Design and properties of human D-amino acid oxidase with covalently attached flavin

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An ''artificial flavinylation'' approach was developed to replace a native noncovalent flavin prosthetic group with a covalently attached flavin analogue in recombinant human D-amino acid oxidase. The protein residue Gly-281 was replaced with Cys by site-directed mutagenesis, followed by reaction between mutated apoenzyme and the thiol-reactive flavin analogue, 8-methylsulfonyl FAD. The stoichiometric process of flavin attachment was accompanied by gain in enzymatic activity, reaching up to 26% activity of the recombinant native enzyme. The steady-state kinetic data together with the results of limited proteolysis and benzoate-binding studies suggest that, although mutation perturbs protein structural and catalytic properties, the flavinylation alone does not have any negative impact. We conclude that, despite the implemented restraints on its mobility, the covalently attached flavin is properly positioned within the protein active site and acts efficiently during D-amino acid oxidase catalytic turnover.

Flavin (flavin adenine dinucleotide, FAD; flavin mononucleotide, FMN; riboflavin) is a prosthetic group of flavoproteins and serves in biological systems as a versatile electron carrier by means of its unique physical and chemical properties (1, 2). Although the majority of flavoproteins contain noncovalently bound flavin, there is a distinctive group of enzymes where the flavin ring system is covalently linked to an amino acid residue such as Cys, Tyr, or His. Despite recent advances in the understanding of the chemistry of covalent attachment of flavin to protein (3), it is still unclear why evolutionary pressure was applied to create the complex mechanism of protein flavinylation. One of the approaches to this problem could be to study the properties of flavoprotein models where the native, noncovalently bound flavin is replaced with a covalently attached one. This, in principle, could be achieved by a combination of methods such as rational protein design, site-directed mutagenesis, and the use of reactive flavin analogues such as 8-methylsulfonyl flavin. The latter is a highly specific thiol-reactive affinity probe (4) that has been previously applied toward a number of flavoproteins to identify a reactive cysteinyl residue within the flavin-binding site (5–7) and also, in a recent study on the alternative flavinylation of 6-hydroxy-D-nicotine oxidase (8).

The subject of the present study was the flavoprotein D-amino acid oxidase (DAAO) from human kidney (hDAAO). The enzyme contains noncovalently bound FAD and catalyzes the oxidative deamination of D-amino acids to their corresponding imino acids, whereas molecular oxygen (second substrate) undergoes reduction to hydrogen peroxide. The structural-functional properties of the enzyme from pig kidney, in particular, and various other sources have been extensively studied (9). In addition, the crystal structure of pig DAAO has been established recently (10, 11). The cDNA of hDAAO has been determined and revealed an encoded protein with an amino acid sequence more than 80% identical to that of pig DAAO (12). Although the sequence alignment data indicate that the human and pig enzymes should exhibit very similar structural and catalytic properties, the human enzyme has not been isolated and characterized.

We have obtained recombinant hDAAO with covalently attached flavin as a result of the reaction between 8-methylsulfonyl FAD and the protein with a Cys introduced at residue 281 by site-directed mutagenesis. The catalytic and spectral properties of the flavin-linked protein construct are described and compared with those of the recombinant wild-type enzyme.

Materials and Methods

D-alanine, FAD, PMSF, and 1–1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin were from Sigma. Premixed SDS/PAGE standards were from Bio-Rad. The automated oligonucleotide synthesis and DNA sequence analyses were performed at the University of Michigan Biomedical Research Core Facilities on Applied Biosystems instruments (Models 394 and 377).

Cloning and Expression. The plasmid containing a hDAAO cDNA (12) inserted into a pET11b vector (pET-hDAAO) was constructed by using a unique *Nde*I site of pET11b (Novagen) as ATG cloning site according to the procedure described by Studier *et al.* (13). The IMPACT T7 one-step protein purification system with the pTYB2 vector was obtained from New England Biolabs. The hDAAO cDNA was subcloned from pET-hDAAO into a pTYB2 vector as follows: pET-hDAAO was subjected to an 18-cycle PCR with *Pfu* DNA polymerase (Stratagene) by using hDAAO-specific oligonucleotide primers, 5′-ATATA-*CATATG*CGTGTGGTGGTGA-3′ (forward) and 5′-GAGGT-GGGATGGTGGCATT-3′ (reverse). The forward primer design allowed us to introduce a unique *Nde*I site (above italics) at the 5′ end of the PCR product. The amplified 1-kb PCR product was digested with *Nde*I enzyme (New England Biolabs), purified by agarose gel electrophoresis, and ligated into a pTYB2 vector that was predigested with *Nde*I and *Sma*I enzymes. The ligation reaction was conducted overnight at 16°C by using a T4 DNA ligase (New England Biolabs) according to the manufacturer's protocol. The resulting pTYB-hDAAO construct was isolated by transformation into an *Escherichia coli* nonexpression host DH5α supercompetent cells (GIBCO/BRL) followed by selective growth on ampicillin/Luria broth agar plates at 37°C. Single colonies were picked up, grown overnight at 37°C in 5 ml of standard ampicillin/Luria broth liquid media, plasmids were isolated with QIAquick kit (Qiagen, Chatsworth, CA), and analyzed for the presence of a 1-kb insertion by *Nde*I and/or *Nco*I enzyme digest followed by agarose gel electrophoresis. The

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Abbreviations: DAAO, D-amino acid oxidase; hDAAO, human kidney DAAO; bicine, *N,N*-bis(2-hydroxyethyl)glycine. This work was supported by National Institutes of Health Grant GM 11106.

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isolated pTYB-hDAAO plasmid was transformed into an *E. coli* BL21(DE3) strain (Novagen) as an expression host and single colonies were subsequently isolated and grown in 5 ml of ampicillin/Luria broth media as described above. The plasmid-containing *E. coli* cultures were stored in 15% glycerol at −70°C until further use. The hDAAO Gly-281 → Cys mutation required a single-base substitution (**G**GC → **T**GC) in pTYB-hDAAO plasmid and was conducted by using a QuickChange Mutagenesis kit (Stratagene) according to Stratagene protocol. The substitution was verified by plasmid DNA sequence analysis.

The hDAAO expression was induced by adding 0.3 mM isopropyl β-D-thiogalactoside to plasmid-carrying *E. coli* BL21(DE3) cells during exponential growth in ampicillin/Luria broth media ($A_{600} \approx 0.6$) and was performed for 5 h at 37°C. Cells were harvested by centrifugation, washed with cold 3% NaCl solution, and kept frozen at −20°C.

Purification Procedure. The hDAAO purification was conducted at 4°C. Normally, 10 g of frozen cell paste was resuspended in 80 ml of cold 50 mM Tris·HCl buffer (pH 8.0) containing 1 mM sodium benzoate, 1 mM EDTA, 1 mM ATP, and 1 mM PMSF. After sonication, the free-cell extract was cleared by centrifugation and mixed with NaCl (to a concentration of 1 M) before applying onto the 5-ml affinity column (Chitin Beads from Stratagene) equilibrated with 50 mM Tris·HCl buffer (pH 8.0) containing 1 mM sodium benzoate/1 mM EDTA/1 M NaCl (buffer A). The sample loading was followed by subsequent wash steps with 100 ml of buffer A and 20 ml of buffer B (buffer A minus NaCl). Then, 10 ml of buffer C (buffer B containing 50 mM hydroxylamine) was applied and the column was left in buffer C overnight at 4°C to cleave hDAAO off the intein fusion partner. After overnight cleavage with hydroxylamine, the hDAAO was eluted from the column with 10 ml of buffer B, concentrated on Centricon 30 to 1 ml volume, passed through a Bio-gel P-6 desalting column (Bio-Rad) equilibrated with 50 mM *N*,*N*-bis(2-hydroxylethyl)glycine (bicine) buffer (pH 8.3) containing 1 mM EDTA, and finally stored in this buffer on ice until further use.

Spectroscopy. Absorbance data were recorded by using a Cary 3 spectrophotometer. The concentration of the wild-type hDAAO was estimated based on the extinction coefficient of 11,300 M^{-1} cm^{-1} per enzyme-bound flavin at 455 nm as reported for pig DAAO (14). The concentration of apoprotein was determined by using the extinction coefficient of 73,000 M^{-1} cm^{-1} at 280 nm that in turn was calculated from hDAAO amino acid sequence data with the PROTEAN program (DNAstar, Madison, WI). 8-Methylsulfonyl FAD was prepared according to Raibekas *et al.* (4). The concentration of 8-methylsulfonyl FAD was estimated based on the extinction coefficient of 11,200 M^{-1} cm^{-1} at 450 nm (4).

Anaerobic samples were prepared in anaerobic cuvettes by applying 10 cycles of evacuation and flushing with oxygen-free argon. The anaerobic photoreduction in the presence of 1 mM EDTA was performed as described by Massey and Hemmerich (15). The specific oxidase activity with D-alanine as a substrate was measured spectrophotometrically in 50 mM bicine buffer (pH 8.3) and 25°C similar to that described (16). The apoprotein of the wild-type hDAAO was prepared by extensive dialysis of the sample against 50 mM bicine buffer (pH 8.3) containing 1 mM EDTA and 1% (wt/vol) of activated charcoal.

Results and Discussion

Protein Design Strategy. The general strategy was to identify an amino acid residue at the shortest distance from the 8-methyl group of the flavin ring based on the available protein structural data and replace this residue with Cys. The latter would serve as a flavin attachment site on protein reconstitution/reaction with 8-methylsulfonyl FAD and could presumably place the attached flavin in a correct position within the protein active site to permit catalysis. The target residue was identified in hDAAO as Gly-281. Although the crystal structure of hDAAO is unknown, this protein shows 84% identity to the pig enzyme based on the amino acid sequence comparison data and Gly-281 as a conserved residue (12). Furthermore, the crystal structure of pig DAAO has been determined (10, 11) and it has been noted that Gly-281 is in van der Waals contact with the 8-methyl group of the flavin ring (10) (Fig. 1). By using pig DAAO structural data, we built a three-dimensional model of the human enzyme with the simulated Gly-281 → Cys mutation. As depicted in Fig. 1, this mutation could bring the thiol group within 1 Å distance to the 8-methyl group of the isoalloxazine ring of FAD in hDAAO.

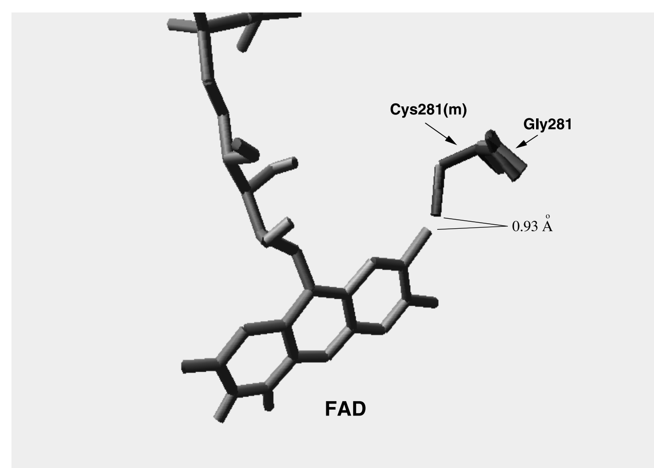

Fig. 1. The suggested position of Cys-281 with respect to the flavin ring in the modeled hDAAO structure. The structure was drawn with the SWISS-PDBVIEWER program. The hDAAO structure was modeled with the SWISS-MODEL program by using a pig DAAO Protein Data Bank coordinate file 1AA8 as a template. The Gly-281 is shown according to the pig DAAO structure, whereas Cys-281(m) is shown as a result of a simulated mutation of Gly-281 → Cys in the modeled hDAAO structure. The mutation was simulated with the SWISS-PDBVIEWER tool.

Protein Expression and Purification. The study was initially focused on the estimation of the total amount of enzyme expressed in *E. coli* cells carrying the pET-hDAAO expression vector. The DAAO activity was measured in free-cell extracts after 4 h of isopropyl β-D-thiogalactoside induction at 37°C with purified pig DAAO as a standard. This resulted in estimated expression as low as 1 mg enzyme/liter culture, making isolation of pure enzyme a very difficult task. Variable growth conditions were attempted, such as different temperature, isopropyl β-D-thiogalactoside concentration, and induction time, but they did not increase the enzyme expression level. Consequently, a new construct was made by using a T7 promoter-based pTYB2 vector (see *Materials and Methods*). This permits expression of hDAAO as a fusion partner with intein carrying a chitin-binding domain and the achievement of a one-step affinity purification of enzyme together with nonenzymatic removal of the intein–chitin-binding domain tag (17). The pTYB-hDAAO vector-derived expression system was therefore employed in these studies as a routine source for obtaining wild-type enzyme and G281C hDAAO. The purification was conducted as described in *Materials and Methods* and normally yielded 0.3–0.5 mg protein/liter culture. The protein was about 95% pure and ran as a 39-kDa band as judged by SDS/PAGE. The contaminating 60-kDa band that was originally found in the purified enzyme solution was efficiently separated when 1 mM ATP was added to the lysis buffer and was therefore attributed to an *E. coli*

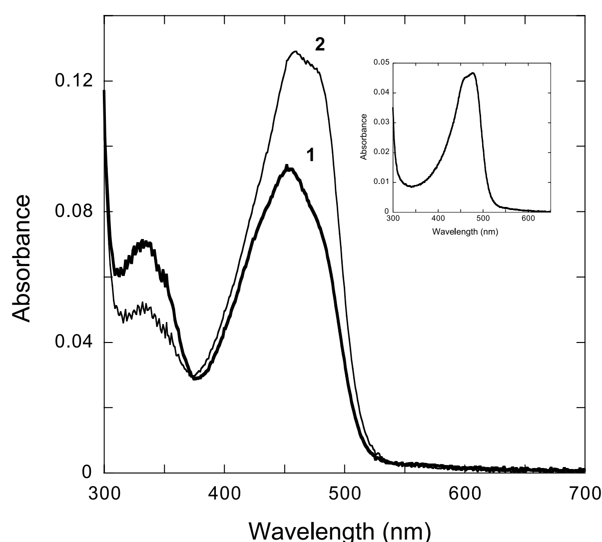

Fig. 2. Absorbance spectra generated during the reconstitution of 8-methylsulfonyl FAD with G281C hDAAO. G281C apoprotein (4 μM) in 50 mM bicine buffer (pH 8.3) containing 1 mM EDTA was incubated with 8 μM 8-methylsulfonyl FAD at 15°C. Spectra were taken after a 2-min (curve 1) and 5-h (curve 2) incubation. (*Inset*) Final sample after passing through the P-6 desalting column (not corrected for dilution).

chaperone GroEL interacting with the recombinant enzyme (data not shown). The wild-type enzyme was purified in the flavin-bound form (holoprotein), whereas the isolated mutant enzyme contained no flavin (apoprotein).

Reconstitution with 8-Methylsulfonyl FAD. Because the purified mutant enzyme was present in inactive apoprotein form, it was directly used in the reconstitution experiments with a twofold excess of the flavin. As anticipated, the covalent attachment ($t_{1/2}$ = 50 min) proceeded through an increase in the flavin absorbance in the 400–500 nm region and was nearly complete after a 5-h incubation at 15°C (Fig. 2). The spectrum of the final sample (Fig. 2 *Inset*), taken after the excess of the unbound flavin was removed by passing it through the P-6 column, displayed properties of a covalently attached flavin (-S-FAD) with an absorbance peak at 478 nm and pronounced shoulder at 460 nm (5). When reconstituted protein in 1 ml buffer solution was precipitated at denaturing conditions with 5% TCA followed by centrifugation, the resulting supernatant was colorless and displayed no flavin absorbance, whereas the protein pellet was yellow, indicating that all flavin was covalently attached to the protein. The protein pellet, washed with 1 ml of H_2O and redissolved in 1 ml of 6 M guanidinium·HCl (pH 8.3), exhibited an absorbance spectrum very close to that observed for nondenatured protein (data not shown). The extinction coefficient for the nondenatured FAD-S-enzyme of 23,000 M^{-1} cm^{-1} at 480 nm was calculated by using a value reported for free 8-(*N*-acetylcysteinyl) FAD (ε_{480} = 25,200 M^{-1} cm^{-1}) (18) and the difference in extinction at 480 nm between denatured and nondenatured FAD-S-enzyme. The extent of covalent incorporation was estimated as 0.95 mol flavin/mol protein with extinction values at 280 and 480 nm. The protein extinction value at 280 nm was corrected for flavin contribution ($\varepsilon_{280,\ corr}$ = 92,000 M^{-1} cm^{-1}) similar to that described earlier (5). As shown in Fig. 3, the hyperbolic kinetic process of flavin attachment was accompanied by the reestablishment of enzymatic activity. The specificity of flavin attachment was confirmed in a control experiment where wild-type apoprotein was reconstituted with 8-methylsulfonyl FAD and analyzed for the presence of the covalent flavin.

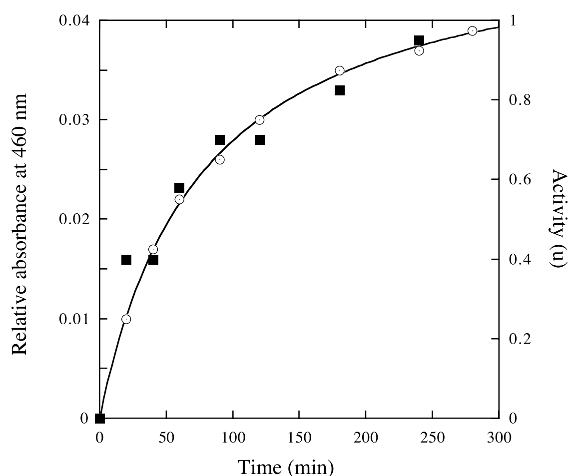

Fig. 3. Simultaneous flavin attachment and appearance of catalytic activity on reconstitution of mutant enzyme with 8-methylsulfonyl FAD. The flavin attachment was monitored by the increase in absorbance at 460 nm (○) under the conditions described in the legend of Fig. 2. During this process, 5-μl aliquots were withdrawn and immediately assayed for DAAO activity (■).

Practically no covalent incorporation (less than 0.03 mol flavin/mol protein) was observed and there was no detectable activity. The data clearly demonstrate that the flavin attachment to the mutant protein proceeds via a stoichiometric reaction with Cys-281 and that as soon as the flavin molecule was covalently attached to the protein molecule, the latter expressed its catalytic activity.

Steady-State Kinetic Properties. The steady-state kinetic data for air-saturated solutions are summarized in Table 1. The apparent k_{cat} and K_m values for the wild-type hDAAO with D-alanine as a substrate were similar to the corresponding values determined earlier for pig DAAO (19). The FAD-S-mutant enzyme displayed a turnover number 26% that of the wild-type enzyme and was about 40% higher than the turnover number determined for the mutant enzyme saturated with FAD. Conversely, the apparent K_m value with D-alanine for the mutant enzyme was about 40% lower than that of the covalently linked flavin enzyme, whereas both K_m values were an order of magnitude higher than that of the wild-type enzyme. Overall, the steady-state kinetic properties between FAD-saturated mutant protein and FAD-S-mutant protein were quite similar and differed (but not dramatically) from those displayed by the wild-type enzyme. The estimated dissociation constant (K_d) values for FAD binding indicated that the mutant protein binds FAD about nine times

Table 1. Apparent steady-state parameters of the human D-amino acid oxidase reaction with D-alanine

Sample	k_{cat}, s^{-1}	K_m (D-ala), mM	K_d (FAD), μM
Wild type	10.0*	1*	11
Mutant enzyme	1.5*	8*	90
FAD-S-mutant enzyme	2.6	13	n/a

The initial rates were measured at 436 nm and room temperature by using a coupled *o*-dianisidine/peroxidase assay (see *Materials and Methods*). The steady-state values that were obtained for the samples preincubated with a 100-fold excess of FAD are indicated by asterisks. Apparent K_d values for flavin binding were determined when either wild-type apoprotein (7 μM) or mutant protein (12 μM) were preincubated with various (1 μM to 2 mM range) concentrations of FAD for 20 min at room temperature followed by the assay. n/a = not applicable.

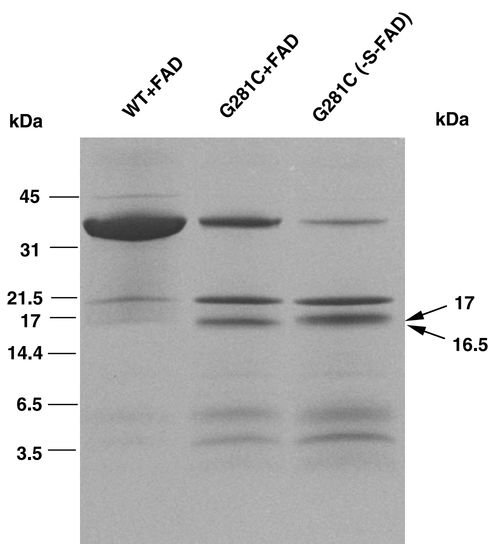

Fig. 4. Limited proteolysis of wild-type enzyme, mutant enzyme, and FAD-S-enzyme. Wild-type and mutant enzymes (but not FAD-S-enzyme) were preincubated with a 100-fold excess of FAD before the addition of trypsin. The 4-h digestion was conducted in the dark at room temperature in 50 mM bicine buffer (pH 8.3) containing 1 mM EDTA, and with a protease to substrate ratio of 1:200. The reaction was stopped by adding 20 mM PMSF. The digests were analyzed by SDS/PAGE according to Schagger and von Jagow (21). WT, wild-type enzyme; G281C, mutant enzyme; and G281C (-S-FAD), FAD-S-enzyme.

weaker compared with the wild-type enzyme, a result expected from the fact that the mutant enzyme was isolated in the apoprotein form. Moreover, the wild-type protein-FAD interaction was itself rather weak, allowing preparation of its apoprotein simply by dialysis against low-salt buffer.

Limited Proteolysis Studies. To investigate whether there was any difference in conformation between wild-type and mutant enzymes because of the mutation and/or covalently attached flavin, we tested the protein susceptibility toward proteolytic attack with trypsin followed by SDS/PAGE analysis. In fact, a significant difference was observed between samples after a 4-h tryptic digestion at room temperature (Fig. 4). Although the wild-type enzyme showed almost complete proteolytic resistance, both mutant- and FAD-S-mutant enzymes showed a similarly higher tryptic sensitivity, generating a digestion pattern of 21-, 17- (16.5-), 6-, and 4-kDa fragments. It must be noted that the FAD-S-mutant enzyme digest produced a slightly longer fragment (17 kDa) compared with that of the apoprotein (16.5 kDa) that could be attributed to the effect of the covalently attached flavin.

The limited proteolysis studies were in good accord with the steady-state kinetic data and suggest that although the mutant protein had a less packed conformation than that of the wild-type enzyme, which resulted in an active site with a decrease in catalytic activity and flavin affinity, the covalent attachment of the flavin did not have a negative impact on the catalytic properties of protein.

Benzoate Binding. Benzoate is known as a competitive inhibitor of pig kidney DAAO that binds to the enzyme active site (K_d = 3 μM) and perturbs the flavin absorbance spectrum in the 480–520 nm region, presumably because of an increase in the nonpolar environment around the flavin ring (20). Fig. 5 illustrates the effect of benzoate on the FAD-S-mutant and wild-type enzymes. When FAD-S-mutant enzyme was mixed with a large excess of benzoate, there was a dramatic effect on the 460–480 nm region of the spectrum resulting in a 30–40% decrease in absorbance and an appearance of a new peak at 470 nm and a weak shoulder at 490 nm (Fig. 5*A*). There was also a significant absorbance increase in the 310–390 nm region of the spectrum. These spectral changes indicate a considerable increase in the hydrophobic environment of the flavin on binding of benzoate, because similar changes are found with model 8-SR-flavins on changing the solvent from aqueous to apolar in nature (5). The spectral changes on benzoate binding to native DAAO are also consistent with this interpretation (20). The reversibility of binding was confirmed by restoration of the initial spectrum after the benzoate was removed from the sample mixture by gel filtration (data not shown). With respect to the wild-type enzyme, the spectral changes observed on benzoate binding were similar to those reported for the pig enzyme (20) and were accompanied by (*i*) a decrease in shorter wavelength peak absorbance (maximum at 373 nm); (*ii*) an increase in absorbance in the 450–500 nm region; (*iii*) a 4-nm shift in longer wavelength absorbance maximum (from 455 to 459 nm); and (*iv*) development of a pronounced shoulder around 484 nm (Fig. 5*B*). The K_d values for benzoate binding at pH 8.3 and 15°C were estimated as 5 and 200 μM for the wild-type

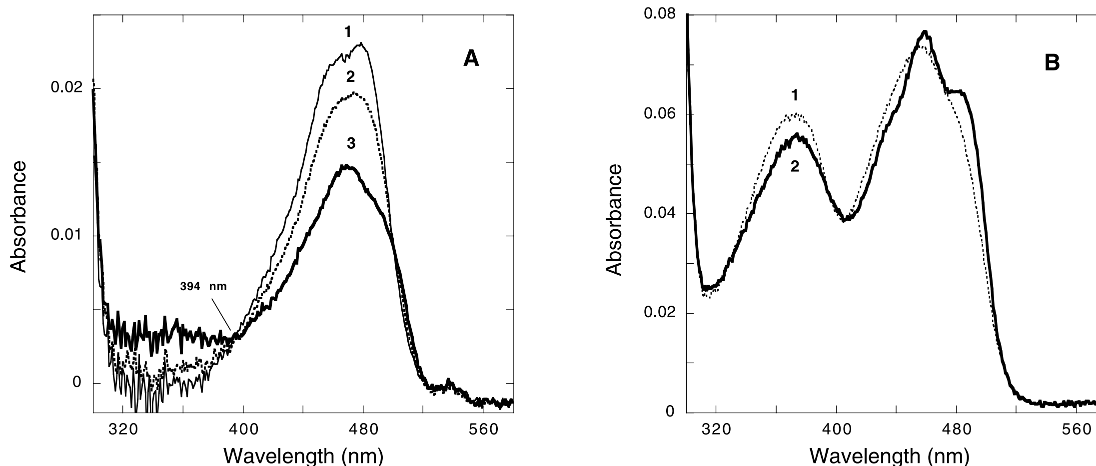

Fig. 5. Perturbation of the enzyme absorbance spectrum on benzoate binding. (*A*) 1 μM of FAD-S-enzyme in 50 mM bicine buffer (pH 8.3) (curve1) was mixed with 2 mM sodium benzoate (1 M stock solution) and spectra were taken immediately (curve 2) and after a 24-min incubation (curve 3) at 15°C. An isosbestic point is observed at 394 nm. (*B*) 6 μM of wild-type enzyme in 50 mM bicine buffer (pH 8.3) (dotted line) was mixed with 40 μM sodium benzoate and incubated for 10 min at 15°C (solid line).

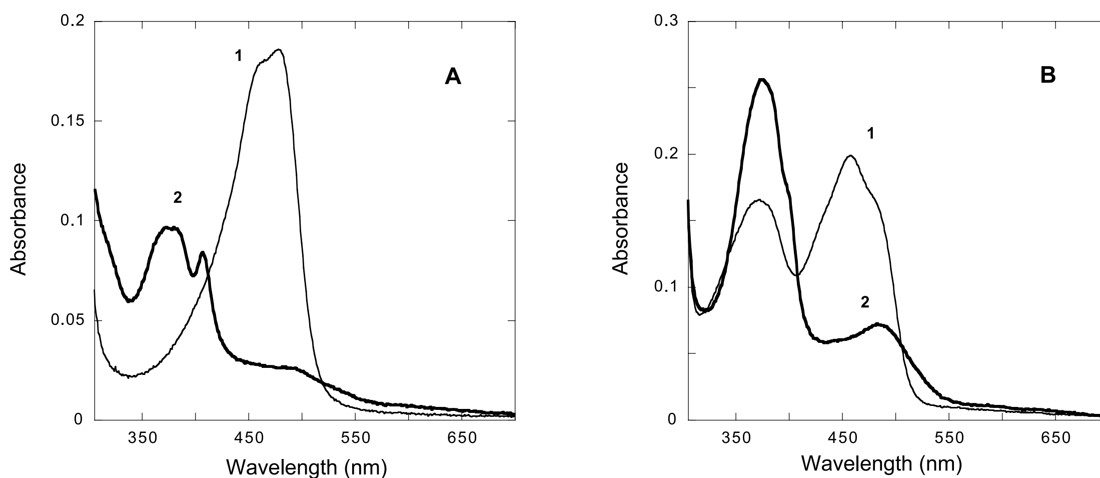

Fig. 6. Anaerobic photoreduction of FAD-S-mutant and wild-type enzymes. Absorbance spectra were recorded at 15°C in 50 mM bicine buffer (pH 8.3) containing 1 mM EDTA with 8 and 18 μM solutions of FAD-S-mutant and wild-type enzymes, respectively (see *Materials and Methods*). (*A*) FAD-S-enzyme before (curve 1) and after 4-min irradiation (curve 2). (*B*) Wild-type enzyme before (curve 1) and after 5-min irradiation (curve 2).

enzyme and FAD-S-mutant enzyme by monitoring the absorbance change vs. different benzoate concentrations at 500 and 478 nm, respectively. Thus, the wild-type enzyme displayed a benzoate binding mode similar to that of pig DAAO, whereas the interaction of benzoate with the FAD-S-mutant enzyme was 40-fold weaker than that with the wild-type enzyme. This difference is likely to be caused by the differences in protein conformation inferred from the limited proteolysis experiments.

Anaerobic Reduction Studies. Under anaerobic conditions, all DAAOs form an anionic (red) flavin semiquinone on photoreduction (9). The results of anaerobic photoreduction studies with FAD-S-enzyme and the wild-type enzyme are summarized in Fig. 6. Both proteins undergo complete semiquinone formation on light irradiation in the presence of EDTA (Fig. 6 *A* and *B*). The flavin red semiquinone of the FAD-S-enzyme displayed two peaks with maxima at 376 nm (ε_{376} = 12,300 M^{-1} cm^{-1}) and 407 nm (ε_{407} = 10,700 M^{-1} cm^{-1}) and a weak shoulder around 493 nm, whereas the flavin semiquinone of the wild-type enzyme showed a spectrum similar to that of pig kidney DAAO with characteristic peaks at 375 nm (ε_{375} = 14,700 M^{-1} cm^{-1}) and 486 nm (ε_{486} = 4,000 M^{-1} cm^{-1}).

In summary, we have engineered an hDAAO that contained covalently attached flavin and demonstrated that it exhibited enzymatic properties comparable with those of the wild-type enzyme. We conclude that the introduction of the restraints on possible flavin movement within the enzyme active site as a result of the covalent linkage did not interfere with the process of hDAAO catalytic turnover. The "artificial flavinylation" strategy presented in this work can be applied generally to study the functional implications of the attachment, especially with respect to controlled flavin mobility in flavoproteins, such as that recently described with *p*-hydroxybenzoate hydroxylase (22, 23).

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