UNCOUPLING OF OXYGEN ACTIVATION FROM HYDROXYLATION IN A BACTERIAL SALICYLATE HYDROXYLASE

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Summary

Benzoate stimulates DPNH oxidation in a bacterial flavoprotein, salicylate hydroxylase (salicylate, DPNH : oxygen oxidoreductase (1 - hydroxylating, 1-decarboxylating)) with a higher K_m than that of salicylate. Whereas salicylate is hydroxylated to catechol, with formation of H_2O, benzoate is unchanged and hydrogen peroxide is released during the course of DPNH oxidation. V_{max} for salicylate hydroxylation and benzoate-stimulated DPNH oxidation are identical. The results suggest that benzoate binds to the salicylate site as a pseudo-substrate and "uncouples" oxygen activation from hydroxylation.

Japanese workers (1 - 8) have described a salicylate hydroxylase, purified from a strain of Pseudomonas putida, and have shown it to be a flavoprotein. Its mechanism of action has been extensively studied using spectrophotometric, fluorometric, and more recently (6), stopped flow techniques. We wish to report the purification of a flavoprotein salicylate hydroxylase from a different, but as yet unidentified, soil organism. Our enzyme has different physical properties from that described by the Japanese workers, and also has the distinctive property of catalyzing a benzoate-stimulated DPNH oxidation, whereas the enzyme of Yamamoto et al.(8) is unaffected by benzoate. The characteristics of the benzoate effect suggest that in the presence of this compound, oxygen is activated in a "normal" fashion but hydroxylation cannot proceed.

Results

1. Isolation and growth of organism. The organism was isolated from a soil sample located near a freshly creosoted telephone pole. It was selected by its ability to grow in a medium containing 0.2 % sodium salicylate as sole carbon source, and purified by selection of single colonies from salicylate-agar. For large-scale preparations, the organisms were grown at 37°C. with vigorous aeration in 40 l. carboys, in a medium containing 0.2 % sodium

salicylate but otherwise essentially similar to that reported by Katagiri et al.(1). Cultures turned black at advanced stages of growth; some of the black pigment was associated with sedimented bacteria, and some was excreted into the medium.

2. Purification of enzyme. Organisms were centrifuged and resuspended in four volumes of 0.033 M potassium phosphate, pH 7.0, and the slurry was sonicated for up to 20 minutes. The purification procedure was modeled on that of Yamamoto et al.(8), but we found that activity sedimented with the protamine sulfate precipitate, and could be eluted by the above buffer made 0.05 M in NaCl. This step gave about 8-fold purification over the sonic extract. The eluate was then subjected to ammonium sulfate fractionation between 47 and 65 % saturation and chromatography on DEAE cellulose using a 0.02 to 0.2 M potassium phosphate, pH 7.0, gradient. The peak fractions were homogeneous in polyacrylamide gel and cellulose acetate strip electrophoresis, and gave a single peak in the analytical ultracentrifuge. The purified enzyme had a specific activity about 14 times that of the sonic extract, indicating that the salicylate hydroxylase comprised about 7 % of the extractable cellular protein. The absorption spectrum was that of a typical flavoprotein.

3. Physical studies. Sedimentation velocity measurements gave an $s_{20,w}$ of 5.91, as compared to 3.4 reported by Yamamoto et al.(8). Sedimentation equilibrium by the method of Yphantis (9) gave a molecular weight of 91,000 using a partial specific volume of 0.728 cc./gm. derived from amino acid analysis (10). Sedimentation equilibrium in 6 M guanidine-HCl of protein treated with β-mercaptoethanol and alkylated with iodoacetamide (11) gave a subunit molecular weight of 46,000. A minimum molecular weight of 48,700 was obtained on the basis of flavin content, assuming a millimolar extinction coefficient of 11.3 for FAD at 450 mμ, and protein determination by the microbiuret method (12). Thus, the enzyme contains two subunits of apparently identical molecular weight. Since the enzyme contains two flavins per mole, this suggests that each subunit binds one flavin. Chromatography of supernatant from boiled

enzyme against standards indicated FAD as the prosthetic group.

In contrast, the enzyme reported by Yamamoto et al.(8) has a molecular weight of 57,000 and contains one FAD per mole. The latter finding suggests that the enzyme of the Japanese workers consists of a single unit.

4. Assay. The standard assay mixture used contained 1 mM EDTA, 133 μM sodium salicylate, 147 μM DPNH and 0.02 M potassium phosphate buffer pH 7.62 in a final volume of 3 ml. Addition of FAD or omission of EDTA had no effect on the activity. Potassium phosphate was used in place of tris-HCl (8) because of observed competitive inhibition of the enzyme by Cl^- ion. One unit of activity represents the oxidation of one μmole DPNH per minute measured at 340 mμ and 27° C.

5. Kinetic analysis. Lineweaver - Burk plots using the standard assay but varying salicylate or DPNH gave a K_m of 2.7 μM for salicylate and 16.7 μM for DPNH. In the absence of salicylate, V_{max} for DPNH oxidation was 3 % of that with salicylate, with a DPNH K_m of about 700 μM.

Salicylate could be replaced with benzoate with resultant K_m's of 2 mM for benzoate and 164 μM for DPNH. Routine assays used benzoate concentrations of 0.03 M. The V_{max} for DPNH oxidation was determined at several benzoate concentrations and extrapolated to infinite concentrations of both reactants. The resultant V_{max} for DPNH oxidation, 193 units/ml., is indistinguishable from that in the presence of salicylate, 195 units/ml. This value corresponds to a turnover number of 850 moles per mole flavin per minute.

These kinetic data indicate that both salicylate and benzoate facilitate DPNH binding, with the former having greater effect. The identical V_{max}'s for DPNH oxidation in the presence of saturating benzoate or salicylate indicate that the reactions share the same rate-limiting step.

6. Product study. Conversion of salicylate to catechol was demonstrated (Figure 1) using a coupled assay consisting of salicylate hydroxylase, yeast alcohol dehydrogenase, ethanol and a catalytic quantity of DPNH. Change of the salicylate spectrum into that of catechol can be seen as the reaction pro-

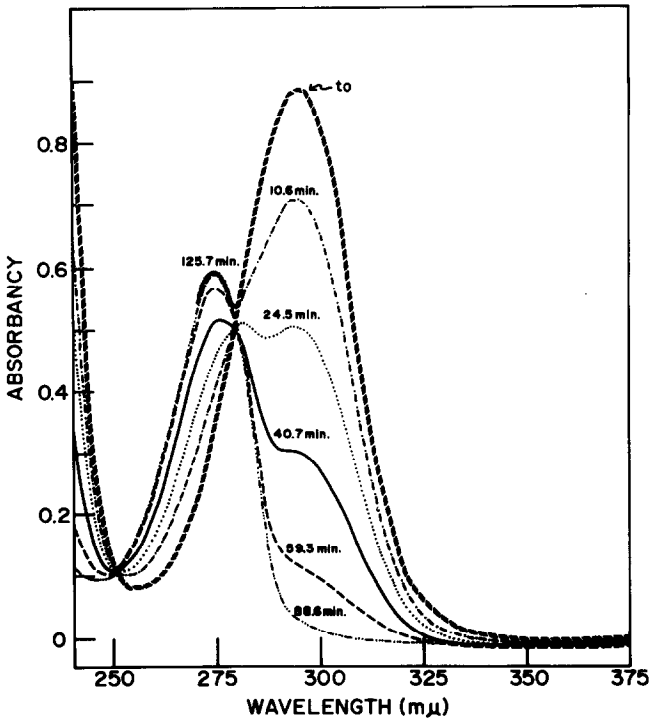

Figure 1 - Enzymatic conversion of salicylate to catechol. Both blank and sample cuvettes for the Cary 14 contained, in 3 ml., 7.4 μg crystalline Sigma yeast alcohol dehydrogenase, 7.7 μg salicylate hydroxylase of 10.9 units/mg specific activity, 0.5 M ethanol and 0.02 M potassium phosphate buffer pH 7.62. Sodium salicylate was added to the sample cuvette to 267 μM concentration, and the reaction was initiated with the addition of 15 μmoles DPNH to both cuvettes. Spectra were recorded at periodic intervals and the time noted as the pen passed over the 296 mμ point.

ceeds. The same experimental approach was attempted with benzoate but no changes in the benzoate spectrum could be detected with even 10 times the levels of enzymes used with salicylate. Attempts were made to isolate a product from a large scale benzoate reaction mixture by acidification and repeated ether extraction, but the only species found was benzoic acid. The finding that the V_{max} for DPNH oxidation was identical in the presence of benzoate or salicylate despite the fact that benzoate was unchanged suggested that the benzoate was bound at the salicylate site, and permitted the prediction that benzoate should be a competitive inhibitor for salicylate hydroxylation. This was indeed found to be the case, as shown in Figure 2. The K_i for benzoate,

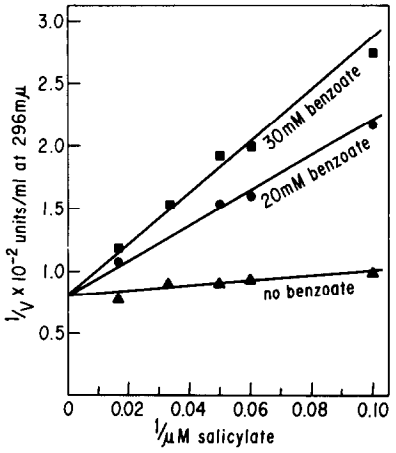

Figure 2 - Competitive inhibition of salicylate hydroxylation by benzoate. Reaction conditions were similar to that described in the standard assay, but with addition of FAD to 6.7 μM. Reactions were initiated by the addition of 1.5 μg of the same salicylate hydroxylase described in the legend of Fig. 1. The units of the ordinate represent μmoles salicylate disappearance per minute; the observed absorbance change at 296 mμ includes a contribution from DPNH oxidation.

3.1 mM, compares with the benzoate K_m of 2 mM in the standard assay at 340 mμ. This discrepancy between K_m and K_i is not considered serious, since the DPNH concentration used in the standard assay was not saturating for the benzoate-stimulated oxidation, although DPNH was present at 9 times its K_m concentration for salicylate hydroxylation.

7. Oxygen electrode experiments. Assays were repeated using a Gilson model KM Oxygraph to measure changes in oxygen tension (Figure 3). In all cases, DPNH was limiting. Addition of catalase to a benzoate-mediated DPNH oxidase assay caused the amount of oxygen consumed to decrease by half (curve B). Addition of catalase after oxygen uptake was complete (curve A) caused re-release of one-half of the O_2 consumed. It can be seen that catalase addition did not affect O_2 uptake in the presence of salicylate (curves C and D). These data indicate that the product of oxygen reduction in the benzoate-mediated reaction is hydrogen peroxide with a 1 : 1 stoichiometry between DPNH and H_2O_2, whereas for salicylate, water and catechol rather than peroxide are the products.

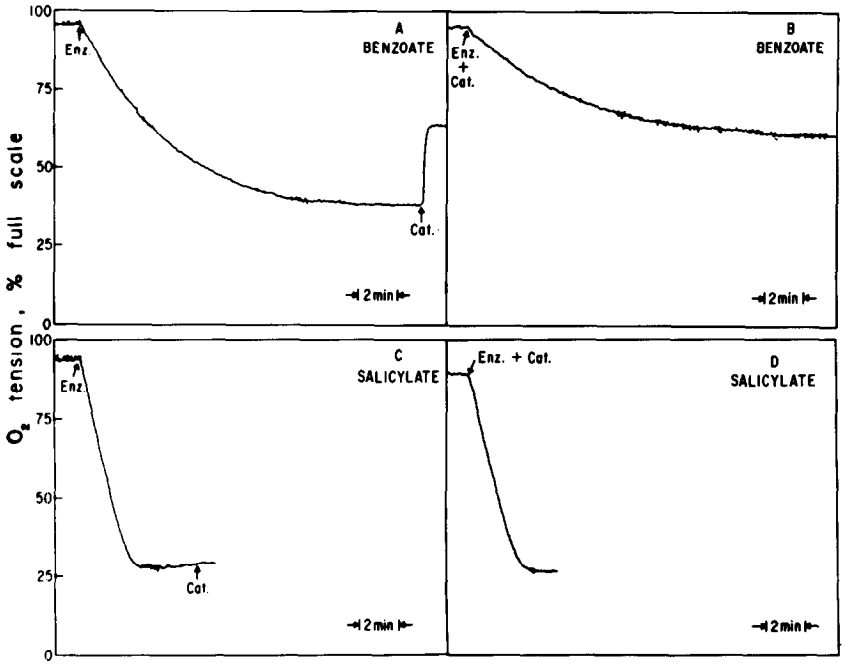

Figure 3 - Consumption of oxygen by reaction mixtures. O_2 consumption was measured with a Gilson model KM Oxygraph equipped with a Clark oxygen electrode. The total chamber volume of 2 ml contained 6.7 μM FAD, 200 μM DPNH, 0.02 M potassium phosphate pH 7.62 and either 2 mM sodium benzoate (curves A and B) or 400 μM salicylate (curves C and D). Reaction was initiated by the addition of 15.4 μg salicylate hydroxylase, and 1500 units Worthington crystalline beef liver catalase was added as shown by the arrows, either initially (B and D) or after reaction was complete (A and C).

Discussion

The above data suggest the following sequence of events during salicylate hydroxylation and benzoate-stimulated DPNH oxidation: Aromatic substrate adds first to enzyme, facilitating binding of DPNH and reduction of enzyme (3,4,6). In the enzyme described by Yamamoto et al.(8), benzoate had no effect; in the enzyme herein described, both benzoate and salicylate bind at the same site, as shown by the competitive inhibition of salicylate hydroxylation by benzoate. The reduced (enzyme) : (salicylate or benzoate) complex can now be considered as being in the proper conformation to permit oxygen activation. With salicylate, the substrate is then hydroxylated. Benzoate, however, is an "unsuitable" substrate and the activated oxygen, since it cannot hydroxylate substrate,

decomposes through unknown intermediates to H_2O_2. Takemori et al. (6) have shown, in their salicylate hydroxylase, that the rate-limiting portion of the reaction mechanism is the interaction of reduced enzyme with oxygen and substrate to yield hydroxylated product. If this holds true for our enzyme, consideration of the rate-limiting step for both the salicylate and benzoate reactions can be narrowed to the oxygen activation process, since the V_{max}'s for DPNH disappearance are identical for benzoate and salicylate while transfer of oxygen to substrate can occur only with salicylate. It should be noted that the V_{max} for DPNH oxidation in the absence of substrate is much lower, indicating that this reaction may be fundamentally different.

The benzoate-stimulated DPNH oxidation may be considered as an "uncoupling" of oxygen activation from hydroxylation. As such, it may provide new opportunities for seeking oxygen intermediates. We have thus far been unable to detect the superoxide anion radical (13, 14), but efforts to detect this or other intermediates are continuing.

Acknowledgements

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References

1. Katagiri, M., Yamamoto, S., and Hayaishi, O., J. Biol. Chem., 237, PC 2413 (1962).
2. Katagiri, M., Maeno, H., Yamamoto, S., Hayaishi, O., Kitao, T., and Oae, S., J. Biol. Chem., 240, 3414 (1965).
3. Katagiri, M., Takemori, S., Suzuki, K., and Yasuda, H., J. Biol. Chem., 241, 5675 (1966).
4. Takemori, S., Yasuda, H., Mihara, K., Suzuki, K., and Katagiri, M., Biochim. Biophys. Acta, 191, 58 (1969).
5. Takemori, S., Yasuda, H., Mihara, K., Suzuki, K., and Katagiri, M., Biochim. Biophys. Acta, 191, 69 (1969).
6. Takemori, S., Nakamura, M., and Katagiri, M., in H. Kamin (Editor) "Flavins and Flavoproteins, Proceedings of the Third International Symposium", University Park Press, Baltimore, 1970, (in press).
7. Suzuki, K., Takemori, S., and Katagiri, M., Biochim. Biophys. Acta, 191, 77 (1969).
8. Yamamoto, S., Katagiri, M., Maeno, H., and Hayaishi, O., J. Biol. Chem., 240, 3408 (1965).
9. Yphantis, D. A., Biochemistry, 3, 297 (1964).
10. Schachman, H. K., in S. P. Colowick and N. O. Kaplan (Editors), "Methods in Enzymology", Vol. 4, Academic Press, New York, p. 32 (1957).
11. Fish, W. W., Mann, K. G., and Tanford, C., J. Biol. Chem., 244, 4989 (1969).

12. Zamenhof, S., in S. P. Colowick and N. O. Kaplan (Editors), "Methods in Enzymology", Vol. 3, Academic Press, New York, p. 696 (1957).
13. McCord, J. M., and Fridovich, I., J. Biol. Chem., 244, 6049 (1969).
14. Massey, V., Strickland, S., Mayhew, S. G., Howell, L. G., Engel, P. C., Matthews, R. G., Schuman, M., and Sullivan, P. A., Biochem. Biophys. Res. Commun., 36, 891 (1969).