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The design of an alternative, covalently flavinylated 6-hydroxy-D-nicotine oxidase by replacing the FAD-binding histidine by cysteine and reconstitution of the holoenzyme with 8-(methylsulfonyl)FAD

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Abstract The cofactor of several flavoenzymes is autocatalytically bound to the polypeptide via a histidyl(N_3)-(8α)-FAD linkage which makes the generation of apoenzyme difficult. We introduced an alternative covalent protein-FAD bond at the active site of 6-hydroxy-D-nicotine oxidase (6HDNO) by replacing the FAD-binding histidine with cysteine. The resulting mutant enzyme was expressed with noncovalently attached cofactor. Incubation with 8-(methylsulfonyl)FAD, and less efficiently with 8-chloro-FAD, resulted in the spontaneous replacement of the noncovalently bound FAD by the flavin derivative and the formation of an 8-(*N*-acetylcysteinyl)FAD linkage. The flavinylated 6HDNO.cys exhibited close to wild-type activity levels. This strategy may be generally applicable to the attachment of artificially designed flavin derivatives to the active site of covalently flavinylated enzymes.

Key words: Flavoenzyme; Site-directed mutagenesis; Covalent flavinylation; FAD

1. Introduction

An increasing number of flavoenzymes with covalently attached cofactors are being discovered [1]. The most common linkage between polypeptide and flavin is a histidyl(N_1 or N_3)-(8α)FAD bond. The bond is formed in an autocatalytic process during the folding of the enzyme inside the cell and once formed it is not possible for it to be hydrolyzed without degradation of the protein. The spontaneous attachment of the cofactor to the protein would represent a convenient way of introducing tightly bound FAD derivatives at the active centre of these enzymes. However, the difficulty in generating apoenzyme hampers this approach. An alternative covalent protein-FAD interaction consists of a cysteinyl-(8α)FAD bond, as realized in monoamine oxidase [2]. The related linkage 8-(*N*-acetylcysteinyl)FAD may form spontaneously between a protein cysteine and the highly reactive FAD derivatives 8-chloro-FAD [3] and, more efficiently, 8-(methylsulfonyl)FAD [4]. A prerequisite for the spontaneous formation of this bond is a proper steric position of the cysteinyl SH group with respect to the 8-position of the isoalloxazine ring [3]. Likewise, a proper steric interaction between histidyl(N_3) and 8α-CH_3 of FAD must be postulated for the autoflavinylation mechanism as demonstrated for 6-hydroxy-D-nicotine oxidase (6HDNO) [5]. By replacing the FAD-binding histidine of 6HDNO with Cys we tested on this model enzyme the prediction of a sterically favorable interaction between an amino acid side chain in this position and the isoalloxazine ring with the aid of 8-(methylsulfonyl)FAD and 8-chloro-FAD. We demonstrate that upon incubation with 8-(methylsulfonyl)-FAD the noncovalently bound FAD in the mutant 6HDNO.cys was spontaneously replaced by covalently bound 8-(*N*-acetylcysteinyl)FAD. The reconstituted enzyme showed enzyme activity close to wild-type levels. By this method it should be possible to generally introduce various functional flavin derivatives into the active site of His-FAD enzymes.

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Abbreviations: 6HDNO, 6-hydroxy-D-nicotine oxidase; PAGE, polyacrylamide gel electrophoresis

2. Material and methods

2.1. Chemicals

8-Cl-FAD was a kind gift from Dr. Massey, Michigan; all other chemicals were commercial products of the highest purity available.

2.2. Expression and purification of 6HDNO proteins

The replacement of the FAD-binding His_{71} of 6HDNO with Cys by site-directed mutagenesis has been described previously [6]. The *6hdno* wild-type and *6hdno.cys* genes were cloned into the expression vector p6HEx3 [7] as a translational fusion with an N-terminal extension of 6 histidine residues. This allowed the one-step purification of the fusion proteins from cell extracts of transformed *E. coli* JM109 cells on a nickel chelating Sepharose column [7]. The 6HDNO protein fraction eluted from the Ni^{2+} affinity column was over 95% pure, as judged from SDS-PAGE analysis.

2.3. Synthesis of FAD derivatives, reconstitution of holoenzyme and spectrometry

8-Cl-FAD was transformed into 8-(methylsulfonyl)FAD according to [4]. Purified 6HDNO.cys (0.25–0.3 μg/μl) was incubated with 10 μM 8-chloro- or 8-(methylsulfonyl)FAD in 40 mM HEPES, pH 7.4, 0.5 M NaCl, 0.1 M imidazole at room temperature for various times. Wild-type, 6HDNO.cys mutant and protein reconstituted with flavin derivatives were precipitated with 5% trichloroacetic acid, at 4°C, washed with 5% trichloroacetic acid by resuspension and the precipitate dissolved in 3 M guanidinium hydrochloride, 0.1 M Tris-HCl, pH 7.4, 3 mM EDTA. Visible spectra were recorded with the aid of a Spectrophotometer Ultrospec Plus, Pharmacia LKB (Freiburg, Germany); flavin fluorescence emission spectra (excitation at 440 nm) with a Perkin Elmer Luminescence Spectrometer LS 50B at a scan speed of 90 and an excitation and an emission slit with of 5 mm. 6HDNO activity was determined according to [8].

3. Results and discussion

The mutant protein, overexpressed and purified from *E. coli* cells, exhibits a yellow color and a visible absorption spectrum characteristic of a flavoenzyme (Fig. 1, spectrum 1). Treatment with trichloroacetic acid results in a colorless protein precipitate and a yellow supernatant (Fig. 1, compare spectra 2 and 3) demonstrating that 6HDNO.cys contains the FAD noncovalently bound to the protein. A dissociation constant K_d for FAD of 1.8×10^{-5} M^{-1} could be determined from

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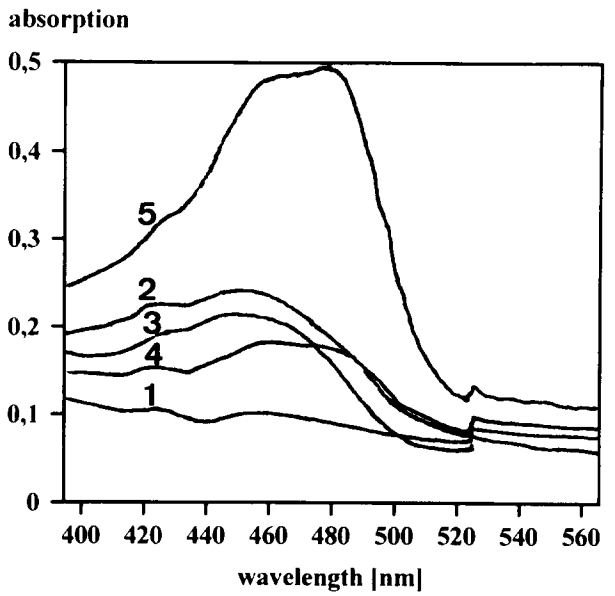

Fig. 1. Visible absorption spectra of 6HDNO.cys and 6HDNO.cys covalently modified with 8-(methylsulfonyl)FAD and 8-chloro-FAD. 4 ml of a 6HDNO.cys solution (0.3 mg/ml) and 4 ml of the same 6HDNO.cys solution that had been reacted with 8-(methylsulfonyl)-FAD or 8-chloro-FAD for 24 h at room temperature, were precipitated with 5% trichloroacetic acid, the protein pellets washed by resuspension in 5% trichloroacetic acid and dissolved at 1.2 mg protein/ml of 3 M guanidinium hydrochloride. Spectra: 1, 6HDNO.cys at 1.2 mg/ml prior to trichloroacetic acid precipitation; 2, supernatant of the trichloroacetic acid-precipitated 6HDNO.cys sample; 3, guanidinium hydrochloride solubilized 6HDNO.cys pellet obtained by trichloroacetic acid precipitation; 4, guanidinium hydrochloride solubilized pellet of 8-chloro-FAD reacted 6HDNO.cys; 5, guanidinium hydrochloride solubilized pellet of 8-(methylsulfonyl)FAD reacted 6HDNO.cys protein.

dialysis against increasing volumes of 20 mM Tris-HCl, pH 7.4, 100 mM NaCl buffer.

When the 6HDNO.cys protein was incubated at room temperature for 24 h in the presence of 10 μM 8-(methylsulfonyl)FAD, followed by precipitation with trichloroacetic acid the resulting protein pellet remained colored. The trichloracetic acid washed and guanidinium hydrochloride dissolved protein exhibited a visible absorption spectrum typical of 8-(*N*-acetylcysteinyl)FAD [9], with an absorption maximum at 480 nm (Fig. 1, spectrum 4). From the extinction coefficient of ε_{480}=25 200 M cm^{-1} for 8-(*N*-acetylcysteinyl)FAD [9] a molar ratio of 1:1 of protein and bound FAD was estimated. Thus, 8-(methylsulfonyl)FAD completely replaced FAD from the mutant protein by formation of a covalent protein-FAD adduct. Incubation under the same conditions of 6HDNO.cys with 8-chloro-FAD resulted in a less pronounced spectral shift (Fig. 1, spectrum 5). Evaluation of the fluorescence emission spectra of the trichloroacetic acid precipitated and guanidinium hydrochloride dissolved proteins revealed that only about 20% of 6HDNO.cys incorporated covalently 8-chloro-FAD as compared to the covalent incorporation of 8-(methylsulfonyl)FAD (Fig. 2). This finding is in agreement with the higher reactivity of the methylsulfonyl derivative as compared to the chloro substituted FAD [10,11].

The specific enzyme activity of the 6HDNO.cys and 6HDNO 8-(*N*-acetylcysteinyl)FAD determined during the first 5 min of the reaction was approx. 80% of that of the wild-type protein expressed and purified under the same conditions as the mutant protein. It has been demonstrated previously that the 6HDNO.cys activity in cell extracts shows saturation kinetics [6]. Similar kinetics were found for the methylsulfonyl-FAD substituted 6HDNO.cys (Fig. 3, open and closed circles). The linearity of the reaction with 6HDNO.cys and 6HDNO 8-(*N*-acetylcysteinyl)FAD was between 0.95 and 0.96 as compared to a linearity of always 1 for the wild-type enzyme. Formation of the histidyl(N_3)-(8α)FAD bond in the wild-type enzyme is stimulated in the presence of glycerol 3-phosphate [5]. The spontaneous formation of the cysteinyl-FAD bond in 6HDNO.cys was not significantly stimulated by glycerol 3-phosphate. However, the compound had a marked effect on the linearity of substrate turnover by the mutant and methylsulfonyl-FAD reconstituted enzyme, which became 0.99–1 (Fig. 3, open and closed triangles). Apparently, glycerol 3-phosphate intervened in some stage of the enzyme reaction preventing its inhibition.

The results presented in this communication demonstrate on a model flavoenzyme that replacement of the FAD-binding histidine by cysteine allows the spontaneous, covalent incorporation of 8α-substituted FAD at the active site. This strategy of introducing FAD derivatives into the active site may be generally applicable to naturally occurring covalently flavinylated enzymes of the histidine-FAD type. The data show that the histidine and the cysteine residue introduced into its place in the mutant protein are in a proper steric position to interact with the 8α- or 8-position of the isoalloxazine ring of FAD giving additional support to the autocatalytic mechanism of enzyme flavinylation [5,12-14].

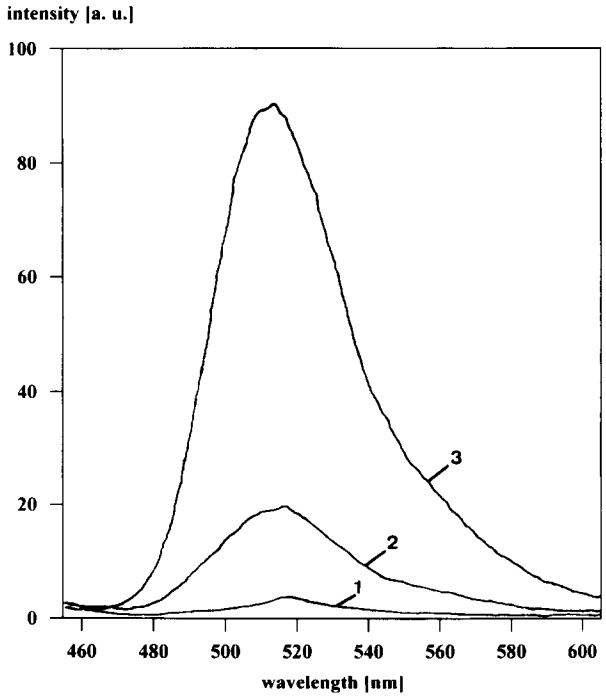

Fig. 2. FAD fluorescence emission spectra of 6HDNO.cys prior to and after reaction with 8-(methylsulfonyl)- and 8-chloro-FAD. The spectra of trichloracetic acid precipitated and 3 M guanidinium hydrochloride solubilized samples (prepared as described in the legend to Fig. 2) were recorded from: 1, 6HDNO.cys; 2, 6HDNO.cys reacted with 8-chloro-FAD; 3, 6HDNO.cys reacted with 8-(methylsulfonyl)FAD.

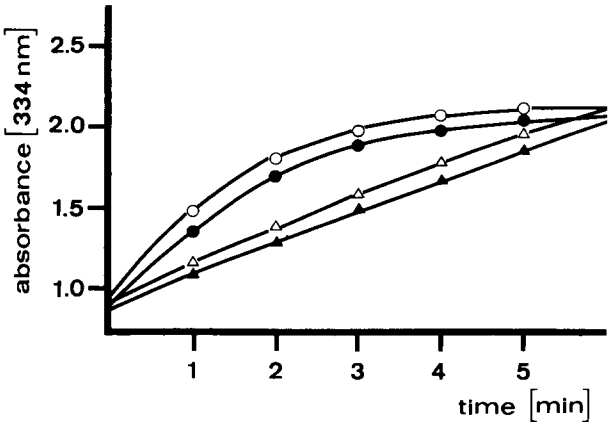

Fig. 3. Enzyme kinetics of unmodified and of 8-(methylsulfonyl)-FAD modified 6HDNO.cys. Enzyme activity of unmodified and modified 6HDNO.cys was determined without (open and closed circles, respectively) or after incubation with 1 mM glycerol 3-phosphate for 30 min at 30°C (open and closed triangles, respectively).

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