Probing the Mechanism of 2-Methyl-3-hydroxypyridine-5-carboxylic Acid Oxygenase by Using 8-substituted-FAD Analogs.

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Introduction

2-Methyl-3-hydroxypyridine-5-carboxylic acid (MHPC) oxygenase (MHPCO) is involved in the degradation of vitamin B_6 by the soil bacterium, *Pseudomonas* MA-1 [1]. MHPCO catalyzes an oxygenation and a ring-cleavage reaction of its substrate, 2-methyl-3-hydroxypyridine-5-carboxylic acid (MHPC), to yield α-(N-acetylamino methylene)-succinic acid. Previous studies have shown that MHPCO belongs to the aromatic flavoprotein hydroxylase class of enzymes [2-5]. The gene of MHPCO has been cloned and the enzyme can be obtained in reasonable quantitites by expressing in an *E. coli* system, permitting the enzyme to be a good model for studying how the oxygenation of nicotinate-related compounds can be catalyzed by a flavoenzyme. Our research project is aimed to investigate the reaction mechanism of MHPCO by using FAD-analogs substituted at the 8-position of the isoalloxazine ring.

Materials and Methods

Apoenzyme preparation and FAD analog reconstitution

Native FAD was removed from the enzyme by ammonium sulfate precipitation at pH 3.0. All working buffers during the preparations contained 0.3 mM EDTA, 1 mM DTT, and 200 μM 5-hydroxynicotinate (5HN). The enzyme in 50 mM MOPS buffer, pH 7.0 was mixed with saturated ammonium sulfate solution (pH 3.0) with enzyme:ammonium sulfate 1:8 (v:v). The mixing process was carried out in a salt-ice bath and time exposure of the enzyme to acid ammonium sulfate was minimized. The suspension was centrifuged at 17,000xg for 4 min and the yellow supernatant was discarded. The pellet was resuspended with saturated ammonium sulfate solution (pH 8.0) and centrifuged at 17,000xg for 4 min. The pellet containing apoenzyme was resuspended with 30 mM MOPS buffer, pH 7.0 and precipitation at pH 3.0 was repeated for 2 additional cycles to convert all of the holoenzyme to the apoenzyme.

Apoenzyme was desalted by passing through a Sephadex G-25 column to remove the excess ammonium sulfate. For reconstitution with high redox potential flavin analogs (8-Cl-FAD and 8-CN-FAD), the column was equilibrated with 50 mM MOPS buffer pH 7.0 in the absence of DTT and the process was kept out of room light. Excess flavin analog (1.5–fold of enzyme) was added to the desalted apoenzyme and the mixture was passed through a Sephadex G-25 column. The reconstituted enzyme was stored at -80 ^{0}C in a freezer until used.

Methods for determining redox potential values and dissociation constants for enzyme-substrate complexes are described in references [2,6]. The pre-steady-state and anaerobic experiments were performed as described in [2,3,5].

Results and Discussion

Thermodynamic properties of MHPCO reconstituted with FAD analogs

Replacing FAD of MHPCO with four different FAD analogs having substituents at the 8-position did not greatly change enzymatic properties when compared with the native enzyme. Apoenzyme of MHPCO can bind tightly to the FAD analogs with K_d values less than 1 μM (results not shown). The binding resulted in perturbation of the flavin absorbance (Figure 1) and the fluorescence, implying that the isoalloxazine of FAD has close contact with the microscopic environment of the protein. Replacement of FAD slightly affected the binding affinity of the substrate (MHPC) or substrate analog (5HN) to the enzyme. MHPC binds to reconstituted enzymes less tightly than when it binds to the native enzyme. The K_d for binding of 5HN to MHPCO varies from 1 μM–20 μM (Table 1). Although the values of ligand binding are different when compared to those of native enzyme, these K_d values still indicate good binding of the enzyme with substrate or substrate analog.

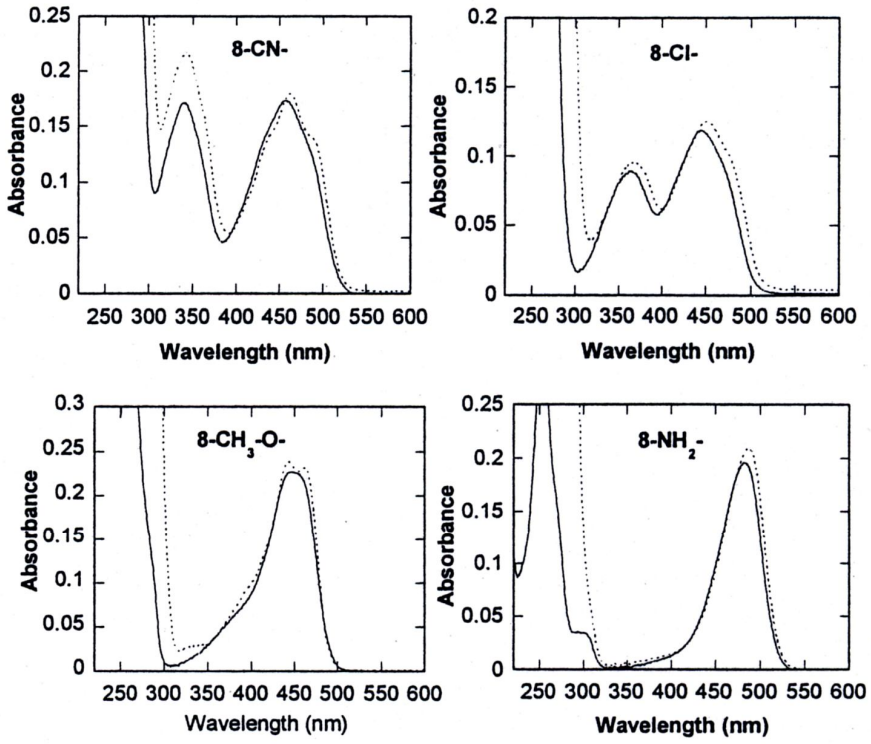

Figure 1 Molar absorption coefficient for MHPCO reconstituted with FAD analogs. The absorption spectra of free FAD analogs are shown in solid lines while dotted lines represent the spectra of the FAD analogs with saturating concentrations of apoenzyme. The concentration of free FAD analogs used were: 17.6 μM of 8-CN-FAD, 11.8 μM of 8-Cl-FAD, 10.6 μM of 8-methoxy-FAD, 4.5 μM of 8-NH_2-FAD.

Table 1 Dissociation constants for binding to enzymes reconstituted with FAD analogs in the presence of substrate (MHPC) or a substrate analog (5HN)

FAD analog	K_d for MHPC (μM)	K_d for 5HN (μM)
8-NH_2-MHPCO	82±2	0.35±0.07
8-Cl-MHPCO	50±3	9.3±0.8
8-CN-MHPCO	56±4	3.8±0.5
8-CH_3-O-MHPCO	159±7	16±1
Native enzyme	9.2±0.6	5.2±0.4

The redox potential values of FAD-analogs are shifted to more positive values when bound to MHPCO. Increases in redox potential values of flavins when bound to the enzyme were also observed in studies of *p*-hydroxybenzoate hydroxylase [7] and lactate oxidase [8]. The binding of MHPC or 5HN to the reconstituted MHPCO resulted in a slight increase of redox potential values for enzyme-substrate complexes, which is similar to the native MHPCO [2].

Table 2 Redox potential values of ligand-free and ligand-bound forms of reconstituted MHPCO.

FAD Analog	E_m^0 free (mV)				E_m^0 bound (mV)	
					MHPC	5HN
Native enzyme			-85		-78	-81
8-NH_2-FAD			-242		-233	-232
8-OCH_3-FAD			-210		-184	-204
	E^0_1	E^0_1	E^0_m	Semiquinone stability (%)		
8-Cl-FAD	-36	+20	-8	14.4	+11	+6
8-CN-FAD	+90	+40	+65	56.9	+80 - +219*	+80 - +219*

(*The redox potentials of ligand-bound 8-CN-MHPCO cannot be determined accurately by using available dyes. The values of the redox potentials were estimated to be in range of +80 to +219 mV by comparing with *N*-methylphenazinium methosulfate and 2,6-dichlorophenol indolephenol respectively).

Catalytic properties of the reconstituted enzyme.

Using stopped-flow spectrophotometry, the oxidized enzyme was mixed with various concentrations of NADH, and the reaction was followed at wavelengths between 450 nm–480 nm to monitor the fraction of oxidized enzyme. The substituent at the 8-position of FAD markedly affects the reduction rate of the enzyme-bound FAD. Rate constants of the hydride transfer steps are 20.8 s^{-1} for 8-Cl-FAD, 12.3 s^{-1} for native FAD [2], and 0.44 s^{-1} for 8-OCH_3-FAD (Figure 2). It is clearly shown that FADs with higher redox potentials are reduced faster than those with lower redox potentials. Studies of *p*-hydroxybenzoate hydroxylase with FAD analogs have shown similar observations [9] .Studies of the oxidative half-reaction have also been carried out to determine rate constants for the hydroxylation step of the MHPCO reconstituted with FAD analogs. Preliminary results show that the reaction of MHPCO reconstituted with 8-CN-FAD, 8-Cl-FAD, 8-OCH_3-FAD have rates for the hydroxylation step of 1.98, 1.69, and 1.39 s^{-1} respectively. The corresponding value for the native enzyme under the same conditions is 0.83-0.9 s^{-1} [3]. All of the reactions of FAD analogs exhibit spectra of C(4a)-hydroperoxyflavin and C(4a)-hydroxyflavin intermediates.

This indicates that the reaction of MHPCO reconstituted with FAD analogs goes through hydroxylation mechanisms similar to those for native enzyme. Hammett analysis has shown that the rate of hydroxylation is increased when the pKa of C(4a)-hydroxy flavin is decreased. This result indicates that MHPCO employs an electrophilic aromatic substitution mechanism for the hydroxylation reaction [7-8].

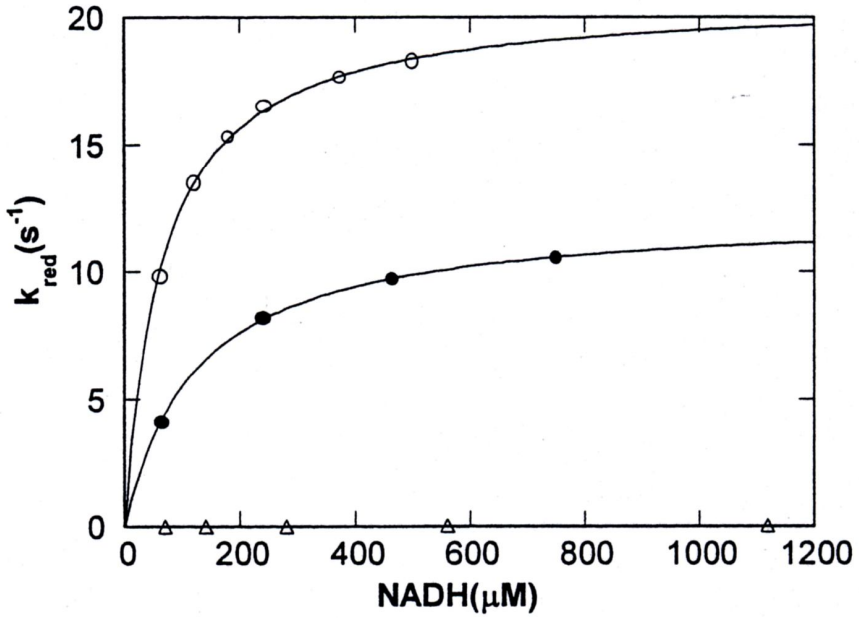

Figure 2 Reduction rate of MHPCO-bound FAD or FAD analogs by NADH. The reduction rate of MHPCO-bound FAD or FAD analogs were plotted versus NADH concentration. The rate values for 8-Cl-FAD, FAD, and 8-OCH_3-FAD enzymes were represented by empty circles, filled circle, and empty triangle respectively.

Acknowledgement

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References

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