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Conformational changes in monoamine oxidase A in response to ligand binding or reduction

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Abstract

The structure of monoamine oxidase B [Nat. Struct. Biol. 9 (2002) 22] revealed three aromatic amino acid residues within contact distance of the flavin cofactor and a large number of aromatic residues in the substrate binding site. Circular dichroism (CD) spectroscopy can detect alterations in the environment of aromatic residues as a result of ligand binding or redox changes. CD spectra of MAO A indicate that a small inhibitor such D-amphetamine perturbs the aromatic residues very little, but binding of the larger pirlindole (2,3,3a,4,5,6-hexahydro-8-methyl-1*H*-pyrazino[3,2,1-j,k]carbazole hydrochloride) causes spectral changes consistent with the alteration of the environment of tyrosine and tryptophan residues in particular. Reduction of the flavin cofactor induces large enhancement of the CD signals in the aromatic region (260–310 nm). When covalent modification of the flavin by clorgyline accompanies reduction, the perturbation is even greater. In contrast to the static picture offered by crystallography, this study reveals changes in the aromatic cage on ligand binding and suggests that reduction of the cofactor substantially alters the environment of aromatic residues presumably near the flavin. In addition, the covalently modified reduced MAO A shows significant differences from the substrate-reduced enzyme.

Keywords: Circular dichroism; Aromatic amino acid; Difference spectra; Flavin reduction; Covalent modification; Monoamine oxidase A

1. Introduction

Human monoamine oxidases (MAO) A and B are flavoprotein amine oxidases that have been intensively investigated due to their roles in metabolism of the neurotransmitters, serotonin and dopamine, and of other biogenic amines [1]. Although 70% homologous in sequence, they are differentially expressed in cells and tissues, exhibit different substrate specificities, and can be specifically inhibited (reviewed in Ref. [2]).

The crystal structure of MAO B [3,4] has revealed the importance of hydrophobic and aromatic residues in the substrate-binding cavity around the flavin in keeping with the hydrophobic pharmacophore delineated by inhibition studies [5]. The aromatic residues in the active site are conserved in all MAO A and B sequences. In addition, Tyr 398 and Tyr 435 in MAO B, thought to orient the substrate, lie perpendicular to the flavin in an orientation also seen for the equivalent aromatic pair in polyamine oxidase [6]. Tyr 60 lies close to the flavin with the backbone carbonyl forming a hydrogen bond to N3 on the flavin. Other aromatic residues located in the active site of MAO B include Phe 168, Tyr 188, Phe 343, Tyr 383, Trp 388, Tyr 401, Phe 402, and Trp 432, all of which are all strictly conserved in MAO A. A schematic diagram of the aromatic residues surrounding the flavin and substrate site in MAO B is shown in Fig. 1.

Mutations of the Trp 388 that stacks with the flavin and the two substrate orienting tyrosines (Tyr 398 and Tyr 435) clearly indicate that the aromatic moieties in these positions are essential for activity [7]. These three residues are all within contact distance of the flavin. Mutation of the equivalent residues in MAO A (Trp 397, Tyr 407, and Tyr 444) gave the same results consistent with a conserved role for these residues.

Ligand binding alters the visible spectrum of the 8-α-cysteinyl-FAD in MAO A [8,9] indicating that the environment of the flavin has been altered. This was suggested to be due to stacking of inhibitor on top of the isoalloxazine ring of

Abbreviations: MAO, monoamine oxidase; CD, circular dichroism

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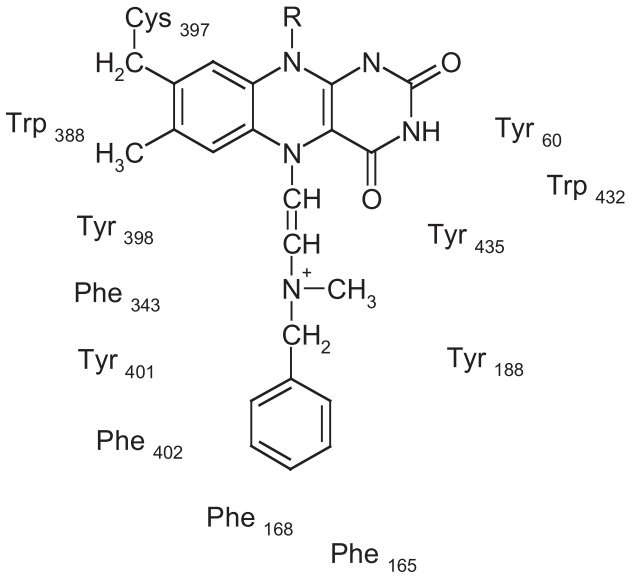

Fig. 1. Schematic representation of the active site of MAO. The structure of the flavin co-factor of MAO after modification by pargyline is shown with the conserved aromatic residues found around the active site in the structure of MAO B [3].

the flavin [10], but the crystal structure of MAO B revealed that inhibitors bind perpendicular to the flavin, although stacking was reported for the phenyl ring of one covalent inhibitor [4]. Ligands also affect the kinetic and redox properties of the enzyme. Re-oxidation of the flavin by O_2 is up to 100-fold faster in the presence of substrate, whereas in the presence of inhibitor it is slower [11]. Inhibitors stabilize the semiquinone form of the flavin and cannot be reduced further, in contrast to unliganded enzyme where only 35% semiquinone is formed before full reduction is observed [8]. In the presence of substrate, semiquinone is never observed [12–14]. The molecular basis of these redox effects is not known.

Given the aromatic-rich active site, circular dichroism (CD) offers a different approach to explore the interactions of ligands with the active site of MAO A. The differential absorption of polarized light is very sensitive to changes in the environments of aromatic residues within the protein [15,16]. CD can provide valuable information about structural changes induced by ligand binding or reduction that affect the position or interactions of phenyalanine, tyrosine and tryptophan. Thus, spectral alterations particularly in the aromatic region (260–310 nm) might be expected after ligand binding or reduction of the flavin if the conformation of these residues is perturbed. Although comparisons of the MAO B structures have not so far shown any change after reduction [3,4], the nonplanar conformations of the carbonyls in the isoalloxazine ring suggest considerable strain in the flavin site. If reduction alters the flavin shape or perturbs the aromatic residues around it, changes in the CD spectra might be expected.

The CD spectra for MAO A reported here did indeed show major changes in the aromatic region after reduction, with differences between the enzyme reduced by dithionite, substrate, or as a result of suicide inhibition. This functional study indicates that the environment of the active site in MAO A is altered more than might be expected from a comparison of the MAO B structures with noncovalent (where the enzyme is assumed to be oxidised) and covalent (where the enzyme is reduced) inhibitors in the active site. Whereas ligand binding appears to result in only a slight increase in the rigidity, reduction and covalent modification induce large changes in the aromatic residues of the active site of MAO A.

2. Materials and methods

2.1. Enzyme purification and visible absorption spectroscopy

Monoamine oxidase A (human liver form) was purified after expression in *Saccharomyces cerevisiae* [12]. The enzyme was stored at −20 °C in a solution of 50 mM potassium phosphate, pH 7.5, containing 0.8% *n*-octyl-β-D-glucopyranoside, 1.5 mM dithiothreitol, 0.5 mM D-amphetamine, and 50% glycerol. Before use, dithiothreitol, D-amphetamine, and glycerol were removed by centrifugal gel filtration in a disposable column (Pierce Biotechnology Inc.). MAO A in 50 mM potassium phosphate, pH 7.2, containing 0.05% Brij-35, was made anaerobic in an anaerobic UV–visible cuvette or CD cell by cycling with argon. The concentration of MAO A was calculated from the absorbance at 456 nm using an extinction coefficient of 12 800 M^{-1} cm^{-1} [12]. Absorbance spectra were recorded in a 1-cm pathlength quartz cell adapted for anaerobic work in a Shimadzu 2101PC spectrophotometer.

2.2. CD spectroscopy

CD spectra were recorded at 20 °C using either a JASCO J-600 or a JASCO J-810 spectropolarimeter. Samples were prepared in 50 mM potassium phosphate, pH 7.2, containing 0.05% Brij-35 and spectra were recorded in quartz cells of 0.5-cm pathlength. Appropriate blanks were subtracted from each spectrum. In each case, two scans were averaged, with a scan rate of 20 nm/min and a time constant of 2 s.

3. Results

3.1. Visible absorption changes after ligand binding and/or reduction

Perturbation of the visible absorbance spectrum of the flavin cofactor of MAO A by D-amphetamine, the small substrate-analogue inhibitor, is shown in Fig. 2a. The difference spectrum shows considerable fine structure, characterised by an increase in the absorbance at 508 nm and a

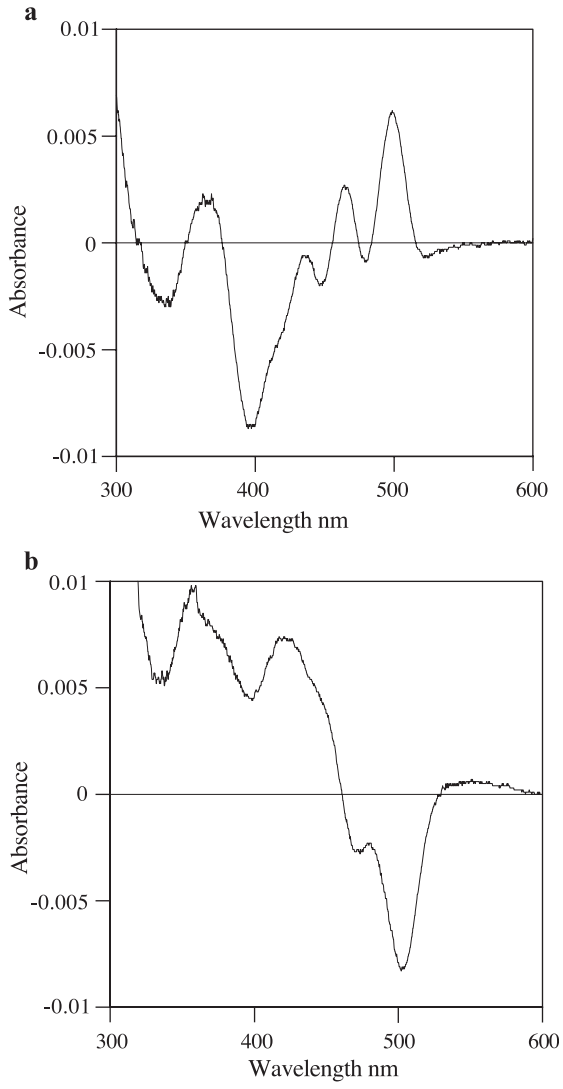

Fig. 2. D-Amphetamine and pirlindole induce changes in the absorbance spectrum of MAO A. (a) The spectrum for MAO A (11.2 μM) alone was subtracted from that for the same enzyme sample mixed with 314 μM D-amphetamine to obtain the difference spectrum. (b) The difference spectrum for MAO A (12.2 μM) with pirlindole (29.5 μM) was obtained in the same way.

decrease at 400 nm. The larger rigid molecule, pirlindole (2,3,3a,4,5,6-hexahydro-8-methyl-1*H*-pyrazino[3,2,1-j,k] carbazole hydrochloride), induces a different change (Fig. 2b), with a clear decrease in absorbance at 500 nm. Such perturbations could arise from stacking interactions [10] or from perturbation of the environment of the flavin [17]. In earlier work, the lack of correlation between the extinction coefficient for ligand-induced decrease at 500 nm and K_i values for the inhibitors [9] suggested that perturbation of protein was the more likely explanation.

The presence of inhibitor in the active site also has an effect on the redox properties of the flavin. Fig. 3 contrasts the partial generation of semiquinone, as indicated by the small peak at 412 nm, during reduction of free MAO A by dithionite (Fig. 3a) with the appearance of only semiquinone (large 412 nm peak) in the presence of D-amphetamine. Further reduction is not observed for the enzyme–inhibitor complex [8].

3.2. Circular dichroism changes after ligand binding

The structure of MAO B [3] revealed that both the flavin and the substrate cavity were surrounded by aromatic residues, so we have used CD to probe the alterations in the environment of aromatic residues in MAO A in the absence and presence of ligands. The CD spectrum of MAO

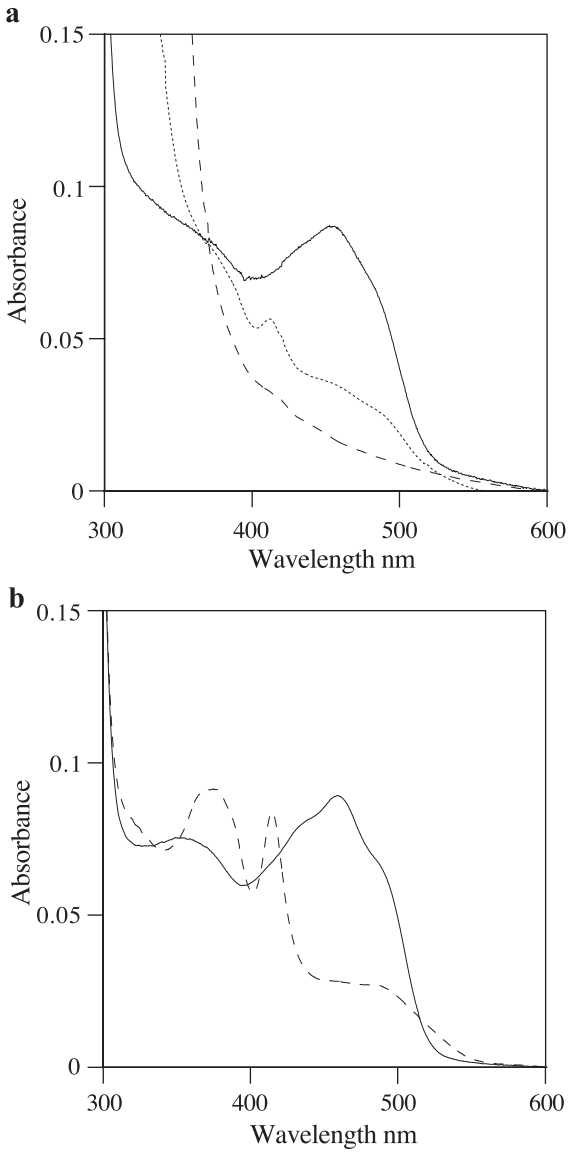

Fig. 3. UV–visible absorption spectra of oxidised and reduced MAO A. (a) Oxidised MAO A (13.2 μM, solid line) was reduced by the addition of dithionite (in the presence of 1 μM methyl viologen as a mediator) to obtain a partial yield of semiquinone (dotted line), and further reduced by more dithionite to the fully reduced form (dashed line). (b) In contrast, the mixture of oxidised MAO A (13.2 μM) and D-amphetamine (1010 μM) (solid line) is only reduced to the anionic semiquinone even with excess dithionite (dashed line).

A is shown in Fig. 4 (solid line). There are only relatively small, broad signals in the flavin region (350–520 nm, Fig. 4) but, in contrast, distinctive fine structured signals with at least three peaks are observed in the aromatic region (260–310 nm; Fig. 4, inset). The classical inhibitor D-amphetamine and the antidepressant pirlindole were added to MAO A at concentrations to ensure saturation. Fig. 4 shows that there is relatively little difference between the CD spectra for the free enzyme and the enzyme–inhibitor complex in the flavin region and only minor alterations in the aromatic region with the principal spectral features being preserved. The tyrosine peak at 285 nm is somewhat decreased in the presence of D-amphetamine but is enhanced together with the tryptophan peak at 293 nm in the presence of pirlindole. These data indicate that inhibitor binding does not greatly perturb the aromatic cage around the active site, but there may be subtle differences between the orientations of pirlindole and amphetamine bound within this active site cage.

3.3. CD changes on reduction

When the MAO A–amphetamine complex is reduced to the semiquinone form, there are small reductions in the broad signals due to flavin (Fig. 5). However, a dramatic change is observed in the aromatic region of the CD spectrum, such that the CD signal is more than doubled below 290 nm. The changes are seen in the signals due to tyrosine and phenylalanine but not in that arising from tryptophan at 293 nm. Fully reduced enzyme (in the absence of ligands) showed an even greater increase in the CD signal for the aromatic region with all peaks being enhanced (Fig. 5, inset).

When MAO is reduced by substrate, no 412 nm peak is ever detected in the visible absorption spectrum [12–14]. Benzyamine was used as the substrate to look for initial changes due substrate binding before reduction. However, the time required for data collection meant that considerable reduction had already occurred by the end of the run, so this was not possible. Fig. 6 shows the CD spectrum of MAO A fully reduced by a 300-fold excess of benzylamine (3 mM). The spectrum is similar to that for the fully reduced free enzyme but there are small alterations in the broad negative signal in the flavin region and in the aromatic region. In the presence of substrate, the signal is more negative than for the dithionite-reduced enzyme, both in the flavin region and in the 265–285 nm region associated with phenylalanine and tyrosine residues. Reduction is also a consequence of the covalent modification of the flavin by the suicide inhibitor, clorgyline [18]. The CD spectrum of this form of the reduced enzyme (Fig. 6, dash and dot line) is dramatically different from either the free reduced enzyme (dashed line) or the substrate-reduced enzyme (dotted line). For the covalently modified reduced enzyme, there is no difference from oxidised enzyme in the flavin region (360–500 nm), in contrast to the other reduced forms. However, there is a large negative CD peak between 310 and 360 nm. This is not seen in the other reduced forms of MAO A, so may be due to constraints on the flavin as a result of the N5 modification. There is also a large increase in the positive CD signal in the aromatic region, with the tryptophan (293 nm) and tyrosine (280 nm) peaks increased to a level similar

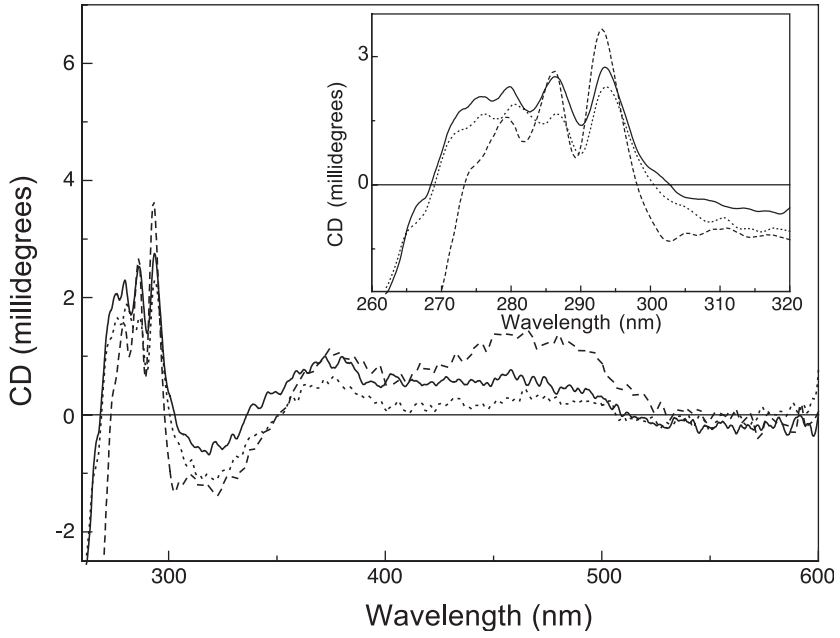

Fig. 4. CD spectra of MAO A. CD spectra of oxidized MAO A (13.2 μM, solid line), MAO A (13.2 μM) with D-amphetamine (0.5 mM (25 times K_i), dotted line), and MAO A (14.3 μM) with pirlindole (30 μM (600 times K_i), dashed line) were recorded in 0.5-cm pathlength anaerobic cells. The inset expands the aromatic region of the spectrum.

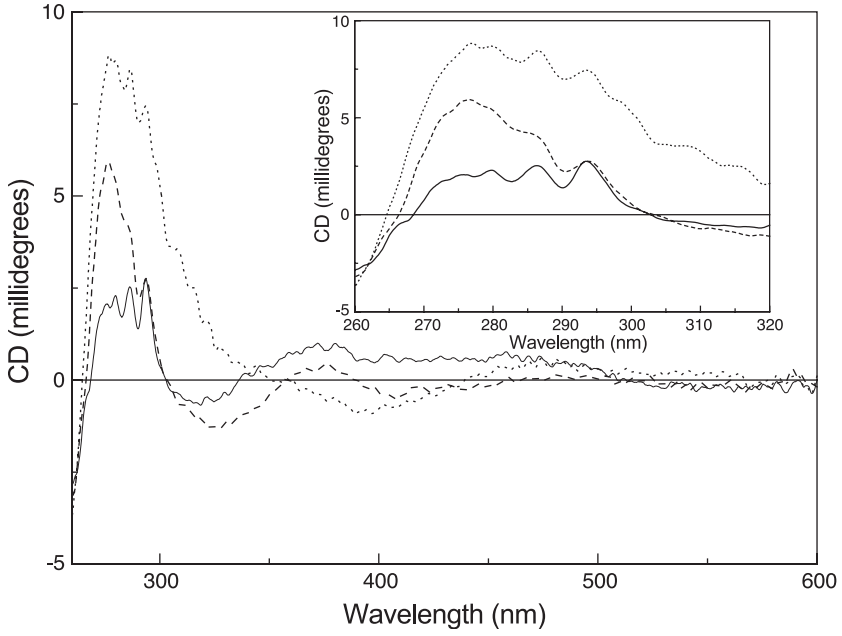

Fig. 5. Reduction of MAO A alters the CD spectrum. The CD spectra of MAO A (13.2 μM) in 0.5-cm pathlength cell: oxidised MAO A (as in Fig. 3, solid line), the MAO A–amphetamine (0.5 mM) complex reduced to semiquinone (dashed line), and fully reduced MAO A (dotted line). The inset expands the aromatic region of the spectrum.

to the free reduced enzyme (Fig. 6, inset). The most obvious difference in this region is that the amplitude of the lowest wavelength peak in the aromatic region (phenylalanine, 265–275 nm) is more than twice that seen for free dithionite-reduced MAO A or for the enzyme reduced by and complexed with substrate. Thus, the environment of aromatic residues is affected by this covalent ligand to the flavin.

4. Discussion

Proximity of substrate to the flavin in MAO A is required for catalysis. Competitive inhibitors are assumed to occupy the same site as substrate and this has been confirmed by X-ray crystallography of MAO B with the competitive inhibitor, isatin [4]. It is not surprising, therefore, that the inhibitors used here perturb the spectrum of

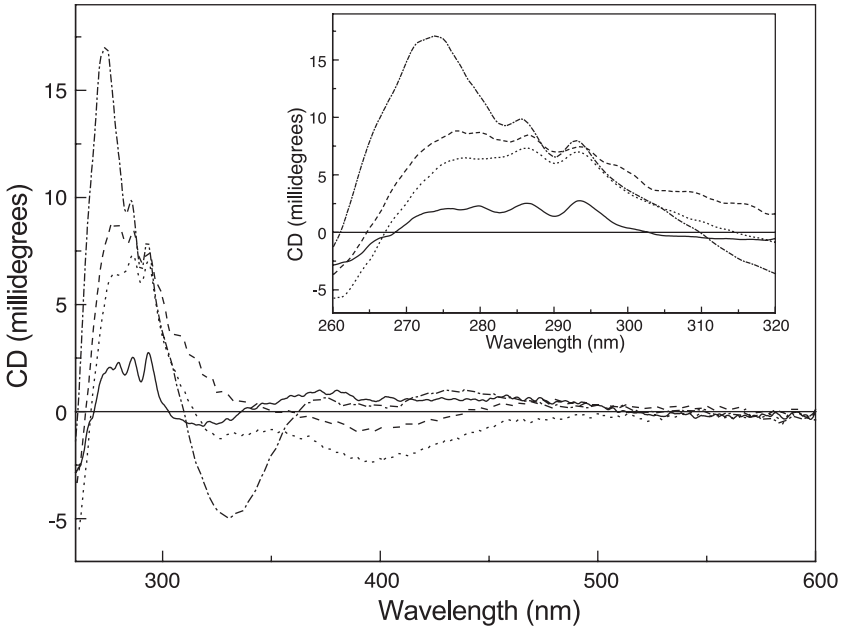

Fig. 6. Comparison of the CD changes induced by reduction. The CD spectra of MAO A (13.2 μM) in 0.5-cm pathlength cell: oxidised MAO A (from Fig. 3; for comparison, solid line); MAO A reduced by dithionite (dashed line); MAO A reduced by 3 mM benzylamine (dotted line); and MAO A (14.3 μM) reduced and covalently modified by 30 μM clorgyline (dotted-dashed line). The inset expands the aromatic region of the spectrum.

the flavin as reported for various other inhibitors [8,9]. The small pseudo-substrate, D-amphetamine, and the large pirlindole molecule, which is rigid and close to the dimensions of the active site determined by structure–activity studies, give quite different perturbations of the flavin spectrum of MAO A (Fig. 2). CD was used to determine whether the substrate-orienting aromatic amino acid side chains, presumably close to both inhibitors, or the aromatic cage (see Fig. 1), presumably within contact distance at least for the larger inhibitor, pirlindole, are perturbed by inhibitor binding. Fig. 4 indicates that D-amphetamine gives a small alteration to the 282 nm CD signal associated with tyrosine residues but does not otherwise perturb the aromatic residues. This pattern is consistent with D-amphetamine located between the two tyrosines and oriented towards the flavin as observed for isatin in MAO B [4]. In contrast, pirlindole induces an increased aromatic CD signal, indicating greater order across all the aromatic species, with the effects on the tyrosine peak (282 nm) and the tryptophan peak (293 nm) being the largest. The larger pirlindole presumably perturbs the many aromatic residues that form the aromatic cage of the substrate binding cavity as seen in MAO B [3,4].

Although free flavin can change shape on reduction, constraints to enzyme-bound flavin could prevent adoption of the lowest energy state and result in altered CD either of the flavin or of nearby aromatic residues. Fig. 5 indicates that there are alterations to the flavin around 400 nm for the reduction from oxidised to semiquinone to fully reduced forms of the enzyme. The dramatic increase in CD signal in the far UV (260–310 nm) indicates that the aromatic environment is indeed greatly perturbed by the reduction of the flavin. The environment of tryptophan is not much altered in the semiquinone form of the MAO A complexed with D-amphetamine, but all three peaks (for phenylalanine, tyrosine and tryptophan) are enhanced in the non-liganded, fully reduced enzyme.

This sensitivity of the CD signal for MAO A to ligand binding and reduction makes it a useful tool to probe differences between covalent and normally modified enzyme. Fig. 5 compares substrate-reduced MAO A, non-liganded reduced MAO A (dithionite reduced), and MAO A reduced and covalently modified by clorgyline. There is a particularly large difference in the 265–275-nm region associated with phenylalanine. Whether this is due to the phenylalanine residue observed near the flavin in the MAO B structure or to a phenylalanine residue in the aromatic cage remains to be established. The difference could either be a result of altered interactions between the flavin and a phenylalanine (as suggested by the position of Phe 343 in contact with flavin in the MAO B structure) or interaction of the covalent modifier with a phenylalanine in the aromatic cage. If the latter were correct, perturbation of the other aromatics (Tyr and Trp) would have been expected too but this is not seen. The lack of difference in the CD signals between the benzylamine- and clorgyline-reduced samples in the Tyr region (282 nm) suggests again that these residues are not greatly perturbed by the benzylamine aromatic ring.

The other large difference between the reduced MAO A samples is seen at 330 nm for the clorgyline-reduced MAO A. This may be due to the N5 modification of the flavin since it is not seen in the other samples. The MAO B structure with pargyline [3] shows the adduct covalently bound to N5 of the flavin (see Fig. 1) with the aromatic moiety of the inhibitor displaced from the expected substrate position between the 2 tyrosines (Tyr 398 and Tyr 435 in MAO B). Such a position would be consistent with the lack of effect on the CD spectrum in the flavin region.

This CD study demonstrates that changes in the aromatic amino acid side chains in the active site of MAO A occur on ligand binding and on reduction, suggesting an important functional role for these residues in modulating binding specificity and redox behaviour of the enzyme. When taken in conjunction with structural studies of the MAO complexed with inhibitors, the spectroscopic investigations provide additional insights into the dynamics of binding to MAO.

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