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Overexpression of Salicylate Hydroxylase and the Crucial Role of Lys[163] as Its NADH Binding Site[1]

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Expression systems for the *sal* gene encoding salicylate hydroxylase from *Pseudomonas putida* S-1 were examined and some constructs were expressed in these systems. By cultivation of *Escherichia coli* BL21 (DE3)/pSAH8 in LB medium at 37°C with isopropyl-β-D-thiogalactopyranoside as the inducer, salicylate hydroxylase was overexpressed mainly in the form of inclusion bodies. Lower temperature cultivation at 20°C after induction resulted in a large amount of the enzyme in the soluble form. The *E. coli* clone harboring the recombinant plasmid produced a 45 kDa protein that appeared to be electrophoretically and immunochemically identical to the *P. putida* enzyme and contained the same N-terminal amino acid sequence. This recombinant DNA product also exhibited properties characteristic of a flavoprotein and was fully functional as salicylate hydroxylase. Based on chemical modification of the salicylate hydroxylase from *P. putida*, Lys163 was previously proposed to be the NADH binding site. In this study, to obtain a better understanding of the predicted role of Lys163, this residue in the active center of salicylate hydroxylase was replaced with Arg, Gly, or Glu by conventional site-directed mutagenesis. Kinetic studies using these mutant enzymes and the recombinant enzyme revealed increases in apparent K_m values for NADH in the order of wild-type enzyme > K163R > K163G > K163E, with some decreases in V_{max}. Examination of the recombinant enzyme and K163G indicated that the pH dependency of K_m on NADH with pK_a 10.5 is lost by mutation despite the lack of changes in V_{max} values, suggesting a requirement for the lysine residue as the NADH binding site. Based on these results, Lys163 is proposed to play a role in the binding of NADH at the active site through an ionic bond rather than playing a role in catalysis.

Key words: inclusion body, Lys, NADH binding site, salicylate hydroxylase, site-directed mutagenesis.

Salicylate hydroxylase [EC 1.14.13.1], isolated from *Pseudomonas putida* S-1 grown with salicylate as the sole carbon and energy source, is a single polypeptide with a molecular mass of 45,288 Da (421 amino acids) (*1*, *2*) that contains 1 mol of FAD per mol of protein as the prosthetic group (*3*). This enzyme catalyzes the decarboxylative hydroxylation of salicylate to produce catechol by consuming stoichiometric amounts of NADH and molecular oxygen. NADPH is less effective as an external electron donor (*4*). The reaction mechanism of this enzyme has been analyzed (*5*). The tertiary structure of salicylate hydroxylase has been studied by NMR (*6*) and X ray analysis (*7*). The gene encoding salicylate hydroxylase has recently been isolated from the chromosomal DNA of *P. putida* S-1 cloned in *Escherichia coli* (*1*). The genes encoding this enzyme from other sources have also been isolated from plasmids of *P. putida* PpG7 (*8*) and *P. putida* KF715 (*9*).

The chemical modification of salicylate hydroxylase with TNBS has previously suggested that Lys163 is the NADH-binding site and that the side chains interact with the pyrophosphate groups of NADH (*10*). *p*-Hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* belongs to a similar family of FAD-dependent aromatic monooxygenases that use an external electron donor, and its three-dimensional structure has been reported (*11*). Its NADPH binding domain, however, has not been characterized by crystallography but from the structure of the enzyme/substrate complex, Arg166 and Arg269, and possibly His162, are suggested to form a salt bridge with pyrophosphate (*12*). The involvement of Arg44 in NADPH binding was proposed by site-directed mutagenesis (*13*). In salicylate hydroxylase, we proposed that Lys163 is the NADH binding site based on chemical modification in the preserved sequence of ADGIKSL (sequence no. 159–165). From our proposal that Lys163 is involved in the NADH binding site of salicylate hydroxylase, and the multiple alignment studies of 50 flavoprotein hydroxylases, a novel conserved sequence motif

[1] The sequence data (*sal*) reported in this paper have been registered with DDBJ, EMBL, and GenBank under the accession No.: AB010714.

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside: TNBS, 2,4,6-trinitrobenzenesulfonic acid.

has been proposed to play a dual function in both FAD and NAD(P)H binding (*14*). In *p*-hydroxybenzoate hydroxylase from *P. fluorescens*, the sequence from position 153–166 plays a role as an NADPH binding site in which His162 corresponds to Lys163 of salicylate hydroxylase of *P. putida* S-1 (*14, 15*).

We report here the development of a system for the expression and site-directed mutagenesis of the *sal* gene, followed by purification and characterization of the recombinant and mutant enzymes in which Lys163 was replaced by Arg, Gly, or Glu. Our results are in agreement with those obtained by chemical modification, indicating that Lys163 is involved in the NADH binding site and suggesting that it functions in NADH binding rather than in catalysis.

MATERIALS AND METHODS

General Chemicals—Restriction enzymes were purchased from Boehringer Mannheim. T4 DNA ligase and competent *E. coli* cells were from Toyobo. The DNA ligation kit and recombinant Taq DNA polymerase were from Takara. pET28a(+) and pTrc99A were from Novagens and Amersham Pharmacia Biotech, respectively. Other enzymes and chemicals used in this study were obtained from commercial sources and were of reagent grade or of the highest quality available.

Expression Constructs—The *sal* gene contains 1,266 base pairs from position 1,435 to 2,700 in the genomic sequence of *P. putida* (accession No.: AB010714). In this study, the first base of the start codon, ATG, was defined as position number 1. Primer 1 was designed by modification of the nucleotide sequence from position –9 to 11 by replacement of TCATGA with CCATGG at positions –2 to 4 to make an *Nco*I site; 5′-GGTGCCCCCATGGGCAAATC-3′ where the underlined residues represent the *Nco*I site. Primer 2 was an antisense primer from position 539 to 520; 5′-ATGCCGTCTGCAGCGATCGC-3′, where the underlined residues represent the *Pst*I site. PCR was performed with pSAH1 (4.2 kb), which is a pUC18 derivative with a 4.2 kbp *Sal*I–*Eco*RI fragment containing *sal* and *salR* of *P. putida* S-1 (*1*) as the template, primer 1 and primer 2. The product was digested with *Nco*I and *Pst*I, and the resulting DNA fragment was subcloned into the similarly digested vector pTrc-99A (this plasmid was named pSAH1A, 4.7 kb). pSAH1 was then digested with *Pst*I and *Hin*dIII and the resulting DNA fragment (1,120 bp) from position 473 to 1,601 was ligated with similarly digested pSAHIA to produce pSAH5 (5.7kb) (position –1 ~ 1,601). For the construction of pSAH8 (6.8 kb), pSAH5 (5.7 kb) was digested with *Nco*I and *Bam*HI and ligated to similarly digested pET28a(+) (5.4 kb).

Mutation was carried out by PCR splice overlap mutagenesis with pSAH8 as the template. The first primer for the wild-type enzyme was 5′-TCGCTGCAGACGGCATC**A**-**AG**TCCTCC-3′, where the underlined and boldface residues are the *Pst*I site and Lys163 codon, respectively. Lys-163 was replaced with GGG(Gly), AGG(Arg), or GAG(Glu) in the mutant enzymes. The second primer for these enzymes was 5′-TACCAAGGATCCTGATGGC-3′ where the underlined residues represent the *Bam*HI site. PCR conditions were 94°C, 1 min; 60°C, 1 min; 72°C, 2 min; 30 cycles. The PCR products were digested with *Pst*I and *Bam*HI, and ligated to pSAH8 digested with *Pst*I and *Bam*HI. Recombinant clones were confirmed by sequencing.

Expression of Enzymes—The recombinant plasmids were transformed into T7 RNA polymerase-containing *E. coli* BL21 (DE3). Precultures were prepared by inoculating individual colonies into 100 ml of LB medium supplemented with kanamycin (50 μg/ml) and shaken overnight at 37°C. Then, the overnight culture (15–25 ml) was diluted to 1.5 liters in LB and shaken at 37°C for 5 h. Protein expression was induced by adding IPTG to 200 μM (final concentration) and shaking overnight at 20°C. The cultures were harvested by centrifugation at 10,000 ×*g* for 10 min and the resulting paste was stored at –20°C.

Enzyme Activity and Purification of Enzymes—Enzyme activity was measured by the reported method (*16*). Apparent K_m and V_{max} values were estimated from Lineweaver-Burk plots. The pH dependency of the kinetic parameters was determined using 50 mM buffers: pH 5.7 – 8.3, potassium phosphate; pH 8.7 – 11.0, glycine-NaOH. The coupling ratio of the NADH oxidase reaction or the ratio of the oxygenase reaction per NADH oxidase reaction was determined as described previously (*16*). The recombinant and mutant enzymes were purified by a method similar to that described previously for the wild-type salicylate hydroxylase (*17*).

Protein Determination, Sequencing, and Protein Chemistry—Total protein was determined using BCA reagent (Pierce). Automated Edman analysis was carried out using an Applied Biosystems model 610A protein sequencer.

Cofactor Binding Studies—The fluorescence of the enzyme was measured on a Hitachi F-3000 fluorometer at room temperature in 0.03 M potassium phosphate buffer, pH 7.0 (*18*). Emission at 340 nm was measured at an excitation wavelength of 280 nm. The dissociation constants for the apoenzyme-salicylate complexes and the apoenzyme-NADH complexes were derived from the observed decrease in protein fluorescence on titration by adding salicylate and NADH, respectively, based on the Stern-Volmer equation (*19*). The fluorescence of the holoenzyme was measured at 525 nm when activated at 450 nm.

Spectra—Light absorption spectra were obtained on a Hitachi U-3000 spectrophotometer. The difference spectra were obtained by placing the enzyme into a cell, adjusting to zero absorption, and then adding of a small amount of salicylate solution into the cell.

Electrophoresis—SDS/PAGE (10% polyacrylamide gels) was performed as described previously (*20*). Molecular mass standards were purchased from Nacalai Tesque: myosin (200 kDa), β-galactosidase (116 kDa), albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Gels were stained with Coomassie Brilliant Blue G-250.

RESULTS

System for sal Gene Expression—Some expression vectors encoding salicylate hydroxylase were constructed and examined for their levels of expression. *E. coli* JM109/pSAH1 on pUC18 produced only small amounts of salicylate hydroxylase after induction by 0.1% salicylate (0.27 unit/g of cells). The *sal* gene of pSAH1 was introduced into the pTrc99A vector to produce plasmid pSAH5. *E. coli* JM-109/pSAH5 was expressed using 200 μM IPTG as the

inducer but the activity did not increase significantly. When the *sal* gene in pSAH5 was ligated into pET28a(+) downstream of the T7 promoter (pSAH8), *E. coli* BL21 (DE3)/pSAH8 cultivated aerobically at 37°C produced salicylate hydroxylase at 8.4 units/g of wet cells in the presence of 200 μM IPTG and 1.0 unit/g in the absence of IPTG. The band of salicylate hydroxylase detected on SDS-PAGE analysis of the crude extract was, however, smaller than that for whole cells (Fig. 1), perhaps because of the inclusion bodies. Growth of the cells at lower temperatures after induction by IPTG solved this problem, as shown in Fig. 1 and Table I. The salicylate hydroxylase activity of the crude extract per g of wet cells obtained at 20°C was more than 80-fold higher than that obtained at 37°C (Table I). At 20°C, the activity per g of wet cells was the highest at 8 h after induction of IPTG (Table II). The activity per g of cells (7.5 units/g) was optimal at 600 μM IPTG when cultivation was performed at 20°C for 12 h. The system for *sal* gene expression was improved to give an activity of 10–80 units/ g of cells in the crude cell extract. The activity was dependent on other cultivation conditions such as aeration.

Purification of the Recombinant and Mutant Salicylate Hydroxylases—Large amounts of salicylate hydroxylase were produced by IPTG induction in the lysate of *E. coli* BL21(DE3)/pSAH8 cells harboring wild-type, K163R, K163G, and K163E salicylate hydroxylase–containing expression plasmids.

These enzymes were purified from IPTG-induced *E. coli* cells by a procedure similar to that reported previously (*17*). As shown in Table III, K163R was purified 8-fold with a yield of 21%. The yields of these enzymes, except K163E, were 80–100 mg from 40 g of cell paste, while the K163E mutant showed a lower yield (7 mg from 40 g of cell paste), largely due to its higher instability during the preparation procedure. The purity of the final preparation was analyzed by SDS–polyacrylamide gel electrophoresis (Fig. 2), and only fractions showing a single band at 45 kDa were collected. The N-terminal amino acid sequence of the recombinant salicylate hydroxylase was GKSPLRVAVIGGGIA, which was almost the same as that of the wild-type enzyme, except for replacement of the first amino acid serine (S) with glycine (G), because of the introduction of an *Nco*I site into the recombinant enzyme. Despite the amino acid replacement, the kinetic (Table V) and electrophoretic properties (not shown) of the recombinant enzyme were similar

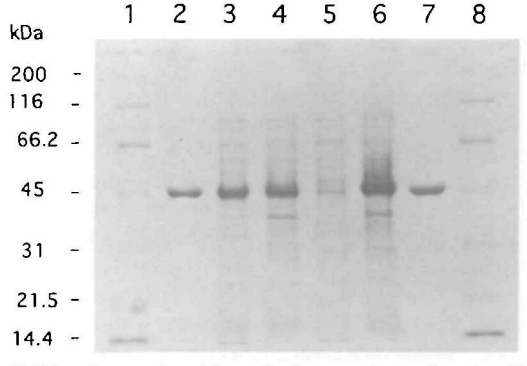

Fig. 1. **SDS–polyacrylamide gel electrophoresis of cells and cell-free extracts.** Lanes 1 and 8, molecular mass markers; lanes 2 and 7, wild-type salicylate hydroxylase; whole cells cultivated at 20°C after induction with IPTG (lane 4) and their cell-free extracts (lane 3); whole cells cultivated at 37°C (lane 6), and their cell-free extracts (lane 5). Samples (protein content 7–10 μg) were electrophoresed on an SDS 10% PAGE gel and stained with Coomassie Brilliant Blue. The mobilities of the standard proteins are indicated to the left of the gel.

TABLE I. **The dependence of salicylate hydroxylase activity in cell-free extracts on cultivation temperature after IPTG induction.** *E. coli* BL21(DE3)/pSAH8 were grown at 37°C with aeration in 30 ml LB medium to an optical density of 5 at 550 nm. After the addition of 200 μM IPTG, cultures were grown for approximately 4 h at 37 or 30°C, or for 12 h at 20°C. Cells were harvested by centrifugation at 10,000 ×*g* and ground with aluminum oxide in 30 mM potassium phosphate buffer, pH 7.4. Cell-free fractions were obtained by centrifugation at 10,000 ×*g*, and the supernatant fractions were used for the assay of salicylate hydroxylase.

Temperature (°C)	Cell weight (g)	Activity (unit)	Protein (mg)	Activity/ cell (unit/g)	Activity/ protein (unit/mg)
37	0.34	0.03	14	0.09	0.002
30	0.39	1.5	16	3.81	0.094
20	0.42	3.0	13	7.11	0.231

TABLE II. **The dependence of salicylate hydroxylase in cell-free extracts on the cultivation time after IPTG induction.** *E. coli* BL21(DE3)/pSAH8 were grown as described for Table I. After the addition of IPTG, cultures were grown at 20°C for the indicated times.

Time (h)	Cell weight (g)	Activity (units)	Protein (mg)	Activity/cell (units/g)	Activity/protein (unit/mg)
0	0.45	0.48	13	1.1	0.04
1	0.4	1.3	12	3.2	0.10
2	0.46	1.5	14	3.3	0.11
4	0.42	2.3	13	5.4	0.18
8	0.39	2.9	13	7.5	0.23
12	0.42	3.0	13	7.1	0.24
20	0.39	2.3	10	5.8	0.22

TABLE III. **Purification of the K163R enzyme.**

Protein sample	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)
Crude extract	285	2,040	3,370	0.61
Ammonium sulfate 38–75% sat.	115	2,070	2,990	0.69
1 st DEAE	57	754	170	4.4
2 nd DEAE	32	435	90	4.8

Purification started with 40 g of cells (wet weight).

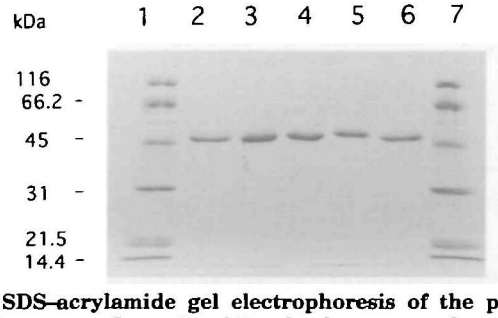

Fig. 2. **SDS–acrylamide gel electrophoresis of the purified mutant enzymes.** Lanes 1 and 7, molecular mass markers; lanes 2 and 6, wild-type salicylate hyroxylase (0.9 μg); lane 3, K163R (0.9 μg); lane 4 , K163G (1.1 μg); lane 5, K163E (1.1 μg). The mobilities of the standard proteins are indicated to the left of the gel.

to those of the wild-type enzyme.

Spectral Characteristics and Formation of the Enzyme-Substrate Complex—The optical spectra of the oxidized forms of K163R, K163G, and K163E salicylate hydroxylases are similar to that of the wild-type protein, showing absorption maxima at 375 and 450 nm (*17*). The absorption spectrum of salicylate hydroxylase is perturbed by the addition of substrate and the peaks at 375 and 450 nm shift to 385 and 455 nm, respectively, with a marked shoulder at around 480 nm (*17*). ES complex formation was demonstrated by titration of the difference spectrum around 480 nm (*21*). Figure 3 shows the absorption difference spectra recorded upon the addition of salicylate to wild-type or mutant salicylate hydroxylase. The shapes of the four difference spectra were similar, indicating no induced change around FAD or the substrate binding sites by mutation. The dissociation constants for salicylate were determined by titration of the absorbance at 484 nm with salicylate. Their K_d values were bound to be similar (Table IV). Apo-salicylate hydroxylase has also been shown to bind substrate by fluorometric analysis (*18*). A fluorescent spectrum showing a maximum at 340 nm following excitation at 280 nm was observed, and the intensity was quenched by the addition of substrate. Formation and dissociation constants for the complex were determined by fluorescence quenching (Table IV). These observations show that mutation of Lys-163 has no effect on substrate-binding.

Steady-State Turnover Kinetics—The kinetic parameters for the enzymes are shown in Table V. The apparent K_d for FAD and apparent K_m for salicylate were not markedly altered by mutation from the data for the wild-type enzyme. However, the apparent K_m for NADH was similar between those of the wild-type enzyme and K163R mutant. This value for the K163G mutant in which the basic Lys residue is replaced by an uncharged Gly was increased by 7–8-fold relative to that of the wild-type enzyme. Furthermore, the value for K163E, in which the basic Lys residue is replaced by acidic Glu, was more than 100-fold higher than that of the wild-type enzyme. These results indicate that the cationic charge on Lys163 interacts with the anionic charge of NADH. The V_{max} values observed were half of the wild-type value for K163R and a third for K163G and K163E. The wild-type enzyme catalyzes the hydroxylation of salicylate to produce catechol using NADH. Under the ordinary assay conditions, 90–98% of the NADH was used for hydroxylation (Table V) with 10–2% NADH for NADH dehydrogenation (*16*). The coupling ratio between NADH oxidation and hydroxylation was determined for the mutant enzymes. The results show that these enzymes have coupling ratios similar to that of the wild-type enzyme (Table V). These results indicate that Lys163 plays a major role in determining the binding affinity with salicylate but not in the catalytic activity.

pH Dependency of the K_m Values for Substrate and NADH—The K_m of the wild-type salicylate hydroxylase for its substrate, salicylate, remained unchanged between pH7 and pH11 (data not shown). As shown in Fig. 4, the K_m of the wild-type enzyme for NADH increased as the pH increased with $pK_a \cong 10.5$, similar to the change in V_{max}. When similar experiments were carried out with K163G, the V_{max} value changed similarly to V_{max} of the wild-type enzyme in this pH range, but the K_m for NADH increased markedly through all pH ranges examined (Fig. 4). These results suggest that Lys163 plays a role in the NADH binding of salicylate hydroxylase but not in catalysis.

Complex Formation of the Apoenzyme with NADH—To investigate the interaction of the apoenzyme with NADH, the quenching of the protein fluorescence was titrated with NADH and the dissociation constants unexpectedly showed similar values for the wild-type and mutant enzymes: 21, 19, 28, and 20 μM for wild-type, K163R, K163G, and K163E, respectively. These results are different from the K_m values for NADH determined by steady-state kinetics, where much larger K_m values for K163G and K163E were observed (Table V). This difference may be due to the differences in the binding mechanisms of the apo- and holoenzymes, and NADH may prefer to bind to the FAD binding site of the apoenzyme. We, therefore, examined this possi-

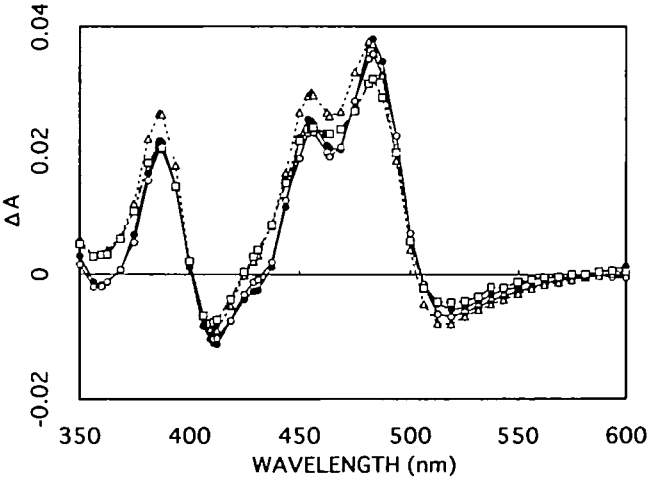

Fig. 3. **Difference absorption spectra of the wild-type and mutant salicylate hydroxylases in the presence and absence of salicylate.** The difference absorption spectra were determined with 20 μM salicylate hydroxylase in 30 mM potassium phosphate buffer, pH 7.0, by the addition of 1 mM salicylate; wild-type (●), K163R (○), K163G (△), and K163E (□).

TABLE IV. **Dissociation constants of salicylate hydroxylase–salicylate complexes.** The holoenzyme was titrated in 30 mM potassium phosphate buffer, pH 7.0, with salicylate. The protein fluorescence of 1 μM apo-salicylate hydroxylase was titrated in 30 mM potassium phosphate buffer, pH 7.0, with salicylate. Dissociation constants were determined from fluorescence values at 340 nm excited at 280 nm based on the Stern-Volmer equation.

Enzyme	Holoenzyme (μM)	Apoenzyme (μM)
Wild-type	3.8	2.5
K163R	4.1	3.4
K163G	3.7	3.3
K163E	3.9	5.1

TABLE V. **Kinetic parameters of the wild-type, recombinant and mutant salicylate hydroxylases.**

Enzyme	K_m′(μM)			V_{max} (min^{-1})	Coupling ratio[b] (%)
	FAD[a]	Salicylate	NADH		
Wild-type	0.10	0.87	4.2	671	92
Recombinant	0.10	1.3	4.0	680	92
K163R	0.07	1.63	3.2	346	95
K163G	0.26	4.96	29.3	229	97
K163E	0.38	9.62	675	197	92

[a] K_d of FAD. [b] The ratio of product formed per NADH oxidized.

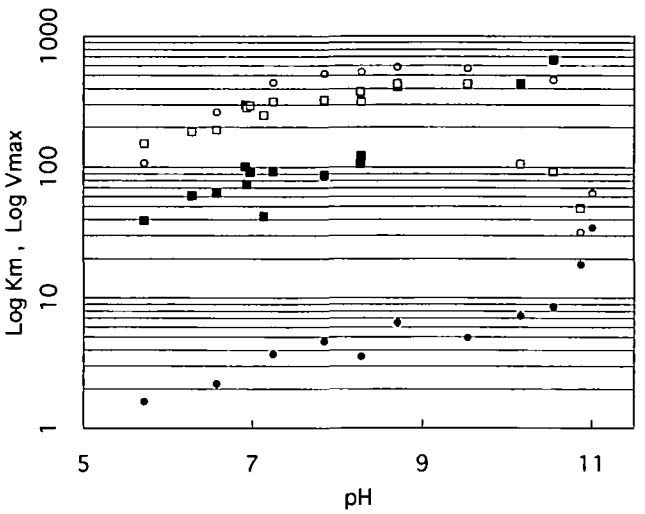

Fig. 4. **Effects of pH on the kinetic parameters of wild-type and mutant salicylate hydroxylases.** Salicylate hydroxylase was used for the wild-type (0.61 μM) and mutant K163G (3.11 μM) in 50 mM potassium phosphate buffer, (pH 5.7–8.3) and glycine-NaOH buffer (pH 8.9–11). K_m for NADH (●: wild-type; ■: K163G) and V_{max} (○: wild-type; □: K163G) were determined with NADH (3–75 μM for wild-type and 20–1,000 μM for K163G).

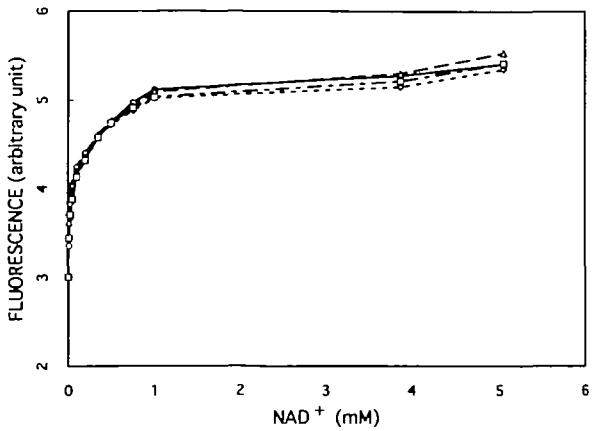

Fig. 5. **Flavin-fluorescence of wild-type and mutant salicylate hydroxylase upon the addition of NAD⁺.** The flavin-fluorescence (525 nm) excited at 450 nm of 1 μM wild-type and mutant salicylate hydroxylases (0.5 ml) was determined in 30 mM potassium phosphate buffer, pH 7.0, and titrated with NAD^+ (0–5 mM); wild-type (○), K163R (△), K163G (◇), and K163E (□).

bility. The fluorescence at 525 nm of free FAD (1 μM) excited at 450 nm was largely quenched by the addition of apo-salicylate hydroxylase (1.5 μM) to 17% (*18*). The FAD fluorescence of the wild-type holoenzyme (0.5 μM) at 525 nm increased twofold by the addition of 1 mM NAD^+, similar to the mutant enzyme (Fig. 5), corresponding to about half of the free FAD fluorescence. The K_d of the apoenzyme-NAD^+ complex (180 μM) (*18*) and of the apoenzyme-FAD complex (0.1 μM) (Table V) suggest competitive binding of NAD^+ with FAD on the FAD binding site. Therefore, NADH would bind the FAD binding site in the apoenzyme.

DISCUSSION

We constructed a system for the overexpression of recombinant salicylate hydroxylase in *E. coli* cells. After several trials, utilizing the strong IPTG-inducible T7 promoter of *E. coli* BL21(DE3)/pSAH8, a system was constructed to overexpress the recombinant enzymes in these cells. Cultivation of the bacteria at 37°C, however, brought about less enzyme activity in the crude extract as compared with the higher level of activity observed in the whole cells, as determined by SDS–polyacrylamide electrophoresis (Fig. 1), suggesting insertion of the expression product into the inclusion bodies (*22*). As high temperature, early induction, high salt concentration and low aeration are known to favor an increase in the number of visible inclusion bodies, *E. coli* were grown at a lower temperature (20°C) for 15 h after induction. This treatment significantly increased the quantity of soluble salicylate hydroxylase (Table I).

Lys163 has been proposed to be the NADH binding site by chemical modification with TNBS (*10*). The inactivation follows pseudo-first order kinetics and one of the 17 lysine residues was specifically modified with TNBS by reaction at pH 8.5 and 25°C. In protection experiments, lysine163 was shown to represent a basic residue that interacts with an anionic group of NADH in the binding site of the enzyme.

In this study, we changed Lys163 to Arg, Gly, and Glu by site-directed mutagenesis. These mutant enzymes had molecular properties similar to those of the recombinant or wild-type enzyme. As the absorption spectra of the enzyme-salicylate complex and the enzymes were similar to all wild-type and mutant enzymes (Fig. 3), these mutations appear not to induce any conformational changes around the FAD- or substrate-binding sites of the enzymes. The effects of replacing of Lys163 on the NADH-binding kinetics of salicylate hydroxylase were, however, observed in the order of Arg < Gly < Glu (Table V). The most intriguing result from this study is that replacement of Lys163 not with Arg but with Glu substantially altered the kinetics. K163R has kinetic properties similar to those of the wild-type enzyme, suggesting the necessity of a positive charge, irrespective of the difference in size of the side chain, for interaction with NADH. Gly has no charge and showed an intermediate affinity for NADH. Glu, which has a negative charge, showed the weakest affinity for NADH. Therefore, a positive charge rather than the size of the side chain of Lys163 seems to be important for NADH binding. This is consistent with the role of Lys163 suggested by modification with TNBS and the role of His162 in *p*-hydroxybenzoate hydroxylase (*15*). The V_{max} values were not markedly different between the wild-type and mutant enzymes. In addition, the coupling ratios of oxygenation and NADH oxidation were not changed.

For the wild-type enzyme, the K_m for NADH and the V_{max} are dependent on pH with $pK_a \cong 10.5$, which would be due to the Lys residue on salicylate hydroxylase (Fig. 4). When Lys163 was changed to Gly (K163G), the K_m for NADH increased by about 100-fold over the pH range examined. the V_{max}, however, did not change markedly over this pH range. On the other hand, the K_m of the wild-type enzyme for salicylate did not change between pH 7 and 11 (data not shown). These results provide further evidence for a role of Lys163 in salicylate hydroxylase in NADH binding but not in catalysis.

In the case of *p*-hydroxybenzoate hydroxylase, Arg44 has been reported to be indispensable for NADPH binding and

optimal catalysis (*13*). Subsequently, His162 and Arg269, which are located at the entrance of the interdomain cleft that leads toward the active site, have been proposed to play a role in NADPH binding without affecting the efficiency of substrate hydroxylation. Lys163 in salicylate hydroxylase would correspond not to His162 and Arg269 rather than Arg44 of *p*-hydroxybenzoate hydroxylase. Eppink *et al.* (*14*) proposed a novel nucleotide binding motif functioning in both FAD and NAD(P)H binding. In *p*-hydroxybenzoate hydroxylase, the sequence motif (residues 153–166) includes strand A4 and the N-terminal part of helix H7. In salicylate hydroxylase 154-DVAIAADGIKSS-MR-167 corresponds to the motif where Lys163 may play the same role as His162 in *p*-hydroxybenzoate hydroxylase.

The apoenzyme forms of salicylate hydroxylase and its mutants all bind NADH with similar affinities, as shown by the protein fluorescence quenching of the apoenzymes. This result appears to be inconsistent with the K_m values for NADH, which are increased significantly by the conversion of Lys163 to Gly or Glu. Under ordinary assay conditions, the K_m for NADH was 4.2 μM (Table V) in the presence of salicylate and 1.1 mM in the absence of salicylate (*23*). In the absence of FAD, the apoenzyme binds NAD^+ (II) and NADH with a K_d of 180 μM (*18*) and 21 μM (this study), respectively, which seemed to be significantly low compared to the K_m for NADH in the absence of salicylate (1.1 mM). This contradiction can be resolved by a hypothesis in which NAD^+ or NADH binds not only the NADH binding site but also the FAD binding site on the enzyme. If this mechanism is correct, the following equilibria will occur:

$$E\text{-}F = E + F \quad (I)$$

$$E + NAD^+ = E\text{-}NAD^+ \quad (II)$$

$$E\text{-}F + NAD^+ = E\text{-}NAD^+ + F \quad (III)$$

where E and F denote the apoenzyme and FAD, respectively. In the titration experiments with the holoenzyme, NAD^+ was shown to increase flavin fluorescence (Fig. 5). Upon the addition of 1 mM NAD^+, the fluorescence intensity increased to the level expected from the dissociation of a half concentration of the holoenzyme, indicating the dissociation of FAD from the holoenzyme by the shifting of equilibrium reaction (III) to the E-NAD^+ complex formation side. This suggestion was strengthened by comparing the higher values of K_m for NADH in the presence of FAD and in the absence of salicylate (1.1 mM) (*15*) with the lower values of K_d for the apoenzyme-NADH complex (10 μM). These results suggest another binding site for NADH and a similarity between the sites of FAD and NADH binding on the enzyme. On *p*-hydroxybenzoate hydroxylase, the analogue of NADPH or FAD, adenosine 5′-diphosphoribose, displaces FAD in the active site, suggesting that NADPH binds to the FAD binding site (*12*).

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