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Identification of a Lysine Residue in the NADH-Binding Site of Salicylate Hydroxylase from *Pseudomonas putida* S-1

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Salicylate hydroxylase from *Pseudomonas putida* S-1 was irreversibly inactivated by trinitrobenzenesulfonic acid (TNBS). The reaction was linearly dependent on TNBS concentration and the second-order rate constant was 120 $M^{-1} \cdot min^{-1}$ for the holoprotein at pH 8.5. Modification of one mole of lysine residue per mole of enzyme caused a large loss of the activity, and the enzyme was no longer able to show NADH-dehydrogenase activity after uncoupling. The presence of NADH, NAD^+, ATP, or AMP afforded protection against the inactivation. The enzyme modified at a single lysine residue was isolated by hydrophobic chromatography as an apoprotein form and characterized. It could bind FAD with the same K_d value for that of native apoprotein. The apparent Michaelis constant of the enzyme was increased 13-fold for NADH, but not for salicylate. V_{max} for NADH oxidation was decreased to one-fifth of that of the native enzyme. A peptide containing one trinitrophenyl-lysine residue was isolated from the chymotryptic digest of the modified enzyme and its amino acid sequence was determined to be TADVAIAADGIKSSM, which is homologous to the sequence from R-154 to I-168 of salicylate hydroxylase from *P. putida* PpG7. The lysine in the peptide may represent a basic residue interacting with an anionic group of NADH in the binding site of the enzyme.

Key words: chemical modification, lysine residue, NADH-binding site, *Pseudomonas putida* S-1, salicylate hydroxylase.

Salicylate hydroxylase [EC 1.14.13.1] [salicylate, NADH: oxygen oxidoreductase (1-hydroxylating, decarboxylating)], isolated from *Pseudomonas putida* S-1 grown with salicylate as a sole carbon and energy source, is a single polypeptide with a molecular size of 54,000 and contains 1 mol of FAD per mol of protein as the prosthetic group (*1-4*). The enzyme catalyzes the decarboxylative hydroxylation of salicylate to produce catechol with the stoichiometric consumption of NADH and molecular oxygen. Enzymes catalyzing the same reaction were purified from *Pseudomonas* species ATCC 29352 (*5*), and ATCC 29351 (*6*), *Pseudomonas cepacia* (*7, 8*), *Trichosporon cutaneum* (*9*), and *Pseudomonas putida* PpG7 (*10*). The reaction mechanisms of these enzymes have been studied in detail (*11-17*), and the primary structure of the enzyme from *P. putida* PpG7 was clarified by sequencing the *nahG* gene (*18*). The interaction and state of FAD in the enzyme protein were studied by NMR (*19*).

To understand the reaction mechanism in more detail, information about the structure of the active site of salicylate hydroxylase from *P. putida* S-1 was required. Previous studies provided evidence of the presence of an arginine residue in the NADH-binding site of the hydroxylase and of the appearance of NADH-dehydrogenase activity by uncoupling the oxygenase activity during glyoxal modification (*20, 21*). Here, we have sought to identify other amino acid residues functioning in the active site of the hydroxylase. The present paper reports on the role of a lysine residue in the NADH-binding site of the enzyme. The residue was modified with TNBS. A peptide containing TNP-lysine was isolated and its amino acid sequence was determined.

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Abbreviations: NaMN, nicotinic acid mononucleotide; NMN, nicotinamide mononucleotide; TFA, trifluoroacetic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TNP, trinitrophenyl; TPCK, L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone.

MATERIALS AND METHODS

Materials—TNBS and pyridoxal-5′-phosphate, biochemical grade, were purchased from Wako Pure Chemicals. Chymotrypsin and TPCK-trypsin were from Sigma. All common reagents were of the highest purity commercially available. Salicylate hydroxylase was purified to homogeneity from *P. putida* S-1 by the previously described methods (*1*). The apo-enzyme was prepared by the use of acidic ammonium sulfate (*1*).

Assay of Salicylate Hydroxylase—Enzyme assays were conducted by the reported methods (*21*) using a Union SM-401 spectrophotometer. Spectral experiments were performed using a Hitachi U-3400 spectrophotometer. The amount of protein was determined by the biuret method (*22*) using bovine serum albumin as the standard. The dissociation constant of FAD from the enzyme preparation was determined by assay of the recovered hydroxylase activity after titration of the apo-enzyme with FAD (*3*).

TNBS-Modification of Salicylate Hydroxylase—Solutions of TNBS were prepared by dissolving an appropriate

amount of the reagent in distilled water. The enzyme was incubated with TNBS in 100 mM potassium phosphate buffer, pH 8.5, at 25°C in the dark. Control incubations were made routinely without the reagent. The effect of pH on the reaction was examined using 100 mM potassium phosphate buffers, pH 6-9, and 100 mM sodium bicarbonate buffers, pH 8.5-11. Other conditions used are specified in the legends of tables and figures. The number of TNP residues bound to the enzyme protein was estimated by measuring the absorbance at 344 nm using a millimolar extinction coefficient of 14.5 $mM^{-1} \cdot cm^{-1}$ (*23*).

Modification of Salicylate Hydroxylase with Pyridoxal-5'-Phosphate—The enzyme (20 μM) was allowed to react with various concentrations of pyridoxal-5'-phosphate (0.3-10 mM) in 100 mM potassium phosphate buffer, pH 7.0, at 25°C in the dark. The reaction was monitored by measuring salicylate-dependent NADH oxidase activity. The reaction was terminated by the addition of 1 mg of solid sodium borohydride to the incubation mixture. The enzyme preparation was passed through a Sephadex G-25 column (0.7×20 cm) pre-equilibrated with 100 mM potassium phosphate buffer, pH 7.0, to remove free reagent. The amount (mol) of pyridoxal-lysine residues in the modified enzyme was estimated spectrophotometrically using the millimolar extinction coefficient of 10.7 $mM^{-1} \cdot cm^{-1}$ at 325 nm (*24*).

Preparation and Isolation of TNBS-Modified Salicylate Hydroxylase—A reaction mixture containing 20 μM salicylate hydroxylase, 100 μM TNBS, 7 μM FAD, and 100 mM potassium phosphate buffer, pH 8.5, in 100 ml (total volume) was incubated at 25°C for 60 min in the dark. The reaction was terminated by the addition of 100 μmol of glycine. The protein was precipitated with solid ammonium sulfate at 75% saturation and collected. The modified enzyme preparation was dissolved in 38% saturated ammonium sulfate, pH 7.0, and applied to a column of TSK gel HW-65 (1×89 cm) pre-equilibrated with 38% saturated ammonium sulfate-30 mM potassium phosphate buffer, pH 7.0. After washing of the column with 100 ml of the same buffer, protein was eluted with a reversed linear gradient (total volume, 200 ml) of 38-10% saturated ammonium sulfate in 30 mM potassium phosphate buffer, pH 7.0, at a flow rate of 0.12 ml/min, and 1.4-ml fractions were collected. The elution pattern was monitored by measuring the absorbances at 280 and 344 nm and by assay of NADH-oxidation activity. The modified protein fractions were pooled, concentrated by ammonium sulfate precipitation, and analyzed by polyacrylamide gel electrophoresis. In these procedures, the enzyme protein was changed to the apo-protein by release of FAD. The fractions containing the apo-enzyme modified with 1 mol of TNP per mol of protein were combined, concentrated, and dialyzed against 30 mM potassium phosphate buffer, pH 7.0, overnight. The isolated, modified enzyme preparation was stored at −80°C.

Digestion of TNBS-Modified Enzyme with Chymotrypsin and Isolation of TNP-Peptide—A reaction mixture containing 19 μM modified enzyme, 0.2 M Tris-HCl buffer, pH 8.0, 7 M guanidine HCl, 10 mM EDTA, and 18.5 mM DTT in a total volume of 10 ml was incubated at 25°C for 2 h under a nitrogen atmosphere. A neutralized solution of monoiodoacetic acid (90 μmol) was added to the mixture and the incubation was continued for a further 30 min. The protein preparation was dialyzed against 2 liters of distilled water overnight, freeze-dried, and dissolved in 0.95 ml of 0.2 M ammonium carbonate, pH 8.2. Chymotrypsin (9.6 nmol, molar ratio of protein and protease = 20 : 1) was added to the carboxymethylated protein solution and the mixture was incubated for 2 h at 37°C. The digest was freeze-dried and dissolved in 0.2 ml of 0.1% TFA. An aliquot of 0.1 ml of the digested peptide mixture was applied to an Inertsil ODS column (GL Science, 4.6×250 mm) and developed with a linear gradient of 0.1% TFA-water and 0.05% TFA-acetonitrile at a flow rate of 0.4 ml/min at 35°C. The chromatogram was monitored at 344 nm to detect TNP-peptide. The TNP-peptide fractions were pooled, combined, and concentrated by evaporation.

Digestion of Native and TNBS-Modified Enzymes with Trypsin and Chymotrypsin—Native and TNBS-treated enzyme preparations (20 nmol) were carboxymethylated as described above and incubated with 55 μg of TPCK-trypsin (molar ratio of protein and protease = 25 : 1) in 0.1 ml of 0.1 M NH_4HCO_3 at 37°C, respectively. After incubation for 6 h, the same amount of TPCK-trypsin was added to each vial and the digestion was continued for a further 15 h. The digests were freeze-dried, and the residues were dissolved in 0.1 ml of 0.1% TFA, and applied separately to an Inertsil ODS column (4.6×250 mm). The column was developed by the methods described above and eluates were monitored at 214 nm. From the digest of the native enzyme, two peptide fractions eluted at 29 and 74 min were collected separately, and freeze-dried. These peptides were not found in the digest of the TNBS-modified enzyme. The amino acid sequence of the 29-min peptide was determined. The 74-min peptide was dissolved in 0.1 ml of 0.2 M ammonium carbonate, pH 8.2, and digested with chymotrypsin (molar ratio of peptide and protease = 20 : 1) for 2 h at 37°C. The digest was freeze-dried, dissolved in 0.1 ml of 0.1% TFA, and applied to an Inertsil ODS column. Elution was carried out by a linear gradient method with 0.1% TFA and acetonitrile as described above. Three peptide fractions with retention times of 50, 54, and 60 min were collected separately and freeze-dried. The amino acid sequences of the 50- and 54-min peptides were analyzed.

Amino Acid Sequencing of Isolated Peptides—Each peptide preparation was loaded on an Applied Biosystems model 470A/120A protein sequencer to determine the amino acid sequence.

RESULTS

Inactivation of Salicylate Hydroxylase by TNBS—Incubation of salicylate hydroxylase with TNBS in 100 mM potassium phosphate buffer at neutral pH rapidly inactivated the enzyme. TNBS, a reagent reacting specifically with amino groups such as the ε-amino group of lysine in protein, is an inactivator as effective as glyoxal for the enzyme (*21*). Although the inactivation rate of hydroxylase increased at more alkaline pH in the range of 6 to 11, the highest ratio of activity loss against the number of modified lysine residues was observed at pH 8.5. The rate of inactivation was linearly dependent on TNBS concentrations at pH 8.5 and 20°C, and it followed pseudo-first order kinetics (Fig. 1A). The secondary plot of k' *versus* the concentration of TNBS gave a straight line with the second-order rate constant of inactivation of 120 $M^{-1} \cdot min^{-1}$ (Fig. 1B). The

3). During the column chromatography FAD dissociated and the enzyme was obtained as the apo-protein. The modified enzyme was obtained in about 20-30% yield from the native enzyme.

Molecular and Enzymic Properties of TNBS-Modified Enzyme—The absorption spectrum of the modified hydroxylase has maxima at 280 and 350 nm and a shoulder at 415 nm (Fig. 5). This indicates that the enzyme is in an apo-protein form, and the absorbance intensities reflect the presence of one mole of bound TNP group per protein. Apo-protein from native enzyme has only an absorption peak at 278 nm. The modified apo-enzyme could bind FAD with the dissociation constant of 0.13 μM (Table II), which is the same as that of the native enzyme.

Kinetic parameters of the modified enzyme listed in Table II indicate a decrease of V_{max} to 20-30% of that of the native enzyme and an increase of K_m value for NADH. In contrast, the K_m value for salicylate showed no change after the modification. Catalytic efficiency (V_{max}/K_m) for NADH of the modified enzyme was only 1.63% of that of the native enzyme. The dissociation constants of salicylate for native

Fig. 3. **Polyacrylamide gel electrophoresis of isolated TNBS-modified salicylate hydroxylase.** Lane 1, native enzyme; lane 2, modified enzyme before purification; and lane 3, modified enzyme after purification. The electrophoresis was carried out on 7.5% polyacrylamide gel (*26*) and proteins were stained with Coomassie Blue.

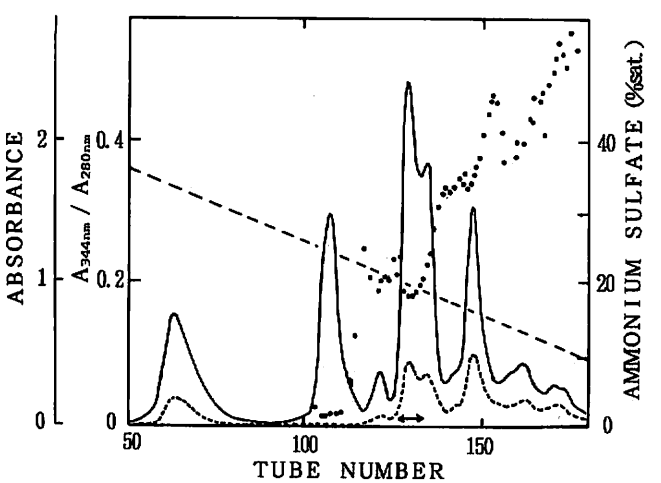

Fig. 4. **Separation of modified salicylate hydroxylase by hydrophobic chromatography.** Experimental conditions are given under "MATERIALS AND METHODS." Absorbances at 280 nm (—) and 344 nm (- - -), the ratio of absorbances at 344 and 280 nm (●), and concentration of ammonium sulfate (·····) were measured. Fractions indicated by the arrows were pooled and referred to as the modified enzyme.

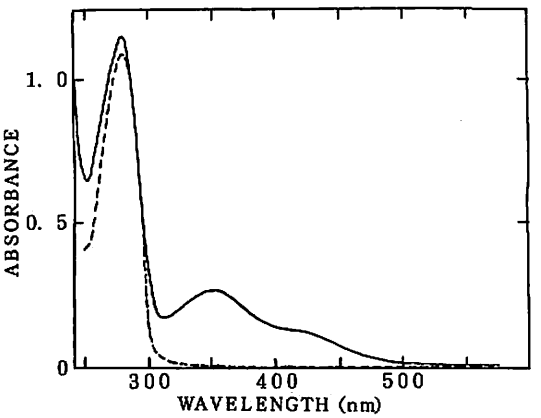

Fig. 5. **Absorption spectra of apo-enzyme of salicylate hydroxylase.** Spectra of (·····) native apo-enzyme, and (—) TNBS-modified apoenzyme in 30 mM potassium phosphate buffer, pH 7.0. The concentration of each enzyme was 15 μM.

TABLE II. **Kinetic parameters of TNBS-modified enzyme.** K_m values for salicylate and NADH were determined using the standard assay system with 1-10 μM salicylate and 1-10 μM NADH for the native enzyme and 10-100 μM NADH for the modified enzyme. Double-reciprocal plots of the data were made to estimate the kinetic parameters. The dissociation constant of FAD was determined by activity-titration of apo-enzyme preparations with FAD (0.01-0.1 μM). For determination of K_d for salicylate, salicylate hydroxylase (30 μM) was titrated with salicylate (3-120 μM) in 30 mM potassium phosphate buffer, pH 7.0, and changes of the absorption at 480 nm were monitored (*1*). ND, not determined.

	Native enzyme			Modified enzyme		
	K_m (μM)	K_d (μM)	V_{max} (min^{-1})	K_m (μM)	K_d (μM)	V_{max} (min^{-1})
Salicylate	1.6	1.7	830	1.8	2.1	ND
NADH	3.7	ND	830	49	ND	170
FAD	—	0.13	—	—	0.13	—

TABLE III. **Amino acid sequence analysis of TNP-peptide of modified salicylate hydroxylase.** Numbers in parentheses denote yields (pmol). X is an unidentified amino acid, which was identified separately as TNP-lysine, as described in "RESULTS."

Cycle	Amino acid	
1	Thr	(354)
2	Ala	(335)
3	Asp	(202)
4	Val	(124)
5	Ala	(104)
6	Ile	(72.0)
7	Ala	(82.4)
8	Ala	(79.8)
9	Asp	(54.6)
10	Gly	(27.6)
11	Ile	(22.0)
12	X	
13	Ser	(9.8)
14	Ser	(8.7)
15	Met	(1.5)

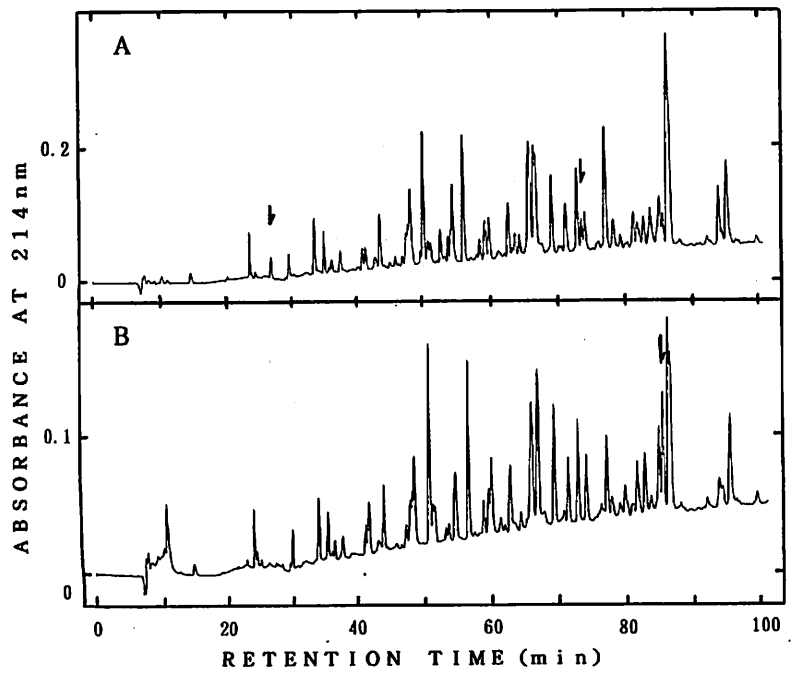

Fig. 6. **HPLC separation profile of tryptic digest of salicylate hydroxylase.** The experimental conditions are given under "MATERIALS AND METHODS." The chromatograms were monitored at 214 nm. (A), tryptic digest of untreated enzyme; (B), TNBS-treated enzyme protein. Arrows in the figure indicate 29- and 74-min peptide fractions in (A) and 96-min peptide fraction in (B).

p-Hydroxybenzoate hydroxylase (*P. fluorescens*)	^{149}TFERDGERLRL^{155}DCDYLAGCD^{159}G^{162}FHGISR
Salicylate hydroxylase (*P. putida* PpG7)	LFT-D^{150}GTEYR--CDLLI^{163}GADGI^{165}KSALR
Salicylate hydroxylase (*P. putida* S-1)	NFA-DGSTYT--ADVAIAADGI*KSSMR

Fig. 7. **Amino acid sequences of TNP-peptide of salicylate hydroxylase from *P. putida* S-1, of L-146 to R-169 of salicylate hydroxylase from *P. putida* PpG7 (*18*), and of T-140 to R-166 of *p*-hydroxybenzoate hydroxylase from *P. fluorescens* (*31*).** The amino acid sequence of the TNP-peptide was compared with those of the two enzymes, especially the sequences around arginine residues, and the sequences homologous to the TNP-peptide are shown in this figure. * indicates the TNBS-modified Lys residue.

and modified enzyme preparations were only slightly different.

Chymotryptic Peptide from Modified Enzyme—The modified enzyme was carboxymethylated and then digested as described in "MATERIALS AND METHODS." Peptides were separated by reverse-phase HPLC with monitoring at 344 nm, and a large peak fraction eluted at 54 min was isolated. This peptide had the sequence TADVAIAADGIX-SSM (Table III). Residue X could not be identified. When tryptic digests of native and modified enzymes were chromatographed as described under "MATERIALS AND METHODS," similar elution patterns were obtained (Fig. 6, a and b), but a few differences were seen. One peptide, eluted at 96 min, in the digest of the modified enzyme was not present in that of the native protein. Two peptides eluted at 29 and 74 min were seen only in the native protein digest and not in the modified protein digest. The 74-min peptide fractions were collected, digested again with chymotrypsin, and separated by HPLC into three peptide fractions with the retention times of 50, 54, and 60 min. Amino acid sequences of the 54-min peptide and 50-min peptide were determined to be TADVAIAADGIK and NFADGSTY, respectively. The sequence of the 54-min peptide is the same as that of the peptide from the TNBS-modified enzyme described above, in which the unidentified residue was confirmed to be lysine. The amino acid sequence of the 29-min peptide from the native protein was determined to be SSMR (Fig. 7).

DISCUSSION

We observed a decrease of the catalytic activity of salicylate hydroxylase to 20% of that of native enzyme upon incubation with TNBS. The inactivation followed pseudo-first order kinetics and one of the 17 lysine residues was specifically modified with TNBS under the reaction conditions at pH 8.5 and 25°C. The electrophoretic mobility of the TNBS-modified enzyme depended upon the number of lysine residues modified. This finding suggests that the reactive lysine residues are located at or close to the surface near the active site of the enzyme. This property was useful for separation of the enzyme having one TNP-lysine/molecule by hydrophobic chromatography (Fig. 4).

The modified enzyme showed a large change of K_m for NADH but not for salicylate. NADH, NAD, ATP, or AMP protects the enzyme against TNBS-inactivation, but adenosine, NaMN, or NMN does not. Based on these results, we consider that the TNBS-modified site is located at or near the NADH-binding site and the modification changes the affinity of hydrogen donors for the site through changes of the interaction of the negatively charged oxygen atoms of phosphate in the AMP-part of NADH with the lysine residue of the enzyme. It is interesting that the modified enzyme preparation showed no detectable NADH-dehydrogenase activity. The activity was observed in the case of glyoxal inactivation (*21*). The results indicate differences in the effects of TNBS and dicarbonyl reagents on the NADH-binding site of the enzyme.

The overall structure of *p*-hydroxybenzoate hydroxylase of *P. fluorescens* has been studied in detail, including the substrate binding and FAD binding sites (*27*). The binding site of pyridine nucleotide has not yet been located, but some information was obtained (*28, 29*). The pyridine ring interacts with the *re*-side of the isoalloxazine ring, as in glutathione reductase (*30*), and the pyrophosphate forms salt bridges with basic amino acid residues. If the structure of the active site of salicylate hydroxylase, in relation to the bindings of flavin and pyridine nucleotide molecules, is similar to that of *p*-hydroxybenzoate hydroxylase, an interaction of the lysine residue with the pyrophosphate moiety of NADH molecule is probable, as discussed above.

A peptide containing TNP-lysine isolated from TNBS-modified salicylate hydroxylase after digestion with chymotrypsin was composed of 15 amino acids, and the determined sequence has some homology to that of the region of Arg-154 to Ile-168 of salicylate hydroxylase from *P. putida* PpG7 with eight identical amino acid residues in 15 residues (Fig. 7). The sequence ADGIKS (161-166) is found in both enzymes and Lys-165, which corresponds to the residue modified with TNBS, is replaced with His-162 in *p*-hydroxybenzoate hydroxylase of *P. fluorescens* (Fig. 7) (*32*). Evidence that the histidine residue (162) and arginine residues (166 and 269) make salt bridges with the oxygen atoms of pyrophosphate of NADPH was provided by crystallographical analyses (*28*). Chemical modification studies of the hydroxylase with diethylpyrocarbonate and dicarbonyl reagents also revealed the presence of one or two histidine (*32, 33*) and arginine residue(s) (*34*) in the NADPH-binding site.

Modification of one in twenty arginine residues of salicylate hydroxylase with dicarbonyl reagents converted the original oxygenase activity into salicylate-dependent NADH dehydrogenase activity using free FAD as an electron acceptor (*21*). The enzyme having one modified arginine residue exhibited a 7-fold increase of apparent K_m for NADH, a 27-fold increase of dehydrogenase activity, and a decrease to 13% of the oxygenase activity of the native enzyme. These data indicated that the arginine residue located at or near the NADH-binding site is modified by dicarbonyl reagents and its interaction with NADH is changed. The location of this arginine residue in the molecule remains to be determined. This is another basic amino acid residue interacting with NADH and may correspond to Arg-166 or 269 of *p*-hydroxybenzoate hydroxylase. At least two basic amino acid residues contribute to the interaction of NADH in the active site of salicylate hydroxylase. The roles of these amino acid residues of the enzyme will be clarified by crystallographic and site-directed mutagenesis studies.

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