Multisubstrate Inhibition of 4-Hydroxybenzoate 3-Monooxygenase

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4-Hydroxybenzoate 3-monooxygenase (*p*-hydroxybenzoate hydroxylase, PHBH; EC 1.14.13.2), isolated from *Pseudomonas fluorescens,* is a very well-characterized NADPH-dependent flavin-monooxygenase[1–5] which catalyzes the conversion of *p*-hydroxybenzoate to 3,4-dihydroxybenzoate (Scheme 1). Extensive enzyme kinetics,[6] inhibition studies,[7–9] and X-ray crystallographic studies[10,11] have been published. The enzyme mechanism has been characterized as a random sequential addition of the two substrates *p*-hydroxybenzoic acid (1) and NADPH to the enzyme and oxidized flavin cofactor.[6] After the NADPH reduces the flavin ring and leaves the active site, molecular O_2 enters to form the oxidizing species, flavin hydroperoxide.[3,12] Oxidation of the aromatic ring proceeds, thereby fulfilling the catalytic cycle of the enzyme. We wished to study this enzyme as a model for kynurenine 3-monooxygenase (EC 1.14.13.9), which is an important enzyme on the pathway from tryptophan to the neurotoxin quinolinic acid.[13] In particular, we wished to design effective inhibitors of the enzyme. Since PHBH uses two substrates which randomly enter the active site of the enzyme, we felt that we could rationally design a multisubstrate, competitive inhibitor of PHBH.

Multisubstrate inhibitors are attractive for two major reasons. First, they are frequently very selective since the inhibitor combines features of both substrates, so there should be little or no inhibition of enzymes which utilize only one of the substrates. Second, the potency of these types of inhibitors can be extremely high, by taking advantage of binding interactions at both substrate sites simultaneously as well as potential enthalpic gains due to reduced molecularity.[14] A number of good examples are discussed in a recent review.[15]

We have relied heavily on published X-ray studies of PHBH complexed with the product 3,4-dihydroxybenzoate[10,11] to design a potent inhibitor. These and other studies have shown that substrate or product lies perpendicular to the plane of the flavin while the NADPH fills a cleft on the *re* face of the flavin.[16] In our modeling experiments, the reaction product was converted from 3,4-dihydroxybenzoate to *p*-hydroxybenzoate, FAD was modeled in its oxidized form, and NADPH was docked with the appropriate hydrogen of its six-membered ring on the *re* face of the B flavin ring. The entire structure was then minimized[17] in order to remove unfavorable contacts of the NADPH. Figure 1a shows the Cα trace of PHBH, the cofactor, and both
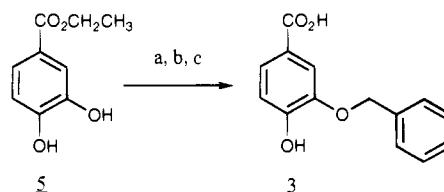
Scheme 1
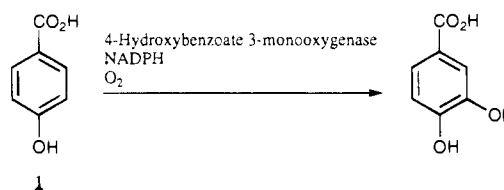
Scheme 2
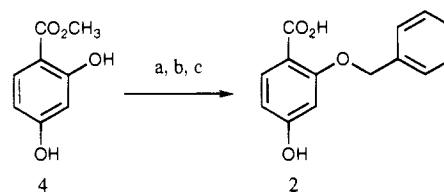
Conditions: (a) Ac_2O, Et_3N, DMAP, Et_2O, room temperature, 1 h; 80%; (b) NaH, benzyl bromide, DMF, room temperature, 5 h; 87%; (c) LiOH, THF, H_2O, 50 °C, 5 h, 46%.

Scheme 3

Conditions: (a) benzyl bromide, K_2CO_3, acetone, reflux, 16 h; (b) silica gel chromatography (10% EtOAc/hexane); (c) LiOH, THF, H_2O (1/1), 40 °C, 16 h, 10% overall yield.

substrates after minimization. The actual distance (measured from the model) of the hydrogen which is transferred to the flavin nitrogen at position 5 is 2.8 Å. Figure 1b shows the cofactor and the two substrates in the same orientation as shown in Figure 1a, except with the Cα trace of the enzyme removed. In designing new inhibitors, we sought to simply mimic NADPH with an unsubstituted phenyl ring with the anticipation that there would be a stabilizing $\pi-\pi$ interaction between the aromatic rings of this group and the flavin nucleus. A methyleneoxy moiety was incorporated to link this simplified NADPH mimic to *p*-hydroxybenzoic acid. Thus, the ether derivatives 2 and 3 were modeled in the active site of the enzyme.

In both modeling experiments, the potential inhibitors (2 and 3) were placed in the enzyme site; the dihydroxybenzoic acid portion of 2 or 3 was oriented in the region previously occupied by 1, and the benzyl ether aromatic ring was located on the *re* face of the flavin cofactor. The x-ray structure was used as the starting point for both simulations, and the same method of minimization which was used for the substrates was applied to both inhibitor systems. The results are shown in parts a and b of Figure 2 for inhibitors 3 and 2, respectively, with the orientation being the same as that of the substrates. Inhibitor 2 remained stacked under the flavin's B ring with its position very similar to the *p*-hydroxybenzoate substrate, whereas inhibitor 3 had moved considerably out of plane, suggesting that 2 would potentially be the better of the two inhibitors due to the favorable $\pi-\pi$ interaction.

The syntheses of 2 and 3 are shown in Schemes 2 and 3, respectively. Acetylation of the more reactive phenol of methyl 2,4-dihydroxybenzoate (4) followed by benzyl-

a

b

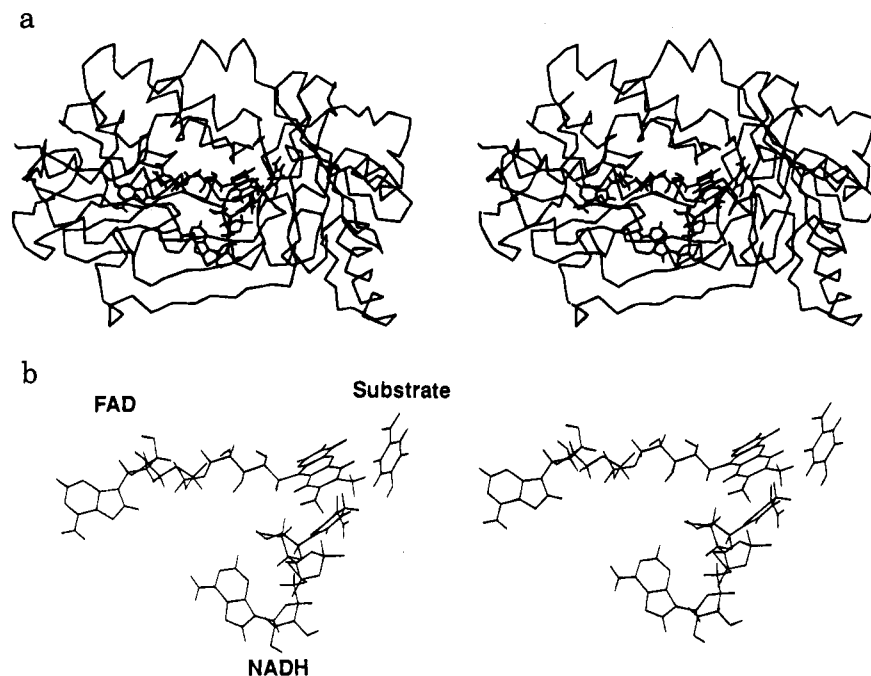**FAD**   **Substrate**

NADH

Figure 1. (a) Stereoview of the minimized[17] structure of p-hydroxybenzoate hydroxylase (PHBH), with cofactor FAD and bound substrates NADH and 4-hydroxybenzoate. Only the Cα trace of the enzyme is shown for clarity. (b) Same as part a except the Cα trace was removed to clearly show the orientation of the cofactor and substrates.
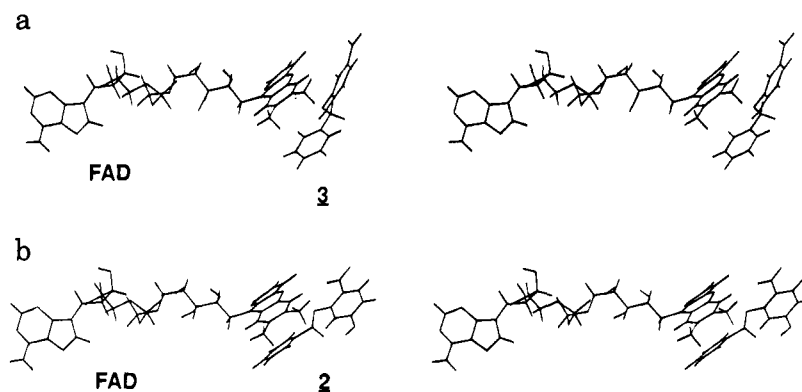
a

FAD

3

b

FAD   **2**

Figure 2. (a) Stereoview of minimized[17] structure of PHBH, cofactor, and compound **3**. Again the enzyme Cα trace was removed for clarity. (b) Same as part a except compound **2** was used.

ation and deprotection and hydrolysis led to **2** in good yield. Since the phenolic groups in ethyl 3,4-dihydroxybenzoate (**5**) were of similar reactivity, **3** was prepared by nonselective benzylation, separation, and hydrolysis. Elemental analysis, ^{1}H and ^{13}C NMR, and mass specra of all compounds were consistent with the assigned structures.

The compounds **2** and **3** were evaluated as inhibitors of PHBH using the method described by Entsh et al.[3] (Figures 3 and 4). In agreement with the molecular modeling predictions, **2** had a significantly higher affinity for the enzyme than the isomeric **3** ($IC_{50} = 100$ μM) and clearly showed a competitive inhibition pattern against both substrates. In fact, **2** was shown to be the most potent, competitive inhibitor thus far reported for this enzyme, with K_i values of 59 ± 6 and 69 ± 12 nM versus p-hydroxybenzoic acid (**1**) and NADPH, respectively.[18] In addition, these values demonstrate a large increase in affinity of **2** compared to the normal substrates, p-hydroxybenzoic acid (420-fold; $K_m = 21$ μM) and NADPH (800-fold; $K_m = 57$ μM). This increase in binding affinity is remarkable especially considering the simplification of the NADPH substrate to a benzyl
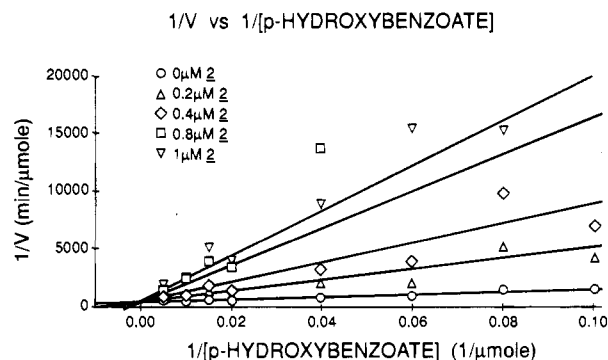
1/V vs 1/[p-HYDROXYBENZOATE]

1/[p-HYDROXYBENZOATE] (1/μmole)

Figure 3. Lineweaver–Burk plot of $1/V$ vs $1/[p$-hydroxybenzoate]. Concentration of substrate was varied from $0.5K_m$–$10K_m$ with a fixed NADPH concentration of 200 μM. p-Hydroxybenzoate: $K_m = 21$ μM, $V_{max} = 0.0021$. Compound **2** vs p-hydroxybenzoate: $K_i = 59$ nM.

moity. Presumably, more potent inhibitors could be obtained by utilizing aromatic compounds which incorporate substitutions that can take advantage of other residues on the enzyme which interact with the NADPH.
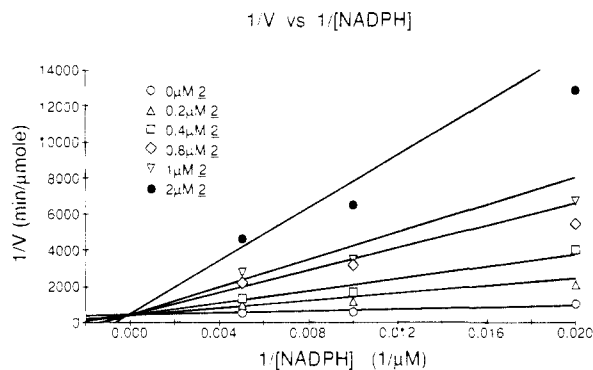
Figure 4. Lineweaver−Burk plot of $1/V$ vs $1/$[NADPH]. Concentration of substrate was varied from $1K_m$–$3.5K_m$ with a fixed p-hydroxybenzoate concentration of 100 μM. NADPH: $K_m = 57$ μM, $V_{max} = 0.0023$. Compound 2 vs NADPH: $K_i = 69$ nM.

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(17) Minimizations were done using Discover 2.8 (Biosym Technologies Inc., San Diego, CA). Each minimization loop employed 100 iterations of steepest descents followed by 900 iterations of conjugate gradients and was repeated until a maximum derivative criterion was met. In loop 1, only hydrogens were minimized until the maximum derivative was less than 1.0 kcal/Å. In loop 2, only hydrogens and all water molecules were minimized until the maximum derivative was less than 1.0 kcal/Å. In loop 3, all hydrogens, all water molecules, and all side chains of PHBH were minimized until the maximum derivative was less than 1.0 kcal/Å. In loop 4, everything was minimized except the backbone of PHBH until the maximum derivative was less than 1.0 kcal/Å. Finally, in loop 5, the entire system was minimized until the maximum derivative was less than 1.0 kcal/Å. The following variable parameters were used: cutoff = 11.0 Å, cutdis = 10.0 Å, swtdis = 1.5 Å, dielectric = 2.0·R. Default settings were used for all other variable parameters.

(18) Competitive inhibition was established by fitting data to Dr. Dexter Northrop's Megabasic adaption of Duggleby's nonlinear regression Basic program. Duggleby, R. G. *Comput. Biol. Med.* **1984**, *14*, 447−455.