ANTIOXIDANTS & REDOX SIGNALING
Volume 3, Number 5, 2001
Mary Ann Liebert, Inc.

Forum Original Research Communication

Substrates but Not Inhibitors Alter the Redox Potentials of Monoamine Oxidases

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ABSTRACT

The midpoint potentials for the reduction of the cysteinyl–flavin adenine dinucleotide (FAD) in monoamine oxidases (MAO) A and B in the absence and presence of ligands have been determined. Both MAO A and MAO B can be reduced chemically in two steps, the first generating a semiquinone spectrum and the second the spectrum of fully reduced FAD, each of which requires two electron equivalents. The midpoint potentials for the oxidized/semiquinone and semiquinone/reduced couples were −159 ± 4 mV and −262 ± 3 mV for MAO A and −167 ± 4 mV and −275 ± 3 mV for MAO B. After modification with a thiol reagent, direct reduction from the oxidized to fully reduced form was observed with no semiquinone and without change in the overall midpoint potential. In the presence of substrate, no semiquinone was formed, but the midpoint potential for full reduction of the flavin was positively shifted by up to 500 mV, depending on the substrate. This shift in potential could permit a more thermodynamically favorable transfer of electrons from the amine substrates to oxygen. In contrast, stable products and inhibitors did not cause a shift in potential and did not prevent the formation of semiquinone.
Antioxid. Redox Signal. 5, 723–729.

INTRODUCTION

MONOAMINE OXIDASES (MAO) A and B oxidize amines in both brain and peripheral tissues as part of the pathways of degradation of neurotransmitters and xenobiotics (for review, see 13). A long-standing problem in understanding the chemical mechanism of MAO has been the great difference the redox potentials of flavin adenine dinucleotide (FAD) [−0.208 V in its free form (1)], amine (around +1 V(10)], and oxygen [+0.295 V for O_2/H_2O_2 (10)]. A further mechanistic puzzle is how the amine substrate influences the reaction of the reduced flavin with oxygen (2, 4, 5). Free reduced enzyme is oxidized slowly, but reduced enzyme with substrate (not product) bound is reoxidized many times more rapidly. The stimulation of reoxidation depends on the amine substrate used, varying from fivefold with 5-hydroxytryptophan to 120-fold for kynuramine with MAO A (11). As a first step toward an explanation for this conundrum, we have measured the midpoint potentials of the cysteinyl-FAD in each enzyme in the absence and presence of substrates and inhibitors.

MATERIALS AND METHODS

Enzymes and reagents

Human liver MAO A expressed in yeast (14) and beef liver MAO B (9) were purified and as-

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sayed as before (11). α-Methylbenzylamine, benzylamine, serotonin, D-amphetamine, indigo disulfonate, and indigo trisulfonate were purchased from Sigma Chemical Co., 2,6-dichlorophenolindophenol (DCIP) from General Biochemicals, Inc., and anthraquinone-2-sulfonate from Aldrich.

Determination of the redox potentials

The redox potentials in the absence of substrate were determined by the xanthine oxidase method (3). The reduction of the enzyme alone from the oxidized to the semiquinone form ($FAD_{ox} \rightarrow FAD_{sq}$) was determined with 5–10 μM MAO A or B in 50 m*M* potassium phosphate, pH 7.2, containing 0.02% Brij-35, 30 m*M* glucose, 1–2 μM methyl viologen, 7–17 μM dye, 200 μM xanthine, 1 unit/ml glucose oxidase, 24 units/ml catalase, and 50 μl of xanthine oxidase (to give 12–96 n*M*). The spectral changes were recorded in a Hewlett–Packard diode array spectrophotometer at 25°C for 30 min to 2 h, depending on the level of xanthine oxidase used. The concentrations of the reduced and oxidized dye were calculated from the absorbance at 610 nm for indigo disulfonate or 600 nm for indigo trisulfonate. The concentrations of the oxidized and semiquinone FAD of the MAO were calculated from the absorbance changes at the isosbestic points of the dyes: 457 nm for indigo disulfonate and 486 nm for indigo trisulfonate.

When the formation of semiquinone was complete (no further change at 412 nm) and the first dye was completely reduced, the second dye [anthraquinone-2-sulfonate (to give 5–10 μM)] was mixed in from the sidearm and spectra recorded again. The spectral picture was complicated because the spectra of the dye and the enzyme-bound FAD overlapped. To calculate the concentration of reduced and oxidized forms of both the dye and the FAD, a nonlinear regression program, NONLIN (shareware by Phillip H. Sherrod, 4410 Gerald Place, Nashville, TN 37205-3806, U.S.A.), was used to analyze the spectra based on the known spectra of the pure forms of each component.

For the determination of the redox potential in the presence of a substrate, the anaerobic cuvette was prepared as above, except that the dye was DCIP and xanthine and xanthine oxidase were omitted. Instead, substrate was placed in the side arm (to give a concentration of at least $20 \times K_m$).

For accuracy, we measured the redox potentials of the dyes in the specific buffer system used and in the presence of detergent necessary to maintain optical clarity for the duration of the experiment. The redox potentials were measured by cyclic voltammetry using electrochemical cell model C1B-120 (BioAnalytical Systems, Inc.) with a silver electrode (+0.202 V) as the reference, a gold working electrode, and a platinum auxiliary electrode. The values obtained were very close to the literature values (10): anthraquinone-2-sulfonate, −250 mV; indigo disulfonate, −137 mV; indigo trisulfonate, −91 mV; DCIP, +215 mV. Neither detergent nor substrate affects the redox potential of the dyes.

Redox potentials for two of the substrates were determined in the same way by cyclic voltammetry. The value obtained for benzylamine was +1.11 V, and for α-methylbenzylamine it was +1.35 V in agreement with literature values (10).

RESULTS

Redox potentials for the enzyme alone

The spectral changes observed after addition of xanthine oxidase to the experimental mixture with MAO A and the reference dye, indigo disulfonate, are shown in Fig. 1. The calculated ratios of oxidized/semiquinone FAD are plotted against those for the oxidized/reduced dye in Fig. 1 (inset). The redox potential of the enzyme was calculated by using the modified Nernst equation shown in the legend to Fig. 1. The difference between the potential of the reference dye and the enzyme was calculated at the value when ln(oxidized/reduced dye) is zero.

From the Nernst equation, the slope of the double logarithmic plot of the oxidized/reduced ratios is 1 when equal numbers of electrons are transferred onto both the enzyme and the dye. Indigo disulfonate accepts two elec-

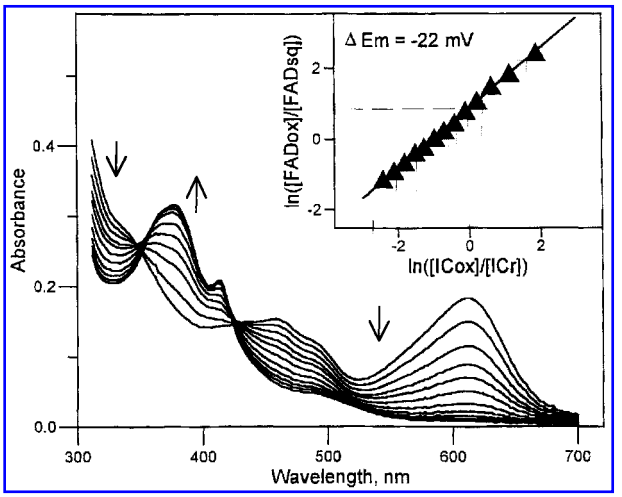

FIG. 1. Selected spectra for the reduction of MAO A flavin in the presence of indigo disulfonate. The enzyme (9.5 μM) and the dye (11 μM) were incubated under anaerobic conditions at 25°C in 50 mM phosphate buffer, pH 7.2, with xanthine oxidase (60 nM), methyl viologen (1 μM), and xanthine (200 μM). The spectra shown were recorded every 12 min over the period of 120 min. The arrows indicate the direction of the changes in absorbance. **Inset:** Determination of the redox potential for FAD_{ox}/FAD_{sq} redox couple from the double logarithmic plot. The difference between the potential for the enzyme and the potential for the reference dye was calculated by using the modified Nernst equation: $\ln([E_{ox}]/[E_r]) = -E_m(N_e\,F/RT) + N_e/N_d\,\ln([D_{ox}]/[D_r])$, where E_{ox} and E_r are the oxidized and reduced forms of the enzyme; D_{ox} and D_r are the oxidized and reduced forms of the reference dye; and N_e and N_d are the number of electrons transferred onto the enzyme and the dye, respectively.

trons, so that for the one-electron reduction of FAD_{ox} to FAD_{sq} indicated by the spectral changes, the expected slope in the inset to Fig. 1 would be 0.5. Instead, the slope obtained from the double logarithmic plot was 1 (Fig. 1, inset), suggesting that two electrons are required for the reduction that produces the semiquinone spectrum for MAO A shown in Fig. 1. Similar data were obtained with indigo trisulfonate and for MAO B with indigo disulfonate (6). That the same slope was obtained with either dye and at different concentrations of xanthine oxidase indicates that the value obtained is not due to a lack of equilibrium between the enzyme and the dye, but rather to the number of electrons required to produce the semiquinone.

When the yield of flavin semiquinone had stabilized at its maximum value, a second reference dye was added and the reduction of enzyme and dye for the second phase of the reduction (FAD_{sq}/FAD_{red}) followed spectrophotometrically as before. The inset to Fig. 2 is the double logarithmic plot for the semiquinone/reduced FAD ratio against the ratio of oxidized/reduced dye. Again, we obtained a slope of 1, as was found for the first phase of the reduction.

From the midpoint potentials relative to the dyes and the absolute values (relative to the standard hydrogen electrode) determined for the dyes from cyclic voltammetry, values for the redox potentials for MAO A and MAO B in the absence of substrate were obtained as shown in Table 1. Note that, in contrast to the values determined here for the bound flavin in

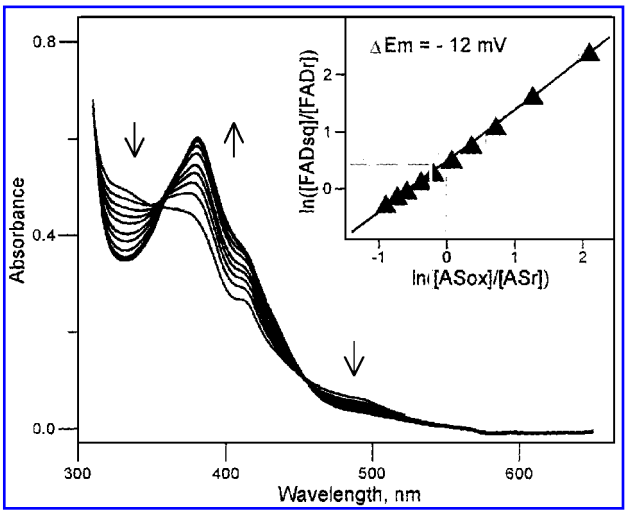

FIG. 2. Selected spectra showing the reduction of MAO A flavosemiquinone in the presence of anthraquinone-2–sulfonate and reduced indigo disulfonate. At the end point of the reaction shown in Fig. 1, anthraquinone-2-sulfonate (10 μM) and xanthine oxidase (30 nM) were added to the reaction mixture, and the reaction was monitored for a further 100 min. The spectra shown were recorded every 10 min. **Inset:** Determination of the redox potential for the FAD_{sq}/FAD_{red} redox couple from the double logarithmic plot. Concentrations of reduced and oxidized forms of the dye and the flavin were calculated by a linear regression from the equation: $A_i = E_{sq} * A_{i,e,sq} + E_o * A_{i,e,o} + D_r * A_{i,d,r} + D_o * A_{i,d,o}$, where A_i is the value of absorbance of the reaction mixture at a wavelength i; $A_{i,e,sq}$, $A_{i,e,o}$, $A_{i,d,r}$, and $A_{i,d,o}$ are the absorbances of the pure forms of the semiquinone enzyme, oxidized enzyme, reduced dye, and oxidized dye, respectively, at a wavelength i; E_{sq} and E_r are concentrations of the semiquinone enzyme and the reduced enzyme; and D_r and D_o are concentrations of the reduced dye and the oxidized dye. **Inset:** The double logarithmic plot for the determination of the redox potential for the semiquinone-fully reduced couple. The difference between the potential for the enzyme and the potential for the reference dye was calculated as described in the legend to Fig. 1.

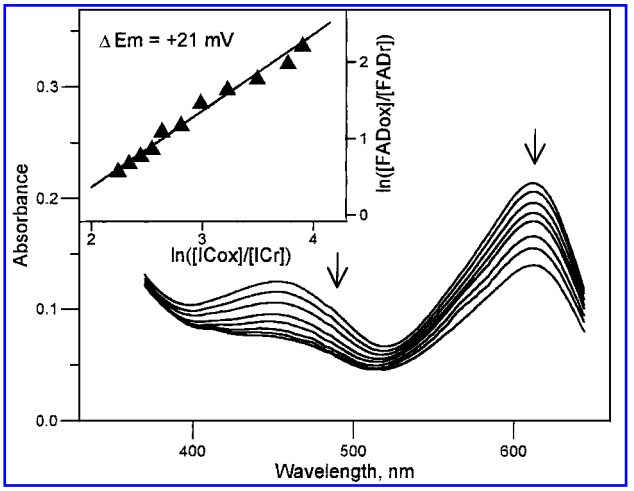

FIG. 3. Selected spectra showing the reduction of MAO A flavin (8.2 μM) by α-methylbenzylamine (50 mM) in the presence of indigo disulfonate (11 μM). The spectra were recorded over a period of 200 min. **Inset:** The double logarithmic plot for the determination of the redox potential for the oxidized–reduced redox couple in the presence of substrate. The difference between the potential for the enzyme and the potential for the reference dye was calculated as described in the legend to Fig. 1.

MAO, the values for free FAD in solution are −0.236 V for the first electron and −0.176 V for the second electron (1).

Redox potential of MAO after modification with the thiol reagent, 4,4′-dipyridyl disulfide (DPDS)

No semiquinone was formed during the reduction of DPDS-modified MAO A (data not shown). The midpoint potential for the full reduction of the flavin (FAD_{ox}/FAD_{red}) was −210 ± 20 mV, the same as for free FAD and the same as that calculated for FAD_{ox}/FAD_{red} in the unmodified enzyme. The slope of the double log plot was 1, consistent with a two-electron transfer to both the enzyme and the dye.

Redox potentials for MAO in the presence of substrate

No semiquinone has ever been detected during reduction by substrate. The overall midpoint potentials for FAD_{ox}/FAD_{red} in the unliganded enzymes, calculated from the redox potentials of the two phases (Table 2), would be −210 mV for MAO A and −221 mV for MAO B. We determined the potentials of both MAO A and B in the presence of both substrates and inhibitors. For the inhibitor–enzyme complexes, the xanthine oxidase method was used to supply electrons as for the free enzyme. However, substrates reduce the flavin so no external source of electrons is required. The rate of reduction must be slow enough that the redox state of the MAO flavin remains in equilibrium with the reporter dye, so only relatively slow substrates can be used.

Figure 3 shows the reduction of MAO A and indigo disulfonate by α-methylbenzylamine over a period of 200 min. As expected, there is no sign of semiquinone formation. The double logarithmic plots of the oxidized/reduced ratios for the enzyme and dye are linear with a slope of 1, consistent with the two electron reduction of both the enzyme and the dye. The calculated redox potentials for both MAO A and B in the presence of substrates are shown in Table 2. Each substrate raises the potential of the flavin but the values obtained are different for each substrate and for each enzyme. For example, α-methylbenzylamine-saturated MAO A has a redox potential of −116 mV, but α-methylbenzylamine-saturated MAO B has a redox potential of +281 mV.

The redox potentials for MAO A and B in the presence of saturating concentrations of inhibitors were also determined. 1-Methyl-4-styrylpyridinium is the four-electron oxidation product of 1-methyltetrahydrostilbazole and is a competitive inhibitor of the steady-state turnover of both MAO A (K_i = 6 μM) and B (K_i = 100 μM) (8). It has no effect on the redox potentials for either enzyme (Table 2) nor does it prevent the formation of the semiquinone during the xanthine oxidase-reduction procedure. The slopes of the double logarithmic plots are

TABLE 1. MIDPOINT POTENTIALS FOR FAD_{ox}/FAD_{sq} AND FAD_{sq}/FAD_{red} IN MAO A AND MAO B

	*Redox potential (mV ± SD)**	
Redox couple	*MAO A*	*MAO B*
Oxidized → semiquinone	−159 ± 4	−167 ± 4
Semiquinone → reduced	−262 ± 3	−275 ± 3

The redox potentials were measured in the reductive direction.

*Standard deviation for n = 3.

TABLE 2. MIDPOINT POTENTIALS FOR MAO A AND MAO B IN THE PRESENCE OF LIGANDS

Ligand	Redox couple	Redox potential ($mV \pm SD$[a]) MAO A	MAO B
None	Oxidized → reduced	−210[b]	−221[b]
With substrate[c]			
5-Hydroxytryptamine	Oxidized → reduced	ND[d]	+194 ± 9
α-Methylbenzylamine	Oxidized → reduced	−116 ± 4[e]	+281 ± 12
Benzylamine	Oxidized → reduced	+263 ± 15	ND[d]
With other ligands (and xanthine oxidase)			
D-Amphetamine[b]	Oxidized → semiquinone	−176 ± 5	−119 ± 3
1-Methyl-4-styrylpyridinium	Oxidized → semiquinone	−158 ± 4	−162 ± 7
α-Methylbenzylamine	Oxidized → semiquinone	−130 ± 6[f]	+245 ± 7[e]
	Semiquinone → reduced	−102[g]	+317[g]

[a]Standard deviation for $n = 3$.
[b]Calculated from the two one-electron potentials.
[c]Substrate is the reductant (no xanthine oxidase present).
[d]ND, not determined because the rate of reduction of this enzyme by this substrate was too fast to be certain of equilibrium between the enzyme and the dye.
[e]The flavin was prereduced by the substrate, and then the reference dye added.
[f]The reduction of MAO A by α-methylbenzylamine is exceedingly slow. When the xanthine oxidase reduction system was induced, semiquinone was formed and the slope of the double logarithmic plot was 0.5.
[g]Calculated from the experimentally determined FAD_{ox}/FAD_{red} midpoint potential and that for the FAD_{ox}/FAD_{sq} couple.

1 for the FAD_{ox} to FAD_{sq} transition for both MAO A and MAO B, just as found for the free enzymes. The other inhibitor used was D-amphetamine, which inhibits the oxidation of kynuramine by MAO A with a K_i of 14 μM (14) and of benzylamine by MAO B with a K_i of 0.74 mM (12). In contrast to the observations with the product, the slope of the double logarithmic plots with amphetamine was 0.5, consistent with the expected electrochemical stoichiometry for the FAD_{ox}-FAD_{sq} redox couple. The redox potentials are given in Table 2. Unlike 1-methyl-4-styrylpyridinium, amphetamine raises the potential of the cysteinyl-FAD in MAO B where it can act as a slow substrate, but not in MAO A.

DISCUSSION

The linearity of the double logarithmic plots over a range of xanthine oxidase concentrations established that the equilibrium between the redox states of the enzyme and reference dyes was achieved. Thus, reliable redox potentials were obtained, albeit only for the reductive direction, a limitation of this method (3).

The slopes of the double logarithmic redox plots for the free enzyme indicated that two electrons are required for each phase of the reduction of MAO. This stoichiometry was confirmed directly by quantitative titration of MAO with dithionite (7), which demonstrated that four electrons were required for full reduction. This stoichiometry suggested the presence of an additional redox group that accepts two electrons in addition to the two required for the flavin reduction. The spectra in Fig. 1 clearly indicate that FAD semiquinone (a one-electron reduction of FAD) is formed. The dye, indigo carmine, is a two-electron acceptor, so a slope of 0.5 would be expected. However, the slope of the plot in the inset of Fig. 1 (and of Fig. 2, for the second step) is 1. One possibility is that the second redox group in the active site could accept one electron, giving an amino acid radical. This radical could then stabilize the flavin semiquinone generated by one electron reduction of the FAD moiety itself. The stoichiometry of such a two-electron reduction of

MAO would be consistent with the slope of the inset to Fig. 1.

Reductive titration of MAO before and after modification of the thiols (7) has suggested that the second redox group is a protein disulfide. In the native enzyme, a semiquinone spectrum is evident after two electron equivalents and four electron equivalents are required for full reduction of the flavin, consistent with the slope of 1 for FAD_{ox}/FAD_{sq} in the inset to Fig. 1 and for FAD_{sq}/FAD_{red} in Fig. 2. After thiol modification, only two electrons were required for full reduction and no semiquinone formation could be detected (7). In this modified enzyme, the slope of the redox plot was 1 as expected for two-electron transfer to both enzyme and dye. Thus, native enzyme is reduced in two two-electron steps in each of which one electron must go to a protein redox group and one to the flavin, whereas the modified enzyme has only one redox group (the FAD) and undergoes two-electron reduction to the fully reduced form (FAD_{ox}/FAD_{red}).

The data summarized in Tables 1 and 2 for the midpoint potentials for the reduction of MAO A and B in the absence and presence of ligands support some general conclusions. First, the midpoint potentials for the first and second electron reductions (Table 1) are inverted compared with those for free FAD, indicating that the covalent attachment and/or the groups around the flavin have altered the redox properties of the FAD moiety. Second, the substrates used here (*e.g.*, benzylamine with MAO A and α-methylbenzylamine with MAO B in Table 2) induce a large positive shift in the potential in both MAO A and MAO B, bringing the redox potential to above +0.25 V. Third, inhibitors do not induce the positive shift observed with substrates and do not prevent the formation of the semiquinone of FAD. A small positive shift (+0.05 V) was observed in the midpoint potential for MAO B with amphetamine. Partial reduction of the flavin in MAO B by amphetamine was obtained after overnight incubation under anaerobic conditions, so the shift could be due to amphetamine acting as an extremely slow substrate for MAO B. There was no shift in the potential for MAO A for which no reduction was observed.

To summarize, the redox potential in the enzyme–substrate complex is positively shifted toward the potential of the amine substrate. This shift is different for each substrate and each enzyme, so the values for the midpoint potentials for MAO A and B in the presence of physiological substrates remain to be determined. The substrate site influences the flavin very specifically depending on the ligand, in both MAO A and B. Thus, in the cell, the amine substrates present will determine the redox properties of their own disposal mechanism.

ACKNOWLEDGMENTS

This research was supported by the National Institutes of Health (HL-16251) and by the Department of Veterans Affairs. We dedicate this article to Professor T.P. Singer, who died in 1999, in recognition of his contribution to science over the 50 years of his research career.

ABBREVIATIONS

DCIP, 2,6-dichlorophenolindophenol; FAD, flavin adenine dinucleotide; MAO, monoamine oxidase.

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Received for publication September 28, 2000; accepted February 25, 2001.

This article has been cited by:

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