

Near-IR CD/MCD Spectral Elucidation of Two Forms of the Non-Heme Active Site in Native Ferrous Soybean Lipoxygenase-1: Correlation to Crystal Structures and Reactivity

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Lipoxygenases (LOs) are non-heme iron enzymes which catalyze the hydroperoxidation of *cis,cis*-1,4-pentadiene containing fatty acids. Mammalian LOs catalyze the conversion of arachidonic acid to leukotrienes, which mediate hypersensitivity and inflammation, and lipoxins, which inhibit cellular immunity.^{1a} LOs are also implicated in the oxidation of low-density lipoprotein to its atherogenic form, which leads to growth of atherosclerotic lesions.^{1b} Ferrous soybean lipoxygenase-1 (SLO-1) has been studied by various spectroscopies² which have described the active site geometry as 6-coordinate distorted octahedral. Two recent X-ray crystal structures have identified four common amino acid active site ligands: His₄₉₉Ne, His₅₀₄Ne, His₆₉₀Ne, and Ile₈₃₉OT2. One crystallographic active site description³ is 4-coordinate, with a ligand arrangement described as distorted octahedral with two adjacent unoccupied positions. The other crystal structure⁴ has an additional amino acid ligand (Asn₆₉₄Oδ1) with a possible sixth water-based ligand. In this study, near-infrared circular and magnetic circular dichroism (NIR CD/MCD) of SLO-1 in its native, glycerol-added, and substrate (linoleate for plant LOs)-added forms shows that native ferrous SLO-1 exists as an approximately equal mixture of two ferrous sites, one 5-coordinate and the second 6-coordinate. Linoleate or glycerol added to SLO-1 produces a pure 6-coordinate species which is identical to the 6-coordinate native component.

SLO-1 was purified⁶ to a specific activity of >210 units/mg. All samples were 1–2 mM in protein. A 2× excess of dithionite was added to anaerobic native MCD samples to reduce a <1% ferric heme impurity and to native + linoleate CD samples to reduce any ferric SLO-1 present, thus preventing turnover. NIR CD/MCD spectroscopies were performed as described earlier.⁷

A high-spin ferrous free ion has a ⁵D ground state which is split into a ⁵T_{2g} ground state and a doubly degenerate ⁵E_g excited state at 10Dq_o, higher energy in an octahedral ligand field (LF). The ⁵E_g excited state will further split in energy in a manner dependent on the coordination geometry. This will produce spin-allowed d–d transitions in the NIR region with a splitting pattern characteristic of the ferrous site geometry which can be directly probed by CD/MCD spectroscopies.⁸ An octahedral site with N and/or O ligands will have two transitions at ~10 000 cm⁻¹, split by ~2000 cm⁻¹. A 5-coordinate square

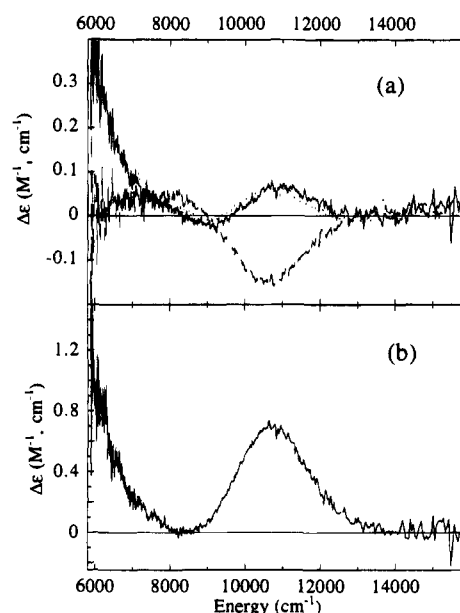


Figure 1. Circular dichroism spectra at 3 °C of (a) native SLO-1 in pD 7 0.1 M MES buffer (—), native SLO-1 + 50% glycerol-*d*₆ (---), and native SLO-1 in saturated sucrose solution (· · ·); (b) native SLO-1 – 60% (native SLO + 50% glycerol-*d*₆), renormalized.

pyramidal site will have two transitions, one at >10 000 and one at ~5000 cm⁻¹, while a trigonal bipyramidal center will have one transition at <10 000 cm⁻¹ and one at ~5000 cm⁻¹. A tetrahedral LF will split the ⁵D ground state by an energy of 10Dq_{Td} = (–4/9)(10Dq_o), which results in a ⁵E → ⁵T₂ spin-allowed transition at ~5000 cm⁻¹.

In Figure 1a (solid) the CD spectrum of native SLO-1 appears to exhibit two positive d → d transitions, one at <6000 cm⁻¹ and a second at 10 700 cm⁻¹, typical of a ferrous ion in a 5-coordinate LF.⁸ Note that the spectrum also exhibits a small positive shoulder at ~8000 cm⁻¹ and weak negative intensity at 9200 cm⁻¹. Additional of glycerol, Figure 1a (dashed), gives a CD spectrum which when Gaussian resolved shows a positive band at 8600 cm⁻¹ and a negative feature at 10 300 cm⁻¹. Two bands at ~10 000 cm⁻¹ split by ~2000 cm⁻¹ is typical of a 6-coordinate ferrous site splitting pattern.⁸ Addition of glycerol as a glassing agent thus perturbs the native ferrous active site of SLO-1. Alternatively, we find sucrose (>50%) to be a suitable glassing agent for the native enzyme as it has no effect on the CD of native SLO-1 (Figure 1a, dotted). Figure 2a (solid) shows the MCD spectrum of native SLO-1 in sucrose. The native enzyme exhibits three features in its 4.2 K MCD spectrum, at <5500, 8600, and 10 500 cm⁻¹. This pattern shows clearly that the ferrous site of native SLO-1 exists as more than one form as no single ferrous LF environment would exhibit more than two d → d transitions in the 5000–15 000 cm⁻¹ region.⁸ The MCD spectrum of glycerol added to SLO-1, Figure 2a (dotted), gives two positive bands at 8600 and 10 300 cm⁻¹, which are at the same energy as in the native SLO-1 + glycerol CD spectrum. Comparison of the two MCD spectra in Figure 2a indicates the possibility that the 6-coordinate species is also contributing to the native SLO-1 spectra. Further examination of the native SLO-1 CD spectrum in Figure 1a (solid) shows that the weak shoulder at ~8000 cm⁻¹ is at the same energy as the positive intensity of the native + glycerol CD spectrum (Figure 1a, dashed). Also the band shape of the native SLO-1 CD spectrum from 9000 to 12 000 cm⁻¹ is indicative of the combination of a positive and a negative feature with offset energies. Taken together, the CD and MCD data suggest that the same 6-coordinate species in the glycerol-added form is a component in native SLO-1. Subtraction of 60% of the native

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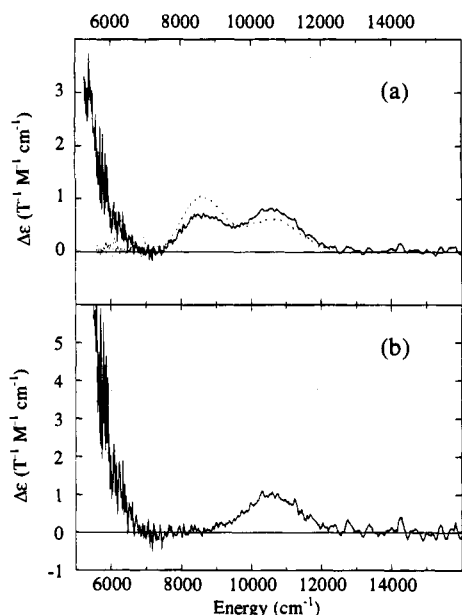


Figure 2. Magnetic circular dichroism spectra at 6 T, 4.2 K of (a) native SLO-1 in pD 7.0.1 M MES and saturated sucrose solution (—) and native SLO-1 + 50% glycerol- d_6 (---); (b) native SLO-1 + 60% (native SLO + 50% glycerol- d_6), renormalized.

+ glycerol signal from the native SLO-1 spectra of Figures 1a and 2a (solid) and renormalization gives the CD and MCD spectra of the second species present in the native enzyme, Figures 1b and 2b. Subtraction of any more or less than $60 \pm 5\%$ produced a three-peak difference spectrum which is not appropriate for a single ferrous site. Simultaneous Gaussian fitting analyses of the native CD and MCD spectra confirm the presence of four bands with a ratio of 6- to 5-coordinate forms of $\sim 3/2$. The transitions in the spectra in Figures 1b and 2b are at <5500 and $10\,600\text{ cm}^{-1}$, a LF band splitting pattern corresponding to a limiting square pyramidal ferrous site geometry.⁸

Addition of $12\times$ excess linoleate substrate, 50% ethylene glycol, or $750\times$ excess ethanol to native SLO-1 gives a CD spectrum identical to that of native + glycerol, Figure 1a (dashed). This indicates that each of these agents binds to the substrate binding pocket shifting the iron active site geometry from a mixed 5- plus 6-coordinate to a pure 6-coordinate form. Varying the pD from 5.6 to 11 results in no change in the native SLO-1 CD spectra, ruling out a pH dependent equilibrium of the two forms. The effect of approaching the two crystallization conditions^{3,4} on the native site was also investigated. SLO-1 in pD 7.20 mM MES with 1 M sodium formate, 0.7 M ammonium acetate, and 0.6 M lithium chloride caused no change in the native CD spectrum; any higher concentrations of these salts caused precipitation of the protein. SLO-1 in pD 5.6 0.2 M sodium acetate buffer also had the same CD spectrum.

Addition of PEG-3400, -1000, or -400 caused precipitation of the protein.

The description of the native SLO-1 active site developed from the above NIR CD/MCD studies is a 60/40% mixture of 6- and 5-coordinate forms, respectively. The two crystallographic descriptions are (1) 4-coordinate³ and (2) 5- or 6-coordinate⁴ with the sixth possible ligand being water-based. Such a ligand is proposed from EPR line broadening studies on the ferric enzyme.⁵ The main difference in the two crystal structures of SLO-1 is the coordination of the side chain of Asn₆₉₄, which is 3.3 \AA from the iron in the Boyington *et al.* structure,³ but bound through O δ 1 in the Minor *et al.* structure.⁴ The coordination difference between the two forms in native SLO-1 in solution may relate to differences in the binding of this ligand. Asn₆₉₄ is at the end of a cavity which starts at the surface of the enzyme³ and could lead to the fatty acid binding site in SLO-1. When linoleate or alcohols are present at the fatty acid binding site, a change in the active site environment occurs, perhaps breaking of hydrogen bonds to the side chain of Asn₆₉₄ in the 5-coordinate component, allowing the O δ 1 of Asn₆₉₄ to bind to the iron, converting the active site to the purely 6-coordinate form.

Catalysis of SLO-1 involves the oxidation of the native ferrous active site to ferric and subsequent reaction with substrate. Ferric SLO-1 gives a complex EPR signal with two forms contributing to the spectrum. When straight chain alcohols⁹ or glycerol¹⁰ is added, the EPR signal arises solely from the more axial form, which is indicated to be 6-coordinate from MCD studies.¹¹ In ferrous SLO-1, we can now see the direct effect on the iron coordination due to the presence of alcohols and fatty acid substrate binding. Thus the ferrous and ferric forms exhibit similar behavior upon binding of alcohols: the conversion of a mixed to a pure form, which may indicate parallel active site coordination between ferrous and ferric SLO-1.

Previous CD/MCD studies^{2c} on SLO-1 described the glycerol-added form as 6-coordinate distorted octahedral but did not investigate the native form or the LF features at lower than 9000 cm^{-1} . By extending the spectral range studied and using a nonperturbing glassing agent for MCD, we demonstrate that native SLO-1 in solution exists as a mixture of two forms, 5- and 6-coordinate, the 6-coordinate form being catalytically relevant as it is the only form present when fatty acid substrate is bound to the protein.

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