-ketoglutarate (2 mM), modified by cysteine (30) modifies three sulfhydryl groups/subunit and the modification of one of them (identified as Cys 390) reduces aminotransferase activity to less than 5% of the initial value. Yet, this N-ethylmaleimide treatment does not prevent the loss of NDS probe signal. These results indicated that the decrease in signal observed in the EPR spectrum of NDS in the presence of enzyme is not primarily due to oxidation of accessible cysteinyl residues.

Displacement of NDS from Aspartate Aminotransferase by Substrates and Analogues—The addition of different lipids such as substrates and analogues to the NDS-enzyme system displaced appreciable amounts of bound NDS (Table 1). Furthermore, the presence of substrates in the sample not only displaces the probe from the enzyme complex but also protects against destruction of the probe signal. For example,
Transaminase Active Site Tryptophan

FIG. 1. Paramagnetic resonance spectra of NDS (0.2 mM) in the absence (A) and presence (B) of aspartate aminotransferase (0.21 mM). Modulation amplitude 0.5 gauss; gain $2.5 \times 10^3$.

Fig. 2. Scatchard plot of the paramagnetic resonance data for NDS binding to cytoplasmic aspartate aminotransferase obtained as explained in the text.

Under the conditions given in the legend of Table I, 6.5% destruction of NDS signal was observed over a 6-min period in the absence of other ligands. When 50 mM $\alpha$-ketoglutarate was added, only 2.6% signal decay occurred over the same period of time. This behavior suggests that the reduction of NDS by oxidizable amino acid residues in the enzyme takes place mainly when NDS is bound to the enzyme.

Similar results regarding NDS displacement from the binding site and protection against signal destruction were obtained when the enzyme was previously incubated with the substrates or even when the ligands were added to the NDS-enzyme system if a correction is made for the percent signal lost during the period of observation.

Inactivation of Aspartate Aminotransferase by Incubation with NDS—Incubation of aspartate aminotransferase with NDS produces a loss of enzymatic activity which follows first order kinetics (Fig. 3). Less than 1% of the initial activity remains after a 10-h incubation with 2 mM NDS. Activity is not restored by dialysis, gel filtration, or treatment with thiol compounds or pyridoxal-P. The inactivation exhibited saturation kinetics as can be observed upon plotting the first order inactivation rate constants as a function of reciprocal NDS concentration. As shown in the inset in Fig. 3, extrapolation of the line to the abscissa gives a $K_{inact}$ (reagent concentration yielding one-half the maximum rate of inactivation) of 1.2 mM. The maximum rate of inactivation calculated from the same plot was 0.04 min$^{-1}$.

The loss in activity is not caused by modification or loss in the coenzyme pyridoxal-P. In fact, under the same experimental conditions, the apoenzyme's rate of inactivation is identical with that of the holoenzyme (data not shown). Furthermore, the pyridoxal-P content of enzyme modified to a 20% residual activity was found to be 90% that of native enzyme. On the other hand, as shown in Fig. 4, NDS can act as a competitive inhibitor ($K_I = 0.725$ mM) of aspartate aminotransferase when assayed at a fixed concentration of L-aspartate (10 mM) at different concentrations of $\alpha$-ketoglutarate (0.05-0.25 mM) and in the presence of 2-5 mM NDS. The value obtained from these data agrees with the $K_I$ for the dissociation of NDS from the probe-enzyme complex calculated from the direct binding data.

All of the substrates and analogues tested protected the

| Substrate or analogue | Concentration (mM) | NDS bound (mM) | Percent of NDS displaced$^*$ |
|-----------------------|-------------------|---------------|-----------------------------|
| None                  | 0.102             |               |                             |
| Oxalacetate           | 50                | 0.053         | 51                          |
| $\alpha$-Ketoglutarate| 50                | 0.04          | 61                          |
| 2-Methyl-DL-aspartate | 100               | 0.014         | 86                          |

$^*$ Similar results were obtained by previous addition of the ligands and a correction made for the per cent signal lost during the period of observation before addition of the ligands.

**Table I**

**Displacement of NDS bound to aspartate aminotransferase by substrates and analogues**

Each sample (100 pl) contained 0.08 $\times 10^{-5}$ M enzyme, 0.7 mM NDS, and the indicated concentrations of substrates in 50 mM potassium phosphate buffer, pH 7.5. Five or more EPR spectra of 0.05-ml samples were obtained over a period of 10 min. The value obtained for the line amplitude after extrapolation to the time of NDS addition was used for the calculation of the concentration of bound NDS and expressed as a percentage of the concentration in the absence of ligands (NDS-enzyme system).
enzyme against inactivation, the best being α-ketoglutarate and α-methylaspartate (Fig. 5).

Spectral Changes in the Modified Enzyme—The absorption spectra of aspartate aminotransferase modified by incubation with 0.2 mM NDS after removal of the excess of reagent by dialysis are compared to those of untreated enzyme in Fig. 6. At high pH, native enzyme absorbs at 360 nm as modified enzyme does, although it is decreased slightly in magnitude. The ultraviolet absorption spectra show an increase in absorbance in the region of the 250-nm absorption minimum, suggesting the breakdown of tryptophan (34) that appears as a maximum at 245 nm in the difference spectrum shown in the inset of Fig. 6. On the other hand, an increase in absorbance is observed in the 320-nm region. The rate of increase in absorbance at 320 nm was closely correlated with the loss in activity (Fig. 7). This absorbance increment at 320 nm is not due to the formation of a derivative of the coenzyme pyridoxal-P because it remains after removal of pyridoxal-P and appears in apoenzyme modified under identical conditions. Its appearance must be related to the modified amino acid residue(s) responsible for the inactivation of the enzyme. Furthermore, incubation of the holoenzyme with NDS, but in the presence of 50 mM α-ketoglutarate, protects the enzyme against both inactivation and increase in absorbance at 320 nm. Samples in the absence and presence of the protective ligand were incubated for 4 h at 25 °C in 0.05 M potassium phosphate buffer, pH 7.5, and passed through a Sephadex G-25 column to remove the excess of NDS and stop the reaction.

As has been described (19, 20), NDS oxidizes specifically both tyrosine and tryptophan residues free or incorporated in a protein. NDS treatments of model compounds, N-acetyl-L-tyrosine ethyl ester and N-acetyl-L-tryptophan ethyl ester,
Fig. 6. Absorption spectra of the pyridoxal form of cytoplasmic aspartate aminotransferase before (solid curve) and after incubation with 0.2 mM NDS (dashed curve) to 25% residual activity. Difference spectrum is shown in the inset.

Fig. 7. Time course for inactivation (○), absorption increment at 320 nm (△), and NDS reduction (●) resulting from incubation of aspartate aminotransferase (0.021 mM) with a 10-fold molar excess of NDS in 50 mM potassium phosphate buffer, pH 7.5, at 25 °C. At various time intervals, aliquots of 1 μl were withdrawn and determined for enzyme activity. Absorption and electron paramagnetic spectra were recorded in aliquots of 1 ml and 50 μl, respectively, at different times. $A_s$ and $A_m$ denote absorbance (320 nm) and line amplitude of the EPR spectra of NDS for the indicated periods; $A_s$, $A_m$, and $A_s$, $A_m$ denote the same parameters at initial and final values.

were carried out in order to detect possible changes in absorption spectra. The spectrum of the tryptophyl derivative most closely resembled the one from the modified enzyme with a broad increase in absorbance around 320 nm which progressed with length of incubation. The spectrum from tyrosyl derivative incubated with NDS under similar conditions also showed an absorbance increment at 320 nm, yet the main characteristics were the appearance of two new absorption maxima at 300 and 390 nm. These latter maxima are absent in NDS-treated aspartate transaminase.

pH Dependence for Inactivation of Aspartate Aminotransferase with NDS—The inactivation of aspartate aminotransferase with a 10-fold molar excess of NDS was followed in a series of buffered solutions from pH 5.6-9. The experiments could not be performed at lower pH values because of the instability of NDS at acidic pH. The rates of inactivation were essentially independent upon the hydrogen ion concentration over the pH range studied.

Loss of Amino Acid Residues Accompanying the Inactivation of Aspartate Aminotransferase by NDS—The amino acid composition of native and modified aminotransferase with a 10-fold molar excess of NDS to 25% residual activity was determined after hydrolysis for 22 h at 110 °C in methane sulfonic acid in order to avoid the destruction of tryptophyl residues. No appreciable loss of the theoretically susceptible tyrosine is observed and only a decrease in the number of tryptophyl residues is detected when compared with the composition of a sample of native enzyme treated in a similar way. It has been reported (21) that 3 mol of NDS must be consumed/mol of indole residue oxidized. Hence, the destruction of approximately 1.3 Trp residues/subunit agrees with both the stoichiometry of the amount of NDS reduced per mol of enzyme (6 ± 0.5/dimer) (Fig. 8) and the changes in the absorption spectrum concurrent with the modification.

Isolation and Characterization of the Tryptic Peptide(s) Containing the Modified Residue(s)—Treatment of the enzyme for 4 h with a 10-fold molar excess of NDS results in destruction of 70-75% of its original activity. These samples,
peptide were identified by manual Edman degradation. Partic acid was found as the N-terminal residue and threonine as the second amino acid. This confirms the identity of the peptide with that including residues 130–153 in the amino acid sequence of native transaminase since it is the only tryptic fragment in the complete sequence that has this composition and those 2 amino acid residues in the NH$_2$-terminal sequence. This peptide includes tryptophan 140.

**DISCUSSION**

As reported for citrate synthase (23), the free radical NDS also binds to cytoplasmic aspartate aminotransferase. However, in citrate synthase, NDS appears to act solely as a reversibly binding analogue of substrate oxalacetate, but, in the aminotransferase, binding leads to inactivation. In this sense, it appears to act as an active site-directed modifying agent, as shown for cytoplasmic aspartate aminotransferase modified with NDS to 25% remaining activity. The enzyme was reacted with a 10-fold molar excess of NDS at pH 7.5, 35°C. Approximately 5 nmol of the tryptic digests of each sample were chromatographed on a Varian Micro Pak MCH 10 C-18 reverse-phase column. For more details, see “Experimental Procedures.”

![Fig. 8. Loss of aspartate aminotransferase catalytic activity as a function of the concentration of reduced NDS. Extrapolation of the line to the abscissa gives 6 ± 0.5 mol of NDS reduced/mol of enzyme (94,000 molecular weight) for complete inactivation. Conditions of the reaction were the same as those described in the legend to Fig. 7.](http://www.jbc.org/)

![Fig. 9. HPLC chromatograms of tryptic digests of cytoplasmic aspartate aminotransferase modified with NDS to 25% remaining activity. The enzyme was reacted with a 10-fold molar excess of NDS at pH 7.5, 35°C. Approximately 5 nmol of the tryptic digests of each sample were chromatographed on a Varian Micro Pak MCH 10 C-18 reverse-phase column. For more details, see “Experimental Procedures.”](http://www.jbc.org/)

**Table II**

| Amino acid | No. of residues $^*$ |
|------------|---------------------|
| Asp        | 3.1 (3)             |
| Thr        | 3.9 (4)             |
| Ser        | 2.5 (2)             |
| Glu        | 1.4 (1)             |
| Gly        | 2.1 (2)             |
| Ala        | 0.9 (1)             |
| Val        | 3.2 (3)             |
| Tyr        | 1.5 (1)             |
| Phe        | 2.4 (2)             |
| His        | 0.9 (1)             |
| Lys        | 1.0 (1)             |

$^*$ Composition was determined after hydrolysis for 24 h at 110°C in 6 N HCl. Numbers in parentheses are numbers of residues expected for the corresponding tryptic peptide 130–153 from native aspartate aminotransferase sequence (35).
agent which is extremely efficient because, as a substrate analogue, it is site specific and, thus, effective at low concentrations. In turn, the low concentrations of modifier employed render it ineffective as a general modifier of tyrosyl and tryptophyl residues for which this agent is known to be an avid oxidizer in aqueous solution. Evidence supporting our conclusion that there is interaction of NDS with the active site region of aspartate aminotransferase is (a) the NDS radical is displaced by different substrates and analogues such as dicarboxylic acids inhibitors as observed from the binding data obtained by EPR and (b) NDS is also a substrate-competitive inhibitor \( K_{i} = 0.72 \text{ mm} \) of the enzyme. Furthermore, the affinity for the binding site \( K_{D} = 0.7 \text{ mm} \) is similar to the one displayed by substrates such as oxalacetate and \( \alpha \)-ketoglutarate \( (36) \) and by other dicarboxylic acids inhibitors such as succinate \( (37, 38) \). The irreversible inactivation of the enzyme seems to be due to active site modifications because inactivation follows the reversible complex formation between reagent and active site of the enzyme with \( K_{mi} \), \( K_{I} \), and \( K_{D} \) values for NDS all in the 0.7–1.2 mm range. Finally, the first order inactivation kinetics, the rate saturation with respect to NDS concentration, and the protective effect of different substrates and analogues against loss of activity are consistent with an active site-specific modification.

The results of the present study show that the loss in activity of aspartate aminotransferase by incubation with NDS is accompanied by destruction or modification of a tryptophyl residue. Comparison of the amino acid composition of native and modified enzyme shows that other amino acid residues theoretically susceptible to NDS oxidation, especially tyrosine and cysteine, as well as the coenzyme are not appreciably affected. Several lines of evidence support the result gleaned from amino acid analysis. It has been reported that NDS is a stable free radical which, after oxidizing tyrosine and tryptophan residues, free or incorporated in proteins \( (20, 39) \), induces the appearance of characteristic changes in the absorption spectra. The spectral changes observed during incubation of the enzyme with NDS (appearance of an absorption peak at the 320-nm region) follow the same time course as the loss in enzyme activity and the decrease in the 280/260-nm absorbance ratio. Similar spectral changes upon oxidation of tryptophyl residues with different reagents have been reported in the literature \( (34, 40–42) \). In addition, we found a stoichiometric ratio of 6 ± 0.5 mol of NDS consumed/mol of enzyme \( (M_{r} = 94,000) \) for complete enzyme inactivation. Thus, the stoichiometry of the reaction is close to 3 mol of NDS consumed/mol of subunit which is in close agreement with the value of 3 mol of NDS reduced in the modification of each tryptophan in studies on isolated molecules \( (19) \) and on some proteins \( (21) \). By contrast, in the oxidation of tyrosine by NDS, be it free, peptide-bound, or incorporated into a protein, 2 mol of NDS/mol of tyrosine were reported to be consumed \( (20, 21) \).

The amino acid composition and partial sequence of the tryptic peptide which contains the residue modified during the inactivation allow its identification in the known amino acid sequence of cytoplasmic aminotransferase \( (35) \). This tryptic peptide in the native enzyme includes tryptophan \( 140 \) which has been located by x-ray crystallographic studies in the active site region of the enzyme near the lower portion of the pyridine component of pyridoxal-P.

The inactivation of aspartate aminotransferase by NDS probably proceeds by a two-step process. The reagent first binds to the substrate binding site as a noncovalent Michaelis complex. The protective effect of different substrates supports this view. Binding is the likely result of the two sulfonates mimicking the carboxylate groups of the substrates in their association with specific residues in the active center of the enzyme. As candidates for carboxylate groups binding sites, lysyl \( (38) \), histidyl \( (11) \), and arginyl residues \( (7–9) \) have been suggested. NDS could bind to the enzyme through one or both sulfonate groups through electrostatic interactions with positively charged amino acid side chains in such manner that the potentially reactive nitroso group of the reagent could be placed within striking range of Trp 140. The second step of inactivation would proceed with the oxidation of the indole ring with possible formation of the 5-hydroxyindole and/or the 4,5-indolequinone derivatives, both of which have been reported as products of the oxidation of indole derivatives by free NDS radical \( (19) \).

A variety of ionizable amino acids has been located in the vicinity of pyridoxal-P in this transaminase. Some of these residues, including Arg 292 and Lys 258, have also been assigned roles in the active center through chemical modification studies. On the other hand, the implication of tryptophan residue as influencing the pyridoxal-P region had only been inferred through physical studies including enzyme tryptophan fluorescence quenching \( (15–17) \), circular dichroism of the aromatic region \( (18) \), and, more recently, x-ray crystallography \( (14) \). Other pyridoxal-P-dependent enzymes also appear to contain buried tryptophyl residues capable of influencing the electronic structure of pyridoxal-P \( (40) \) and, through a circumstantial association of ideas, tryptophan and pyridoxal-P have been implicated in cause-effect relationships between these two aromatic groups in pyridoxal-P enzymes in general \( (43) \).

Little is known regarding the role of tryptophyl residues in enzymatic active centers. A wealth of information appears to center on physical observations such as fluorescence studies of the behavior of rotational freedom and exposure or movement of tryptophans in many enzymes upon binding of ligands. Also, perturbations induced through modifications of tryptophyl residues with reagents such as bromosuccinimide have been informative in pointing out probable roles of this residue in structure-function relationships in proteins. On the other hand, we are unaware of specific examples in which a tryptophyl residue may be directly implicated in an enzyme-active center with an assigned function in an enzyme reaction mechanism. In crystalline aspartate transaminase, free holoenzyme Trp 140 lies in front of the pyridoxal-P coenzyme, at about a 70° angle and van der Waals contact with the pyridine ring \( (13, 14) \). Modification without insertion of a bulky modifier and without significant change in the overall charge of the modified indole can now be accomplished by using NDS. What is the role of the apparent interactions of indole and pyridine rings in the active center? The indole could assist in the electron delocalization process in the well known reaction sequence of transamination. Furthermore, the forward movement of the pyridine upon formation of external aldimes with substrates \( (13) \) should be accompanied by parallel displacements of the indole ring of Trp 140. If, in any way, the new indole derivative produced by NDS oxidation impedes this movement, it could affect catalytic events and possibly even the complex stereochemistry of exposure of external aldimes to added reducing agents such as NaBH₄ \( (44) \). These challenging questions and many others now appear to be testable both in solution and in crystals of aspartate transaminase since enzymes differing only in the nature of the indole at Trp 140 can be prepared. Since the aminotransferase enzyme is well known because of the ease with which events at the active center in the reaction sequence can be followed by a variety of spectroscopic means \( (12, 13, 18, 38, 44) \), the answer to many questions regarding a possible role of tryptophan appears to be an attainable goal.
Transaminase Active Site Tryptophan

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