Methylation of the Active Center Histidine 217 in D-Amino Acid Oxidase by Methyl-p-nitrobenzenesulphonate*

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Incubation of D-amino acid oxidase with excess methyl-p-nitrobenzenesulphonate results in a pseudo-first order, irreversible loss of 95% of the assayable activity using p-phenylglycine as substrate. The rate of inactivation reaches a limiting value of 0.021 min⁻¹ (pH 7.7, 22 °C) as the concentration of inhibitor is increased, consistent with a two-step mechanism with complex formation prior to covalent inactivation. This rate decreases with decreasing pH in a manner that suggests that the basic form of a group within the enzyme, with an apparent pKₐ of 6.7, is required for inactivation. Approximately three [¹⁴C]methyl residues are covalently incorporated at maximal inactivation. The competitive inhibitor benzoate prevents inactivation and the incorporation of approximately 1 methyl residue. Histidine 217, the residue modified by 5-dimethylaminonaphthalene-1-sulfonyl chloride (Swenson, R. P., Williams, C. H., Jr., and Massey, V. (1983) J. Biol. Chem. 258, 497–502), was identified as the major residue modified in the absence of benzoate. Both the 1-methyl and 3-methyl derivatives of histidine 217 were observed in a molar ratio of 0.64:0.36.

We recently reported on the presence of a specific histidine residue (histidine 217) in the active center of pig kidney D-amino acid oxidase (1). The identification was based on the differential labeling of histidine 217 by dansyl chloride in the presence and absence of the competitive inhibitor benzoate (1, 2). The inactivation of the enzyme by this reagent in the absence of benzoate may be entirely the result of the demonstrated blockage of the substrate (ligand) binding pocket by the bulky dansyl residue (2); thus the question of whether histidine 217 actually participates in catalysis remains unanswered.

A large collection of physicochemical and mechanistic information for this model flavoprotein dehydrogenase/oxidase suggests roles for a number of amino acid residues in catalysis. For example, extensive ligand-binding studies have demonstrated that the ionization of two amino acid residues in addition to that of the 3-methyl group of the FAD affects ligand affinity (3). Also, the mechanism proposed for catalysis requires the presence of a basic residue in the enzyme to facilitate proton transfer during the transient formation of a carbocation on the α-carbon of the substrate in electron transfer to the flavin (4, 5). Subsequent proton transfer from the α-amino group of substrate to complete electron transfer may also be facilitated by the enzyme. Does histidine 217 participate in ligand binding and/or in any of these proton transfer steps? In an effort to determine the role that this residue may occupy in catalysis, we sought a means of chemically modifying histidine 217 without the introduction of bulky chemical substituents.

There are few reagents that are specific for histidine and that also introduce minimal modification (6). However, a report by Nakagawa and Bender describing the inactivation of chymotrypsin by methylation of histidine 57 with the substrate analogue MNBS suggested a possible approach (7). This reagent seemed suitable for use with D-amino acid oxidase for the following reasons. 1) MNBS is a good methylating reagent because of the strong electron-withdrawing properties of aryl sulfonic acids. Upon reaction with the enzyme the large active site-directed portion would be free to diffuse out of the binding site leaving the small methyl residue covalently attached to a specific amino acid residue. 2) MNBS has been used effectively in the methylation of specific active site histidine residues in at least three other proteins, chymotrypsin (7), phospholipase A2 (8), and chicken liver carboxyesterase (9). 3) Nitroaromatic compounds bind to D-amino acid oxidase causing perturbations in the flavin spectrum in a manner similar to many other competitive inhibitors of this enzyme such as benzoate and o-amino benzoate (10). Binding is undoubtedly the result of the nitro group mimicking the carboxylate, an essential element in most, if not all, D-amino acid oxidase ligands (11). Fluorodinitrobenzene has been used to label two residues in the active center of D-amino acid oxidase in a mutually exclusive manner, tyrosine 55 and lysine 204 (12). Thus, MNBS should also be active site directed. 4) Dansyl chloride seems also to be directed toward the active center of D-amino acid oxidase and to histidine 217. The sulfonyl portion of the molecule may interact in such a manner as to bring about the specificity observed. The similar functional group in MNBS may be directed toward the histidine as well. 5) The methyl group is small and should intro-
DUPLICATE minimal steric hindrance factors; however, the acid/base properties and specific interactions of the residue methylated may be affected. 6) Unlike many other histidine derivatives, methylhistidine is resistant toward hydrolysis, providing an enzyme derivative more amenable to characterization and further study.

We report here the results of the modification of d-amino acid oxidase with MNBS. The reagent was found to preferentially methylate histidine 217 in the absence of benzoate, altering its catalytic activity. Methylated d-amino acid oxidase should provide a good system for further study of the mechanism of d-amino acid oxidase and in the determination of the catalytic role of histidine 217.

**EXPERIMENTAL PROCEDURES**

**Materials**

D-Amino acid oxidase was purified from pig kidneys as described previously (1). Methyl-p-nitrobenzenesulfonylate was obtained from Pierce Chemical Co. (recrystallized from hexanes before use) or synthesized as described under “Methods” using p-nitrobenzenesulfonyl chloride (Aldrich), [35S]methanol (Amersham Corp.), and sodium methoxide (Matheson Coleman and Bell). d-(-)-phenylglycine, FAD, and 1- and 3-methylhistidine were purchased from Sigma. L-1-Tosyl-2-phenylethyl chloromethyl ketone-treated trypsin was from Worthington. Acenitritile (UV spectral grade) was obtained from Burdick and Jackson. Sequencer chemicals were from Beckman and Pierce Chemical Co. All other chemicals were analytical reagent grade.

**Methods**

**Synthesis of [35S]Methyl-p-nitrobenzenesulfonate**—Methyl-p-nitrobenzenesulfonate radiolabeled at the methyl position was synthesized by a modification of the method reported by Morgan and Cretcher (13). Sodium methoxide was used to dilute the specific activity of the [35S]methanol and allowed to react with a 2-fold excess of p-nitrobenzenesulfonyl chloride in ethyl ether for 3-4 h at 22°C. Solvent was removed by evaporation with a nitrogen stream and the residue suspended in a 1:1 mixture of H2O and chloroform. The aqueous layer was extracted with an equal volume of chloroform at least three additional times, the chloroform fractions combined, and solvent removed by evaporation under a partial vacuum (water aspirator). The residue was analyzed by HPLC and found to contain significant amounts of starting sulfonyl chloride; thus the [35S]MNBS was further purified by preparative HPLC using a water/acetonitrile gradient. The purified radiolabeled reagent had a retention time on the HPLC and a UV spectrum (λmax = 292 nm) comparable to that of the commercial reagent.

**Enzyme Assay**—Enzymatic activity during chemical modification was assayed spectrophotometrically using D-phenylglycine as substrate (14). The standard assay mixture consists of 4.5 mM d-phenylglycine and 3.3 μM added FAD in 50 mM sodium pyrophosphate, pH 8.5, saturated with air. The change in absorbance at 292 nm was monitored continuously for several minutes after addition of enzyme in a spectrophotometer thermostated at 20 or 25°C.

**Enzyme Modification**—Enzyme was routinely modified at pH 7.4-7.6 in 50 mM sodium phosphate buffer at 22-25°C. Benzofeate-free d-amino acid oxidase was adjusted to a final concentration of 0.1-0.2 mM and made 0.1 mM in free FAD. Recrystallized or HPLC-purified MNBS in stock solutions of 20-35 mM in acetonitrile was added in a 5-fold molar excess over enzyme-bound FAD to initiate the reaction. Because of the destruction of reagent by hydrolysis during the course of the modification, an additional 5-fold excess reagent was added at 65-75 min, and a 2.5-fold excess added at 180 and at 280 min. After a total reaction time of 7-8 h, residual reagent and the sulfonic acid product were removed by dialysis at 4°C against 50 mM sodium pyrophosphate, pH 8.5.

**Tryptic Digestion, Peptide Mapping, Isolation, and Characterization**—Modified protein was denatured in guanidine HCl and cysteine thiols carboxymethylated as described in detail previously (12) except that dithioerythritol in a 10-fold molar excess over protein thiols was included in a 5.5-h incubation at pH 8.0 (30°C) prior to the addition of recrystallized iodoacetate (1.05-fold molar excess over total thiols in the reaction mixture). Digestion of the modified protein by trypsin as well as analytical peptide mapping and peptide isolation by reverse phase HPLC were carried out as described elsewhere (1, 12, 15). Amino acid sequences were determined by the automated liquid phase Edman degradation procedure and phenylthiohydantoin amino acid derivatives identified as described previously (1, 12, 15).

**Amino Acid Analysis and the Identification of Methylhistidine**—Amino acid compositions were determined by conventional ion exchange amino acid analysis (1, 2) and/or as described below. The methyl derivatives of histidine are not resolved from histidine under the conditions used on our amino acid analyzer; therefore, an HPLC method was developed to resolve 1- and 3-methylhistidine. The acid hydrolysate (6 N HCl, 110°C 24 h) of the purified peptides was derivatized with o-phthalaldehyde/mercaptoethanol reagent using a method similar to that reported by Hill et al. (16) prior to injection onto a Waters Associates μBondapak phenyl column (0.39 × 30 cm) equilibrated in 12.5 mM sodium phosphate, pH 7.4, acetoniitile (95:5, v/v) and eluted with a three-part linear acetonitrile gradient (see Fig. 6). The fluorescent amino acid derivatives were detected and quantitated with a Waters Associates model 420-C fluorescence detector (filters: excitation, 340-nm bandpass; emission, 440-nm cutoff) interfaced with a model 730 Diamodule.

**RESULTS**

**Inhibition of D-Amino Acid Oxidase by Methyl-p-nitrobenzenesulfonate**—Incubation of d-amino acid oxidase with a 5-fold molar excess of methyl-p-nitrobenzenesulfonate at pH 7.5, 22°C in the absence of benzoate resulted in a time-dependent loss of enzymatic activity as monitored by the standard spectrophotometric assay using D-phenylglycine as substrate. The rate of inactivation did not follow pseudo-first order kinetics nor was complete inactivation observed (<50%) when only the one addition of reagent was made; however, further inactivation was observed if additional reagent was added. Nakagawa and Bender (7) have reported the spontaneous hydrolysis of this reagent by solvent (t0 of hydrolysis of 100 min at pH 7.5, 25°C), thus introducing a competing side reaction causing the destruction of reagent on a similar time scale as enzyme inactivation. The above results are consistent with this observation. If additional reagent is added at approximately each half-life of the reagent, inactivation proceeds in an apparent pseudo-first order manner until approximately 95% of the activity has been lost (Fig. 1 and inset).

In previous work we have made use of the differential effect of...
of the presence and absence of the competitive inhibitor benzoate on inactivation and covalent labeling as one criteria for the modification of active site amino acid residues (1, 2, 12). As in those studies, the inclusion of benzoate in the reaction mix results in a much slower rate of inactivation as shown in Fig. 1.

Evidence for Complex Formation Prior to Inactivation—Nitroaromatic compounds are ligands for D-amino acid oxidase (10); therefore, MNBS could form a complex with D-amino acid oxidase prior to covalent reaction, resulting in a modification more specific for active site residue(s). The reaction scheme can be summarized as follows,

\[ E + I \rightleftharpoons [E-I] \rightarrow E_{inactivation} + P. \]

If the equilibrium between enzyme and the complex is rapid relative to the rate of inactivation then the following expression holds (17),

\[ \frac{1}{k_{obs}} = \frac{k_1 k_2 [I]}{k_1 [I] + k_{-1}}. \]

A plot of \(1/k_{obs}\) versus \(1/[I]\) should be linear with the ordinate intercept equivalent to \(1/k_0\) and the ratio of slope to intercept giving the \(K_1(k_{-1}/k_1)\) for the complex. Fig. 2 shows the effect of MNBS concentration on the initial pseudo-first order rate of inactivation of D-amino acid oxidase at pH 7.7 and 6.1. Only the initial portion of the inactivation reaction was used in the determination of the inactivation rate, i.e. at times less than 20 min where <15% of the reagent had been hydrolyzed. At either pH, the double reciprocal plot is linear and does not pass through the origin; therefore, the data is consistent with the two-step mechanism proposed (17). At pH 7.7 the maximal rate was determined (by extrapolation to a saturating concentration of MNBS) to be 0.021 min\(^{-1}\) and the apparent \(K_1\) was 1.3 mM. Lowering the pH slowed the inactivation rate to 0.0075 min\(^{-1}\) but had little apparent affect on the binding of MNBS. The experimentally determined rates could not be saturated due to the limited solubility of the reagent in aqueous solution and the relatively high \(K_1\) for the enzyme.

The effect of pH on the inactivation rate was investigated in more detail by incubating the enzyme with 1.0 mM MNBS in buffers of various pH values. The inactivation rates as determined from the initial portion of the reaction decreased with decreasing pH in a manner that suggests that the unprotonated form of an ionizable residue with an apparent pK\(_a\) of 6.7 was required for inactivation (Fig. 3).

Incorporation of \(^{14}C\)Methyl Residues—The degree of methylation associated with the inactivation reaction was determined using reagent synthesized with a \(^{14}C\)-labeled methyl residue in the ester. An average of 3.2 methyl residues per D-amino acid oxidase monomer had been covalently incorporated at maximal inactivation under the conditions described in Fig. 1. In a similar experiment but one in which 5 mM benzoate was included in the reaction mixture a total of 2.6 methyl residues were incorparated. These results suggest that the incorporation of approximately a single methyl residue is responsible for inactivation.

Determination of the Site of Methylation Responsible for Enzyme Inactivation—The site of methylation responsible for inactivation was determined by comparison of HPLC tryptic peptide maps of protein modified with \(^{14}C\)MNBS in the absence and presence of benzoate. Representative chromatograms from reverse phase HPLC monitored at 220 nm are shown in Fig. 4. The radioactivity associated with each peptide fraction is also indicated by the histogram plotted with each chromatogram. Several observations can be made. As expected, under both experimental conditions radio-label was distributed throughout the tryptic peptide map. However, the peptide fraction eluting at 32.5 min was preferentially labeled in enzyme modified in the absence of the competitive inhibitor (Fig. 4A). The ratio of label found in this fraction in the absence of inhibitor as compared to the benzoate control was 7:1. None of the other radioactive fractions displayed this large difference in labeling. These fractions must represent the many random methylation reactions that occur during the inactivation of the enzyme. The benzoate control retained approximately 80% of the enzymatic activity. Much of the activity loss could be accounted for by the observed level of methylation in the 32.5-min fraction in this control; therefore, these nonspecific modifications are responsible for little, if any, inactivation of the enzyme.

These results strongly suggest that the 32.5-min peptide fraction contains an active site amino acid residue which, when methylated, results in the inactivation of enzyme activity. This fraction was characterized further.

Isolation and Characterization of the Major Radiolabeled Fraction—The 32.5-min peptide fraction was isolated by preparative HPLC from the trypic digest of protein modified by
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Fig. 4. High performance liquid chromatographic peptide maps of tryptic digests of D-amino acid oxidase modified with [14C]MNBS in the absence (A) or presence (B) of 5 mM sodium benzoate as described in Fig. 1. A portion (2.0-2.5 nmol) of the 12-h tryptic digests of reduced and carboxymethylated protein were chromatographed in 0.1% phosphoric acid on a Waters Associates μBondapak C18 reverse phase column using a linear acetonitrile gradient. Peptide elution was monitored by the absorbance at 220 nm (full scale sensitivity; 0.04 and, after 29 min, 0.1). The histograms represent the radioactivity associated with each peak.

[14C]MNBS to a residual catalytic activity of approximately 20% that of the initial unmodified enzyme, using HPLC conditions similar to those described in Fig. 4. Based on previous observations (15), it was suspected that this fraction may contain at least one other peptide; therefore, the 32.5-min peptide fraction was rechromatographed on the C18 reverse phase column equilibrated at pH 7.4. As shown in Fig. 5, three major peptide peaks were observed. Radioactivity was associated only with the peak eluting at 24 min. The peaks with retention times of 22.5 and 24 min had similar amino acid compositions and represent unmodified and methylated tryptic peptide T22, respectively (see below). The large nonradiolabeled peak, eluting at 30.5 min, had a composition consistent with tryptic peptide T21 (peptide nomenclature is from sequence results (15)). Thus, the methylated and unmodified forms of T22 coelute during reverse phase liquid chromatography at pH 2.2 but are resolved at pH 7.4.

Peptide T22 contains histidine 217 which has been shown to reside in the active center of D-amino acid oxidase by chemical modification with dansyl chloride (1, 2). It seemed quite likely that it was this residue that was preferentially modified in methylated T22. Thus, an HPLC method was developed to resolve the fluorescent o-phthalaldehyde/mercaptoethanol derivates of 1- and 3-methylhistidine from histidine and the other amino acids present in the 24-h acid hydrolysate of the methylated peptide (see under "Methods").

Fig. 5. Rechromatography at pH 7.4 of the major radiolabeled peptide fraction (equivalent to the peak with retention time of 32.5 min in Fig. 4A) isolated initially from digests of protein modified with [14C]MNBS to a residual activity of approximately 20% by HPLC under conditions described in Fig. 4. Combined preparative fractions were rechromatographed on a Waters Associates μBondapak C18 column in 1.0 mM sodium phosphate, pH 7.4, using a linear acetonitrile gradient. Peptide elution was monitored by the absorbance at 220 nm. OD, optical density.
peptides (see Fig. 6). The values in parentheses are the number of chromatography at pH lysine derivative, which elutes after 30 min, was not observed in the “...”

Sequence: 

NH₂-Asn-Phe-Ile-Ile-Thr-His-Asp-Leu-Glu-Arg-COOH

The results of the analyses are shown in Fig. 6. Trace A represents the elution profile for the acid hydrolysate of the methylated T22 fraction (retention time of 24 min, Fig. 5). Histidine was not present; however, two peaks with retention times identical to standard 1- and 3-methylhistidine were observed. These results are in contrast to trace B, the chromatogram from the analysis of the hydrolysate of the unmodified T22 peptide fraction (retention time of 22.5 min, Fig. 5), in which histidine, but not the 1- or 3-methylhistidine derivative, was observed. The rest of the chromatogram was similar to that of methylated T22.

The quantitative results from these analyses are summarized in Table I. The composition of each peptide fraction is again consistent with the expected composition from the sequence of T22 (15). The 1- and 3-methylhistidine in methylated T22 together account for the equivalent amount of histidine observed in the unmodified peptide, i.e. approximately 1 residue. The relative proportion of 1- to 3-methylhistidine was 64 to 36%, respectively.

The results from the sequence analysis of 14C-methylated T22 are consistent with the composition data reported in Table I and with the sequence of peptide T22 reported previously (15). An identifiable PTH derivative was observed for each Edman degradation cycle except cycle 6. Histidine is the sixth residue in T22; however, its PTH derivative was not observed. A peak having a slightly longer elution time than that of PTH histidine appeared instead. The majority of the radioactivity was found in cycle 6 with a lesser amount appearing in cycle 7 (some out-of-stepness was observed in the later stages of the analysis). Although the PTH derivatives of 1- and 3-methylhistidine were not synthesized to confirm their elution times, the sequence data described, together with the amino acid analysis, demonstrate unambiguously that it is the histidine in tryptic peptide T22 that is methylated preferentially in native D-amino acid oxidase in the absence of benzoate.

**DISCUSSION**

Histidine 217 has been identified as one of the amino acid residues in D-amino acid oxidase that is located in or near the active center of the enzyme. This identification was made previously by chemical modification of the enzyme with dansyl chloride (1, 2). Though dansylation resulted in the complete inactivation of the enzyme, the bulkiness of this reagent made it difficult to exclude the possibility that histidine 217 resides in the periphery of the substrate-binding site and is, therefore, not directly involved in catalysis. This work, however, reports that the introduction of the small methyl residue onto the imidazole ring of histidine 217 by methyl-p-nitrobenzene sulfonate causes a substantial irreversible loss of activity in this enzyme. In addition to confirming our previous conclusion that this amino acid residue exists in the active center of D-amino acid oxidase, these results strongly suggest that histidine 217 functions in catalysis.

Several comments should be made about the modification reaction itself. The overall inactivation reaction proceeds more slowly than that of chymotrypsin. The maximal inactivation rate for D-amino acid oxidase (as determined by extrapolation to saturating concentration of MNBS) was 0.021 min⁻¹ at pH 7.7. Under similar reaction conditions, the pseudo-first order rate of inactivation of chymotrypsin was reported to be 0.12 min⁻¹ (7). It was not apparent if this rate was determined under conditions in which chymotrypsin was saturated with MNBS; therefore, it can only be stated that the rate of methylation is at least 6-fold faster than that for D-amino acid oxidase. The slower rate of methylation in the oxidase may be the result of histidine 217 being inherently less reactive because of differences in the local environment or the result of a less favorable orientation of the reagent relative to this histidine in the enzyme. Comparison to the inactivation rates for phospholipase A₂ and the chicken liver carboxylesterase is not possible due to differences in reaction conditions or lack of data.

The reaction with MNBS was less specific for D-amino acid oxidase than for chymotrypsin, for which the reaction has been reported to be stoichiometric, with the majority of the label found on histidine 57 and much lesser amounts on histidine 40 and tyrosine 171, or for equine phospholipase A₂, in which 1.5 methyl residues were incorporated per mol of...
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protein. The reaction with D-amino acid oxidase results in up to 2.5 methyl residues being incorporated nonspecifically at maximal inactivation, in addition to the methylation of histidine 217. The greater degree of nonspecific labeling in D-amino acid oxidase may be the consequence of its slower inactivation rate, remaining in the presence of reagent for a longer period of time than was the case for the other proteins. Histidine 217 is the major single position of attack by MNBS. Methylation was markedly reduced in the presence of the competitive inhibitor benzoate. This specificity must be, at least in part, the result of MNBS being active site directed, forming a noncovalent complex prior to inactivation. The apparent \( K_r \) for this complex is similar to the \( K_r \) determined for complexes between D-amino acid oxidase and related nitroaromatic compounds (10). A small, rapidly formed perturbation of the bound PAP-visible spectrum, similar to that produced by nitrobenzene, was observed upon titration of D-amino acid oxidase with MNBS, suggesting that this reagent binds near the flavin isovaloxazine ring system (data not shown). The \( K_r \) for the complex, as determined from these spectral changes, is similar to the apparent \( K_r \) obtained from the inactivation studies.

Both the 1- and 3-methyl derivatives of histidine 217 were observed in methylated D-amino acid oxidase. The presence of both derivatives is in contrast to methylated chymotrypsin in which only the 3-methyl derivative was observed and in methylated phospholipase A\(_2\), in which only 1-methylhistidine was present (7, 8). The methylation of chicken liver carboxylesterase by MNBS, although not fully characterized, appears to result in both methyl derivatives of histidine (9). X-ray crystal structures of chymotrypsin and bovine phospholipase A\(_2\) both indicate that the methylated histidine is strictly oriented in the active site through interaction of the nonreactive imidazole nitrogen with the carbonyl of the side chain of aspartate 102 in chymotrypsin and aspartate 99 in the phospholipase (7, 8). The lack of positional specificity in D-amino acid oxidase may indicate a lack of such orientation in the native enzyme or that the binding of MNBS disrupts existing interactions. The latter situation may be a prerequisite to methylation. For example, MNBS functions as a substrate analogue with chymotrypsin; thus the reactive ester is most likely positioned near the N-3 of histidine and the hydroxyl of serine 195 in much the same way as for the structurally related general acyl ester substrates such as \( p \)-nitrophenyl acetate. Though MNBS is structurally related to known ligands of D-amino acid oxidase, it is not strictly a substrate analogue; thus the exact positioning of the ester relative to histidine 217 cannot be predicted. It may be that the ester binds over the plane of the imidazole in such a way that movements of the side chain of this residue are required in order for reaction at either nitrogen to occur. The slower rate of modification of D-amino acid oxidase relative to chymotrypsin is consistent with such a mechanism. Methylation of the N-1 position of histidine 217 is preferred by approximately 2:1 over N-3, thus a certain degree of orientation can occur; however, it should be noted that some preference for methylation of N-1 of histidine free in solution by MNBS at pH 7.9 has been reported (7). We previously isolated peptide T22 dansylated at histidine 217 from two discrete peptide fractions from the tryptic digest of D-amino acid oxidase inactivated with dansyl chloride in the absence of benzoate (1). Characterization of these peptide fractions gave no indication as to the structural difference between them; thus we speculated that each fraction represented the two different imidazole adducts of histidine 217 in T22. The ratio of the two fractions as isolated ranged between 1.4 and 1.8 to 1.0, which is similar to the ratio of 1- to 3-methylhistidine 217 reported here. Thus, our assumption about the dansyl derivatives may be correct and suggests that both imidazole nitrogen in histidine 217 may be susceptible to dansylation to a similar degree as to methylation by MNBS.

Methylation of histidine 217 in D-amino acid oxidase results in irreversible enzyme inactivation. Steady state turnover studies, to be reported elsewhere, indicate that this loss of activity is the result of changes in both \( V_{\text{max}} \) and \( K_m \), when D-phenylglycine is used as substrate. It is difficult to rationalize how the introduction of the small methyl group onto a residue in the periphery of the substrate-binding site could inactivate the enzyme, especially in view of the fact that D-amino acid oxidase is capable of binding ligands and substrates differing greatly in size (11). Thus, the inactivation caused by methylation of histidine 217 would indicate that this residue participates directly in catalysis, either by facilitating proton transfer or by stabilizing the local conformation of the active center through hydrogen bonding. We are now in the process of determining the effect of the methylation of histidine 217 on ligand binding and on specific steps in catalysis.

REFERENCES

Methylation of the active center histidine 217 in D-amino acid oxidase by methyl-p-nitrobenzenesulfonate.
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