

A free-radical intermediate in the reduction of plant Cu-amine oxidases

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The reaction with substrate of plant amine oxidases from either *Euphorbia latex* or lentil seedlings in the presence of cyanide led to the appearance of a free-radical type of ESR spectrum with well-resolved hyperfine structure, which is very likely due to coupling with various protons over an aromatic ring. It was established that the free-radical spectrum was not derived from the substrate, since either aromatic or aliphatic substrates gave rise to identical spectra. The presence of copper is essential to the appearance of the free-radical ESR signal.

Amine oxidase Free radical ESR Enzyme mechanism Copper protein

1. INTRODUCTION

Copper-containing amine oxidases (Cu-AOs) oxidize primary amines yielding the corresponding aldehyde, ammonia and hydrogen peroxide [1]. Copper is essential to the catalysis but a second prosthetic group, tentatively identified as pyridoxal phosphate [2], is also needed. In fact, either removal of the copper or covalent modification of the enzyme with carbonyl reagents (phenylhydrazine, semicarbazide, etc.) leads to inactivation of Cu-AOs. Moreover the reaction with carbonyl reagents gives rise to optical spectra similar to those observed, under the same conditions, with authentic pyridoxal phosphate or pyridoxal phosphate-enzyme [3].

The catalytic mechanism of Cu-AOs is of the ping-pong type [4–6]. The enzyme is reduced by the amine substrate and liberates the corresponding aldehyde in the absence of oxygen. Ammonia is produced in the reoxidation step, which requires oxygen or other electron acceptors of suitable redox potential (e.g., cytochrome *c*, see [7]). Reduction and reoxidation of the enzyme apparently do not imply valence changes of the copper [8,9], as detectable by ESR.

2. MATERIALS AND METHODS

Amine oxidases from *Euphorbia characias* latex (ELAO) and *Lens culinaris* seedlings (LSAO) were purified as in [10,11]. *p*-Dimethylaminomethylbenzylamine (DABA) was synthesized according to [12]. Putrescine (1,4-diaminobutane · HCl) was obtained from Fluka.

Optical spectra were recorded on a Beckman model 5230 spectrophotometer at room temperature. ESR spectra were obtained at 9 GHz with a Varian E-9 spectrometer equipped with the Varian variable temperature accessory. For the room temperature spectra a high sensitivity E-238 cavity resonating in the TM₁₁₀ mode and the Varian E-258 liquid slution flat cells for large volumes were used. When necessary, a Varian C-1024 time-averaging computer was used to resolve weak hyperfine lines of room temperature, low-power ESR spectra. Anaerobic spectra were obtained using photometric cuvettes or low-temperature ESR tubes sealed with a Thunberg device, into which reagents were added with a syringe through a rubber cap.

3. RESULTS AND DISCUSSION

Addition of substrate to ELAO or LSAO in the absence of air completely bleached the visible absorption of the former enzyme, while with the latter one gave rise to new absorption bands at 370, 430 and 465 nm. The low temperature ESR spectra of the two enzymes which are typical of amine oxidase copper [8], were only slightly and similarly modified under these conditions (fig.1a). In particular, no reduction of signal intensity could be observed. By analogy to other Cu-AOs [8] such changes are likely to be due to conformational effects rather than to direct binding of substrate to copper. The similarity of ESR spectra despite the difference of absorption spectra, is further evidence that the visible chromophore is not associated with the copper component [13,14].

In the presence of 3 mM KCN no effect on the absorption spectrum of the 'resting' enzyme was observed, while the ESR spectra changed into a line shape that is typical of a CN^- -copper adduct as found in other copper proteins [15]. These results indicate that CN^- binds to the copper site of the enzymes. Addition of excess substrate to the cyanide-treated enzyme led to the appearance of an

additional signal at $g = 2$ (fig.1b) which reached a maximum height in 5 min incubation at room temperature and then was stable in the frozen sample or in anaerobic liquid solutions. Incubation in air led to a gradual disappearance of this signal. At room temperature and low microwave power only the signal at $g = 2$ was detectable and, at field modulation amplitudes lower than 1 G, it showed a well-resolved hyperfine spectrum (fig.2). These features are indicative of a free radical. The spectrum displays a certain degree of anisotropy, suggesting the binding of the radical to the protein. This makes the spectrum difficult to interpret, however, the value of the large splitting is compatible with coupling of the unpaired spin with an aliphatic proton, while the smaller splitting can be assigned to coupling to various protons over an aromatic ring (cf. [16,17]). Although a coupling to nitrogen cannot be excluded, only an isotopic substitution approach [17], presently impracticable, would allow identification of the radical. More information was obtained from simpler biochemical tests. Identical spectra were produced by incubation with either putrescine or DABA (this test was made with LSAO only, the latter molecule not being a substrate for ELAO). Since the latter substrate contains an aromatic nucleus, the splitting would have been strongly affected, were the

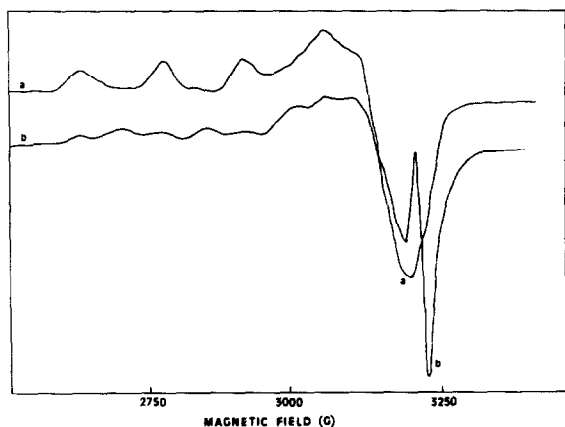


Fig.1. Low-temperature ESR spectra of AOs. a, LSAO (100 μM) in 100 mM K-phosphate buffer (pH 7.0) in anaerobiosis after addition of 2 mM substrate (DABA). b, as a, after addition of 3 mM KCN. Under these conditions, the enzyme is not saturated by CN^- and both the native enzyme and the CN^- -adduct spectra are apparent. ESR conditions: microwave power, 20 mW; modulation amplitude, 10 G; microwave frequency, 9.15 GHz; temperature, -150°C .

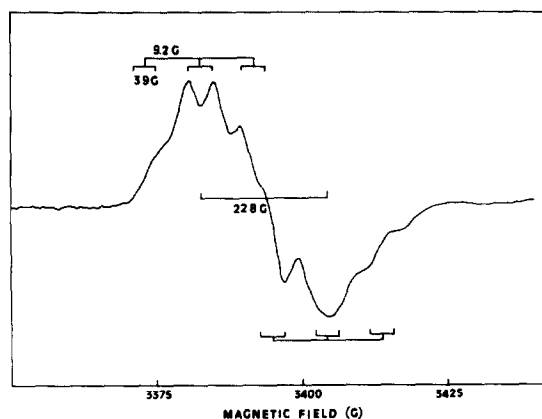


Fig.2. Room-temperature ESR spectrum of the AOs free-radical signal. The spectrum was obtained under the conditions of curve b of fig.1 and was recorded at 10 mW microwave power, 0.5 G modulation amplitude, 9.38 GHz microwave frequency and 25°C temperature. The pattern of hyperfine splittings is shown on the two halves of the major doublet.

unpaired spin centered on the substrate. This result confirms that the radical species is bound to the protein. It is tempting to speculate that this spectrum is associated with the organic cofactor of the enzyme, perhaps an aromatic moiety located in the active site region. In any case, the formation of the free radical is related to the reduction of the enzyme by the substrate.

Binding of cyanide to the copper may slow down reoxidation of the reduced enzyme and thus trap a semiquinoid form of the reduced enzyme. Rapid mixing experiments have shown that aldehyde is released by LSAO in the absence of air and that the release is only slightly affected by the presence of cyanide (unpublished). In fact, admission of oxygen leads to the disappearance of the radical signal, although initial presence of oxygen was compatible with its formation.

The copper-free enzyme, prepared as previously described [18], did not show the free-radical spectrum under the same conditions, while it is still able to form the product aldehyde.

It should also be emphasized that controls on Cu-AOs from animal sources did not show the formation of a radical under the same experimental conditions. However, other factors, like variations of the stability constant of the CN^- adduct or of kinetic parameters, could explain this finding, without implying different mechanisms for different amine oxidases. In this context it should be recalled that plant amine oxidases are characterized by a higher catalytic activity [10,11] than animal amine oxidases.

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