Chemical Modification of D-Amino Acid Oxidase

AMINO ACID SEQUENCE OF THE TRYPTIC PEPTIDES CONTAINING TYROSINE AND LYSINE RESIDUES MODIFIED BY FLUORODINITROBENZENE

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D-Amino acid oxidase can be inactivated by covalent modification of predominantly tyrosine residue(s) at pH 7.4 by a low molar excess of fluorodinitrobenzene, which appears to act as an active site-directed reagent (Nishino, T., Massey, V., and Williams, C. H., Jr. (1980) J. Biol. Chem. 255, 3610-3616). Peptide mapping by high performance liquid chromatography of tryptic digests of protein modified with radiolabeled reagent revealed two major radioactive fractions with substantially different retention times which were not observed in protein modified in the presence of benzoate, a potent competitive inhibitor. Isolation and sequence analysis of the major radiolabeled peptides, as well as other direct chemical analyses, are presented which unambiguously demonstrate that these fractions represent modification of two different regions of the protein. The majority of the radiolabel was found within a 61-amino acid residue peptide containing an O-DNP-tyrosine residue exclusively at position 17. The substantial sequence surrounding this tyrosine residue indicates that it is different from that shown to react with N-chloro-D-leucine (Ronchi, S., Galiliano, M., Minchioni, L., Curti, B., Rudie, N. G., Porter, D. J. T., and Bright, H. J. (1980) J. Biol. Chem. 255, 6044-6046). The second fraction consisted of a 12-residue peptide containing an ε-DNP-lysine residue at position 5. Together, these two modified amino acids represented 0.89 mol of DNP incorporated/protein monomer. Both modifications must contribute to inactivation to account for the 90% decrease in enzymatic activity. Evidence is presented which suggests that both groups are within the active center of the enzyme and are modified in a mutually exclusive manner.

The flavoprotein D-amino acid oxidase from hog kidney has been the subject of a large amount of mechanistic research. In an effort to identify specific amino acid residues in the active center which are involved in catalysis, a number of different chemical modifications have been reported (1, and references therein). It has been shown that Sanger’s reagent, 1-fluoro-2,4-dinitrobenzene, will almost completely inactivate the protein during the incorporation of approximately two DNP residues per D-amino acid oxidase monomer (1). Inclusion of the competitive inhibitor, benzoate, prevented inactivation and the incorporation of one DNP residue. Chemical analysis showed that tyrosine was the major amino acid modified in the absence of benzoate. This information suggested that FDNB functions predominately as an active site-directed reagent. In support of this idea, Nishino and co-workers (2, 3) have shown that a number of nitroaromatic compounds bind to d-amino acid oxidase with characteristic spectral changes similar to those caused by benzoate and its analogues. This binding may be the result of the nitro group mimicking the carboxyl group in the binding site of the enzyme (2). Indeed, the carboxyl group is a necessary requirement for substrate and inhibitor binding (3).

Rudie et al. (4) have shown that a specific tyrosine in or near the active site of this enzyme was dichlorinated at the 3 and 5 positions by N-chloro-D-leucine. The catalytic maximum velocity of the modified enzyme was reduced by 70% and the rate of flavin reduction by D-alanine was decreased 200-fold (4). The N-chloro derivatives of a number of other D-amino acids as well as several L-isomers did not modify the protein but did act as competitive inhibitors (4). Recently, the amino acid sequence surrounding the uniquely chlorinated tyrosine has been reported (5).

It is of importance to determine whether Sanger’s reagent modified a specific tyrosine residue in the substrate binding site of D-amino acid oxidase and whether this is the same tyrosine that is chlorinated by N-chloro-D-leucine. Knowledge of the sequence surrounding the residue(s) will allow placement in the primary structure of the protein, which has now been completed (6). This communication reports the isolation and characterization of the tryptic peptides containing two different DNP-amino acid residues modified in the absence of benzoate. A 61-residue peptide containing a single modified tyrosine residue represented the major site of modification, which is different from that chlorinated by N-chloro-D-leucine (5). Additionally, a 12-residue peptide was characterized which contains a lysine residue also modified by FDNB in the absence of benzoate. Evidence is presented which suggests that FDNB modifies, to differing extents, these two amino acid residues in a mutually exclusive manner.

**EXPERIMENTAL PROCEDURES**

**Materials**

D-Amino acid oxidase was purified from pig kidneys by the method described by Brumby and Massey (7) as modified by Curti et al. (8).

The abbreviations used are: DNP, dinitrophenyl; FDNB, 1-fluoro-2,4-dinitrobenzene; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; HPLC, high performance liquid chromatography; PTH, phenylthiohydantoin; rt. retention time; im, imidazole.

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1-Fluoro-2,4-dinitrobenzene was obtained from Aldrich and used to dilute [U-\(^{14}\)C]FDNB from Amersham to the desired specific activity. D-NP-tyrosine, ε-DNP-lysine, D-NP-methionine, and iodoacetic acid were from Sigma. im-DNP-Histidine was a gift from Dr. Fred Sanger, Medical Research Council Laboratory of Molecular Biology, Cambridge, England. L-1-tosylamido-2-phenylthyl chloromethyl ketone (TPCK)-treated trypsin was purchased from Worthington, while Miles was the source of the Staphylococcus aureus V8 protease. Sequencer chemicals were obtained from Beckman. Phenylthiobodyantoin standards were from Pierce and uv spectral grade acetonitrile was obtained from Burdick and Jackson. All other chemicals were analytical reagent grade.

**Methods**

\( \text{D-Amino acid oxidase was modified with FDNB essentially as described by Nishino et al. (1). Four hundred twenty-five nmol of benzoate-free enzyme which had been dialyzed against 50 mM sodium phosphate, pH 7.4, were supplemented with PAD to a final concentration of 0.1 mM. A portion (25 nmol) was set aside for use as a benzoate-free enzyme which had been dialyzed against 10 mM sodium phosphate, pH 7.4, 25 °C. Approximately 2 nmol of the tryptic digest of protein modified in the absence of benzoate (A) that are not present in the benzoate-containing control (B). These two radioactive fractions are associated with two new radioactive peaks, having retention times of 51 and 60 min, which are not present in the benzoate control. A number of minor radioactive fractions are common to both digests and must represent the nonspecific modifications of a number of surface amino acids not essential for enzymatic activity. Many of these fractions are associated with large uv-absorbing peaks normally observed in chromatograms of digests from unmodified protein. The maps are quite reproducible with 85 to 95% of the injected radioactivity recovered from the column.**

**RESULTS**

D-Amino acid oxidase was 90% inactivated in 270 min by a 5-fold molar excess of FDNB at pH 7.4 as previously described (1). Analysis of the radiolabeled protein after removal of excess reagents indicated that 2.1 mol of DNP/mol of d-amino acid oxidase monomer had been incorporated. Enzyme preincubated with the competitive inhibitor benzoate was modified with 0.7 mol of DNP/monomer and retained 99% of its activity. These results are consistent with earlier observations (1).

Digestion of the denatured and alkylated protein with TPCK-treated trypsin in 2 M urea at pH 7.5 was followed by HPLC peptide-mapping analysis. The results after 12 h of digestion are shown in Fig. 1. Peptide elution times as monitored at 220 nm are shown together with a histogram of the radioactivity determined for each peak. Two major radioactive peptide peaks are observed in the digest of protein modified in the absence of benzoate (A) that are not present in the benzoate-containing control (B). These two radioactive fractions are associated with two new 220 nm absorbing peaks, having retention times of 51 and 60 min, which are not present in the benzoate control. A number of minor radioactive fractions are common to both digests and must represent the nonspecific modifications of a number of surface amino acids not essential for enzymatic activity. Many of these fractions are associated with large uv-absorbing peaks normally observed in chromatograms of digests from unmodified protein. The maps are quite reproducible with 85 to 95% of the injected radioactivity recovered from the column.

The kinetics of peptide release during digestion of the modified protein was monitored by HPLC peptide analysis of a portion of the digest at various times after addition of trypsin. The two major radiolabeled peptides show dramatically different release rates during the digestion (Fig. 2). The 51-min peak was maximally released in less than 90 min and remained at a constant level throughout the remainder of the
digest. This type of release is typical of most of the unmodified peptides. The 60-min peak was released more slowly. During the first 2 h of digestion, the radioactive peptide eluting after 59 min was released maximally, while the 60-min peak was present in low levels. During subsequent digestion, the 59-min peak was slowly fragmented, with the concomitant increase in the levels of the 60-min peak. These results suggest a precursor-product relationship between these two radioactive peptides. This conclusion was confirmed by sequence analysis of both peaks. Two nonradiolabeled peptides (rt = 55 and 56 min) displayed similar release kinetics and represent the same tryptic peptide region (again confirmed by sequence analysis) as the 59- and 60-min radioactive peaks only, of course, unmodified by FDNB. Neither the 56- nor the 60-min peaks was fully released even after 12 h of digestion, the final time point analyzed. The large difference in the release rates of the 51- and 60-min radiolabeled peptides suggests that these two peptides come from two different regions of the protein molecule. The implications of these observations will be discussed.

Thiolysis Experiments—FDNB-inactivated D-amino acid oxidase can be partially reactivated by incubation with dithioerythritol (1). Shaltiel (13) has shown that the compounds, O-DNP-Tyr, im-DNP-His, and S-DNP-Cys, are readily thiolized to release the free amino acid, while the DNP derivatives from α- and ε-amino groups are stable. Reactivation of modified D-amino acid oxidase was associated with a decrease in the DNP content of the protein, most notably in the O-DNP-tyrosine levels (1). Similar thiolysis experiments are reported here and were carried out on either the modified protein before trypsin digestion or the trypsic digest itself. Similar results were obtained upon peptide analysis in both cases. The results of incubation of the 12-h trypsin digest (from Fig. 1A) in 10 mM dithioerythritol, pH 8.5, at 20 °C overnight is shown in Fig. 3. Approximately 95% of the 60-min radioactive peak and its digestive precursor were thiolized, as indicated by the near absence of radioactivity and uv absorbance eluting at these times. In contrast, 85% of the radioactivity at 51 min remained. This small decrease, which occurred early in the incubation and then remained constant, may be due to a small amount of thiolizable contaminants co-eluting with the main stable constituent. The large fraction of radioactivity eluting after 45 min is the product of thiolysis, that is, the DNP-dithioerythritol adduct. This was shown by HPLC analysis of the reaction product formed between FDNB and excess dithioerythritol at pH 8.5. Approximately 60% of the total radioactivity incorporated into D-amino acid oxidase in the absence of benzoate was recovered as DNP-dithioerythritol. Of this, 65% was derived from the 60-min peak and its precursor, while the remaining 35% must be contributed by the nonspecific modifications. Indeed, lower levels of the minor radioactive fractions were observed after thiolysis. The absorbance profile at 220 nm is consistent with these results. After thiolysis, the 59- and 60-min peaks are almost completely eliminated, while the areas of the 55- and 56-min peaks increase proportionally, again confirming that these peaks represent the same tryptic peptide region. Approximately 95% of the peak area at 51 min was retained after thiolysis. The two large uv-absorbing peaks at 6 and 10 min are the reduced and oxidized forms of dithioerythritol, respectively. All other peaks were retained in essentially the same proportions.

Peptide analysis of modified protein which had been reactivated by dithioerythritol prior to digestion gave similar results. The 59- and 60-min radioactive peaks were completely thiolized at the point where enzyme reactivation had reached its maximal level; however, essentially all of the 51-min peak remained. Although the majority of enzymatic activity is regained upon thiolysis, not all could be recovered (1). This observation was confirmed in the present work even when complete thiolysis of the 59- and 60-min peaks had occurred. The amount of nonreactivatable activity correlated closely with the level of the nonthiolizable 51-min peak. For example, in an experiment where reactivation proceeded to a maximum of 85% of the original enzymatic activity, 0.16 mol of the 51- min fraction/mol of D-amino acid oxidase monomer was observed.

Stoichiometry—The stoichiometry of FDNB reaction in each of the two major modified peptide regions was determined by the radioactivity recovered from the HPLC column and the specific activity of the FDNB reagent. The 51-min peak accounts for 17% of the radioactivity injected onto the column and represents 0.36 out of the 2.1 residues of DNP incorporated into each D-amino acid oxidase monomer. The thiolysis experiments suggest that this fraction contains approximately 15% thiolizable contaminants; therefore, the main constituent represents 0.31 residue/monomer. Both the structurally related 59- and 60-min peaks together account for 27% of injected radioactivity or 0.58 residue/monomer. Since 90% of the injected radioactivity was recovered during chromatography, these values should reflect closely the actual modification state of the protein. Neither peptide fraction alone is present in levels which could account for the observed 90% inactivation of the protein prior to peptide analysis;
however, together they would account for 0.89 mol or approximately 45% of the radioactivity incorporated.

The above observations, as well as sequence data reported below, indicate that the 56-min peak represents the unmodified form of the same peptide which elutes at 60 min. The relative peak areas of these two forms (including their digestive precursors of rt = 55 and 59 min, respectively) were determined by planimetry. Sixty-four per cent of this peptide area is modified, while 36% elutes unmodified. These results are consistent with the radioactivity measurements. The significance of these results will be discussed.

Isolation and Characterization of the Major DNP-modified Tryptic Peptides—The DNP-modified tryptic peptides were isolated from the digests in levels sufficient for further chemical characterization using the same HPLC procedure as used for the analytical peptide mapping. Greater than 30 nmol of the digest could be fractionated in this manner without a significant loss in resolution or recovery. The major radioactive peptide fractions were pooled and phosphate salts removed by chromatography over short Sephadex G-25 or G-50 columns in 25% acetic acid. This material was suitable for any one of a variety of analyses. The identity of the amino acid(s) modified by FDNB in each radiolabeled peptide was determined by HPLC analysis of the 12-h acid hydrolysate of each peptide. A short gradient elution method was developed which would resolve the DNP-amino acid derivatives that would be expected from a modified protein with a methionine as its NH$_2$-terminal residue (15) (Fig. 4A).

The acid hydrolysate of each peptide was injected together with a mixture of carrier amounts of the authentic DNP derivatives. The standard DNP-amino acid with which the radioactivity co-eluted was determined after collection of peaks and scintillation counting. The results demonstrate that two different amino acid residues have been modified in the absence of benzoate. The major radiolabeled peptide (rt = 60 min) contains a modified tyrosine (B), while the peptide emerging at 51 min contains a modified lysine. Both derivatives were isolated in good yield, as has been reported for these hydrolysis conditions (14). The results were confirmed using an entirely different chromatographic system based on a C18 reverse-phase column (data not shown). These identifications support the thiolysis observations in that the O-DNP-tyrosine adduct in the 60-min radiolabeled peptide is thiolizable, while the ε-DNP-Lys derivative in the 51-min peptide is not.

Both peptides were subjected to amino acid analysis and automated Edman degradation. The results of the amino acid analysis of the 24-h hydrolysates are shown in Table I (columns A and B). The amino acid sequence of the modified tyrosine peptide (rt = 60 min) is shown in Table II and Fig. 5. The peptide was sequenced entirely to the N-terminal arginine in a single run. Some difficulty in interpretation was encountered following the methionine at cycle 48; however, the final 12 residues overlap the NH$_2$-terminal sequence of the CNBr3A peptide reported by Ronchi et al. (15). Repeated sequence analysis of this peptide as well as the unmodified version (rt = 56 min) gave identical results. This peptide, therefore, represents a sizable region (61 amino acid residues out of a total of 347) of the D-amino acid oxidase molecule and contains three tyrosine, one histidine, and no lysine or cysteine residues. Radioactivity from each Edman degradation cycle was measured in an effort to determine the site of modification. The results are included in Table II. Several

![Fig. 4. Identification of the amino acid residues modified by [U-$^3$H]FDNB. A, standard DNP-amino acids chromatographed on a Waters Associates $^3$Bondapak phenyl column using gradient 10 (Waters Solvent Programmer) from 25% to 50% acetonitrile in 0.1% phosphoric acid in 20 min (flow, 1.9 ml/min). B, 12-h acid hydrolysate of the radiolabeled peptide emerging at 60 min in Fig. 1; C, the 12-h acid hydrolysate of the radiolabeled peptide emerging at 51 min (Fig. 1). The recovery of radioactivity eluting with each standard DNP-amino acid peak is given in per cent of the total amount injected.](http://www.jbc.org/)

### Table I

| Residues/mole$^a$ | A$^b$ | B | C | D |
|------------------|------|---|---|---|
| Aspartic acid    | 1.0  | 7.1 | 2.0 | 4.8 |
| Threonine        | 0.3  | 0.0 | 0.5 | 1.8 |
| Serine           | 0.3  | 4.2 | 1.3 | 2.3 |
| Glutamic acid    | 1.1  | 6.1 | 3.5 | 2.3 |
| Proline          | 1.0  | 5.5 | 3.9 | 2.2 |
| Glycine          | 1.0  | 4.5 | 1.2 | 3.3 |
| Alanine          | 1.0  | 4.8 | 1.6 | 2.9 |
| Valine           | 1.0  | 2.5 | 1.0 | 1.3 |
| Methionine       | 0.3  | 1.1 | 1.1 | 1.1 |
| Isoleucine       | 1.3  | 1.2 | 0.2 | 1.4 |
| Leucine          | 1.1  | 4.9 | 0.9 | 3.8 |
| Tyrosine         | 1.8  | 3.1 | 1.7 | 2.2 |
| Phenylalanine    | 4.1  | 2.3 | 1.7 | 2.2 |
| Histidine        | 0.3  | 1.2 | 0.9 | 1.4 |
| Lysine           | 0.7  | 7.2 | 1.0 | 1.0 |
| Arginine         | 1.2  | 1.1 | 1.0 | 1.0 |
| Tryptophan       | N.D.$^b$ | N.D. | N.D. | N.D. |
| Total            | (12) | (6) | (26) | (35) |

$^a$ Compositions were determined after hydrolysis for 24 h at 110 °C in 6 N HCl. Residues are determined based on a calculated average nmol/residue. The values in parentheses are the number of residues determined by sequence analysis.

$^b$ Column A, Peptide containing the FDNB-modified l-lysine residue (rt = 51 min, Fig. 1A); column B, peptide containing the FDNB-modified tyrosine residue (rt = 60 min, Fig. 1A); column C, radioactive fragment from the S. aureus protease digest of the modified tyrosine peptide (peak C, Fig. 6); column D, nonradioactive fragment from the S. aureus protease digest of the modified tyrosine peptide (peak E, Fig. 6).
in cup. Yields expressed in nanomoles. Amount of peptide sequenced, 60 nmol. Initial yield, 88%. Repetitive yield, 95% based on the first 50 fractions contained no significant counts over background. Total radioactivity placed in cup, 336,000 dpm. Residue was observed this analysis) and incomplete initial coupling (88%, as determined to the DNP-modified form. These results are consistent of the PTH derivative of 0-DNP-tyrosine might be anticipated unmodified tyrosine and no unusual peak that could be attributable to the DNP-modified form. These results are consistent with the low recovery of radioactivity in this fraction. Poor yields of the PTH derivative of O-DNP-tyrosine might be anticipated in view of the conditions used during sequence analysis. Each Edman degradation takes 80 min to perform; therefore, the peptide remains at 55 °C for approximately 21 h prior to the 17th cycle. During this time, the peptide is washed repeatedly with chlorobutane containing 0.15% dithioerythritol during extraction of the anilinothiazolinone derivative. Residual levels of dithioerythritol would remain in the sequencer cup and could thiolize the O-DNP-tyrosine derivative during the coupling step. The low levels of radioactivity observed in all of the fractions prior to cycle 17 may reflect the washout of the thiolysis product. Tyrosine is the exclusive amino acid residue modified in this peptide (see Fig. 4); however, this peptide contains three tyrosine residues (at position 17 as well as positions 36 and 57). It was necessary to use a nondestructive method to determine unambiguously the site of modification. The purified peptide was digested by the protease from S. aureus V8, which would be expected to cleave the peptide after the glutamic acid at position 26, thus separating the modified tyrosine at position 17 from the other two putatively unmodified tyrosines in the carboxyl-terminal portion. The results of HPLC analysis of this digest are shown in Fig. 6. Two major uv absorbing peaks (excluding the solvent peak “A”) were observed on HPLC. The peak designated “C” was radioactive and accounted for 67% of the injected radioactivity. Amino acid analysis (Table I, column C) confirms that this peak represents the first 26 residues from the NH2 terminus of the 61-residue tryptic peptide. Tyrosine was not observed in the analysis, which is consistent with its being modified with DNP. Peak “E” was not radioactive and represents the 35-residue carboxyl-terminal portion (Table I, column D). Peak “D” contained 10% of the radioactivity and represents undigested material. Peak “B” contains 11% of the radioactivity and corresponds to amino acid residues 26 through 26 of the modified tryptic peptide. This fragment is the result of the slow proteolysis of the Asp-Val peptide bond, which is known to be quite susceptible to hydrolysis by the S. aureus protease. These results, together with the chemical analysis shown in Fig. 4, indicate that the tyrosine at position 17 is the exclusive amino acid residue in the 61-residue tryptic

### Table II

**Automated Edman degradation of the FDNB-modified tyrosine tryptic peptide**

| Cycle | PTH Amino acid | Yield | Dpm | Cycle | PTH Amino acid | Yield |
|-------|----------------|-------|-----|-------|----------------|-------|
| 1     | Phe            | 36    | 31  | Gln   | 13             |       |
| 2     | Thr            | 5     | 12  | Gln   | 12             |       |
| 3     | Pro            | 4     | 33  | Thr   | 2              |       |
| 4     | Phe            | 4     | 34  | Phe   | 6              |       |
| 5     | Thr            | 17    | 5   | Asn   | 12             |       |
| 6     | Thr            | 5     | 4   | Tyr   | 13             |       |
| 7     | Thr            | 16    | 2   | Leu   | 8              |       |
| 8     | Asp            | 30    | 2   | Leu   | 10             |       |
| 9     | Val            | 33    | 4   | Ser   | N.Q.           |       |
| 10    | Ala            | 41    | 2   | His   | 4              |       |
| 11    | Ala            | 40    | 3   | Ile   | 5              |       |
| 12    | Gly            | 30    | 3   | Gly   | 5              |       |
| 13    | Leu            | 33    | 3   | Ser   | N.Q.           |       |
| 14    | Trp            | 25    | 4   | Pro   | 4              |       |
| 15    | Gin            | 26    | 2   | Asn   | 6              |       |
| 16    | Pro            | 30    | 3   | Ala   | 6              |       |
| 17** | Tyr            | 22    | 120 | Ala   | 7              |       |
| 18    | Thr            | 10    | 10  | Asn   | 5              |       |
| 19    | Ser            | N.Q.  | 4   | Met   | 3              |       |
| 20    | Glu            | 19    | 2   | Gly   | 6              |       |
| 21    | Pro            | 17    | 5   | Leu   | 3              |       |
| 22    | Ser            | N.Q.  | 1   | Thr   | N.Q.           |       |
| 23    | Asn            | 18    | 2   | Pro   | 2              |       |
| 24    | Pro            | 13    | 1   | Val   | 2              |       |
| 25    | Gin            | 15    | 5   | Ser   | N.Q.           |       |
| 26    | Glu            | 12    | 6   | Gly   | 5              |       |
| 27    | Ala            | 14    | 57  | Tyr   | N.Q.           |       |
| 28    | Asn            | 13    | 58  | Asn   | 1              |       |
| 29    | Trp            | 8     | 59  | Leu   | 1              |       |
| 30    | Asn            | 13    | 60  | Phe   | 1              |       |

* Total radioactivity (×10^(-5)) determined in each fraction. Cycles 25 to 61 contained no significant counts over background. Total radioactivity placed in cup, 336,000 dpm.

** Location of the FDNB-modified tyrosine. Due to the instability of the O-DNP-Tyr adduct during sequence analysis, 90% of this residue was observed as unmodified PTH-tyrosine (see text). **

** Indicates beginning of overlap with cyanogen bromide peptide CBNb3A (15). **

Fig. 5. Complete amino acid sequence of the FDNB-modified tryptic peptide emerging at 60 min (Fig. 1). The tyrosine residue at position 17 is the exclusive amino acid residue modified in this peptide.
TABLE III
Automated Edman degradation of the FDNB-modified lysine tryptic peptide

| Cycle | PTH Amino acid | Yield |
|-------|---------------|-------|
| 1     | Gly           | 19    |
| 2     | Gln           | 13    |
| 3     | Ile           | 5.8   |
| 4     | Ile           | 4.2   |
| 5     | "             | "     |
| 6     | Val           | 1.3   |
| 7     | (Asp)         | (0.8) |

* No new PTH derivative observed.

**Fig. 7.** The amino acid sequence of the FDNB-modified tryptic peptide emerging at 51 min (Fig. 1). The lysine at position 51 was modified by FDNB. The sequence of residues 1 through 7 was determined in this work, while residues 8 through 12 are from the complete amino acid sequence of D-amino acid oxidase (6) and is consistent with the amino acid analysis shown in Table I.

The partial sequence of the modified lysine-containing peptide (rt = 51 min) is shown in Table III and Fig. 7. Several attempts were made at sequencing the entire 12-residue peptide, but were frustrated by washout of this hydrophobic peptide from the sequenator cup. Despite this problem, seven cycles could be interpreted. PTH-amino acid derivatives were identified for six of the first seven cycles; however, a new PTH derivative could be identified in cycle 5 using our standard HPLC methodology. This cycle did contain radioactivity, which after acid hydrolysis co-eluted with e-DNP-lysine under the conditions described in Fig. 4. Modification of the ε-amino group of lysine by FDNB would be expected to prevent tryptic hydrolysis. Indeed, the two tryptic peptide regions surrounding the modified lysine have been isolated from unmodified D-amino acid oxidase, sequenced separately, and shown to be juxtaposed by nontryptic overlap peptides (6). The partial sequence analysis of the lysine-modified peptide together with its amino acid analysis are sufficient to place the lysine in the entire sequence of D-amino acid oxidase.

**DISCUSSION**

The results reported in this communication both support and extend our previous observation that the reagent 1-fluoro-2,4-dinitrobenzene serves as an active site-directed inhibitor. Inhibition proceeds by the covalent modification of predominantly a tyrosine residue within the active center of D-amino acid oxidase. Unexpectedly, a second lesser but nonetheless significant modification was observed, that being a lysine residue, which must also be in or near the active site. Several lines of evidence support these conclusions.

(a) HPLC peptide analysis of the tryptic digest of D-amino acid oxidase inactivated by a 5-fold excess of FDNB in the absence (Fig. 1A) as opposed to the presence of the substrate-competitive inhibitor, benzoxae (2), shows the presence of two major new radioactive fractions which have markedly different elution times (51 min versus 60 min).

(b) These two different peptide fractions have quite different release rates during trypsin digestion (Fig. 2). The 51-min peak was released very early and remained at a constant level during subsequent digestion. The peptide region emerging after 60 min was released in precursor form (rt = 59 min) early in the digest. This precursor was fragmented slowly, not being fully digested even after an extended digestion of 12 h. Subsequent sequence analysis showed that the 60-min peak was a legitimate tryptic peptide and was not, therefore, an artifact generated by contaminant proteases such as chymotrypsin. These results suggest that the two major radiolabeled peptides are derived from different regions of the D-amino acid oxidase molecule.

(c) Dithioerythritol partially reactivates the modified protein by thiolysis of many of the protein DNP derivatives (1). Incubation of the tryptic digest in dithioerythritol prior to peptide analysis shows that the 60-min peak and its digestive precursor are readily thiolized, while the majority of the 51-min fraction remains unchanged (Fig. 4). Shaltiel (13) reports that the compounds O-DNP-Tyr, im-DNP-His, and S-DNP-Cys are readily thiolized, while the DNP derivatives of ε- and ε-amino groups are stable (13).

(d) Direct chemical analysis of the 12-h hydrolysate of each of the purified radioactive peptide fractions, by reverse-phase HPLC, demonstrates that a tyrosine within the 60-min peak and a lysine within the 51-min peak were modified by FDNB (Fig. 4). These results are consistent with the thiolysis experiments.

(e) Automated sequence analysis of the two radioactive peptides establishes that both modified amino acid residues are derived from different regions of the primary structure. The 60-min peptide is composed of 61 amino acids and is modified exclusively at the tyrosine in position 17 (Fig. 5). The 51-min peptide contains 11 amino acid residues and a modified lysine at position 5. The primary structure of D-amino acid oxidase has been completed recently (6); thus, it is now possible to locate these residues in the overall sequence. FDNB modifies the tyrosine residue at position 55 and the lysine residue at position 204.

Although these residues are far from one another in the primary structure, a number of observations suggest that both residues are located in the active center of D-amino acid oxidase. Modification proceeds in a mutually exclusive manner, that is, modification of the lysine residue prevents the modification of the tyrosine in the same active center. Evidence to support this conclusion is as follows.

(a) Neither peptide was observed in sufficient amounts to account for the 90% inactivation observed prior to peptide analysis. Only 0.58 mol of the modified tyrosine was observed, while 0.31 mol of the nonthiolyzable lysine derivative was found. Partial recovery during HPLC peptide analysis is an unlikely explanation because approximately 90% of the injected radioactivity was recovered after chromatography. Even in the unlikely event that the nonrecovered portion was derived exclusively from one of the two radioactive fractions, correction for this loss would still not account for the total inactivation observed. The inactivation could be accounted for only if both modifications (0.89 mol/mol) contribute.

(b) If the incorporation of 0.31 mol of DNP-residue into the lysine prevents modification of the tyrosine as postulated, then corresponding levels of the unmodified tyrosine peptide should be present in the peptide analysis. This was in fact demonstrated. The peptide emerging at 56 min represents the unmodified form of the tyrosine peptide, as shown by a number of observations. Both the unlabeled 56-min peak and the radioactive 60-min fraction are released during trypsin digestion at similar rates. Thiolyis of the O-DNP-tyrosine adduct causes the 60-min peak to decrease with a corresponding increase in the 56-min absorbance. Direct sequence analysis of both peptide peaks confirms their identity. The determination of the relative peak areas of the two forms of this peptide region shows that 64% is represented by the modified
peptide and 36% remains unmodified. Thus, the amount of unmodified tyrosine peptide observed correlates well with the 41% that one would predict (10% of the enzyme remained active; 31% was modified at the lysine).

(c) As stated above, both modifications must contribute to the inactivation of the enzyme. The reactivation of D-amino acid oxidase by thiols supports this conclusion. Enzyme reactivated prior to tryptic peptide analysis shows complete thiolysis of the tyrosine adduct, while the lysine modification remains intact. The enzyme could never be reactivated back to its original specific activity. The amount that remains inactive correlates closely with the level of lysine modification observed. The amount of specific lysine modification observed in this work is greater than that expected from the 90% reactivation previously reported (1). It is not obvious at this time why this apparent discrepancy exists. However, one plausible explanation can be put forth. In the reactivation experiments reported previously, the modified protein solution was centrifuged to remove small amounts of aggregated protein prior to gel filtration to remove excess reagents. If a selected population of modified protein was removed in this manner, the supernatant would be enriched with the residual levels of active protein or with the tyrosine-modified enzyme. This would result in a somewhat higher apparent specific activity of the enzyme solution after reactivation. Indeed, if modified protein was reactivated by direct dilution of the solution with an equal volume of buffer containing dithierythritol and benzoate, less reactivation (80 to 85%) was observed. A number of FDNB-modified protein samples have been prepared and analyzed. In every case, both modifications were observed and conformed to the mutually exclusive criteria outlined above; however, some variability in the ratio of lysine to tyrosine modification was observed. It is not apparent at this time why this occurs.

The inactivation of D-amino acid oxidase with FDNB probably proceeds by a two-step process. The reagent first binds to the substrate binding site as a noncovalent Michaelis complex. Nishino et al. (2) have reported that FDNB as well as a number of other nitroaromatic compounds binds rapidly to the enzyme, causing a shift in the visible absorption spectrum. Most notably, o-nitroaniline causes an increase in absorbance in the region of 500 to 640 nm much like o-aminobenzoate (3). Addition of stoichiometric amounts of the nitro compound causes the maximal spectral change, consistent with tight binding. Binding is probably, in part, the result of the nitro group mimicking the carboxylate anion of the substrate, which is essential for binding, in its association with an arginine residue in the active center of the enzyme (1). Because the FDNB molecule has two nitro groups, at least two modes of binding are possible with respect to the final position of the fluoride-leaving group. This is represented schematically in Fig. 8.

The second step of inhibition would proceed with the displacement of the fluoro group by either the tyrosinate anion or the unprotonated lysine in the active center. Because more then one binding mode is possible, at least two different amino acid residues could be modified in a mutually exclusive manner. The ratio of lysine to tyrosine modified would depend on several factors. The Michaelis complexes formed may not be equivalent and could have different association constants; therefore, the distribution of the different enzyme-inhibitor complexes may not be equal. Second, the ratio of the nucleophilic basic forms would depend on their relative $pK_a$ values. Finally, the ratio would depend on rate of nucleophilic displacement exhibited by each base.

This work extends the number of amino acid residues within D-amino acid oxidase that have been chemically modified. The tyrosine residue identified by reaction with FDNB (tyrosine 55) is different from that reported by Ronchi et al. (5) which is dichlorinated by N-chloro-D-leucine (tyrosine 224). Both tyrosine residues must be in or near the active center of the enzyme for the following reasons. The reagents used for modification are substrate-like and appear to be active site-directed. The arguments for FDNB have been presented. The chlorination reaction is quite specific in that only $N$-chloro derivatives of certain D-amino acids are reactive, while other $D$-isomers as well as all L-isomers tested served as competitive inhibitors but did not chemically alter the protein (4). Apparently, this is not the consequence of the inability of D-amino acid oxidase to oxidize these compounds to form a more reactive chlorinating reagent. Chlorination was shown to proceed through a nonoxidative mechanism (4). Second, both tyrosine modifications are inhibited by benzoate. The specific FDNB modifications were prevented by benzoate and the enzyme retained almost all activity. Benzoate reduced the amount of chlorination by $N$-chloro-D-leucine; however, 38% of the reaction still occurred (4). Third, both modifications inactivate the enzyme. FDNB modification does so completely, while chlorination causes a 70% reduction of the catalytic maximum velocity and a 2000-fold decrease in the rate of FAD reduction by D-alanine. Finally, both tyrosine residues may be situated near the enzyme-bound FAD because the visible spectrum is perturbed by either chlorination or modification by FDNB.

Not enough information is available at this time to indicate what roles each tyrosine residue may play in the catalytic mechanism. The chlorination of tyrosine 224 is affecting in some way the FAD reduction step. The inhibition caused by FDNB modification is probably the result of steric blockage of the substrate binding site. It has been proposed that a tyrosine residue is involved in the binding of substrate by electrostatic interaction with its $\alpha$-amino group (1). Binding of the FDNB molecule through the 2-nitro group could position the fluoride-leaving group near the phenolic oxygen of that tyrosine residue.

FDNB also partially modified a specific lysine residue within D-amino acid oxidase which must also be in or near the active center. Modification contributes to inactivation and is prevented by either benzoate or dinitrophenylation of the tyrosine residue. Chemical modifications of lysine residues in this protein have been reported. Sodium borohydride reduction of the enzyme in the presence of $\delta$-alanine results in lysine modifications (16, 17); however, this does not lead to loss of activity (18) as previously reported. Mizon et al. (19, 20) have isolated the peptides containing two different modified lysine residues (lysine 33 and, perhaps, lysine 158) which are different from that reported in this communication. Miyake and Yamano (21) have modified the apoenzyme with pyridoxal 5-phosphate in the presence of sodium borohydride. Two molecules of reagent were incorporated per monomer with partial loss of activity. It is not apparent whether specific lysine residues were modified. The mechanistic role, if any, of the lysine residue modified by FDNB is not yet known.

![Fig. 8. Hypothetical mechanism for the reaction of FDNB with either the lysine or tyrosine residue through two different FDNB-binding modes.](image-url)
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