Determination of the Position of Monoxygenation in the Formation of Catechol Catalyzed by Salicylate Hydroxylase*

Riyad Y. Hamzah and Shiao-Chun Tu†‡

From the Department of Biophysical Sciences, University of Houston, Houston, Texas 77004

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The isolation and characterization of three different forms of salicylate hydroxylase have been previously reported. As the first example of flavoprotein external monoxygenase, this enzyme was discovered in 1962 (1) and subsequently purified from cells of Pseudomonas putida (2, 3). In later years, salicylate hydroxylase was also isolated from soil organisms now maintained by the American Type Culture Collection as Pseudomonas sp., ATCC 29352 (4, 5) and P. sp., ATCC 29351 (6). The P. putida enzyme differs substantially from the latter two with respect to molecular weight, subunit structure, substrate specificity for reduced pyridine nucleotide and salicylate, and kinetic properties (6, 7). While the two hydroxylases with respect to molecular weight, subunit structure, substrate specificity for reduced pyridine nucleotide and salicylate, and kinetic properties (6, 7). While the two hydroxylases differ in their structural properties and substrate specificities, they are significantly distinct in certain kinetic properties and their absorption spectra upon anaerobic reduction by NADH (4-6).

The activity of salicylate hydroxylase is characterized by the substrate decarboxylation as well as hydroxylation (or monooxygenation) in yielding catechol as a product (2, 4). In such a reaction, the site of hydroxylation could be at either position 1 or 3 of the salicylate ring. Furthermore, the three forms of salicylate hydroxylase may not necessarily be identical in this respect. In fact, it is known that there are three forms of m-hydroxybenzoate hydroxylase, with the site of substrate hydroxylation at position 6 in one case (8) and position 1 in the other two (9, 10). For each form of salicylate hydroxylase, the determination of the site of substrate hydroxylation is important for an understanding of the reaction mechanism.

For the P. putida enzyme, pyrogallol was detected chromatographically as a reaction product from 2,3- or 2,6-dihydroxybenzoate, suggesting that the sites for decarboxylation and hydroxylation were the same (2). However, the expected product hydroxyhydroquinone from 2,4- or 2,5-dihydroxybenzoate could not be detected in the same study. In the case of P. sp. (ATCC 29352) hydroxylase, the reported partial substrate activities for 2,3-dihydroxybenzoate and 3-methyl salicylate (4) appeared to suggest that position 1 was the site of hydroxylation. However, the reaction products of these salicylate analogs were not identified, thus providing no direct evidence for decarboxylation and/or hydroxylation. In the present investigation, we have used synthetic [3,5-²H]salicylate as a substrate to examine the hydroxylase from cells of P. sp. (ATCC 29351), the strain previously studied by Presswood and Kamin (6), with respect to the position of hydroxylation. Structural characterization of the reaction product allows us to unequivocally identify position 1 as the site of decarboxylative hydroxylation.

EXPERIMENTAL PROCEDURES

Materials

Pseudomonas sp. 29351 was obtained from American Type Culture Collection and was recently identified in our laboratory as Pseudomonas cepacia. Experimental procedures for cell growth and salicylate hydroxylase purification were essentially the same as previously reported (4). Protein concentrations were determined by the method of Lowry et al. (11) using lysozyme as a standard. One unit of hydroxylase activity is defined as 1 µmol of NADH oxidized/min at 25 °C, pH 7.6, and saturating levels of salicylate. One activity unit for alcohol dehydrogenase is for 1 µmol of ethanol to acetaldehyde conversion per min at pH 8.8 and 25 °C.

Sodium salicylate and catechol were obtained from Fisher. Salicylic acid and ²H₂O (99.8%) were products of Merck and Aldrich, respectively. Horse liver alcohol dehydrogenase (1.9 units/mg of protein) and NAD⁺ were purchased from Sigma.

Methods

Spectrometric Analyses—Absorption spectra were determined using a Perkin-Elmer absorption spectrophotometer 552. NMR spectra were recorded with Varian NMR spectrometer model T-60 at 60 MHz or model FT-80 at 80 MHz. Mass spectra were obtained using a Hewlett Packard mass spectrometer 5930 A.

Synthesis of [3,5-²H]Salicylate—A mixture of salicylic acid and sodium salicylate, 200 mg each, was added to 2 ml of ²H₂O in a test tube. The tube was capped and heated at 100 °C. Once a week, the solvent was removed at room temperature under reduced pressure and replaced with fresh ²H₂O. The degree of deuterium incorporation was monitored by examining the NMR spectra of aliquots withdrawn at different times. After 40 days, the sample was dried under reduced pressure and redissolved in a desired solvent.

Isolation of Enzymatic Reaction Product—The reaction was carried out at 25 °C in 14 ml of 25 mM sodium phosphate, pH 7.6, containing 3.3 units of salicylate hydroxylase, 1.8 units of alcohol dehydrogenase, 0.15 mmol of the deuterated salicylate, 0.11 µmol of NAD⁺, and 5.9 mmol of ethanol. To monitor the disappearance of

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† To whom requests for reprints should be addressed.

salicylate, aliquots were withdrawn at different times, diluted with the same buffer, and changes in absorbance at 296 nm were determined (4). The reaction was stopped at >50% completion by the addition of 2 ml of 3.3 N HCl, and catechol was isolated by the method of Yamamoto et al. (2). The solution was extracted three times with 20-ml portions of ether. The combined ether phase was washed once with 1 ml of H2O, three times with 6-ml portions of 0.5 M sodium bicarbonate to remove the salicylic acid, twice more with 1 ml of H2O, and then dried under reduced pressure. A portion of the dried sample was used for molecular weight determination by mass spectroscopy. Another portion was dissolved in 6H2O for NMR analysis.

RESULTS AND DISCUSSION

The NMR spectrum of salicylate is shown in Fig. 1A, along with the signal assignments (12, 13). Upon heating salicylic acid/salicylate in H2O, a hydrogen/deuterium exchange occurred specifically at positions 3 and 5 (Fig. 1B). The total integrated signal intensity for protons at positions 3 and 5 was measured as a function of time. The exchange reaction followed apparent first order kinetics with a half-life of about 185 h and reached ~97% completion after 40 days. The [3,5-2H]salicylate sample so obtained exhibited two NMR singlets for the protons at positions 4 and 6, with their integrated signal 50% of that for the total protons of a salicylate standard (Fig. 1, A and B). The deuterated sample also showed an absorption spectrum identical with that of a salicylate standard in neutral phosphate buffer.

Using [3,5-2H]salicylate and NADH (generated from ethanol and NAD+) by the activity of alcohol dehydrogenase as substrates for the P. cepacia salicylate hydroxylase, the product catechol was isolated as described under "Methods." The isolated product was identical with a catechol standard in neutral phosphate buffer.

The retention of both deuterium atoms of [3,5-2H]salicylate by the reaction product catechol was further demonstrated by the mass spectroscopic measurement of the latter (Fig. 2). The observed m/e value of 112 for the parent peak of the isolated sample corresponds to the molecular weight of [12H]catechol. Furthermore, the spectrum of the isolated sample closely resembles that for a catechol standard measured under identical conditions, except that all major peaks (i.e., peaks with m/e values >63) of the former have undergone appropriate mass shifts due to the presence of 2 deuterium atoms in the molecule.

The synthetic [3,5-2H]salicylate was also tested as a substrate for kinetic isotope effect. Reactions were carried out at 23°C in 1 ml of 50 mM sodium phosphate, pH 7.6, containing 3 μg of P. cepacia salicylate hydroxylase and saturating amounts of NADH (0.11 mM) and proto- or [3,5-2H]salicylate (0.1 and 0.25 mM). The initial reaction velocities were each determined in four repeated assays by following changes in absorbance at 296 nm as a function of time. The kH/kD values, determined from observed Vcat, H/Vcat, D ratios, at the two saturating levels of salicylate were both found to be 1.00 ± 0.02, indicating that there is no detectable kinetic isotope effect of the deuterated salicylate. Such a finding is consistent with the conclusion that the hydroxylation does not occur at position 3 of salicylate.

As mentioned before, the position of substrate hydroxylation has not been firmly established prior to this work for any one of the three known species of salicylate hydroxylase. The approach of using [3,5-2H]salicylate as a substrate has allowed us to identify unequivocally, for the P. cepacia hydroxylase, position 1 of salicylate as being the site of decarboxylation and hydroxylation. The same approach should be quite applicable to the other two salicylate hydroxylase species.

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REFERENCES


Fig. 1. NMR spectra of [3,5-2H]salicylate and the enzymatic reaction product derived from it, in comparison with those of salicylate and catechol standards. The spectra of a salicylate standard (A) and the deuterated salicylate, obtained after 40 days of the deuterium exchange reaction described under "Methods" (B), were both determined at 60 MHz and a sample concentration of 200 mg/ml. The spectra of a catechol standard (C, 10 μg/ml) and the catechol produced enzymatically from the deuterated salicylate (D, 5 μg/ml) were recorded at 80 MHz. All spectra were determined in 6H2O, and chemical shifts are based on sodium 2,2-dimethyl-2-sila-pentane-3-sulfonate as a standard. Signal assignments are indicated in parentheses. For both the spectra and the corresponding integrated signals, identical vertical scales are used for A and B, whereas one intensity unit for C is 5.38-fold larger than that for D.

Fig. 2. Mass spectrum of catechol produced enzymatically from [3,5-2H]salicylate. The catechol sample was isolated and prepared for the mass spectroscopic measurement as described under "Methods."
Salicylate Hydroxylase Reaction Product