KINETIC MECHANISM OF THE REDUCTIVE HALF OF REACTION CATALYZED BY SALICYLATE HYDROXYLASE

Lee-Ho Wang, Shiao-Chun Tu

Department of Biochemical and Biophysical Sciences, University of Houston-University Park, Houston, Texas 77004, U.S.A.

Introduction

Salicylate hydroxylases have been isolated from Pseudomonas putida (1), Pseudomonas sp. 29351 (2), P. sp. 29352 (3), and Pseudomonas cepacia (4,5). All four species of this hydroxylase exhibit some differences in structural and/or kinetic properties. The kinetic mechanisms for the first three species have been partially resolved (2,6,7). In the present study, we have examined the kinetic mechanism of the P. cepacia enzyme, with a particular emphasis on the reductive half of reaction.

Results and Discussion

Initial velocities of salicylate hydroxylase were examined at various levels of the substrates salicylate, NADH, and O_2. Double reciprocal plots of initial rates versus salicylate concentrations at a constant level of oxygen and several fixed concentrations of NADH yielded a set of linear lines that converged to a common point. Using the same data, double reciprocal plots of initial rates versus varying NADH concentrations at a constant level of O_2 and several constant concentrations of salicylate again yielded a family of converging lines. These results clearly indicate that the hydroxylase is capable of forming a ternary complex containing salicylate and NADH. Double reciprocal plots of initial rates versus NADH concentrations at a fixed concentration of salicylate and several constant levels of oxygen, however, produced a set of parallel lines. Similarly, parallel lines were also obtained from double

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reciprocal plots of initial rates versus varying salicylate concentrations at a constant level of NADH and several fixed concentrations of oxygen. These results indicate that, subsequent to the binding of salicylate and NADH, a product is released and then oxygen binds to an enzyme form which is different from the original oxidized enzyme. Our results are consistent with a reaction mechanism involving, sequentially, the binding of salicylate and NADH to form a ternary complex, the reduction of the enzyme-bound FAD by NADH, the release of NAD^+ as a product, the binding of O_2 to the reduced enzyme:salicylate complex, the formation and release of products, and the regeneration of the oxidized holoenzyme.

Results described thus far, however, do not distinguish a random from a fixed-order binding of salicylate and NADH. This was resolved by comparisons of dissociation constants for salicylate (K_{Sal}) and NADH (K_{NADH}) determined by equilibrium measurements with those deduced from the steady-state kinetic results (8). At 23°C and pH 7.6, K_{Sal} and K_{NADH} were calculated to be 12.5 μM and 0.4 mM, respectively, based on the above described steady-state measurements. Using equilibrium techniques, K_{Sal} and K_{NADH} were found to be 12 μM and 0.45 mM, respectively, corresponding well with those deduced from initial rate determinations. Following the arguments detailed previously (8,9), these observations indicate a random binding of salicylate and NADH to the enzyme.

Such a conclusion is further supported by the examination of tritium isotope effect. Salicylate hydroxylase catalyzes a slow oxidation of NADH to form H_2O_2 in the absence of any benzenoid substrate. Benzoate greatly stimulates the rate of NADH oxidation and leads to stoichiometric formation of H_2O_2 without being hydroxylated itself. Using (4R)-[4-^{3}H]NADH and benzoate as cosubstrates, a tritium isotope effect on V_m/K_m ($^TV/K$) was observed. Such an isotope effect was dependent upon the level of benzoate used (Fig. 1). Based on the analysis described previously (10), $^TV/K$ should be independent of benzoate concentration if benzoate binds to enzyme prior to NADH. On the other hand, $^TV/K$ should be reduced to unity as the benzoate concentration approaches infinity if NADH binds to the enzyme prior to benzoate. Only when benzoate and NADH bind to the hydroxylase in a random order, $^TV/K$ will decrease at higher con-

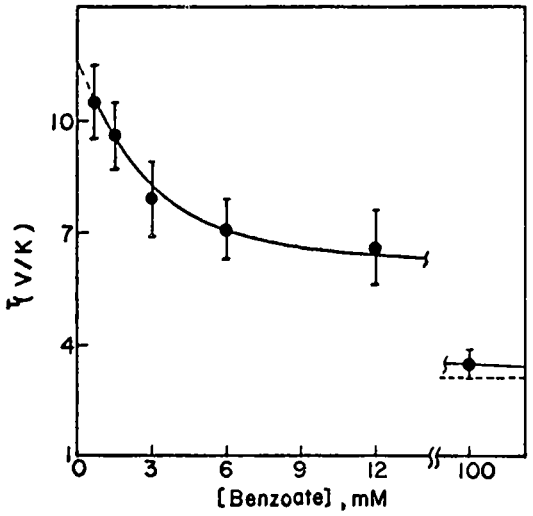

Fig. 1. Dependence of the $^{T}V/K$ effect of (4R)-[4-^{3}H]NADH on benzoate concentration. Isotope effects were determined at 23°C in 0.02 M KPi, pH 7.6, containing 18 nM enzyme, 0.1 mM tritiated NADH, and various amounts of benzoate as indicated.

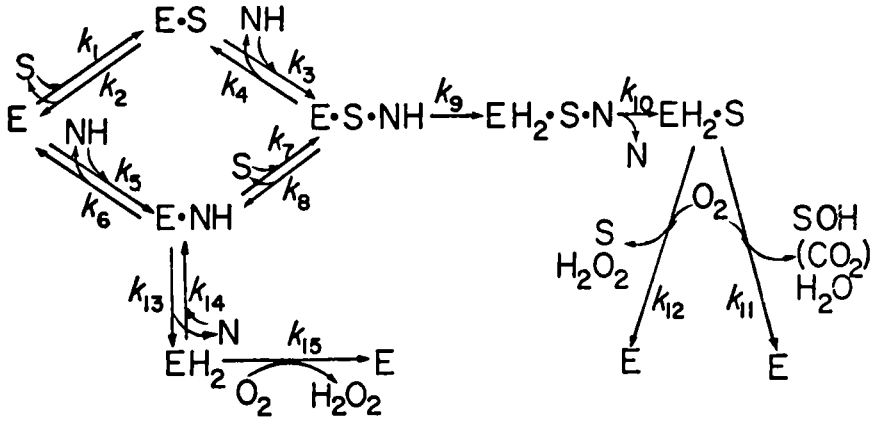

Fig. 2. Kinetic mechanism of salicylate hydroxylase. E and EH_2 are oxidized and reduced enzyme, respectively; N and NH are NAD^+ and NADH, respectively; S and SOH are substrate (or effector) and hydroxylated product, respectively. The isotope-sensitive step is indicated by k_9.

centrations of benzoate but reaches a final level significantly larger than 1 at infinite concentration of benzoate. Our results thus clearly indicate a random binding for NADH and benzoate. At 0.14 mM salicylate, (4R)-[4-^{3}H]NADH also exhibited a $^{T}V/K$ effect of 3.36 ± 0.37.

A mechanism is thus proposed (Fig. 2) to depict both the substrate hydroxylation and H_2O_2 formation activities of salicylate hydroxylase.

Acknowledgment

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