BIOCHEMISTRY
including biophysical chemistry & molecular biology

Article

pubs.acs.org/biochemistry

The Cation−π Interaction between Lys53 and the Flavin of Fructosamine Oxidase (FAOX-II) Is Critical for Activity

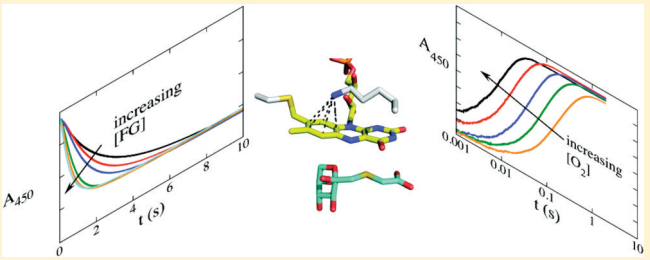
François Collard,[†,§,⊥] Rebecca L. Fagan,[‡,∥,⊥] Jianye Zhang,[†] Ina Nemet,[†,@] Bruce A. Palfey,*[,‡] and Vincent M. Monnier*[,†]

[†]Department of Pathology and Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106-7288, United States

[‡]Department of Biological Chemistry, University of Michigan Medical School, 1150 W. Medical Center Dr., Ann Arbor, Michigan 48109-0606, United States

Supporting Information

ABSTRACT: Fructosamine oxidases (FAOXs) are flavin-containing enzymes that catalyze the oxidative deglycation of low molecular weight fructosamines or Amadori products. The fructosamine substrate is oxidized by the flavin in the reductive half-reaction, and the reduced flavin is then oxidized by molecular oxygen in the oxidative half-reaction. The crystal structure of FAOX-II from *Aspergillus fumigatus* reveals a unique interaction between Lys53 and the isoalloxazine. The ammonium nitrogen of the lysine is in contact with and nearly centered over the aromatic ring of the flavin on the *si*-face. Here, we investigate the importance of this unique interaction on the reactions catalyzed by FAOX by studying both half-reactions of the wild-type and Lys53 mutant enzymes. The positive charge of Lys53 is critical for flavin reduction but plays very little role in the reaction with molecular oxygen. The conservative mutation of Lys53 to arginine had minor effects on catalysis. However, removing the charge by replacing Lys53 with methionine caused more than a million-fold decrease in flavin reduction, while only slowing the oxygen reaction by ∼30-fold.



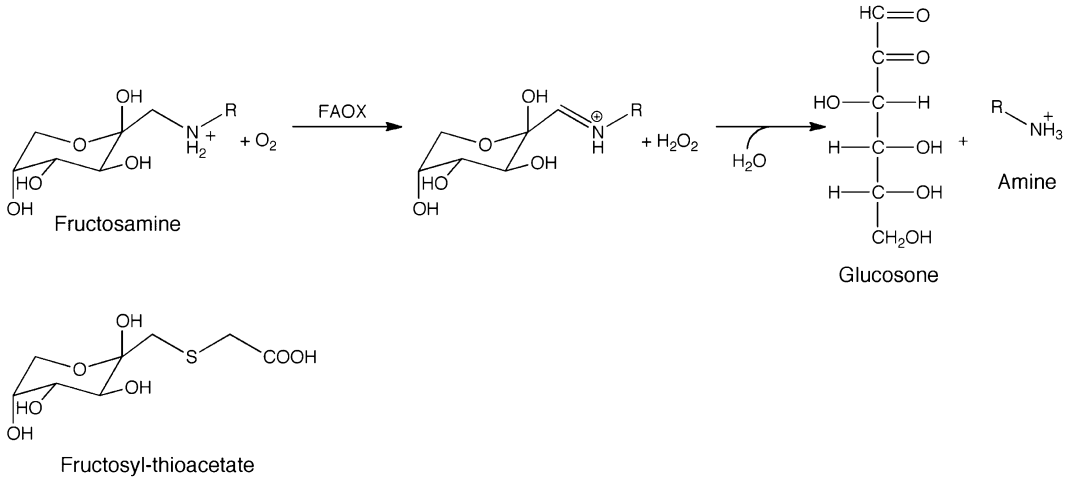
Fructosamine oxidases (FAOXs) catalyze the oxidative deglycation of low molecular weight fructosamines (Amadori products; Scheme 1). Fructosamines are formed by the spontaneous reaction of glucose and amino groups of amino acids.[1] Protein-linked fructosamines are found in all mammalian tissues. In humans, their formation is increased in diabetes and thought to be responsible (in part) for diabetic complications.[1] Some fungi and bacterial genomes contain FAOXs, enabling those organisms to sustain growth on low molecular weight fructosamines as a sole energy source, suggesting that fructosamines are also present in the natural environment.[2] Fungal FAOXs are peroxisomal enzymes containing covalently bound FAD that catalyze the oxidation of the carbon−nitrogen bond between the amino portion of fructosamines and C1 of the fructosyl moiety (Scheme 1). The resulting Schiff base is readily hydrolyzed to glucosone and an amino acid. After reduction, the reduced FAD is oxidized by molecular oxygen with concomitant release of hydrogen peroxide.

The structure of FAOX-II from *Aspergillus fumigatus* was solved in the presence and absence of the inhibitor fructosyl thioacetate (FTA; Scheme 1),[3] a nonmetabolizable analogue of fructosylglycine, the substrate with the lowest apparent K_m. The overall fold is most similar to monomeric sarcosine oxidase (MSOX), its closest homologue. An important difference is that the C-terminal 36 residues of FAOX-II form a loop over the substrate binding site which is missing in MSOX. In the ligand-free FAOX-II structure, no continuous electron density is seen for residues 58−66 and 110−116, revealing that in the absence of ligand these two surface regions are flexible. When inhibitor is bound, these regions become ordered with the ligand located on the *re*-side of the flavin ring system in a conformation that could represent a reactive substrate complex. The interactions made by the ligand with the protein fall into three groups: those involving the inhibitor's carboxylate, those involving the fructosyl group, and those forming a hydrophobic interface along one side of the inhibitor.[3] The FTA carboxylate oxygens hydrogen-bond to the side chains of Tyr60, Arg112, and Lys368. The importance of those interactions for enzyme activity appears to be modest as indicated by the fact the Tyr60Phe, Arg112Glu, and Lys368Met mutants display only 3-, 10-, and 5-fold increases in the apparent K_m of fructosylglycine, respectively, and V_{max} is almost unaffected compared to the wild-type.[3] The fructosyl portion of the ligand is stabilized by a series of hydrogen bonds involving mainly the side chains of Arg411 and Glu280. The latter residue is strictly conserved among FAOXs and is required for enzymatic activity.[3]

The structure of FAOX-II also reveals a unique interaction between Lys53 and the isoalloxazine: the (presumably) cationic ε-amine of the side chain makes van der Waals contact with the aromatic ring of the flavin on the *si*-face. The ammonium

Received: December 28, 2010
Revised: July 14, 2011
Published: July 14, 2011

Scheme 1



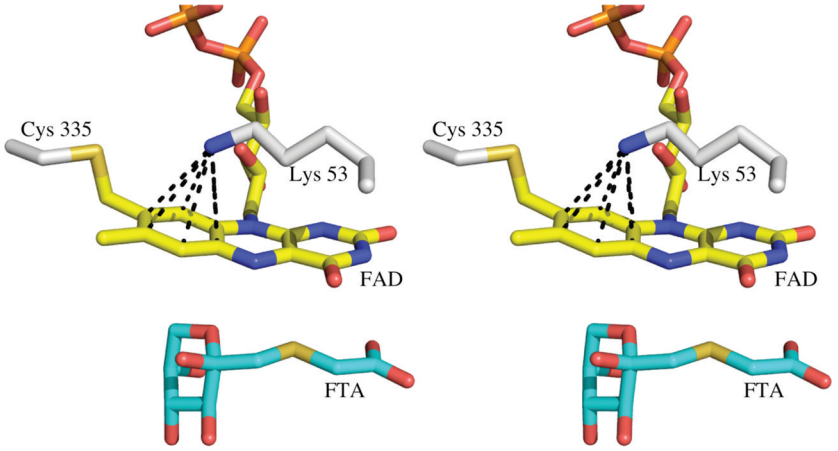


Figure 1. π–cation interaction at the active site of FAOX. Stereo view of the active site of FAOX-II showing the (presumably) cationic ε-amine of Lys53 nearly centered over the benzene ring of the flavin on the *si*-face. The inhibitor, FTA, is bound on the *re*-face of the flavin. Coordinates were taken from PDB entry 3dje.

nitrogen is nearly centered over the benzene ring, with N–C distances ranging from 3.2 to 3.8 Å (Figure 1). Such an ammonium–π interaction had never been reported for a flavoprotein. In MSOX, an Arg residue is in an analogous position, but the delocalized positive charge of the nitrogen atoms of the guanidinium group contacts the flavin both in the aromatic and piperazine rings.

In this paper, we report transient kinetic studies on the reductive and oxidative half-reactions of the wild-type enzyme from *A. fumigatus* and on Lys53 mutant enzymes. We found that the positive charge of Lys53 is critical for enzymatic activity.

EXPERIMENTAL PROCEDURES

Expression and Purification of FAOX-II. FAOX-II was expressed in an *E. coli* BL21pLysS strain carrying the expression plasmid of FAOX-II[4] and purified by PEG fractionation, followed by two steps of anion exchange chromatography on DEAE-Sepharose and Q-Sepharose, as described previously.[3]

Construction and Purification of Mutant Enzymes. Mutagenesis was performed using a Strategene QuikChange kit according to the manufacturer's instructions. The mutant enzymes were expressed under the same conditions as the wild-type enzyme.[3] The purification procedure used for the mutant proteins was a simplified version of the purification method described in ref 3 that involved only the DEAE-Sepharose and the Q-Sepharose purifications steps.

Analysis for Covalently Bound Flavin. To determine whether flavin was covalently linked to the protein, two different assays were used. In the first one, 80 μL of the enzyme solution (20 mg/mL) was mixed with 20 μL of 70% (w/v) perchloric acid (PCA) and centrifuged for 30 min at 12000*g* at 4 °C. The supernatant was neutralized with K_2CO_3, the protein pellet redissolved in 6 M guanidinium hydrochloride, and the absorbance at 450 nm was measured for the neutralized supernatant and the redissolved pellet. In the second assay, 180 μL of the protein solution (20 mg/mL) was mixed with 20 μL of 10% SDS, filtered on an Amicon 10 kDa membrane and washed with 3 × 0.5 mL of water. Proteins were assayed (Bio-Rad assay), and the absorbance at 450 nm was measured in the filtrate, wash fraction, and the retentate.

Synthesis of Substrate and Inhibitor. Fructosylglycine (*N*-(1-deoxy-D-fructos-1-yl)glycine; FG) and fructosyl thioacetate (FTA) were synthesized as described previously.[3,5] The fructosylglycine preparation contained a nonreactive brown-colored contaminant whose absorbance contribution was subtracted from absorbance data in most experiments.

dx.doi.org/10.1021/bi1020666 | *Biochemistry* 2011, 50, 7977–7986

Instrumentation. Absorbance spectra were collected using a Shimadzu UV-2501PC scanning spectrophotometer. Stopped-flow experiments were performed at 4 °C using a Hi-Tech Scientific KinetAsyst SF-61 DX2 stopped-flow spectrophotometer whose flow time was adjusted to equal the dead time so that the time axis of traces represents the time after mixing.

Preparation of Anaerobic Solutions. Enzyme solutions for rapid reaction studies were made anaerobic in glass tonometers by repeated cycles of evacuation and equilibration over an atmosphere of purified argon.[6] Substrate solutions were made anaerobic within the syringes that were to be loaded onto the stopped-flow instrument by bubbling solutions with purified argon. Slow reactions were performed in anaerobic cuvettes[7] and monitored in a standard scanning spectrophotometer. These reaction mixtures were also made anaerobic by repeated evacuation and equilibration with purified argon.

Fructosyl Thioacetate Binding. The dissociation constant of fructosyl thioacetate (FTA) was determined in aerobic titrations. The enzyme of interest in 50 mM KPi, pH 7.4, was titrated with a concentrated solution of FTA. The changes in flavin absorbance caused by the binding of FTA were plotted against the concentrations of FTA to determine the dissociation constant. For wild-type FAOX and the Lys53Arg mutant enzyme, the data were fit to the tight-binding quadratic equation (eq 1),[8] where E_0 is the total enzyme concentration, $[FTA]_0$ is the total added ligand concentration, $\Delta\varepsilon_{max}$ is the maximum extinction change, and K_d is the dissociation constant. For the Lys53Met mutant enzyme, the data were fit to a hyperbola (eq 2) to determine the K_d; ΔA represents the change in absorbance at a particular concentration, and ΔA_{max} is the absorbance change extrapolated to infinite [FTA].

$$\Delta A = \Delta\varepsilon_{max} \times \left[\frac{E_0 + [FTA]_0 + K_d - \sqrt{(E_0 + [FTA]_0 + K_d)^2 - 4E_0[FTA]_0}}{2}\right] \quad (1)$$

$$\Delta A = \frac{\Delta A_{max}[FTA]}{K_d + [FTA]} \quad (2)$$

Reductive Half-Reaction of Wild-Type and Lys53Arg FAOX-II. Kinetics of the reductive half-reactions of wild-type and Lys53Arg FAOX-II were studied in anaerobic stopped-flow experiments. Anaerobic enzyme (~16 μM after mixing) in 50 mM KPi, pH 7.4, was mixed with anaerobic fructosylglycine (FG) (ranging from 0.05 to 1 mM final concentration) in the same buffer. Flavin absorbance at 450 nm was followed as a function of time. Reaction traces were fit to either three exponentials in the case of wild-type FAOX-II or four exponentials in the case of Lys53Arg FAOX-II with Program A (Rong Chang, Chung-Jin Chiu, Joel Dinverno, and David P. Ballou, University of Michigan). Rate constants were estimated by plotting the observed rate constants as a function of substrate concentration and fitting to either a line or a hyperbola in Kaleidagraph (Synergy, Inc.).

Rate constants were refined by global fitting in Berkeley Madonna. Reaction traces at each concentration were averaged, and all concentrations were simultaneously fit to the differential equations describing the reaction schemes shown in the Results. Initial estimates of the rate constants were obtained from the initial analysis relying on individually fit curves (previous paragraph). Both rate constants and extinction coefficients were optimized during the fitting process. An example model is available in the Supporting Information.

Determination of Spectral Intermediates in the Reductive Half-Reaction of Lys53Arg FAOX-II. Anaerobic enzyme in 50 mM KPi, pH 7.4, was mixed with 1 mM FG in the same buffer. Absorbance spectra were collected from 350 to 700 nm for 75 s with an integration time of 1.5 ms using a diode array detector. Data sets were collected with intervals between spectra of 75 ms, 150 ms, 300 ms, 600 ms, and 1.2 s; 160 spectra that covered all reaction phases were combined into a single file for analysis. Absorbance spectra of intermediates were calculated in SpecFit/32 (Spectrum Software Associates) using a three-step reversible model plus a single-step reaction to describe damaged enzyme. Anaerobic enzyme was mixed with buffer without substrate to obtain the spectrum of fully oxidized enzyme. Substrate was mixed with buffer to obtain the spectrum of the contaminant in the FG solution. The spectrum of the contaminant was subtracted from all spectra before analysis.

Reductive Half-Reaction of Lys53Met FAOX-II. Reduction of Lys53Met FAOX-II was extremely slow. Anaerobic enzyme in 50 mM KPi, pH 7.4, was mixed with 2.5 mM anaerobic FG in an anaerobic cuvette, and the reduction was followed in a standard scanning spectrophotometer at 25 °C. The enzyme precipitated after ~1 week, preventing flavin reduction to be followed to completion. A first-order rate constant for reduction was estimated by dividing the initial rate of flavin reduction (obtained from the slope of the absorbance decrease at 450 nm) by the concentration of the enzyme.

Oxidative Half-Reactions of Wild-Type, Lys53Arg, and Lys53Met FAOX-II. Oxygen reactivity of each enzyme was studied in anaerobic stopped-flow experiments at 4 °C. Anaerobic enzyme (~30 μM) was reduced with either 1 equiv of FG (in the case of wild-type and Lys53Arg FAOX-II) or dithionite (in the case of Lys53Met FAOX-II). The fully reduced anaerobic enzyme was mixed with various concentrations of oxygen (ranging from 30.5 to 625 μM, after mixing) in buffer, and flavin oxidation was observed by the increase in absorbance at 450 nm. Reaction traces were fit to one exponential (in the case of wild-type and Lys53Met FAOX-II) or to the sum of two exponentials (in the case of Lys53Arg FAOX-II). The observed rate constants varied linearly with O_2 concentration, allowing bimolecular rate constants to be determined from the slopes of the lines.

Enzyme-Monitored Turnover for Wild-Type and Lys53Arg FAOX-II. The entire catalytic cycle of wild-type and Lys53Arg FAOX-II was studied by enzyme-monitored turnover (EMT) in stopped-flow experiments at 4 °C. Anaerobic oxidized enzyme was mixed with 1 mM FG and 625 μM O_2. (Concentrations are those after mixing.) Flavin absorbance at 450 nm was used to follow the reaction. Traces were analyzed by integration.[9] The values for the K_m of O_2 were too low to be determined; only k_{cat} values were obtained.

RESULTS

FAOX-II is a flavoprotein oxidase that catalyzes the oxidation of the carbon−nitrogen bond of fructosamines (Scheme 1). Here we investigate the reactivity of the enzyme from *Aspergillus fumigatus*. The importance of Lys53, which makes an unusual

cation−π interaction with FAD, was investigated by studying the Lys53Arg and Lys53Met mutant forms of the enzyme.

Properties of Mutant FAOX-II. Mutation of Lys53 did not affect overexpression or stability of FAOX-II. The absorbance spectra of the oxidized wild-type enzyme and the mutant Lys53Arg are nearly identical (Figure S1). However, the absorbance spectrum of the Lys53Met mutant enzyme was noticeably different (Figure 2 and Figure S1). Unlike wild-type, free FAD was detected. Thus, Lys53Arg FAOX contains covalently bound flavin, but in the Lys53Met enzyme, the FAD is not covalently bound. Nonetheless, flavin binds tightly to the Lys53Met enzyme, which is purified as the holoenzyme. Indeed, upon concentrating the enzyme, any apoenzyme present precipitated. Thus, all experiments with the Lys53Met enzyme were with holoenzyme.

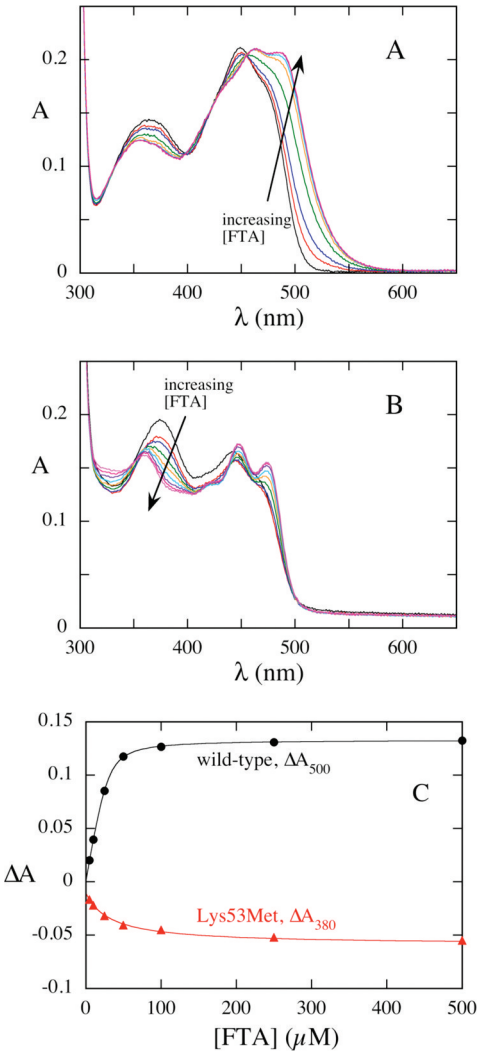


Figure 2. FTA binding titration, pH 7.4, 25 °C. Wild-type (A) and the Lys53Met mutant enzyme (B) were titrated aerobically with FTA (0–500 μM). The maximum change in absorbance due to FTA binding as a function of FTA concentration is shown in (C). Fitting to eq 1 for wild-type enzyme gives a K_d of 3.4 ± 0.1 μM. Fitting to a square hyperbola gives a K_d value of 33 ± 5 μM for the Lys53Met mutant enzyme.

the extinction of the 450 nm peak is lower than that of the 360 nm peak, suggesting important changes in electronic structure of the isoalloxazine.

FAD is covalently bound to wild-type FAOX at the 8α-position via Cys335.[3] Covalent flavin attachment was probed using both SDS and PCA denaturation. The PCA precipitation and the SDS treatment separate the absorbance at 450 nm from the protein in the case of the Lys53Met enzyme. In the cases of wild-type and the Lys53Arg enzymes (data not shown), no

Fructosyl Thioacetate Binding. Aerobic titrations at pH 7.4 were used to determine the affinity of fructosyl thioacetate (FTA), an inhibitor of FAOX-II. Binding of FTA caused the flavin absorbance spectrum to become highly resolved and shifted to the red (Figure 2) to some degree in all three enzyme forms studied (WT and Lys53Arg by 16 nm, Lys53Met by 7 nm). The vibronic bands of flavin absorbance spectra become more resolved as the environment of the flavin (especially the hydrogen bonds) becomes more rigid. The spectra of the FTA complexes, therefore, can be rationalized by the exclusion of water from the active site, decreasing fluxional hydrogen bonds. The largest change in absorbance upon FTA binding to the wild-type and the Lys53Arg enzymes occurred at ~500 nm, while the largest change for the Lys53Met protein was at ~380 nm. The K_d determined for wild-type and Lys53Arg FAOX were nearly identical, 3.4 ± 1 and 5.5 ± 0.5 μM, respectively. In the Lys53Met enzyme, the K_d of FTA was 33 ± 5 μM, around 10-fold higher than in wild-type.

Reductive Half-Reaction of the Wild-Type Enzyme. The reductive half-reaction of wild-type FAOX-II was studied in anaerobic stopped-flow experiments. Anaerobic oxidized enzyme (16 μM, final) was mixed with varying concentrations of anaerobic fructosylglycine (FG) at pH 7.4, and flavin spectral changes were monitored. The experiments were conducted at 4 °C to slow the reaction sufficiently so that it could be observed. During the reductive half-reaction, no spectral evidence of flavin semiquinones (either neutral or anionic) could be observed, nor was charge-transfer absorbance observed. Reactions at pH 9 did not form these intermediates either (data not shown).

At 450 nm, three phases of reduction were observed (Figure 3A). Therefore, the absorbance traces were initially fit to three exponentials to determine the observed rate constants of each phase. The first phase consisted of an initial lag. The observed rate constant of this phase varied roughly linearly with respect to FG concentration, suggesting that this phase represents substrate binding. The very small amplitude change of this phase made it difficult to accurately determine the observed rate constants at each FG concentration. Nevertheless, the data were fit to a line with a slope of ~10^4 M^{-1} s^{-1} and an intercept of ~2 s^{-1} (Figure 3B). The second phase was accompanied by a large decrease in absorbance and represents flavin reduction. The observed rate constant associated with this phase saturated as the FG concentration was increased, allowing the data to be fit to a hyperbola (Figure 3B and Figure S2). The resulting saturating rate constant was 2.6 ± 0.1 s^{-1}, and the half-saturating concentration of FG was 137 ± 18 μM. The final phase was accompanied by a small decrease in flavin absorbance. The observed rate constant of this phase was independent of FG concentration. Averaging the observed rate constants at all concentrations gave a value of 0.39 ± 0.04 s^{-1}, which we assign to product release.

These data indicate a three-step reductive half-reaction, in which FG first binds to the oxidized enzyme, followed by carbon−nitrogen bond oxidation, and finally product release.

dx.doi.org/10.1021/bi1020666 | Biochemistry 2011, 50, 7977–7986

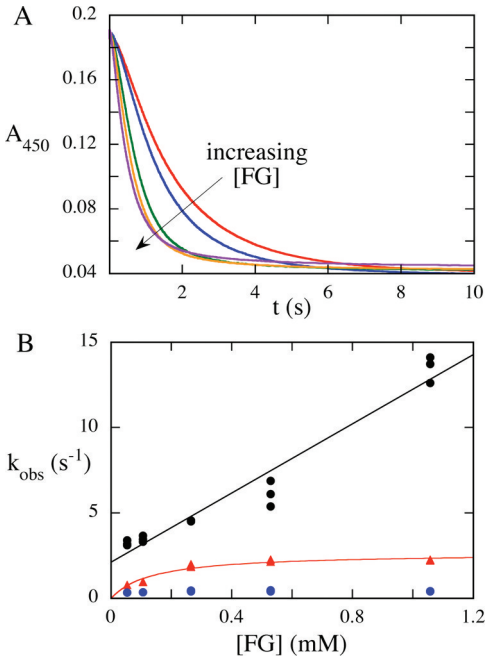


Figure 3. Reductive half-reaction of wild-type FAOX. (A) Averaged reaction traces ($N \geq 3$) at 450 nm observed when ~16 μM enzyme at pH 7.4 was mixed with varying amounts of FG (0.05−1.05 mM final) at 4 °C. The reaction occurs in three phases. The initial lag phase represents substrate binding. The large second phase represents flavin reduction. The small final phase could represent product release. (B) The observed rate constant of the first phase (black circles) is roughly linear with FG concentration giving a bimolecular rate constant of ~10^4 M^{-1} s^{-1} and a reverse rate constant of ~2 s^{-1}. The rate constant of the second phase (flavin reduction; red triangles) saturates with increasing FG concentration, giving a reduction rate constant of 2.6 ± 0.1 s^{-1} and a half-saturating value of 137 ± 18 μM. The observed rate constant of the third phase (blue circles) is ~0.4 s^{-1} and is independent of FG concentration.

To more accurately determine the rate constants for these processes, a global analysis was done by simultaneously fitting averaged absorbance traces at all FG concentrations (six total) to the differential equations describing the three-step model. The rate constants determined by the initial analysis of the absorbance traces were used as initial guesses. The rate constants obtained from this analysis (Scheme 2) were mostly

Scheme 2

$$E_{ox} + FG \underset{0.39\ s^{-1}}{\overset{1.5 \times 10^4\ M^{-1}s^{-1}}{\rightleftharpoons}} E_{ox}{\bullet}FG \xrightarrow{2.2\ s^{-1}} E_{red}{\bullet}imine \underset{\text{not determined}}{\overset{0.49\ s^{-1}}{\rightleftharpoons}} E_{red} + imine$$

similar to the original estimates obtained from classical graphical analysis. The bimolecular rate constant for FG binding was 1.5 × 10^4 M^{-1} s^{-1}, in good agreement with our initial determination. The rate constant for flavin reduction found by global fitting was 2.2 s^{-1}, which is very similar to our initial value of 2.6 s^{-1} obtained from the limiting value of the hyperbola fit to the observed rate constant of the second phase. A rate constant of 0.49 s^{-1} for product release was obtained in the global fit. The notable change from the simpler analysis was the rate constant for the dissociation of FG, which converged to 0.39 s^{-1} in the global fit. This value is considerably lower than our initial estimate of 2 s^{-1} obtained from the intercept of the observed rate constant of the first phase and indicates that FG is actually a sticky substrate. This difference can be understood by considering the exact solution for the two observed rate constants of a two-step reversible reaction (eq 3 and Scheme 3):

$$k_{obs} = \frac{k_1[FG] + k_2 + k_3 + k_4 \pm \sqrt{(k_1[FG] + k_2 + k_3 + k_4)^2 - 4((k_3 + k_4)k_1[FG] + k_2k_4)}}{2} \quad (3)$$

Scheme 3

$$E_{ox} \underset{k_2}{\overset{k_1[FG]}{\rightleftharpoons}} E_{ox,1}{\bullet}FG \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_{ox,2}{\bullet}FG$$

Under appropriate conditions,[10] the observed rate constant of the first phase is approximated by eq 4 (a limiting case of eq 3).

$$k_{obs,1} = k_1[FG] + k_2 + k_3 + k_4 \quad (4)$$

The intercept of the linear concentration dependence is, therefore, the sum of the dissociation rate constant (k_2) and the forward and reverse rate constants of the second reaction step (k_3 and k_4, respectively). Because FAOX-II reduction is very favorable, k_4 is essentially zero, and the intercept is the sum of the dissociation rate constant and the rate constant controlling flavin reduction, giving

$$k_{obs,1} = k_1[FG] + k_2 + k_3 \quad (5)$$

Therefore, the intercept obtained from the plot of $k_{obs,1}$ as a function of [FG] is dominated by the rate constant for flavin reduction.

Reductive Half-Reaction of the Lys53Arg Enzyme. Mutagenesis was used to investigate the importance of the positive charge of Lys53. The reductive half-reaction of the Lys53Arg mutant protein, in which the positive charge is delocalized over the guanidinium moiety, was studied in anaerobic stopped-flow experiments by mixing anaerobic oxidized enzyme with varying concentrations of anaerobic FG at 4 °C. Absorbance traces at 450 nm, the maximum of the oxidized flavin absorbance peak, showed four phases (Figure 4A). As with wild-type, no evidence for flavin semiquinone was observed.

The first phase observed was very similar to that of the wild-type enzyme—a very small increase in absorbance whose observed rate constant increased linearly with respect to FG concentration, indicating that this phase represents binding. Fitting to a line gave a bimolecular rate constant for FG binding of 1.2 × 10^4 M^{-1} s^{-1} (Figure 4B), almost identical to that of wild-type, and an intercept of 0.9 s^{-1}. The second phase was accompanied by a substantial decrease in flavin absorbance, just short of half the total change. The observed rate constants associated with this phase increased to a saturating value, 2.1 ± 0.1 s^{-1}, with increasing FG concentration. We did not use low enough FG concentrations to accurately determine a half-saturating concentration. The third phase of the reaction was accompanied by an absorbance change of a little more than half of the total. The observed rate constant of this phase is independent of FG concentration with an average value of 0.13 ± 0.01 s^{-1}. The fourth phase is accompanied by a small decrease in flavin absorbance. The observed rate constants of this phase are also independent of FG concentration, with an average value of 0.051 ± 0.006 s^{-1}.

dx.doi.org/10.1021/bi1020666 | Biochemistry 2011, 50, 7977–7986

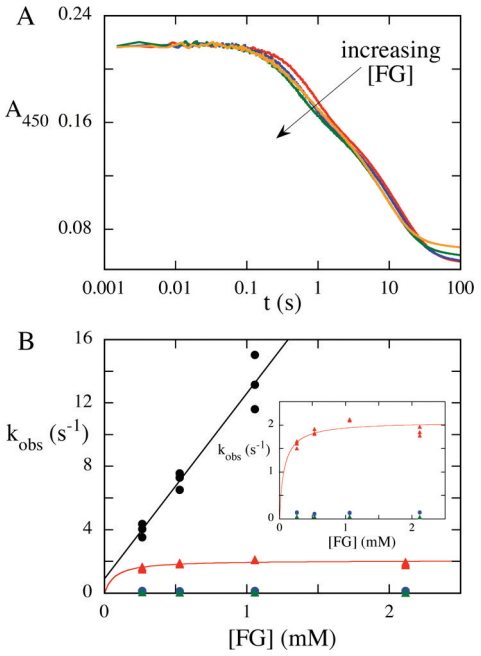


Figure 4. Reductive half-reaction of Lys53Arg FAOX. (A) Averaged reaction traces ($N \geq 3$) at 450 nm observed when ~16 μM enzyme at pH 7.4 was mixed with varying amounts of FG (0.26–2.1 mM final) at 4 °C. The reaction occurs in four phases. Data are plotted on a logarithmic time scale. (B) The observed rate constant of the first phase (black circles) is linear with FG concentration giving a bimolecular rate constant of 1.2×10^4 M^{-1} s^{-1} and a reverse rate constant of 0.9 s^{-1}. The rate constant of the second phase (red triangles) saturates with increasing FG concentration, giving a saturating rate constant of 2.1 ± 0.1 s^{-1}; a hyperbola was fit to the data giving a half-saturating value of 71 ± 20 μM, but FG concentrations low enough for an accurate determination were not used, so the fitted value of the half-saturating concentration is not considered to be accurate. The observed rate constants of the third (blue circles) and fourth (green triangles) phases are independent of FG concentration. The inset shows the concentration dependence of phases two through four for more clarity.

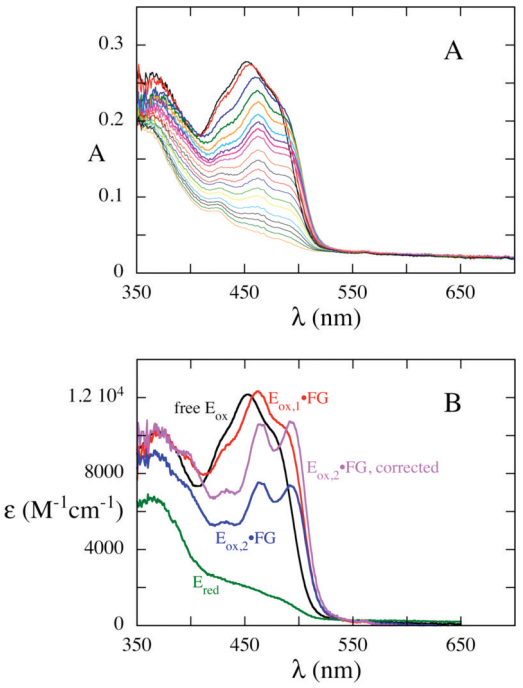


Figure 5. Spectral analysis of the reductive half reaction of Lys53Arg. (A) Anaerobic oxidized Lys53Arg was mixed with anaerobic FG at pH 7.4, 4 °C. Concentrations after mixing were 24 μM enzyme and 484 μM FG. Spectra were collected using a photodiode array detector with a 1.5 ms integration time. Spectra were collected at intervals ranging from 75 ms to 1.2 s, as described in Experimental Procedures. (B) Spectra in the reaction in (A) were calculated by singular-value decomposition and fitting to the reaction scheme and rate constants in Scheme 4. The spectrum labeled as $E_{ox,2}$·FG (blue) was a mixture of oxidized and reduced enzymes, indicating a branched mechanism (Scheme 5). The spectrum of the oxidized intermediate enzyme for ($E_{ox,2}$·FG, corrected; lavender) was calculated by assuming there was 35% reduced enzyme (E_{red}) in the mixture and subtracting it from the spectrum calculated according to the linear mechanism.

To obtain more information on what the phases of this reaction represent, anaerobic oxidized Lys53Arg was mixed with anaerobic FG at 4 °C and spectra were collected from 350 to 700 nm using a diode array detector. The first phase was accompanied by very small spectral changes, in agreement with the single-wavelength data (Figure 5A). The second phase resulted in a species in which the flavin absorbance spectrum became more resolved and clearly had oxidized flavin. The perturbations to the flavin absorbance spectrum caused by the substrate are reminiscent of the large changes observed upon forming the nonreactive FTA complex (Figure 2). This intermediate converts to a species that appears to be mostly reduced. The final phase was accompanied by small spectral changes to give the fully reduced enzyme absorbance spectrum.

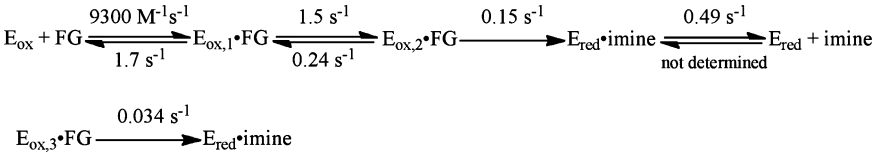
To gain a better understanding of the reductive half-reaction of the Lys53Arg mutant enzyme, Berkeley Madonna was used to globally fit the averaged single-wavelength absorbance traces at different FG concentrations. The data would not fit to a simple four-step reversible model, even though four phases are clearly present in the reductive half-reaction. Therefore, the traces were fit to the four-step reductive half-reaction mechanism (upper line of Scheme 4) with the last step—imine dissociation—held constant to the wild-type rate value, plus a separate single-step reaction for damaged enzyme (lower line, Scheme 4). The rate constant of product release was held constant at 0.49 s^{-1}, the value determined from the wild-type reductive half-reaction data. The data fit very well to this model. The rate constants obtained (shown in Scheme 4) were very similar to those obtained from classical graphical analysis. The rate constant obtained for the reaction of damaged enzyme was 0.034 s^{-1}. This rate constant corresponds to the final phase observed spectroscopically.

This linear kinetic scheme was used with the diode array data to calculate the spectra of intermediates that occurred during the reductive half-reaction. As with the wild-type enzyme, the first observed phase represented substrate binding. This phase resulted in a species with an absorbance spectrum very similar to that of oxidized enzyme but shifted slightly to the red and having a slightly higher extinction (Figure 5B). The second intermediate had a spectrum that appeared to be a mixture of highly resolved oxidized flavin and reduced flavin; this converted to fully reduced enzyme in the next phase.

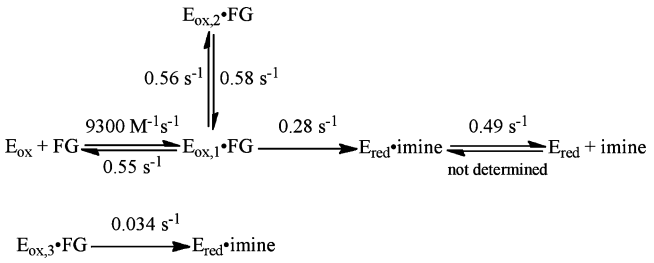
An absorbance spectrum falling between oxidized and reduced flavin is not known in flavin chemistry—a mixture of oxidized and reduced is required to explain the calculated spectrum. A mixture of species requires a branch (Scheme 5). The spectrum of the oxidized enzyme form in the mixture ($E_{ox,2}$·FG,corrected in Figure 5B) was estimated by subtracting 35% reduced enzyme, obtained after the third phase, from the

dx.doi.org/10.1021/bi1020666 | Biochemistry 2011, 50, 7977–7986

Scheme 4



Scheme 5



spectrum of the second spectrum calculated for the linear sequence ($E_{ox,2}$·FG in Figure 5B). The spectrum of the second oxidized enzyme·FG complex obtained ($E_{ox,2}$·FG in Scheme 5) in this way is remarkably similar to the spectrum of the Lys53Met·FTA complex (Figure 2B). Note, though, that the choice of 35% reduced enzyme is only approximate—no unique value can be obtained from the available data.

The branched pathway in Scheme 5 was used as the basis for global fitting in Berkeley Madonna. The extinction coefficients were held constant at the values determined in the linear fit except for $E_{ox,2}$·FG. The extinction coefficient used for this species was calculated by subtracting 35% reduced enzyme from the mixture of oxidized and reduced and dividing by 0.65. During the fit, rate constants for FG binding, product release, and damaged enzyme were held constant to the values determined from the linear fit. The rate constants allowed to fit were those for the dissociation of FG from $E_{ox,1}$·FG, the reaction of $E_{ox,1}$·FG to E_{red}·imine, and the isomerization to and from $E_{ox,2}$·FG. The rate constants in Scheme 5 were obtained after the fit converged. Notably, a rate constant for flavin reduction of 0.28 s^{-1} was obtained, about 8-fold lower than the rate constant for reduction in the wild-type enzyme. It is important to remember, however, that the specific rate constants obtained by this analysis depend on the assumption that 65% of the enzyme partitions to the dead-end oxidized enzyme−substrate complex. While the spectral analysis shows that this is reasonable, the actual partitioning may deviate slightly from this, changing the kinetic analysis.

Reductive Half-Reaction of the Lys53Met Enzyme. The reductive half-reaction of the Lys53Met mutant was also studied. Neutralizing the positive charge slowed reduction substantially. Anaerobic oxidized enzyme was mixed with anaerobic FG (2.5 mM) at pH 7.5 and 25 °C in an anaerobic cuvette, and changes in flavin absorbance were followed in a standard scanning spectrophotometer. The flavin was reduced extremely slowly, and the enzyme precipitated after ~1 week, preventing flavin reduction to be followed to completion. A rate constant of ~10^{-7} s^{-1} was estimated from the initial rate of the absorbance change. This is more than a million-fold slower than wild-type enzyme.

Oxidative Half-Reaction of Wild-Type FAOX-II. FAOX-II is a flavoprotein oxidase; its reduced form is oxidized rapidly by molecular oxygen.[11−13] This reaction was investigated using rapid-reaction kinetics. Anaerobic enzyme was reduced with 1 equiv of FG. The resulting anaerobic reduced enzyme was mixed with different concentrations of oxygen in buffer at pH 7.4 and 4 °C. Reoxidation of the enzyme, monitored at 450 nm, occurred in a single phase (Figure 6A). The observed rate constant varied linearly with oxygen concentration, giving a bimolecular rate constant of (1.65 ± 0.01) × 10^5 M^{-1} s^{-1} (Figure 6B), typical of oxidases. The intercept of the line was 0.9 ± 0.4 s^{-1}, which is essentially zero within experimental error, indicating an irreversible reaction. O_2 always reacts irreversibly with reduced flavoproteins because the two-electron reduction potential of O_2 is so high.

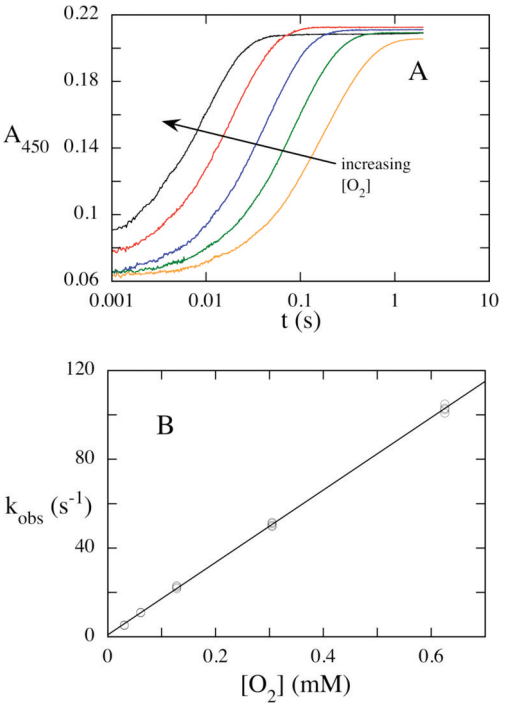


Figure 6. Oxidative half-reaction of wild-type FAOX. (A) Reaction traces at 450 nm observed when enzyme reduced with 1 equiv of FG at pH 7.4 was mixed with varying concentrations of oxygen (30.5−625 μM) in buffer at 4 °C. The oxidation reaction occurs in a single phase. (B) The observed rate constant is linear with oxygen concentration, giving a bimolecular rate constant of 1.65 × 10^5 M^{-1} s^{-1}, typical of flavoprotein oxidases.

Oxidative Half-Reaction of the Lys53Arg and Lys53-Met Mutant Enzymes. The role of Lys53 in the oxidative half-reaction was also probed. The Lys53Arg mutant enzyme was reduced anaerobically with 1 equivalent of FG. The resulting reduced enzyme was mixed with different concentrations of oxygen in buffer at pH 7.5 and 4 °C, and the oxidation of the flavin was monitored at 450 nm. Flavin oxidation was biphasic and thus fit to two exponentials (Figure S3).

dx.doi.org/10.1021/bi1020666 | Biochemistry 2011, 50, 7977−7986

The first phase constituted the majority of the total absorbance change observed. The observed rate constants of this phase increased linearly with oxygen concentration, giving a bimolecular rate constant of $(1.17 \pm 0.01) \times 10^5$ M^{-1} s^{-1}, nearly the same as that of wild-type. The second phase was accompanied by a small change in absorbance—less than 10% of the total change. The observed rate constant of this phase also increased linearly with respect to oxygen concentration, giving a bimolecular rate constant of $(2.0 \pm 0.2) \times 10^4$ M^{-1} s^{-1}. The two phases of flavin oxidation suggests that there are two populations of reduced mutant enzyme, though the reason for the minor population is not known. A small proportion of damaged enzyme was also detected in the reductive half-reaction of this mutant enzyme.

The anaerobic Lys53Met mutant enzyme was reduced by one equivalent of dithionite and reacted with various concentrations of O_2 at pH 7.5 and 4 °C. Flavin reoxidation, observed by the increase in absorbance at 450 nm, was described by a single exponential (Figure S4). The linear variation of the observed rate constant with O_2 concentration indicated an irreversible reaction with a bimolecular rate constant of $(5.6 \pm 0.1) \times 10^3$ M^{-1} s^{-1}.

Enzyme-Monitored Turnover. The entire catalytic cycle of FAOX-II was investigated using enzyme-monitored turnover in a stopped-flow spectrophotometer. Anaerobic oxidized wild-type enzyme was mixed with 1 mM FG and 625 μM oxygen at pH 7.4 and 4 °C. The reaction was monitored at 450 nm to observe changes in flavin absorbance (Figure 7). An initial *increase* in flavin absorbance was observed within the first 2 s, and the absorbance remained this high for the duration of the steady state, indicating that a species with an extinction higher than that of free oxidized enzyme is the predominant enzyme form during turnover. Under the conditions of our experiments, oxygen was the limiting substrate. When oxygen was depleted, a sharp decrease in flavin absorbance was observed. The K_m value of O_2 could not be calculated from the enzyme-monitored turnover experiments because it was too low. Nonetheless, the data were analyzed by integration to determine the turnover number.[9] The apparent k_{cat} observed at 1 mM FG was 0.296 ± 0.005 s^{-1}. Comparing the rate constants determined in each half-reaction with the turnover number suggests that product release is at least partially rate-limiting during catalysis.

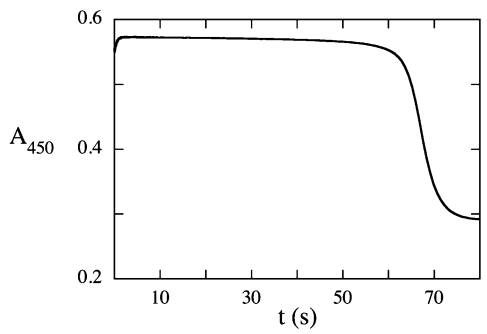


Figure 7. Enzyme-monitored turnover of wild-type FAOX. Reaction trace at 450 nm observed when enzyme was mixed with 1 mM FG and 625 μM of oxygen at pH 7.4, 4 °C. An initial increase in flavin absorbance was observed. Analyzing the data by integration gave a k_{cat} of ~0.3 s^{-1}.

Enzyme-monitored turnover experiments were also conducted with the Lys53Arg mutant enzyme (Figure S5). As with wild-type, the K_m value of O_2 could not be calculated. The apparent k_{cat} observed at 1 mM FG was 0.169 ± 0.005 s^{-1}, very similar to the rate constant obtained for flavin reduction in the half-reaction experiments. Thus, delocalizing the charge of Lys53 changes the rate-limiting step in turnover to the chemical step, flavin reduction.

DISCUSSION

Fructosamine oxidase (FAOX) is a flavin-containing enzyme that catalyzes the oxidation of the carbon–nitrogen bond of fructosamines and the reduction of molecular oxygen to hydrogen peroxide (Scheme 1). The FAD prosthetic group is covalently bound to a cysteine residue at the 8α-methyl group of the flavin. The structure of the enzyme was solved recently[3] and revealed an interesting interaction between a lysine residue and the flavin. Lys53 is found on the *si*-face of the flavin nearly centered over the benzene ring of the isoalloxazine ring system (Figure 1). This ammonium–π interaction is unique to FAOX. Monomeric sarcosine oxidase (MSOX), the closest homologue to FAOX, contains an arginine residue in a similar position;[14] however, the positive charge of the guanidinium group contacts the FAD in both the aromatic and piperazine rings. Here, we investigated the role that the nondispersed positive charge of Lys53 plays in catalysis by FAOX.

The positive charge on the *si*-face of the flavin is important for covalent flavinylation. In the Lys53Arg mutant FAOX protein, the charge is delocalized but still present and covalently bound FAD is still obtained. However, in the Lys53Met mutant protein, the charge is removed and flavinylation is lost. Flavinylation has been very well studied in MSOX,[15,16] the closest homologue of FAOX, which also contains a positively charged residue (arginine) on the *si*-face of the flavin. Analogous to our present results, when Arg49 in MSOX is mutated to lysine, covalent FAD is still obtained, but when the charge is removed by mutation to alanine, no flavinylation occurs.[15] In addition, apoArg49Lys and apo-wild-type MSOX exhibit similar rate constants and mechanisms of flavinylation.[15] The positive charge of Arg49 in MSOX has been proposed to stabilize the thiolate form of Cys315 (the form required for covalent attachment of the FAD) as well to lower the pK_a value of the 8-methyl group of the flavin.[15,16] These postulated functions are not affected by the conservative mutation to lysine. It is very likely that Lys53 plays very similar roles in covalent flavinylation of FAOX, roles that can be accomplished by replacement of Lys53 with arginine, but not with methionine.

The reductive half-reaction of FAOX—fructosamine oxidation with concomitant reduction of the FAD prosthetic group—was studied in anaerobic stopped-flow experiments at 4 °C. Unlike in MSOX[17] or amadoriase I,[18] no charge-transfer absorbance was observed upon substrate binding to FAOX. The ammonium form of the substrate, which would predominate at pH 7.4, would not have a lone pair to donate in a charge-transfer interaction with the flavin. However, no charge-transfer absorbance was observed at pH 9 either, where a significant fraction of the substrate should be in the neutral amine form. Therefore, it appears that the lack of charge-transfer absorbance in FAOX is due to other factors, such as poor flavin–substrate orbital overlap. Instead of charge-transfer formation, a small lag in the absorbance change was observed that had a linear dependence on substrate concentration (Figure 3). This binding phase was

followed by a large decrease in absorbance representing the chemical step and a smaller change in absorbance postulated to represent product release. Thus, our kinetic data are consistent with a three-step reductive half-reaction in which the oxidized enzyme binds the substrate, flavin reduction occurs, and product is released (Scheme 2).

The role of Lys53 in the mechanism of FAOX was studied by site-directed mutagenesis. Interestingly, delocalization of the positive charge at position 53 by substituting arginine changed the mechanism of the reductive half-reaction. A simple linear reaction scheme was not sufficient to explain the spectral and kinetic data. Instead, a branched mechanism in which some enzyme forms a dead-end complex (Scheme 5) seems most likely. The highly resolved spectrum of this complex closely resembles that of the Lys53Met·FTA complex (Figure 2B), suggesting that the charge near the flavin is absent in this complex, perhaps because Arg53 moves away from the flavin. The movement of the arginine in the mutant is plausible. Because the arginine side chain is larger than a lysine side chain, some rearrangement might be needed. Notably, Gln52 occupies two conformations in the wild-type FAOX-FTA structure,[3] suggesting that this region of the protein could be flexible. For whatever reason, a point-charge, borne by lysine in position 53, appears to ensure the integrity of the enzyme−substrate complex.

The importance of a positive charge at position 53 of FAOX was emphasized with the Lys53Met mutant enzyme. This enzyme was very nonreactive with FG. Even at 25 °C, flavin reduction was only ∼10% complete within 1 week. It is estimated that neutralizing the positive charge slowed flavin reduction by more than a million-fold. This suggests that the positive charge lowers the energy of the unoccupied orbital of the isoalloxazine which accepts a hydride (or equivalent) from the substrate.

While mutation of Lys53 had significant effects on the reductive half-reaction, much milder effects were observed on the oxidative half-reaction. Wild-type FAOX reacts with molecular oxygen with a bimolecular rate constant of 1.65×10^5 M^{-1} s^{-1}. Delocalization of the positive charge in the Lys53Arg mutant enzyme had little effect on the oxygen reaction. With this mutant, more than 90% of the enzyme reacted with a bimolecular rate constant of 1.14×10^5 M^{-1} s^{-1}, only ∼1.4-fold slower than wild-type. The remaining 10% of the Lys53Arg enzyme reacted with a bimolecular rate constant of 1.9×10^4 M^{-1} s^{-1}, about an order of magnitude slower than wild-type. We propose that this slower phase represents damaged enzyme (∼10% of the total enzyme population). This damaged enzyme also accounts for the final phase observed in the reductive half-reaction of this mutant, which was also ∼10% of the total amplitude change.

The greatest effect on oxygen reactivity was observed for the Lys53Met mutant enzyme. This enzyme reacts with O_2 with a bimolecular rate constant of 5.3×10^3 M^{-1} s^{-1}, ∼30-fold slower than wild-type. This decrease in reactivity is very mild when compared to the more than a million-fold slowing of flavin reduction observed in this mutant, further indicating that Lys53 is extremely important for the reductive half-reaction. It is unclear what is causing the mild decrease in O_2 reactivity in this mutant. It could be due to changes in electrostatic stabilization of O_2^- or semiquinone, which are obligatory intermediates during oxidation.[11,19] Alternatively, it could be due to a change in reduction potential due to the loss of the covalent FAD.

The complete catalytic cycle was also studied in the wild-type and Lys53Arg proteins via enzyme-monitored turnover. In both cases, an initial increase in flavin absorbance is observed and absorbance remains high throughout the steady state. This indicates that a species with an extinction higher than that of the free oxidized enzyme is the predominant enzyme form during turnover in both proteins. In the absence of a full steady-state analysis of FAOX, it is not known whether a ternary complex forms during turnover. However, it has been shown that molecular oxygen reacts with the E_{red}·P complex in both wild-type and Arg49Lys MSOX.[20,21] Thus, we propose a similar mechanism for FAOX and postulate that the predominant species present during turnover is E_{ox}·P.

The K_m of O_2 was too low in both wild-type and Lys53Arg FAOX to be determined. However, the turnover number could be calculated for both enzymes. The conservative mutation of Lys53 to arginine only reduced k_{cat} by ∼2-fold. This is a much milder effect than was observed for the analogous mutation in MSOX. The Arg49Lys mutant protein had a k_{cat} ∼40-fold lower than wild-type MSOX.[21]

Taken together, our results show that the positive charge of Lys53 is very important for FAOX. Removal of the positive charge renders the enzyme practically dead with respect to flavin reduction. However, it is only the charge and not its distribution that is important—dispersing the positive charge by mutation to arginine had only minor effects on flavin reduction, although a dead-end branch was introduced, indicating that the point-charge helps organize the active site. In an interesting contrast, the corresponding residue in MSOX is arginine; when mutated to lysine, a large decrease in k_{cat}/K_m was observed, indicating that not only the charge but also its distribution was very important for catalysis.[21]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Wild-type, Lys53Arg, and Lys53Met FAOX absorbance spectra, an expanded plot of $k_{obs,2}$ and $k_{obs,3}$ vs [FG] in the reductive half-reaction of wild-type FOAX, reaction traces and plots of k_{obs} vs $[O_2]$ for the Lys53Arg and Lys53Met proteins, enzyme-monitored turnover trace of the Lys53Arg mutant protein, and an example Berkeley Madonna global fitting simulation model. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone (734) 615-2452, Fax (734) 764-3509, e-mail brupalf@umich.edu (B.A.P.); phone (216) 368-6238, fax (216) 368-1357, e-mail vmm3@cwru.edu (V.M.M.).

Present Addresses

[§]de Duve Institute, Université Catholique de Louvain, 75-39, Avenue Hippocrate, 1200 Brussels, Belgium.

[∥]Department of Biochemistry, University of Iowa, 51 Newton Rd., Iowa City, IA 52242.

[@]Division of Organic Chemistry and Biochemistry, Rudjer Boskovic Institute, Bijenicka c. 54, 10 000 Zagreb, Croatia.

Author Contributions

[⊥]These authors contributed equally to this work.

Funding

This work was supported by NIH grants EY07099 (V.M.M.), GM061087 (B.A.P.), NIGMS training grant GM07767 (R.L.F.), the Juvenile Diabetes Foundation, and the Belgian F.N.R.S (F.C.).

■ ACKNOWLEDGMENTS

We thank Dr. Vivien C. Yee for helpful discussions.

■ ABBREVIATIONS

EMT, enzyme-monitored turnover; FAOX, fructosamine oxidase; FG, fructosylglycine; FTA, fructosyl thioacetate; KP_i, potassium phosphate buffer; MSOX, monomeric sarcosine oxidase; PCA, perchloric acid; PEG, poly(ethylene glycol).

■ REFERENCES

(1) Monnier, V. M., Mustata, G. T., Biemel, K. L., Reihl, O., Lederer, M. O., Zhenyu, D., and Sell, D. R. (2005) Cross-linking of the extracellular matrix by the Maillard reaction in aging and diabetes: an update on "a puzzle nearing resolution. *Ann. N.Y. Acad. Sci. 1043*, 533−544.

(2) Gerhardinger, C., Taneda, S., Marion, M. S., and Monnier, V. M. (1994) Isolation, Purification, and Characterization of an Amadori Product Binding Protein from a *Pseudomonas* sp. Soil Strain. *J. Biol. Chem. 269*, 27297−27302.

(3) Collard, F., Zhang, J., Nemet, I., Qanungo, K. R., Monnier, V. M., and Yee, V. C. (2008) Crystal Structure of the Deglycating Enzyme Fructosamine Oxidase (Amadoriase II). *J. Biol. Chem. 283*, 27007−27016.

(4) Takahashi, M., Pischetsrieder, M., and Monnier, V. M. (1997) Molecular cloning and expression of amadoriase isozyme (fructosyl amine:oxygen oxidoreductase, EC 1.5.3) from Aspergillus fumigatus. *J. Biol. Chem. 272*, 12505−12507.

(5) Grandhee, S. K., and Monnier, V. M. (1991) Mechanism of formation of the Maillard protein cross-link pentosidine. Glucose, fructose, and ascorbate as pentosidine precursors. *J. Biol. Chem. 266*, 11649−11653.

(6) Palfey, B. A. (2003) Time Resolved Spectral Analysis in *Kinetic Analysis of Macromolecules: A Practical Approach* (Johnson, K. A., Ed.) Chapter 9, pp 203−228, Oxford UniversityPress, New York.

(7) Williams, C. H. Jr., Arscott, L. D., Matthews, R. G., Thorpe, C., and Wilkinson, K. D. (1979) Methodology employed for anaerobic spectrophotometric titrations and for computer-assisted data analysis. *Methods Enzymol. 62*, 185−198.

(8) Taira, K., and Benkovic, S. J. (1988) Evaluation of the Importance of Hydrophobic Interactions in Drug Binding to Dihydrofolate Reductase. *J. Med. Chem. 31*, 129−137.

(9) Gibson, Q. H., Swoboda, B. E., and Massey, V. (1964) Kinetics and Mechanism of Glucose Oxidase. *J. Biol. Chem. 239*, 3927−3934.

(10) Fleck, G. M. (1971) *Chemical Reaction Mechanisms*, pp 66−100, Holt, Rinehart and Winston, New York.

(11) Palfey, B. A., Ballou, D. P., and Massey, V. (1995) in *Active Oxygen: Reactive Oxygen Species in Biochemistry* (Valentine, J. S., Foote, C. S., Greenburg, A., and Lieberman, J. F., Eds.) Chapter 2, pp 37−83, Chapman andHall, New York.

(12) Palfey, B. A., and Massey, V. (1998) in *Comprehensive Biological Catalysis* (Sinnott, M., Ed.) Vol. *III*, Chapter 29, pp 83−154, Academic Press, New York.

(13) Fagan, R. L., and Palfey, B. A. (2010) Flavin-Dependent Enzymes in *Comprehensive Natural Products Chemistry II* (Begley, T., Ed.) Vol. *7*, Chapter 3, pp 37−114, Elsevier, Oxford, UK.

(14) Trickey, P., Wagner, M. A., Jorns, M. S., and Mathews, F. S. (1999) Monomeric sarcosine oxidase: structure of a covalently flavinylated amine-oxidizing enzyme. *Structure 7*, 331−345.

(15) Hassan-Abdallah, A., Zhao, G., and Jorns, M. S. (2008) Covalent Flavinylation of Monomeric Sarcosine Oxidase: Identification of a Residue Essential for Holoenzyme Biosynthesis. *Biochemistry 47*, 1136−1143.

(16) Hassan-Abdallah, A., Bruckner, R. C., Zhao, G., and Jorns, M. S. (2005) Biosynthesis of Covalently Bound Flavin: Isolation and *in vitro* Flavinylation of the Monomeric Sarcosine Oxidase Apoprotein. *Biochemistry 44*, 6452−6462.

(17) Zhao, G., and Jorns, M. S. (2006) Spectral and Kinetic Characterization of the Michaelis Charge Transfer Complex of Monomeric Sarcosine Oxidase. *Biochemistry 45*, 5985−5992.

(18) Wu, X., Palfey, B. A., Mossine, V., and Monnier, V. (2001) Kinetic Studies, Mechanism, and Substrate Specificity of Amadoriase I from *Aspergillus sp*. *Biochemistry 40*, 12886−12895.

(19) Bruice, T. C. (1984) Oxygen-Flavin Chemistry. *Isr. J. Chem. 24*, 54−61.

(20) Wagner, M. A., and Jorns, M. S. (2000) Monomeric Sarcosine Oxidase: 2. Kinetic Studies with Sarcosine, Alternate Substrates, and a Substrate Analog. *Biochemistry 39*, 8825−8829.

(21) Hassan-Abdallah, A., Zhao, G., and Jorns, M. S. (2008) Arginine 49 Is a Bifunctional Residue Important in Catalysis and Biosynthesis of Monomeric Sarcosine Oxidase: A Context-Sensitive Model for the Electrostatic Impact of Arginine to Lysine Mutations. *Biochemistry 47*, 2913−2922.