Biochimica et Biophysica Acta, 657 (1981) 530—534

BBA Report

BBA 61386

MECHANISM OF SALICYLATE HYDROXYLASE-CATALYZED DECARBOXYLATION

KENZI SUZUKI and MASAYUKI KATAGIRI

Department of Chemistry, Faculty of Science Kanazawa University, Kanazawa 920 (Japan)

(Received August 4th, 1980)

Key words. Decarboxylation; Flavin enzyme; Salicylaldehyde, Salicylate hydroxylase; Oxygenase, (Ps. putida)

Summary

Salicylate hydroxylase (salicylate, NADH: oxygen oxidoreductase (1-hydroxylating, decarboxylating), EC 1.14.13.1) in *Pseudomonas putida* catalyzed hydroxylation of the substrate analogue, salicylaldehyde, to form catechol and formate with stoichiometric consumption of NADH and O_2. Consequently, a study of primary product derived from the carboxyl group of the authentic substrate, salicylate, was undertaken. The experimental results revealed that CO_2 not H_2CO_3, was produced first.

Salicylate hydroxylase (salicylate, NADH: oxygen oxidoreductase (1-hydroxylating, decarboxylating), EC 1.14.13.1) in *Pseudomonas putida* was the first FAD-containing oxygenase to be discovered, which catalyzes hydroxylation of salicylate to form catechol and CO_2 with stoichiometric consumption of NADH and molecular oxygen [1]. The catalyzed reaction 1 apparently fulfils the typical monooxygenase stoichiometry.

$$\text{Salicylate} + \text{NADH} + H^+ + O_2 = \text{catechol} + CO_2 + NAD^+ + H_2O \quad (1)$$

When the substrate salicylate was replaced by the analogues, *o*-nitro-, *o*-iodo- and *o*-aminophenol, the stoichiometry between substrate, NADH and O_2 was found to be in a ratio of 1 : 2 : 1 [2]. These findings indicate that the salicylate hydroxylase apparently has various types of oxygenase functions which are dependent on the nature of the substrate.

We further examined a salicylate analogue, salicylaldehyde, as the substrate. The experiments revealed catechol as the hydroxylated product, as identified

by TLC on silica gel and by the metapyrocatechase method [3,4]. The salicylaldehyde used in this study was shown to be free of salicylate by careful analyses on thin layer plates in different solvent systems.

Possible effects of H_2O_2 on salicylaldehyde oxidation [5] were tested, since it is known that, under certain conditions, the hydroxylase can also catalyze substrate-dependent oxidation of NADH to generate H_2O_2 [6]. However, we found that no catechol was produced on addition of substrate amounts of H_2O_2 (up to 2 mM) to the system in place of NADH, in both the presence and absence of salicylate hydroxylase. We also found that 100 nM beef liver catalase had no effect on the rate of catechol formation in the aldehyde hydroxylase system.

Salicylaldehyde, though only weakly affecting the absorption spectrum of the salicylate hydroxylase flavoprotein, perturbed the fluorescence and CD spectra of the enzyme. Since the formation of salicylaldehyde-enzyme complex is suggested by these results, fluorimetric quenching of the flavoprotein at 342 nm with excitation at 292 nm, on addition of salicylaldehyde, was measured using a method similar to that previously reported for studying salicylate binding to the enzyme [7]. From the titration curves, it was derived that one molecule salicylaldehyde was bound to one molecule of salicylate hydroxylase. The fluorimetric dissociation constant was calculated to be 120 μM in 30 mM potassium phosphate buffer (pH 7.8).

Table I summarizes the kinetic parameters for the enzyme-catalyzed reaction with salicylaldehyde as the substrate. The K_m and V values, except the K_m for O_2, are one to two orders of magnitude higher and lower, respectively, than those obtained in the normal salicylate hydroxylase reaction [8]. The salicylaldehyde hydroxylase reaction was most active at around pH 7.8 under the same conditions.

The experimental results, summarized in Table II revealed that one molecule each of NADH and salicylaldehyde was consumed with formation of one molecule of catechol and formate. The latter product, formate, was further identified according to Kennedy and Barker [9], by paper chromatography in three different solvent systems. The R_F values for the product and for authentic

TABLE I

KINETIC PARAMETERS FOR THE SALICYLATE HYDROXYLASE-CATALYZED REACTION WITH SALICYLALDEHYDE AS SUBSTRATE

The standard reaction mixture contained 200 μM salicylaldehyde/100 μM NADH/7 μM FAD/500 nM salicylate hydroxylase/30 mM potassium phosphate, pH 7.8. Values for salicylaldehyde and NADH were determined based on the rate of NADH consumption at 360 nm, instead of at 340 nm, where salicylaldehyde had some absorption. Values for O_2 were based on the rate of O_2 consumption in a Clark type oxygen electrode cell in an atmosphere of O_2/N_2 mixture.

	K_m (μM)	V (mol/min per mol enzyme)
Salicylaldehyde	87	112
NADH	118	132
O_2	196	93

TABLE II

STOICHIOMETRY OF SALICYLALDEHYDE HYDROXYLATION AND PRODUCT FORMATION

The reactions were carried out at room temperature in optical cuvettes. The incubation system (1 ml) contained 1 mM salicylaldehyde/30 mM potassium phosphate, pH 7.0/2.5 μM salicylate hydroxylase/ 7 μM FAD/indicated amounts of NADH. The reaction was followed at 360 nm. The values for the hydroxylase-coupled NADH consumption were corrected for the formation of H_2O_2 which is derived from the substrate-dependent but hydroxylase-uncoupled oxidation of NADH [6]. Immediately after the reaction was completed, the pH of the reaction mixture was adjusted to pH 8.0 with 1 M NaOH, and the mixture was extracted three times with 2 ml of diethyl ether each time. The solvent fractions were combined and evaporated to dryness. The residue was determined for catechol by the metapyrocatechase method [3,4]. To the water layer was added 0.2 ml 1 M H_2SO_4 and the mixture was extracted 10 times with 2 ml of diethyl ether. After 10 μl 1 M NaOH was added to the combined ether fractions, the solvent was evaporated. The residue was analyzed for formic acid by the method of Grant [10].

NADH added (nmol)	NADH consumed (nmol)	Catechol formed (nmol)	Formic acid formed (nmol)
500	430	440	320
1000	850	800	640
1500	850	960	820

formic acid were identical. A parallel experiment carried out in Clark type oxygen electrode cells indicated stoichiometric consumption of one molecule of oxygen per NADH and substrate molecule. To examine further the possible formation from salicylaldehyde of CO plus H_2O, instead of HCOOH, the hydroxylase reaction was carried out in the presence of 4 μM oxyhemoglobin under similar conditions to those given in Table II. Under the conditions, no change in absorbancy at or close to 420 nm was observed during the entire course of reaction. Since it was confirmed that at least 1 nmol CO was detectable by the absorbance change at 420 nm in the presence of 4 μM oxyhemoglobin, and since this concentration of hemoglobin did not reduce the rate of catechol formation from salicylaldehyde, the possibility was eliminated that salicylaldehyde was metabolized to catechol, CO and H_2O, according to a monooxygenase stoichiometry analogous to that proposed for the hydroxylation of salicylate (reaction 1). Thus, the hydroxylation of salicylaldehyde may be represented by reaction 2.

$$\text{Salicylaldehyde} + \text{NADH} + H^+ + O_2 = \text{catechol} + \text{HCOOH} + \text{NAD}^+ \quad (2)$$

These findings in turn raised the question as to whether the hydroxylation of salicylate, the authentic substrate, was actually catalyzed by a monooxygenase mechanism since reaction 1 could be explained alternatively in a dioxygenase fashion represented by reaction 3.

$$\text{Salicylate} + \text{NADH} + H^+ + O_2 = \text{catechol} + H_2CO_3 + \text{NAD}^+ \quad (3)$$

It is, therefore, of interest to reinvestigate in detail the originally proposed stoichiometry (reaction 1) for salicylate hydroxylase-catalyzed hydroxylation of salicylate.

The method used for distinguishing between the two stoichiometries, i.e., distinguishing between the CO_2 and carbonate produced, was a modification of the procedures applied for the identification of the products of reactions

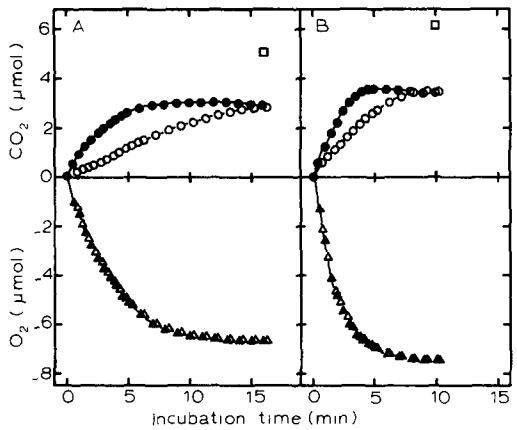

Fig. 1. The salicylate hydroxylase reaction in an atmosphere of air (A) and 100% O_2 (B). The reactions were carried out in Warburg vessels at 10°C and the manometers were shaken vigorously at 240 cycles per min with an amplitude of 55 mm. The reaction mixture contained (final concentration); in the main flask (2.4 ml), 4 mM salicylate/7.5 μM salicylate hydroxylase/7 μM FAD/1.6 μM bovine erythrocyte carbonic anhydrase (Boeringer Mannheim), with (●, ▲) and without (○, △) 1 mM *p*-toluenesulfonamide, in 100 mM potassium phosphate, pH 7.0; and in the side arm (0.1 ml), 3 mM NADH in the same buffer. The CO_2 evolution (●, ○) was measured manometrically by the method described (Chapter III of Ref. 13). The squares (□) represent a sum of CO_2 and H_2CO_3 formed at the end of the reaction. The O_2 uptake (▲, △) was measured in the presence of 0.2 ml of 20% KOH at the center well.

catalyzed by the yeast carboxylase [11,12] and plant α-ketoglutarate decarboxylase systems [11].

The salicylate hydroxylase reaction was carried out in a Warburg manometer at 10°C, in a medium containing salicylate/NADH/salicylate hydroxylase/carbonic anhydrase/with and without addition of a potent inhibitor of the latter enzyme, *p*-toluenesulfonamide [12]. The results shown in Fig. 1 clearly indicate that the first accumulated species is CO_2, which is rapidly equilibrated with H^+ plus HCO_3^- in the presence of carbonic anhydrase. A similar experiment (data not shown), carried out in the presence of Bromophenol blue as an H^+ indicator, showed that the rate of H^+ consumption according to reaction 1 was prevented by the presence of carbonic anhydrase, which should stimulate regeneration of H^+.

The conclusion that the primary product of the salicylate hydroxylase reaction is CO_2 justifies the originally proposed stoichiometry for the catalyzed reaction [1]. The experimental results shown in this report also support our previous hypothesis that apparent mechanisms of an oxygenase may vary depending on the nature of the substrate [14].

We thank Ms. K. Nakada for technical assistance. This work was supported in part by grants from the Ministry of Education, Science, and Culture, Japan and from the Yamada Science Foundation.

References

1 Katagiri, M., Yamamoto, S. and Hayaishi, O. (1962) J. Biol. Chem. 237, 2413—2414
2 Takemori, S., Suzuki, K. and Katagari, M. (1976) in Flavins and Flavoproteins (Singer, T.O., ed.), pp. 178—183, Elsevier, Amsterdam

3 Katagiri, M., Takemori, S., Suzuki, K. and Yasuda, H. (1966) J. Biol. Chem. 241, 5675—5677
4 Takemori, S., Komiyama, T. and Katagiri, M. (1971) Eur. J. Biochem. 23, 178—184
5 Dakin, H.D. (1941) in Organic Syntheses, Collective Vol. I, 2nd edn. (Gilman, H., ed.), pp. 149—150, John Wiley and Sons, Inc., New York
6 White-Stevens, R.H. and Kamin, H. (1972) J. Biol. Chem. 247, 2358—2370
7 Suzuki, K., Takemori, S. and Katagiri, M. (1969) Biochim. Biophys. Acta 191, 77—85
8 Yamamoto, S., Katagiri, M., Maeno, H. and Hayaishi, O. (1965) J. Biol. Chem. 240, 3408—3413
9 Kennedy, E.P. and Barker, H.A. (1951) Anal. Chem. 23, 103—104
10 Grant, W.M. (1948) Anal. Chem. 20, 267—269
11 Conway, E.J. and O'Malley, E. (1953) Biochem. J. 54, 154—163
12 Krebs, H.A. and Roughton, F.J.W. (1948) Biochem. J. 43, 550—555
13 Umbreit, W.W., Burris, R.H. and Stauffer, J.F. (1949) Manometric Techniques and Tissue Metabolism, Burgess, Minneapolis
14 Katagiri, M. and Takemori, S. (1973) Mol. Cel. Biochem. 2, 137—152