Archives of Biochemistry and Biophysics
Vol. 372, No. 2, December 15, pp. 360–366, 1999
Article ID abbi.1999.1530, available online at http://www.idealibrary.com on IDEAL®

Lysine 219 Participates in NADPH Specificity in a Flavin-Containing Monooxygenase from *Saccharomyces cerevisiae*

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Received July 12, 1999, and in revised form September 22, 1999

The flavin-containing monooxygenase from *Saccharomyces cerevisiae* (yFMO) uses NADPH and O_2 to oxidize thiol containing substrates such as GSH and thereby generates the oxidizing potential for the ER. The enzyme uses NADPH 12 times more efficiently than NADH. Amino acid sequence analysis suggests that Lys 219 and/or Lys 227 may act as counterions to the 2′ phosphate of NADPH and to help determine the preference for pyridine nucleotides. Site directed mutations show that Lys 219 makes the greater contribution to cosubstrate recognition. Conversion of Lys 219 to Ala reduces NADPH dependent activity 90-fold, but has no effect on NADH-dependent activity. Conversion of Lys 227 to Ala reduces NADPH-dependent activity fivefold and NADH-dependent activity threefold. Dissociation constants for $NADP^+$ to oxidized yFMO were measured spectroscopically. K_d is 12 μM for the wild-type enzyme and 243 μM for the K219A mutant, consistent with the role of Lys 219 in pyridine nucleotide binding.

Key Words: **flavin-containing monooxygenase; *Saccharomyces cerevisiae;* NADPH recognition.**

Flavin-dependent monooxygenases (FMOs) are a family of enzymes that carry out the oxygenation of small organic compounds. They came to prominence in biochemistry because of their role in detoxifying xenobiotic compounds in mammals (1, 2). To facilitate this activity, mammals have evolved multiple isozyme forms with varying activity and substrate specificity (3) as well as differing tissue distribution (4). A unifying nomenclature for the FMOs has been proposed based on amino acid sequence which breaks mammalian enzymes into five families, FMO1 through FMO5 (5).

In general, FMOs require molecular O_2, NAD(P)H, H^+, and an oxygenatable substrate; NAD(P)H is used to reduce the flavin, which reacts with O_2 to form a stable 4α-hydroperoxyflavin, FAD–OOH. This reacts to oxygenate the organic substrate at a nucleophilic center such as a nitrogen or sulfur atom. After release of the oxygenated product, water and $NAD(P)^+$ are released (6).

Recently we cloned the single yeast gene which codes for an FMO homolog, yFMO (7). The protein was expressed with a His_{10} tag on the amino terminal end. The yeast enzyme, unlike mammalian enzymes, does not catalyze the oxidation of amines, but attacks thiols, including glutathione (GSH), cysteine, and cysteamine. It is likely that a major physiological activity of yFMO is the O_2 dependent oxidation of GSH to GSSG, the glutathione disulfide. GSH and GSSG form the primary redox buffer of eucaryotic cells. The ratio of GSH/GSSG is about 100 in the cytoplasm and about 2 in the ER (8). A deletion mutant yeast strain was used to show that yFMO helps generate the GSSG required to maintain the oxidizing potential of the ER. Yeast lacking the enzyme cannot properly fold plasmid coded proteins that contain disulfide bonds (9). Since yeast contain only this single FMO activity it is likely that this thiol oxygenating enzyme is the ancestral form from which a wide variety of FMOs have evolved.

In this work, we investigate the binding of NADP to yFMO. It has been shown that the flavin binding domain of mammalian FMOs lies at the amino terminus of the protein (10), but that a second nucleotide binding domain lies near the middle of the enzyme. We have identified this sequence and hypothesize that Lys 219 and/or Lys 227 may act as a counterion to the 2′ phos-

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0003-9861/99 $30.00

phate of NADPH during specific cosubstrate binding. Several mutations were created to test this hypothesis.

MATERIALS AND METHODS

Materials. Oligonucleotides for polymerase chain reaction (PCR) were purchased from Life Technologies, Inc. (Gaithersburg, MD). All restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA). The original TA cloning kit was purchased from Invitrogen Corp. (Carlsbad, CA). *Taq* DNA polymerase was from Perkin-Elmer (Norwalk, CT). The Ni–NTA resin was purchased from Qiagen Inc. (Santa Clarita, CA). FAD, NAD, NADP+, ATP, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and cysteamine were purchased from Sigma Chemical Co. (St. Louis, MO).

Bacterial strains and plasmids. *Escherichia coli* strains INVαF′ (Invitrogen Corp.) (F′ endA1 recA1 hsdR17(rk$^-$, mk$^-$) supE44 thi-1 gyrA96 relA1 f80lacZ15D(lacZYA-argF)u169) and BL21(DE3) (11) (Novagen Inc., Madison, WI) (F$^-$ ompT hadSB (rb$^-$,mb$^-$)gal dcm (DE3)) were used for cloning and expression experiments. The expression plasmid pET16b was purchased from Novagen Inc (Madison, WI) and pREP4groESL (12) was provided by Dr. M. Stieger (Hoffman- La Roche, Basel, Switzerland).

Site-directed mutagenesis and construction of expression vectors. To facilitate mutagenesis, the yeast FMO gene was amplified by *Taq* DNA polymerase using pYFMO, clone5 (7) as the template and two primers: a sense primer (5′-GTAACTAGTCATGACAGTGA-3′), having a *Bsp*HI restriction site and the start codon, and the T7 promoter primer (5′-TAATACGACTCACTATAGGG-3′). The PCR product was cloned into pCRII vector to yield a plasmid pYFMO-B. With pHis10-FMO (7) and pYFMO-B as the template for PCR, site-directed mutagenesis was carried out to convert Lys 219 to Ala (K219A), Asn (K219N), and Glu (K219E). Mutagenic oligonucleotides, 5′-TGCTCGCAGGCTCNNNTATACTATTATA-3′, corresponded to the codons for residues 215 to 223 of yFMO. K219A oligonucleotide (AAA → CGA) contained two mismatches, K219N (AAA→TTG) and K219E (AAA → CTG) oligonucleotides contained three mismatches. For the K227A mutant (AAG → CGG), a mutagenic primer, 5′-GATTAACTTGGCCCGCAGTTGATTGCT-3′, was used. Site-directed mutagenesis was carried out using a modified megaprimer method (13). The first round of PCR was performed with pHis10-FMO (7) as the template and two primers—the mutagenic primer and the T7 promoter primer. The second round of PCR was done using the first PCR product and *Bsp*HI-cut pYFMO-B as the templates with the T7 promoter primer. The final PCR products were cloned into the T/A cloning vector, pCRII. Once a recombinant mutant clone was identified, the mutated gene was subcloned into expression vector, pET16b, as previously described (7).

Expression and purification of wild type and mutant yFMO from E. coli. Expression and purification of yeast FMO were done as previously described (7). Briefly, an overnight culture was added to 1 L of 2 × YT supplemented with appropriate antibiotics. The culture was grown at 37°C for 4–5 h with shaking. IPTG, FAD, and ATP were added to a final concentration of 1 mM, 10 μM, and 1 mM, respectively, to induce expression. The culture was grown at 30°C for 3 more h and the cells were harvested. The cell pellet was resuspended in lysis buffer (50 mM K-phosphate, pH 8.0, 10 mM FAD) and the cells were broken in a precooled French Pressure cell (SLM Aminco, Urbana, IL) at 20,000 p.s.i. twice. The lysed cells were subjected to ultracentrifugation in a Ti 60 rotor (Beckman) at 4°C for 1 h at 100,000*g*. The S100 fraction was applied to a 5-ml Ni–NTA column, previously equilibrated in lysis buffer with 5 mM imidazole. After loading, the column was washed with lysis buffer and with lysis buffer containing 60 mM imidazole. The bound proteins were eluted with 0.5 M imidazole in lysis buffer. The eluted proteins were collected in 2-ml fractions. Fractions containing yFMO were pooled and dialyzed into storage buffer (50 mM K-phosphate, pH 8.0, 10% glycerol). The protein concentration was determined by the Bradford method (14). Flavin binding was determined as previously described (7).

Enzymatic assay of yeast FMO. Enzymatic activities were measured by substrate-dependent oxygen uptake at 37°C (15) in a mixture containing various concentrations of NAD^+ or $NADP^+$, 1.25 mM glucose 6-phosphate, 1.0 IU of glucose-6-phosphate dehydrogenase, 50 mM FAD, and 0.1 M K-phosphate buffer, pH 8, in a final volume of 2.0 ml. Enzyme concentrations were 0.1 to 0.5 μM. Oxygen uptake was determined in a 2-ml thermostated oxygraph vessel (Gibson Medical Electronics) fitted with a Clark-type electrode. The signal from the electrode was recorded with a EU-200-02 DC offset module. Cysteine substrate (50 mM) was added in the capillary access port. After 2–3 min of temperature equilibration the enzyme was added through the access port and oxygen uptake was recorded for an additional 2–4 min.

Kinetic analysis. Kinetic constants were calculated from initial reaction velocities using a range of substrate concentrations running 10-fold above and below K_m values. K_m and V_m values were determined by a least squares fit to the kinetic equation using Sigma Plot 2.0 for Windows (Jandel Scientific) and from Lineweaver–Burk plot; the methods gave essentially identical results.

Spectroscopic analysis. Absorption spectra were recorded using a Model 8452A Hewlett-Packard diode array spectrophotometer. Various $NADP^+$ solutions were added to a 30 μM solution of wild-type or K219A mutant protein in 50 mM potassium phosphate (pH 7.4) at room temperature. All spectra were corrected for dilution. The differences between the spectrum of free enzyme and those obtained in the presence of $NADP^+$ were calculated and plotted. The absolute absorbance of the flavin bands, at about 360 and 450 nm, decreased with increasing $NADP^+$; the spectra were plotted as absorbance of the empty enzyme–absorbance of the liganded form to give a positive spectrum change with increasing ligand. The fractional absorbance changes observed at 450 nm were plotted against the added $NADP^+$ concentration, giving a saturable hyperbolic curve; K_d values were determined by a least squares fit of the data to a hyperbolic equation using the program SigmaPlot 2.0 for Windows (Jandel Scientific).

RESULTS

Sequence Alignment

The amino acid sequence of the yeast FMO homolog is 18–23% identical with those of the mammalian FMOs derived from cDNA sequences. Two nucleotide binding domain sequences can be identified, the FAD site at the N terminus of the protein, and the putative NAD(P) site near the middle. This latter sequence is well conserved and located at the same positions in the primary sequences of all known mammalian FMO enzymes as well as other NADP and NAD requiring flavo enzymes (Table I). It is known that the mammalian FMOs can utilize both NADPH and NADH as the cosubstrate, although the K_m of NADH for mammalian FMO is ~10 times higher than that for NADPH (16). In general, it is thought that discrimination between these two pyridine nucleotides is centered on the 2′-phosphate group of the NADPH (17). The residue corresponding to Lys 219 is conserved as a possible cationic counterion to the phosphate group among enzymes using NADP (Table I). The cationic residue corresponding to yFMO Lys 227 is conserved in the

TABLE I

NAD(P)H Binding Domain of FMOs and Other Flavoprotein Oxidoreductases

NADP(H)-binding		
FMO		
S. cerevisiae	195	GNGSSGQDIANQLTTV---AKKVYNSIKEPASNQLKA
Rabbit FMO1	190	GMGNSGTDIAVEASHV---AKKVFLSTTGGAWVISRV
Rabbit FMO2	190	GIGNSASDIAVELSKK---AAQVYISTRKGSWVMSRI
Rabbit FMO3	190	GLGNSGCDIATELSHT---AEQVYISSRSGSWUMSRV
Rabbit FMO4	190	GLGNSGGDVAVELSRV---AAQVLLSTRTGTWVISRS
Rabbit FMO5	191	GIGNSGGDLAVEISHT---AKQVFLSTRRGAWIMNRV
Glutathione reductase		
E. coli	174	GAGYIAVELAGVINGL---GAKTHLFVRKHA--PLRS
Human	194	GAGYIAVEMAGILSAL---GSKTSLMIRHDK--VLRS
Trypanothione reductase		
T. congolense	195	GGGFISVEFAGIFNAYKPNGGKVTLCYRNNP--ILRG
NAD(H)-binding		
Dihydrolipoamide dehydrogenase		
E. coli	180	GGGILGLEMGTVYHAL---GSQIDVVEMFDQVIRAAD
S. cerevisiae	209	GGGIIGLEMGSVYSRL---GSKVTVVEFQPQIGASMD
Human	220	GAGVIGVELGSVWQRL---GADVTAVEFLGHVGGVGI
P. fluorescens	188	GAGVIGLELGSVWARL---GAEVTVLEALDKFLPAAD

Note. The glycine-rich NAD(P) signature sequence is enclosed in the first shaded box; it is common to both NADP- and NAD-dependent enzymes. The second shaded box marks Lys 219 of yFMO and homologs; the third box marks Lys 227 of yFMO. References: FMO, *S. cerevisiae* (7), rabbit FMO1 and FMO2 (25), FMO3 and FMO4 (26), FMO5 (27); glutathione reductase, *E. coli* (28), human (29); trypanothione reductase, *T. congolense* (30); dihydrolipoamide dehydrogenase, *E. coli* (31), *S. cerevisiae* (32), human (33), *P. fluorescens* (34). Sequences were aligned with the aid of the program CLUSTAL (35).

FMO family as well as other NADP using enzymes, but is not common in those using NAD. To investigate the effects of this positively charged residue on the 2′-phosphate binding, Lys 219 of yFMO was mutagenized to Ala (K219A), Asn (K219N), and Glu (K219E). Lys 227 was converted to Ala (K227A) and a double Ala mutant (K219/227A) was created.

Expression of wt and Mutant Yeast FMO in E. coli

The mutant and wild-type proteins were expressed in *E. coli* and purified using Ni–NTA resin. Applying this purification scheme, milligram quantities of homogeneous mutants and wild-type FMO were obtained. The mobilities of the mutant proteins on polyacrylamide gels were identical to that of wild type (data not shown). During purification the proteins exhibited a yellow color, indicating a bound flavin. The flavin contents of purified mutants and wild-type FMOs were 0.90 to 1.12 mol/mol, suggesting that the alterations had not effected binding of the flavin (Table II).

Kinetic Studies of Wild-Type and Mutant FMOs

Kinetic constants were obtained from initial rate measurements by fitting to the kinetic expression and by double reciprocal plots. Figure 1 shows an example of the double reciprocal plots. Figure 1a shows the velocity of the wild type yFMO using NADPH and NADH as cosubstrates. Figure 1b shows the results using the K219A mutant protein; similar plots (not shown) were constructed for each mutant protein. The kinetic constants for the wild-type and all mutant FMOs are summarized in Table III.

A given mutation alters the overall catalytic activity of the enzyme. The best measure of this overall effect is the specificity constant—the ratio of k_{cat} to K_m (18). This value measures the apparent second-order rate constant for the interaction and catalysis of substrate by the enzyme. The specificity constant values are found in the first two columns of Table III. They show that for the wild-type enzyme, NADPH is about 12 times more efficient a cosubstrate than is NADH.

The NADPH dependent activity is reduced by mutation of either Lys 219 or Lys 227. Conversion of Lys219 to Ala reduces overall activity to about 1% of wild-type levels, while conversion of Lys227 to Ala reduces overall activity to about 17%. In other words, yFMO is about 17 times more sensitive to mutation of Lys 219

TABLE II

FAD Content of Purified Wild-Type and Mutant FMOs

Construct	FAD content (nmol/mg protein)	FAD occupancy (%)
Wild-type	20.1 ± 1.95	90.0
K219A	25.5 ± 1.88	112
K219N	24.3 ± 1.94	109
K219E	23.6 ± 4.21	106
K227A	22.5 ± 3.37	99.2
K219, 227A	23.1 ± 2.10	102

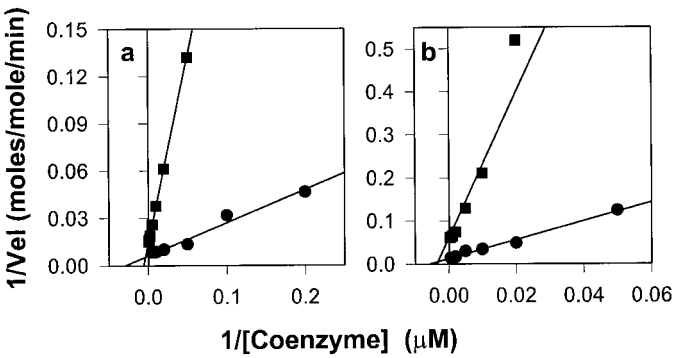

FIG. 1. Steady state kinetics for cofactor-dependent oxidation of Cys by yFMO. (a) Double reciprocal plots for wild-type yFMO oxidation of 50 μM Cys as a function of NADPH (circles) or NADH (squares) concentration. (b) The kinetics using the K219A mutant of yFMO.

than to mutation of Lys 227. The double Ala mutation has an overall activity similar to that of the K219A mutant. NADH-dependent activity is much less sensitive to mutation of these two Lys residues; K219A has wild-type activity and K227A is reduced only about threefold in overall activity.

In addition to the Ala mutations, Lys 219 was converted to the neutral polar residue Gln and to the charged residue, Glu. The K219Q enzymes NADPH-dependent activity was reduced 250-fold and NADH dependent activity was reduced 80-fold. The K219E protein had no detectable NADPH dependent activity and NADH dependent activity was reduced about 90-fold.

Table III also lists the Michaelis constants for the hyperbolic kinetic plots of each yFMO protein. The K_m for NADPH as a wild-type yFMO cofactor is 31 μM, while the K_m for NADH is more than five times higher at 168 μM. Converting Lys 219 to Ala increases the K_m for NADPH 9-fold but has no effect on the K_m for NADH. When Lys 227 was converted to Ala, the K_m for NADPH increased 8-fold and that for NADH increased only 1.4-fold. Compared to this, K_m values are increased when Lys 219 is replaced by electrostatically neutral alanine or asparagine, by 9- and 14-fold respectively. The double mutant had a K_m resembling that of the K219A mutant.

The k_{cat} values for NAD(P) dependent turnover were also affected by these mutations (Table III). Wild-type yFMO has a k_{cat} of 161/min for NADPH-dependent activity and 75/min for NADH-dependent activity. The yFMO K219A enzyme reduces the NADPH constant ninefold but that for NADH is essentially unchanged. Alteration of Lys 227 had no significant effect on NADPH-dependent activity (70% activity) and that for NADH is reduced about twofold.

The ratios of kinetic constants for the NADPH-dependent to NADH-dependent reactions are summarized in Table IV. This succinctly illustrates the comparative effects of yFMO mutations on the utilization of the nucleotide cosubstrates. It shows that for wild type, NADPH is nearly 12 times more effective than NADH. This is due to a 5.4 times better K_m and twofold better k_{cat}. Converting Lys 227 to Ala leaves an enzyme with a sevenfold preference for NADPH. However, converting Lys 219 to Ala gives the enzyme a preference for NADH. Although overall activity is substantially reduced, this K219A mutant has an eightfold preference for NADH. This preference is also observed for the double mutant and for K219Q.

$NADP^+$ Binding

The binding of $NADP^+$ to wild-type and K219A forms of yFMO was observed spectroscopically. Figure 2a shows the difference spectrum for oxidized wild-type yFMO as it titrated with $NADP^+$. Increasing concentration of $NADP^+$ leads to an increasing difference in the flavin absorbance bands, particularly at 370 and

TABLE III

Kinetic Parameters for Cysteine Oxidation by Wild-Type and Mutant FMOs

	Kinetic parameters					
	k_{cat}/K_m ($min^{-1}/\mu M$)		K_m (μM)		k_{cat} (min^{-1})	
Construct	NADPH	NADH	NADPH	NADH	NADPH	NADH
Wild-type	5.2 (100)	0.448 (9)	31.0	168	161	75.3
K219A	0.06 (1)	0.486 (9)	278	177	17.5	86.1
K227A	0.90 (17)	0.134 (2)	124	235	111	31.6
K219, 227A	0.11 (2)	0.247 (5)	310	227	33.6	56.1
K219Q	0.02 (0.4)	0.007 (0.1)	434	672	9.3	4.8
K219E	N/D	0.005 (0.1)	N/D	5890	N/D	27.8

Note. The values for the specificity constant, k_{cat}/K_m, in columns 2 and 3 have also been normalized to percentage of wild type activity; this is indicated in parentheses.

TABLE IV

Ratio of Kinetic Parameters for NADPH- to NADH-Dependent Activities of Wild-Type and Mutant FMOs

Constructs	k_{cat}/K_m	$1/K_m$	k_{cat}
Wild-type	11.6	5.4	2.14
K219A	0.129	0.64	0.203
K227A	6.65	1.9	3.51
K219, 227A	0.440	0.74	0.599
K219N	3.03	0.51	0.645
K219Q	2.86	1.55	1.90

450 nm. Figure 2b plots the change in absorbance at 450 nm against $NADP^+$ concentration for the wild-type enzyme. $NADP^+$ binding is saturable and shows a K_d value of 12 μM. Similar spectra were taken for the titration of the K219A mutant. The difference absorbance at 450 nm is plotted in Fig. 2c. It again shows saturable behavior with a K_d value of 240 μM.

DISCUSSION

A comparison of the amino acid sequence of the mammalian FMO shows that all forms can be assigned to one of five gene subfamilies (5). Yeast FMO has well conserved FAD- and NADPH-binding domains like the mammalian FMOs. Several proteins with FAD and NADPH/NADH binding domain have been crystallized and their tertiary structures determined by X-ray crystallography; these include human glutathione reductase (19), *E. coli* glutathione reductase (20), lipoamide dehydrogenase (21), and *para*-hydroxybenzoate hydroxylase (22).

To take one example, the FAD and NADP binding domains of glutathione reductase exhibit a common pyrophosphate-binding $\beta\alpha\beta$ "nucleotide binding" fold centered around a highly conserved sequence, GXGXXG/A (22). These three conserved residues allow for formation of a tight turn between the first β-strand and the α-helix of the $\beta\alpha\beta$ motif. The specific recognition of the 2′-phosphate of NADPH in human glutathione reductase includes two positively charged residues, Arg 218 and Arg 224 (17); this charge pairing results in a 60-fold greater K_m for NADH than for NADPH (23). Lysines 219 and 227 in yFMO are homologs of arginine residues 218 and 224 in glutathione reductase and our goal was to assess their role in determining specificity of pyridine nucleotide binding.

Analysis of yFMO mutants suggests that our initial hypothesis is correct—lysines 219 and 227 are involved in NAD(P) binding and specificity. Lys 219 is clearly the more important residue of the two. For example, the specificity constant (k_{cat}/K_m) for NADPH-dependent catalysis is decreased 90-fold when it is converted to Ala, but only 6-fold when Lys 227 is converted to Ala. Since these residues are hypothesized to ion pair with the 2′ phosphate of NADPH, it is reasonable to assume that Lys 219 makes the stronger interaction. One would predict that these mutations would have less effect on NADH-dependent activity because NADH lacks the 2′ phosphate. This is, indeed, observed. Mutation of Lys 219 has no effect on the overall NADH dependent activity, and alteration of Lys 227 diminishes the specificity constant only about 4-fold. Converting the important Lys 219 to a Glu should place a negative charge near that of the 2′ phosphate and

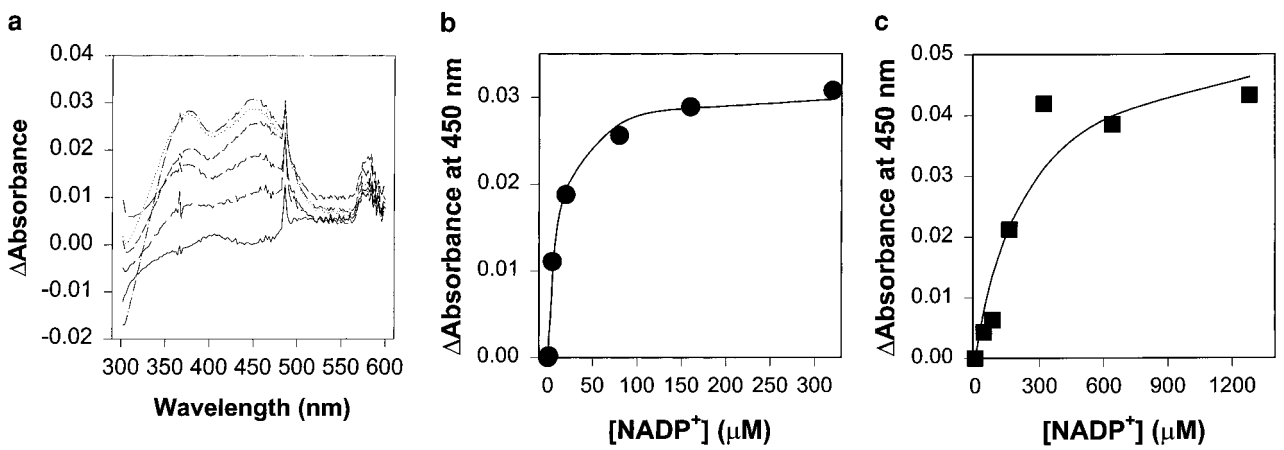

FIG. 2. Binding of $NADP^+$ to yFMO. (a) The difference absorbance spectrum for oxidized wild-type yFMO enzyme in the presence of increasing concentrations of $NADP^+$. The lowest spectral curve corresponds to the difference between empty yFMO and the enzyme with 1 μM $NADP^+$; the curves increase in a monotonic fashion for $NADP^+$ concentrations of 5, 20, 80, 160, and 320 μM. The last few spectra are pulled up slightly on the difference absorbance scale to separate the lines for this display. Similar data, not shown, were taken for the K219A mutant protein. (b) The difference in absorbance at 450 nm, from (a), plotted versus concentration of $NADP^+$ for the wild-type yFMO. The hyperbolic curve has a K_d = 12 μM. (c) The difference in absorbance at 450 nm plotted versus concentration of $NADP^+$ for the K219A mutant of yFMO. The hyperbolic curve has a K_d = 243 μM.

should greatly inhibit NADPH-dependent yFMO activity; in fact no activity can be measured for this mutant. However, the mutation also inhibits NADH-dependent activity 80-fold. It is difficult to interpret this result in terms of specific enzyme NAD or NADP interactions. It may be, however, that the newly generated Glu 219 forms an ion pair with Lys 227 and this interaction perturbs cosubstrate binding. In the absence of an X-ray structure, it is not prudent to overinterpret the kinetic results in terms of molecular structure.

Our kinetic analysis determined K_m and k_{cat} values for NADPH and NADH dependent catalysis by the various yFMO proteins. These may be used to suggest how the mutations effect cosubstrate–enzyme interactions. K_m is the concentration of cofactor allowing half maximal velocity, but it is common to interpret K_m values as a rough measure of substrate affinity. Conversion of Lys 219 to the neutral Ala increased K_m for NADPH by nearly a log. The magnitude of this effect is also observed in titration of oxidized yFMO with $NADP^+$ (Fig. 2). In these experiments a true dissociation constant can be measured between the empty enzyme and the oxidized dinucleotide. The observed K_d for the wild type, 12 μM, is roughly 20 times lower than for binding to the K219A mutant. In the simplest terms this is consistent with the notion that Lys 219 and the 2′ phosphate make a thermodynamically favorable interaction that contributes to the strength of binding. Its contribution to the association effects pyridine dinucleotide binding 10- to 20-fold, suggesting an interaction worth about 1.5 Kcal/mol.

The same mutation did not affect binding of NADH, which lacks the 2′ phosphate. The K227A mutant increased K_m for NADPH 4-fold, suggesting a minor contribution to binding the 2′ phosphate. The K_m for NADH is increased only 1.4-fold, consistent with the notion that Lys 227 also interacts by ion pairing. If both Lys 219 and Lys 227 made independent contributions to NADPH binding, one might expect the effects of a double mutant to be multiplicative. That is, if one residue stabilized binding 10-fold and a second stabilized it 4-fold, then a double mutant would decrease binding 40-fold. In fact, the double mutant had K_m values similar to those of K219A (and the specificity constant is also similar to K219A), suggesting that Lys 219 is the dominant residue for NADPH recognition, and that Lys 227 adds little to the binding affinity.

The mutations also show k_{cat} effects. As shown in Table III, wild-type yFMO exhibits a k_{cat} with NADPH that is more than twice as fast as for NADH. This difference shows that for yFMO, unlike for mammalian FMOs, pyridine nucleotide-dependent reduction of FAD contributes strongly to the rate limiting catalytic step and may be rate limiting. All Lys 219 mutations reduce k_{cat} significantly for NADPH dependent catalysis; for example K219A is reduced ninefold. However the K227A mutant has a k_{cat} value about 70% of wild type. In the absence of an X-ray structure, interpretation of such data is difficult. The altered k_{cat} can be interpreted as reflecting a modification in the rate limiting step(s) of the reaction; since this probably involves hydride transfer from the cosubstrate, a decrease in k_{cat} might reflect an altered orientation of the cosubstrate. The large effect for K219A may indicate that the ion pair not only aids in the attraction of NADPH, but helps orient it most productively.

NADPH binds to human glutathione reductase with a 60-fold lower K_m than NADH (17), and other reductases or dehydrogenases with dual NAD(P) specificity have a similar binding preference. However wild-type FMO has only a ninefold lower K_m value for NADPH than for NADH. This may be due to the presence of lysine residues in the 2′-phosphate binding site instead of arginines. It is known that arginines have a much greater propensity than lysines to bind phosphate (24). As seen in Table I, yFMO is somewhat unusual in having lysines at each of the two cationic sites used by FMOs and other flavoprotein oxidoreductases that use NADP as a cosubstrate.

ACKNOWLEDGMENTS

This work was supported by Grant GM 30048 from the National Institutes of Health, Grant MCB-9601096 from the National Science Foundation, and by grants from the Foundation for Research and the Welch Foundation.

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