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Mutation of surface cysteine 374 to alanine in monoamine oxidase A alters substrate turnover and inactivation by cyclopropylamines

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Abstract—Modification of cysteine (Cys) residues inactivates monoamine oxidases (MAO) yet the crystal structure shows no conserved cysteines in the active site of MAO A (Ma, J. et al. *J. Mol. Biol.* **2004**, *338*, 103–114). MAO A cysteine 374 was mutated to alanine and the purified enzyme characterized kinetically. The mutant was active but had decreased k_{cat}/K_m values compared to the wild-type enzyme. Cyclopropylamine-containing mechanism-based inactivators similarly showed lower turnover rates. Spectral studies and measurement of free thiols established that 1-phenylcyclopropylamine (1-PCPA) formed an irreversible flavin adduct whereas 2-phenylcyclopropylamine (2-PCPA) and *N*-cyclo-α-methylbenzylamine (*N*-CαMBA) formed adducts that allowed reoxidation of the flavin on denaturation and decreased cysteine in both wild-type and mutant MAO A. In the 1-PCPA and *N*-CαMBA inactivations, the partition ratio was decreased by more than 50% in the mutant. The data suggest that mutation of Cys374 influences MAO A catalysis, which has implications for MAO susceptibility to redox damage. These results are compared with previous work on the equivalent residue in MAO B, namely, cysteine 365.

1. Introduction

Monoamine oxidase (MAO) is a FAD-containing enzyme located in the outer membrane of mitochondria that oxidizes a variety of amines to the corresponding imines in both the central nervous system and peripheral tissues. The two forms of MAO, MAO A and MAO B, have more than 70% sequence identity, including seven conserved cysteines. The fact that MAO contains one or more vulnerable cysteine residues was first recognized in 1945.[1] Inactivation of bovine liver MAO B by various sulfhydryl reagents was prevented by substrates and inhibitors. Kinetics and physicochemical observations suggested that two cysteine residues were required for activity.[2] Corroborating this, the kinetics of the inactivation of purified MAO A by 2,2′-dipyridyl disulfide (DPDS) were biphasic and suggested that at least two cysteine residues were modified before activity disappeared.[3] D-Amphetamine, a competitive inhibitor, protected against inactivation, suggesting that the groups modified might be located in the active site of the enzyme. Site-directed mutagenesis of MAO A Cys374 and the corresponding MAO B Cys365 to serine resulted in no activity when expressed in COS cells.[4] However, when these residues were mutated to alanine and expressed in *Pichia pastoris*, both MAO A Cys374Ala and MAO B Cys365Ala mutants were active.[5] The properties of the MAO A Cys374Ala mutant are reported here.

The crystal structures of MAO B and now also of MAO A revealed that there are no cysteines in the active site,[6,7] leaving the reasons for the loss of activity upon cysteine

Abbreviations: MAO, monoamine oxidase; FAD, flavin adenine dinucleotide; DPDS, 2,2′-dipyridyl disulfide; 2-PCPA, 2-phenylcyclopropylamine; 1-PCPA, 1-phenylcyclopropylamine; *N*-CαMBA, *N*-cyclopropyl-α-methylbenzylamine; PCR, polymerase chain reaction; YPD, yeast peptone dextrose; DEAE, diethylaminoethyl; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PEA, phenylethylamine; MPP^+, 1-methyl-4-phenylpyridinium; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PR, partition ratio.

Keywords: Monoamine oxidase; Cysteine modification; Cyclopropylamine; Allosteric effect; Chemical mechanism.

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modification yet to be elucidated. Cyclopropylamines are mechanism-based inactivators of MAO and various analogs modify either the flavin and/or a cysteine residue, depending on the structure of the inactivator.[8,9] Inactivation by these types of inactivators is useful in the study of the catalytic mechanism because they must act as substrates to generate the reactive product that inactivates the enzyme. Therefore, cyclopropylamines were employed in this study to gain insight into the reason why mutation of Cys374 in MAO A does not inactivate the enzyme.

In addition to the inactivation seen with the cyclopropylamine family, several laboratories have reported inactivation by sulfhydryl reagents.[2,3,10] For example, *N*-ethylmaleimide inactivates MAO by modifying several of the sulfhydryl residues.[10] It is puzzling that active site ligands protect the enzyme from inactivation despite the fact that the surface cysteine residues remain accessible to the modifying agent.

To probe the influence of the cysteine residues on catalysis, we compare here the steady-state kinetics of the mutant MAO A Cys374Ala and wild-type enzyme and their mechanism-based inactivation by cyclopropylamines showing that alteration of this single surface residue does alter the kinetics. We compare these results with published data on MAO B and suggest a mechanism for inactivation based on the previously proposed one-electron transfer mechanism for MAO[9] and the new data.

2. Results

2.1. Expression of MAO A and MAO A Cys374Ala in *Pichia pastoris*

Recombinant human liver MAO A and the mutant MAO A Cys374Ala were expressed constitutively in *P. pastoris* and purified as described in the Experimental section. After purification, the samples of MAO A and MAO A Cys374Ala had specific activities of 0.3 and 0.4 U/mg, respectively. Based on the difference in the reduction of the flavin by substrate and by dithionite, the wild-type MAO A preparation was 92% active and the mutant preparation 87% active. The wild-type MAO A showed the expected kinetics (see Table 1 and below), mass (59822 Da), and spectral characteristics (not shown). The only difference from data obtained using MAO A purified after expression in *S. cerevisiae*[11] is the high rate for the oxidation of serotonin (Table 1).

2.2. Substrate steady-state kinetics

The steady-state kinetic parameters for MAO A and MAO A Cys374Ala were determined for several substrates (Table 1). For kynuramine and benzylamine, the K_m of the mutant is significantly higher than for the wild type ($p < 0.05$). The specificity constants (k_{cat}/K_m) for the faster substrates, kynuramine and serotonin, are slightly lower for the mutant. In fact, the slope of the plot of the specificity constants for the mutant against those for the wild-type enzyme is 0.7 (Fig. 1), revealing the same tendency for decreased values of k_{cat}/K_m in the mutant for all substrates. The values of $\Delta\Delta G^{\ddagger}$ (Table 1) reflect this trend, showing that more activation energy is required in the mutant to catalyze the oxidation of amines, and the difference is larger for the substrates that are turned over faster.

2.3. Competitive inhibition

Only some of the K_m values for substrates are significantly different in the mutant relative to wild-type enzyme, suggesting that binding is not likely to have been altered by the mutation (Table 1). The K_i values for competitive inhibitors, D-amphetamine, MPP^+, and harman were also determined. Table 2 shows little variation in the K_i values between wild-type and mutant MAO A for each of these structurally different inhibi-

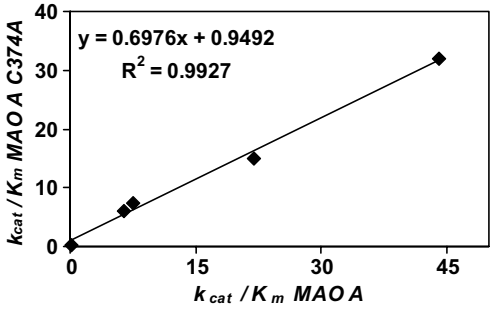


Figure 1. Kinetic differences between MAO A and mutant MAO A Cys374Ala in the specificity constant for kynuramine, benzylamine, MPTP, serotonin and PEA. The values are taken from Table 1.

Table 1. MAO A and MAO A Cys374Ala (C374A) steady-state parameters for substrates[a]

Substrates	K_m (mM)		k_{cat} (s^{-1})		k_{cat}/K_m (s^{-1} mM^{-1})		$\Delta\Delta G^{\ddagger}$ (kJ mol^{-1})
	WT[b]	C374A	WT	C374A	WT	C374A	
Benzylamine[c]	0.42 ± 0.03	0.61 ± 0.03	0.09 ± 0.01	0.079	0.20 ± 0.03	0.22 ± 0.03	−0.24
MPTP	0.033 ± 0.004	0.039 ± 0.003	0.241 ± 0.004	0.29 ± 0.02	7.5 ± 1	7.4 ± 0.6	+0.03
PEA	0.49 ± 0.07	0.44 ± 0.04	3.1 ± 0.4	2.5 ± 0.4	6.4 ± 0.9	6 ± 1	+0.16
Serotonin	0.43 ± 0.02	0.51 ± 0.07	18.6 ± 0.9	16 ± 2	44 ± 4	32 ± 8	+0.80
Kynuramine[c]	0.10 ± 0.02	0.17 ± 0.02	2.2 ± 0.1	2.5 ± 0.1	22 ± 3	15 ± 2	+0.96

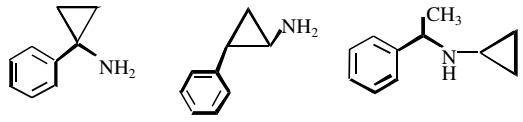
[a] The values represent means ± SEM of at least five determinations.
[b] WT, wild type MAO A.
[c] K_m values for mutant and wild-type enzymes are significantly different using *t*-test—two sample assuming unequal variances, $p < 0.05$.

Table 2. MAO A and MAO A Cys374Ala competitive inhibitor constants[a]

Inhibitor	K_i (μM)	
	WT	Cys374Ala
D-Amphetamine	14.4 ± 0.5	11.5 ± 0.7
MPP^+	3.5	4.5 ± 0.5
Harman	0.128 ± 0.005	0.130 ± 0.002

[a] The values represent the means ± SEM of data from two determinations each using seven inhibitor concentrations.

tors, consistent with a lack of alteration in binding to the active site.



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2.4. Inactivation by cyclopropylamines

The inactivation of MAO A and MAO A Cys374Ala by 1-PCPA (**1**), 2-phenylcyclopropylamine (2-PCPA, **2**) and *N*-cyclopropyl-α-methylbenzylamine (*N*-CαMBA, **3**) was examined (Table 3). The K_I and k_{inact} values are the inactivation equilibrium constant and inactivation rate constant at saturation, respectively. The rates of inactivation (k_{inact}) are essentially the same for both enzymes but the K_I values are 1.6- to 2-fold higher for the mutant than the wild-type enzyme. The k_{inact}/K_I values for the mechanism-based inactivators with the mutant enzyme reflect the same decrease found for substrates, confirming the alterations in the catalytic efficiency.

The partition ratio was determined over a period of 5–10 days for each cyclopropylamine (PR in Table 3). The partition ratio reflects the number of product species generated per covalent modification event that results in inactivation. Table 3 shows that, although the partition ratio with 2-PCPA is not significantly changed, those for *N*-CαMBA and 1-PCPA are lower in the mutant by more than 50%. This means that, for each reactive product molecule generated, inactivation is two-fold more likely to occur in the mutant lacking Cys374 than in the wild type.

The experiments described here show that mutation of Cys374 in MAO A does not prevent inactivation. To determine the target of modification (thiol or flavin) by these inactivators in both the wild type and mutant MAO, the free cysteine content was measured. The cysteine contents of MAO A and MAO A Cys374Ala before and after inactivation by *N*-CαMBA and 1-PCPA are listed in Table 4. Wild-type MAO A contained 8.3 cysteine residues per mol of flavin, in agreement with previous determinations.[3,12] The mutant enzyme, MAO A Cys374Ala, contained only 6.9 free thiols, a decrease of one cysteine, as expected. Both MAO A and the mutant MAO A Cys374Ala inactivated by *N*-CαMBA have one less free cysteine residue than untreated enzyme, showing that one thiol has been modified in both. In view of the variable modification of thiol groups by *N*-ethylmaleimide,[10] it is entirely possible that the observed stoichiometry is statistical and dependent on the conditions used, which were chosen to obtain maximal inhibition with minimum reagent. However, the data show that at least some thiol modification accompanied the inactivation. After borohydride treatment designed to stabilize the proposed cysteine adduct,[13] wild-type and mutant samples were sent for digestion and mass spectrometry to identify the cysteine peptides and any modification. However, two separate mass spectrometry laboratories failed to obtain all of the cysteine peptides, so there was no reliable data to identify which cysteine was modified. The thiol determination could not be done with 2-PCPA because the adduct formed is unstable, releasing cinnamaldehyde,[14] which interferes with the thiol measurement.

Table 4. Cysteine content of MAO A and MAO A Cys374Ala (C374A) before and after inactivation by *N*-CαMBA and 1-PCPA[a]

Inactivator	WT	C374A
None	8.3	6.9
N-CαMBA	7.0	5.8
1-PCPA	8.2	6.8

[a] Values correspond to the number of cysteine residues per enzyme molecule (average of four experiments).

Since inactivation by the cyclopropylamines can also occur by flavin modification that prevents reoxidation of the flavin when the protein is denatured, the spectra after denaturation of the mutant enzyme inactivated by each of the three cyclopropylamines were recorded. Figure 2A shows that the spectrum after denaturation of the 1-PCPA modified mutant is clearly not that of oxidized flavin (Fig. 2A), suggesting the formation of irreversibly modified flavin. The flavin in the MAO A Cys374Ala mutant remains reduced, as is the case for the wild-type enzyme,[12] consistent with modification of the flavin by 1-PCPA. This is in agreement with the find-

Table 3. Kinetic properties for 2-PCPA, *N*-CαMBA and 1-PCPA with MAO A and MAO A Cys374Ala (C374A)[a]

Inactivator	K_I (mM)		k_{inact} (min^{-1})		k_{inact}/K_I (mM min^{-1})		PR[b]	
	WT	C374A	WT	C374A	WT	C374A	WT	C374A
N-CαMBA	1.4 ± 0.3	2.7 ± 0.4	0.9 ± 0.3	1.0 ± 0.1	0.64	0.37	4.7	1.6
1-PCPA	7.3 ± 0.6	12.8 ± 0.8	0.50 ± 0.01	0.61 ± 0.03	0.068	0.048	11.5	5.6
2-PCPA	0.20 ± 0.02	0.31 ± 0.03	4.1 ± 0.4	5.0 ± 0.4	20.5	16.1	3.2	2.9

[a] The values represent the means ± SEM of two experiments.
[b] PR, partition ratio.

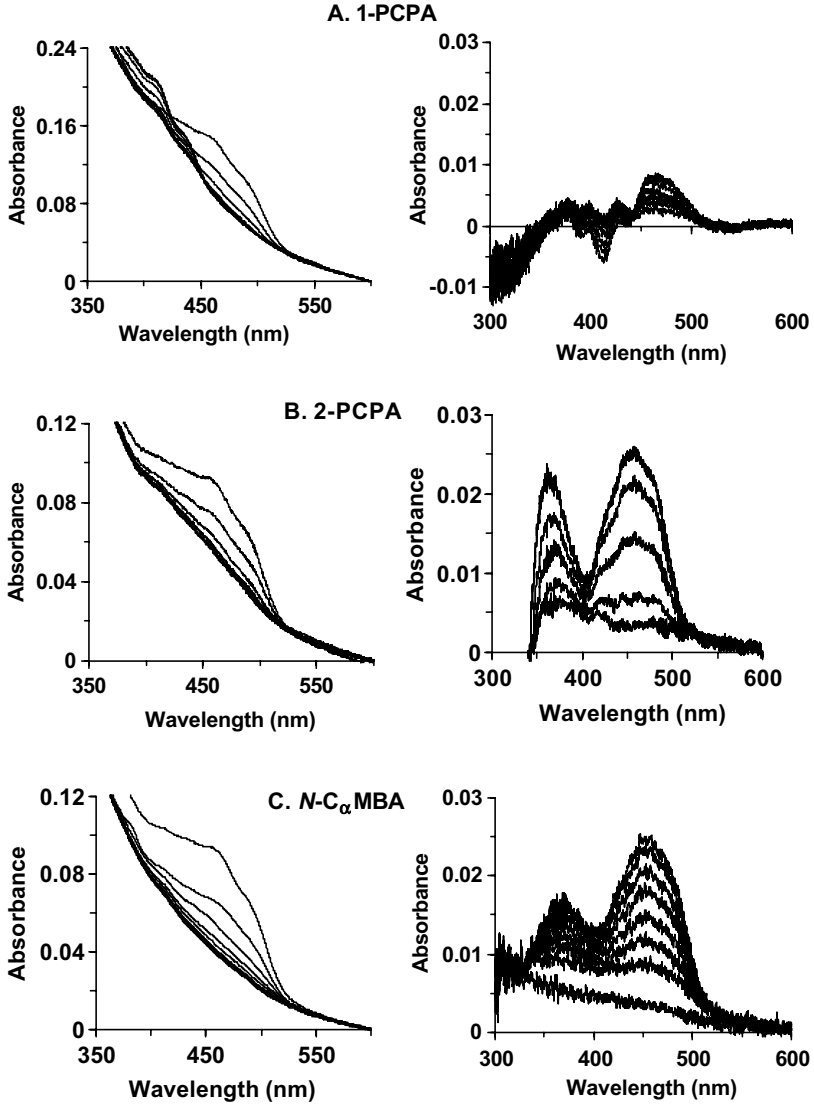


Figure 2. MAO A Cys374Ala flavin spectral changes upon inactivation by cyclopropylamines followed by protein denaturation. Spectra were recorded (left column) during the incubation with the inactivator and activity monitored until >95% inactivation was reached. The enzyme was then denatured by the addition of an equal volume of saturated urea (11 M) and allowed to reoxidise. (A) Left: Reduction of the flavin by 1-PCPA. 1-PCPA (5 mM) was added to 3.9 μM MAO A Cys374Ala in 50 mM potassium phosphate buffer, pH 7.2, 0.1% Brij-35, and spectra were recorded over a period of 5 h. Right: Difference spectra of flavin after denaturation. The flavin remained reduced over a period of 16 h. (B) Left: Reduction of the flavin by 2-PCPA. 2-PCPA (2 mM) was added to 3.4 μM MAO A Cys374Ala as above, and spectra were recorded over a period of 2 h. Right: Difference spectra of flavin after denaturation. The flavin was fully reoxidized after 1 h. (C) Left: Reduction of the flavin by *N*-CαMBA. *N*-CαMBA (2.5 mM) was added to 3.5 μM MAO A Cys374Ala as above, and spectra were recorded over a period of 3 h. Right: Difference spectra of flavin after denaturation. Spectra were recorded over a period of 1 h, after which time the flavin was fully reoxidized.

ings in MAO N where the non-covalent flavin allowed identification of the adduct at N5 of the flavin.[15] On the other hand, in the enzyme modified by 2-PCPA the oxidized flavin spectrum appears after denaturation under mild conditions where both N5 and C4α adducts should be stable (Figure 2B). The same result was obtained with wild-type MAO A (not shown). Thus, with both the wild-type and mutant enzymes, 2-PCPA does not irreversibly modify the flavin, suggesting that the inactivator forms an adduct with an amino acid residue, possibly a cysteine, although this could not be verified directly. The *N*-CαMBA-modified enzyme yielded a fully oxidized flavin spectrum after denaturation (Fig. 2C), indicating that the flavin is not irreversibly modified. The decrease of one free thiol shows that the target of *N*-CαMBA modification must include a cysteine residue.

3. Discussion

The data in the literature related to the influence of thiol groups on the catalytic properties of both MAO A and MAO B is often contradictory. For example:

- Thiol modification inactivates the enzyme[3]
- Thiol modification changes the redox properties of the flavin[16]

- Ligands prevent thiol modification[3]
- Cys to Ser mutants expressed in COS cells retain partial or no activity[4] but Cys389Ala in MAO B was inactive[17]
- Inactivation of MAO B by *N*-CαMBA results in modification of Cys365[12]
- MAO B Cys365Ala and MAO A Cys374Ala mutants expressed in *P. pastoris* retain activity[5]
- The crystal structures of MAO A and MAO B show that Cys374 and Cys365, respectively, are on the surface of the protein and not in the active site[6,7]
- MAO A Cys374Ala, although active, shows small catalytic differences from the wild-type (Tables 1 and 3).

Clearly the cysteine residues in MAO play an important role in maintaining the wild type, fully functional enzyme.

The observation that crystals of MAO A and MAO B were obtained only with ligand in the active site[6,7] suggests that the enzyme is relatively flexible and this has been confirmed by variations in side chain positions in the active site.[18] In addition, circular dichroism studies showed that ligand binding induced conformational changes.[19] Chemical modification of MAO B Cys365 by *N*-CαMBA, now known to be on the surface, 20 Å from the entrance cavity, inactivates the enzyme.[12] Modification of as-yet-unknown cysteines alters the redox properties of the flavin in the active site,[16] despite the fact that in the structure of MAO no cysteine is within contact distance of the flavin.[6,7] This means that the surface cysteines may play an allosteric role in determining the properties of the active site.

3.1. MAO A Cys374Ala mutant is active

The wild-type and mutant enzymes were expressed in *P. pastoris* at levels that permitted analysis of the activity and detection of the MAO protein by western blot (not shown). Both were fully active, in contrast with the data reported for COS cell expression.[4] In the case of COS cells, the expression of the MAO A clones was verified only at the level of mRNA so it is possible that the MAO A Cys374Ser mutant protein reported as having no activity was not translated into protein or that the mutant protein was less stable in COS cells.

3.2. MAO A Cys374Ala has small kinetic differences from the wild-type

After *P. pastoris* expression, both the wild-type and mutant proteins were stable and were purified by the usual method. This permitted a full kinetic characterization of the mutant to see how this residue, presumably on the surface of the enzyme, influences the catalytic activity. Using five substrates (Table 1) with a k_{cat} range from 0.09 to 19 s^{-1}, a clear trend emerged (Fig. 1) that the mutant gave lower specificity constants (k_{cat}/K_m) than the wild-type enzyme. This is most apparent for the substrates with higher turnover numbers. Theories of enzyme catalysis[20] suggest that many enzymes use binding energy to optimize k_{cat} rather than to decrease K_m below the level encountered in the cell. This is achieved by stabilization of the transition state. The differences between the activation energies for amine oxidation by the wild type and the mutant were analyzed for the five substrates in Table 1. The values, which range from −0.24 to +0.96 kJ mol^{-1}, are relatively small but reflect the trend of the k_3 values determined by stopped-flow analysis.[11] It seems that this mutation, although on the surface of the enzyme, does influence the catalytic process in the active site.

Some differences are seen in the K_m values but it should be noted that the K_m for MAO A includes rate constants.[11] The competitive inhibitor constants that were measured give estimates of the binding constants, but even they are not simple constants because D-amphetamine[21] and MPP^{+}[11] bind both to the oxidized and to the reduced form of the enzyme. Inspection of Table 2 shows minor differences in the K_i values, indicating that the influence of the mutation on the active site does not strongly affect the binding. Rather, it is likely that the chemical steps in the catalysis are altered as reflected in the activation energy changes in Table 1.

3.3. MAO A Cys374Ala is still inactivated by cyclopropylamines

Three cyclopropylamines were studied as inactivators of both wild-type enzyme and the Cys374Ala mutant. In previous work, 1-PCPA was shown to modify the flavin of MAO A and *N*-CαMBA a cysteine residue.[12] The results in Figure 2 (the flavin redox state) and Table 4 (the thiol count) confirm this and, further, demonstrate that 2-PCPA does not irreversibly modify the flavin. Mutation of Cys374 to Ala does not block the modification of the enzyme by any of these inactivators (Fig. 2, Table 4). Also, inactivation of both wild-type and Cys374Ala MAO A by these cyclopropylamines gives the same overall results: one cysteine lost with *N*-CαMBA and no cysteines are lost with 1-PCPA. These results are consistent with the absorption spectra after inactivation and after inactivation and urea denaturation (Fig. 2). *N*-CαMBA does not prevent reoxidation of the flavin after denaturation, whereas 1-PCPA does, suggesting that the former does not modify the flavin, but the latter does.

As with the substrates, specificity constants for the mechanism-based inactivators (k_{inact}/K_I) in the mutant were less than in the wild type (Table 3). Since the inactivation depends on the catalytic turnover the same factors should indeed be influenced by the mutation. The alteration in the partition ratio is another matter. For *N*-CαMBA and 1-PCPA the partition ratio is decreased by more than 50% for the mutant. Given that the lifetime of the reactive product of catalysis can be assumed to be unchanged, the residue(s) modified in the mutant must be more susceptible or available than in the native enzyme.

3.4. Cysteine reactivities toward cyclopropylamines in MAO A and MAO B

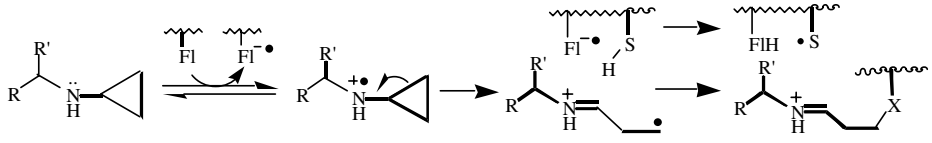
In previous work it was found that there were differences between MAO A and MAO B inactivation

by cyclopropylamines. 1-PCPA attached reversibly to a cysteine residue and irreversibly to the flavin when it inactivated MAO B,[22] while with MAO A, 1-PCPA modified only the flavin.[12] In the cases of *N*-CαMBA and 2-PCPA, both MAO A and MAO B are inactivated by attachment to a cysteine. However, the crystal structure of MAO B covalently modified with 2-PCPA showed that the flavin was modified at C4α of the flavin ring.[18] This was unexpected and is not supported by the chemical and spectral evidence from three different laboratories. Indeed, after inactivation of MAO B with radioactive 2-PCPA, all inhibitor-introduced radioactivity could be separated from the flavin peptide, and the flavin was fully reoxidized when the protein was denatured under conditions where either N5 or C4α adducts would be stable.[14,23]

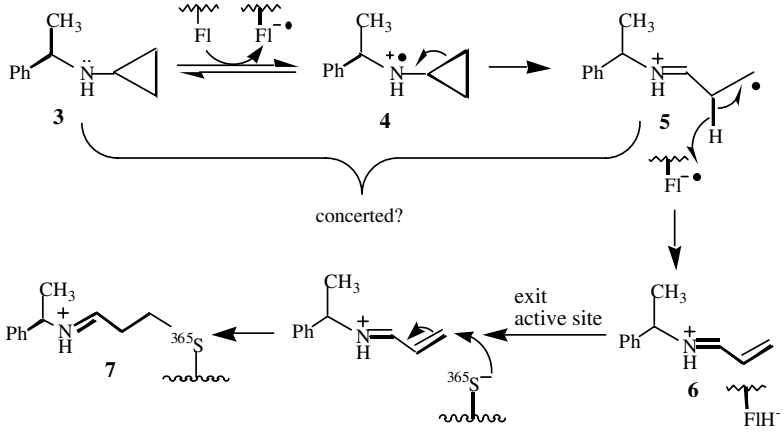
The results regarding modification of the cysteine residues of wild-type MAO A and Cys374Ala by cyclopropylamines are surprising, especially for *N*-CαMBA, because with MAO B, Cys365 was identified as the site of modification by this inactivator,[13] and Cys374 in MAO A is equivalent to Cys365 in MAO B. The Cys-365Ala mutant of MAO B was recently shown to be active.[5] At first sight, this appears to contradict the result that inactivation of MAO B by *N*-CαMBA modifies Cys365.[13] However, Cys365 in MAO B and Cys374 in MAO A are not the only possible sites of modification. Evidence for the reactivity of other cysteine residues comes from the inactivation of MAO B with allylamine[24] and from treatment of MAO A and MAO B with *N*-ethylmaleimide (NEM).[10] A time-dependent incorporation of multiple equivalents of allylamine was observed in MAO B. With *N*-ethylmaleimide, seven MAO A thiol groups were modified in 5 h, whereas only two MAO B thiols were modified after 25 h. Therefore, in the absence of Cys374 (MAO A) and Cys365 (MAO B), other cysteine residues can be accessible for modification.

3.5. Mechanism of inactivation of MAO by cyclopropylamines

Scheme 1 depicts a mechanism for inactivation of MAO B by cyclopropylamines that involves trapping of the radical produced by cyclopropyl ring homolysis with an active-site cysteine radical. According to the crystal structure of MAO B,[7] there is no cysteine residue in the active site, and $Cys365_b$ which is modified by *N*-CαMBA, is more than 20 Å from the active site. So how does modification of Cys365 inactivate MAO B? Scheme 2 proposes a revised mechanism for inactivation of MAO B by *N*-CαMBA. According to this mechanism, electron transfer to the amine radical cation (**4**) leads to homolytic cleavage of the cyclopropyl ring as before. In the revised mechanism this radical (**5**) is further oxidized to the corresponding α,β-unsaturated iminium ion (**6**), which escapes the active site because of the lack of an appropriate nucleophile to react with it. On the way out, it reacts with Cys365, which undergoes a facile Michael addition to give **7**. A similar mechanism could be relevant to the inactivation of MAO A. In the case of the Cys374Ala mutant of MAO A or the Cys365Ala mutant of MAO B, reactive product **6** would bypass the former site of these cysteine residues (now alanine residues in the mutants) and react with different cysteine residues, as evidenced by the loss of one cysteine residue for both wild type and mutant enzymes (Table 4).



Scheme 1.



Scheme 2.

3.6. Implications for MAO A in the cell

The cyclopropylamine data here, together with literature evidence for the inactivation of MAO by thiol reagents, indicate that at least some of the eight free thiols on the surface of MAO are readily modified and that this modification decreases the activity. Several examples of enzyme regulation by thiol modification are already established, for example, in the release of calcium induced by a thiol reagent[25] or the regulation of the adenine nucleotide carrier in apoptosis[26] and in the observation that selenium oxidation of protein thiol groups induced the mitochondrial permeability transition.[27]

The difference in sensitivity of MAO A and MAO B to inactivation by thiol reagents is also notable. The half times for inactivation by *N*-ethylmaleimide were 3 min for MAO A but 8 h for MAO B.[10] This means that MAO A is much more likely to be inactivated by thiol modification or oxidation than MAO B. The alteration of the oxidation of amines in the MAO A Cys374Ala mutant indicates that alteration of even one of the surface cysteines can lower the efficacy of catalysis, particularly noticeable for serotonin oxidation (decreased by 30%). Earlier data clearly show progressive inactivation with multiple thiol modifications. Given the important role of MAO, particularly in neurons, its susceptibility to thiol oxidation or modification with concomitant decrease of activity may have implications for cell function and neurodegenerative disease.

3.7. Conclusions

In conclusion, the results described here suggest a small allosteric effect of cysteine residue Cys374 on catalysis. The distance from where the reactive product of cyclopropylamine oxidation is generated next to the flavin and the location of the nearest Cys is consistent with the hydrophobic and relatively low nucleophilic character of the active site of MAO.[7] Because of this environment, electrophiles generated at the active site are able to escape and modify other parts of the enzyme. The lack of nucleophiles near the flavin in the active site may be imperative so that there is no interference with the radical chemistry that MAO appears to catalyze.[8,9]

4. Experimental

4.1. Construction of MAO A expression vector and Cys374Ala mutant

A cDNA encoding human liver MAO A cloned into the vector pYEDP60 was a gift from Dr. P. Urban, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France. Polymerase chain reaction (PCR) was used to introduce restriction sites for *Sfu* I and *EcoR* I at the 5′- and 3′-ends, respectively, to remove untranslated regions and facilitate cloning into pGAPZa (Invitrogen Corp.). A PCR product corresponding to the 5′-end of MAO A, generated using the primers 5′-GTA*TTCGAA*-CAAGATGGAAAACCAAGAAAAGGC-3′ and 5′-CTG*GAATTC*AGGCGCCCCGAAATGGATA-3′, was cleaved with *Sfu* I–*EcoR* I and cloned into the corresponding sites of pGAPZa. To remove the 3′-untranslated region, a PCR product generated using the primers 5′-CTTGCCCGGAAAGCTGATCGAC-3′ and 5′-CGTGT*GAATTC*TCAAGACCGTGGCAGGAGC-3′ was cleaved with *Kas* I and *EcoR* I and used to replace the corresponding region of pGAPZa containing the 5′-end of MAO A.

Mutation of the codon for Cys374 to alanine was performed by PCR using the immediately adjacent (5′) *Sac* I restriction site. The 5′-ACAAAATCTGCTGGA-CAAAGACTGCTAGG-3′ and 5′-GGGGGGG*GAG-CTC*AGCGATTTTCTTCTTCCTT-3′ primers were used in PCR and the *Sac* I–*Sac* I cleaved product was used to replace the corresponding fragment in wild-type MAO A. The absence of unwanted PCR-derived mutations in the MAO A wild-type and Cys374Ala mutant was verified by sequencing.

4.2. Transformation of *Pichia pastoris* and expression

The pGAPZa plasmids containing wild-type MAO A and MAO A Cys374Ala cDNAs were linearized with *Avr* II and separately transformed by electroporation into a protease deficient strain (SMD 1168 kex1:: SUC2),[28] as described in the Invitrogen product manual. The transformants were screened for multiple integration into the *Pichia* genome by increasing concentrations of zeocin (Invitrogen). The clones resistant to the higher concentrations of antibiotic were allowed to grow in shake flasks containing YPD media at 30 °C. After harvesting, the cells were broken by vortexing with glass beads (425–600 microns, Sigma) and the MAO activity measured using kynuramine as substrate.

4.3. *Pichia* fermentation

The fermentation was performed in a Microferm fermentor (New Burnswick Scientific) with 10 L total volume of basal salts medium with the addition of 2 mL/L of trace salts.[29] The growth was improved with the addition of 0.5% yeast extract, 1% peptone, and 2% glucose, at 30 °C, pH 6–7, agitation from 400 rpm and aeration from 6 L/min. The fermentation was done in two batches with addition of 0.5 L of 40% glucose and increase of agitation (up to 800 rpm) and aeration (up to 14 L/min) at the beginning of the first stationary phase. The pH was kept constant by the addition of filter-sterilized ammonia. The cells were harvested at the start of the second stationary phase and stored at −80 °C.

4.4. MAO purification

The enzyme purification was adapted from the previously described method.[3,30] The cells were broken using glass beads and vortexed in 4 cycles of 1 min duration of vortexing and 1 min of cooling in ice. The mitochondria membranes were separated and incubated at 30 °C in 0.1 M triethanolamine, pH 7.2, at a protein concentration of 12 mg/mL with 25 mM CaCl, 100 mg/L of phospholipase C (Sigma) and 225 μL/L of phospholipase A

(from *Naja naja* venom, 1603 U/mL, specific activity = 254 U/mg). After the Triton X-100 extraction, the enzyme was loaded onto 1000 cm × 2 cm column of DEAE Sepharose CL-6B (Sigma) and eluted with a linear gradient of 20–225 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol, 0.5 mM D-amphetamine, 0.8% β-octylglucoside, 0.5 mM phenylmethylsulfonyl fluoride and 3 mM dithiothreitol. After concentration of the fractions with the highest purity MAO, the enzyme was stored at −20 °C in 50% glycerol (v/v). The fraction of active enzyme was determined spectrally as the amount of the flavin cofactor reduced by substrate compared to the total flavin reduced by dithionite. Anaerobic flavin reduction was measured at 456 nm ($\varepsilon = 10.8\ mM^{-1}\ cm^{-1}$)[3] after addition of 1 mM kynuramine and then after addition of a few crystals of sodium dithionite.

4.5. Steady-state kinetics

All substrates (kynuramine, benzylamine, serotonin, 1-methyl-4-phenyl-1,2,3,6-tetrahydopyridine (MPTP) and PEA) and inhibitors (D-amphetamine, 1-methyl-4-phenylpyridinium (MPP^+) and harman) were purchased from Sigma. Initial rates of amine oxidation were measured in 50 mM potassium phosphate, pH 7.2, with 0.2% Triton X-100, at 30 °C. With kynuramine, the reaction was followed spectrophotometrically at 314 nm, with benzylamine at 250 nm and with MPTP at 343 nm. The oxidation of serotonin and PEA were measured polarographically. Changes in the activation energy for amine oxidation as a result of the mutation were calculated from $\Delta\Delta G^{\ddagger} = -RT \ln[(k_{cat}/K_m)_{mutant}/(k_{cat}/K_m)_{wild\text{-}type}]$.[31] The significance of the kinetic differences between MAO A and the mutant was verified using the *t*-test (two-sample assuming unequal variances) in Microsoft Excel, with a 95% degree of confidence.

The competitive inhibition constants for the inhibitors were obtained using kynuramine as substrate at an enzyme concentration in the cuvette of 20 nM. The reaction was started by the addition of substrate to the enzyme mixture for D-amphetamine and MPP^+. In the case of harman, the enzyme was pre-incubated at 30 °C with inhibitor for 3 min and then added to the assay mixture containing both substrate and inhibitor.

4.6. Inactivation by cyclopropylamines

The syntheses of 1-PCPA[22] and *N*-CαMBA[32] have been reported. 2-PCPA was purchased from Sigma. All experiments with the cyclopropylamines were performed as previously published,[12] except those described below.

The K_I and k_{inact} values were determined by measuring the remaining activity using 1 mM kynuramine during inactivation over a range of concentrations of each cyclopropylamine (0.8–5 mM for 1-PCPA, 0.125–0.6 mM for *N*-CαMBA and 12–100 μM for 2-PCPA). The logarithm of the percentage of activity was plotted against time to calculate the half-life ($t_{1/2}$) for each inactivator concentration. The $t_{1/2}$ versus the reciprocal of the inactivator concentration (Kitz and Wilson plot[33]) gives a straight line where the intercept at the *x*-axis gives the negative reciprocal of the K_I value, and the intercept at the *y*-axis gives $1/k_{inact}$.

The PR was determined using 10 different concentrations of inactivator (0–6 μM for *N*-CαMBA and 2-PCPA and 0–80 μM for 1-PCPA) with a constant amount of enzyme (about 2 μM). The solutions were incubated at 25 °C for up to 15 days and the activity of the samples was expressed relative to the control solution without inactivator.

For the thiol titrations, aliquots (200 μL) of 28 μM MAO A or 20 μM MAO A Cys374Ala were added to 200 μL aliquots of a solution of 1-PCPA (final concentration of 0 or 5 mM) or *N*-CαMBA (final concentration of 0 or 2 mM) in 100 mM potassium phosphate buffer, pH 7.2, containing 20% glycerol and 0.2% Triton-X and were incubated overnight at room temperature. Aliquots were taken for enzyme activity and protein assays. Each enzyme solution was dialyzed for 3 h against three changes (500 mL each) of 100 mM potassium phosphate buffer, pH 7.2, containing 20% glycerol and 0.2% Triton-X and spun at 14,000 g, 4 °C for 10 min. Aliquots were taken for enzyme activity and protein assays. The thiol titrations with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) were performed according to a modification of the literature procedures.[34] A 100-μL aliquot of inactivated MAO A or MAO A Cys374Ala was added to a solution of 480 μL of distilled water with 200 μL of 100 mM potassium phosphate, pH 8. The reference cuvette contained the same solution except with the dialysis buffer substituted for the enzyme. After initiating autozero against the reference cell, aliquots of 20 μL of 4 mg/mL DTNB in 100 mM potassium phosphate, pH 8, were added to the reference cuvette and then to the enzyme solution. The reaction was followed at 412 nm until no change was observed.

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