Volume 214, number 2, 274–278 FEB 04599 April 1987

The generation of an organic free radical in substrate-reduced pig kidney diamine oxidase-cyanide

David M. Dooley, Michele A. McGuirl, Jack Peisach+ and John McCracken+

Department of Chemistry, Amherst College, Amherst, MA 01002 and +*Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461, USA*

Received 30 January 1987

When the cyanide complex of the copper protein, pig kidney diamine oxidase, is reduced anaerobically by cadaverine (1,5-diaminopentane), the broad, 480 nm absorption band characteristic of the resting enzyme is bleached and a new absorption spectrum with features at 457, 429, 403 (shoulder), 360 (shoulder) and 332 nm appears. Concomitantly, the EPR spectrum of the enzyme Cu(II)-CN complex decreases in intensity and a new signal is observed that is attributable to an organic free radical. The g values and hyperfine splittings are similar to those previously assigned to a free radical observed when the cyanide complex of lentil seedling diamine oxidase is reacted with the substrate p-dimethylaminomethylbenzylamine [(1984) FEBS Lett. 176, 378–380]. The optical absorption and EPR spectra of the organic radical observed in both proteins are consistent with the same semiquinone-type structure, as expected if pyrroloquinolinequinone (PQQ) is the bound cofactor found in both enzymes.

Amine oxidase; Organic free radical; EPR; Optical absorption spectrum; Pyrroloquinolinequinone

1. INTRODUCTION

Despite their overall similarity, it has been apparent for many years that the various copper-containing amine oxidases isolated from mammalian and plant sources display intriguing differences in their reactions with substrates [1,2]. Even though each of these enzymes exhibits preferential activity for different substrates, they all nevertheless contain divalent copper and a covalently bound organic cofactor, pyrroloquinolinequinone (PQQ) [3–9]. When amine substrates are added under anaerobic or low-temperature conditions to plant diamine oxidases, a spectral intermediate with characteristic electronic absorption bands at 465, 430 and 360 nm is observed [10–12]. No such intermediate has been definitively detected with mammalian amine oxidases reacted with substrate, although suggestive evidence for such a species has been presented [13]. When the cyanide complex of lentil seedling amine oxidase is reacted with the substrate p-dimethylaminomethylbenzylamine, a new EPR signal appears that is assigned to the enzyme-bound organic cofactor [14]. No analogous EPR signal has been reported for the mammalian enzymes.

Here, we report the generation of a reduced species from the cyanide complex of pig kidney diamine oxidase (PKAO) reacted anaerobically with cadaverine (1,5-diaminopentane). This species displays optical and EPR spectra that bear a strong resemblance to those of analogous forms generated from plant proteins, but formed with different substrates present. As the same spectral species is generated in amine oxidases, independent of their source, it is suggested that the mechanism of formation of this species in plant and mammalian enzymes is similar.

Correspondence address: D.M. Dooley, Department of Chemistry, Amherst College, Amherst, MA 01002, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

2. MATERIALS AND METHODS

PKAO was purified as in [15]. Enzyme preparations used in these experiments had a specific activity of 2.0, determined with the *p*-dimethylaminomethylbenzylamine assay [16]. Substrates were redistilled or recrystallized before use and all other chemicals were the highest purity available commercially.

Enzyme solutions were made anaerobic by gentle exchange against O_2-free Ar gas for 1.5–3 h. Reagent solutions were vigorously purged with purified Ar. Cyanide solutions were prepared immediately prior to each experiment by transferring, via a gas-tight syringe, a known volume of Ar-saturated buffer to a septum-stoppered, Ar-purged, volumetric flask containing solid NaCN. Aliquots of NaCN and substrate solutions were also added to the enzyme with a gas-tight syringe under a constant stream of Ar. Amine oxidase was allowed to react with cyanide for several minutes prior to substrate addition. Further experimental details may be found in the figure legends. A Cary 219 spectrophotometer, interfaced to an Apple IIe computer, was used to record optical absorption spectra. Varian E-9 and E-112 instruments were used for EPR measurements. Field calibrations were made with diphenylpicrylhydrazyl or with a Varian NMR gaussmeter. Frequencies were measured with a PRD model 559-B wavemeter.

3. RESULTS

Resting PKAO and its complex with cyanide display similar optical absorption spectra. The spectral changes that accompany the anaerobic addition of cadaverine to the cyanide complex are shown in fig.1. With time, the 480 nm band of the resting enzyme, previously assigned to the cofactor [13], is bleached while new bands appear at 457, 429 and 332 nm. Shoulders are also evident at 403 and 360 nm. This spectrum is nearly identical to those previously reported for the substrate-reduced pea seedling [10,11] and lentil seedling amine oxidases [12,17]. Under anaerobic conditions the new spectral species prepared from the mammalian enzyme is stable. However, O_2 addition quickly restores the spectrum of the resting enzyme, with its broad absorption near 480 nm.

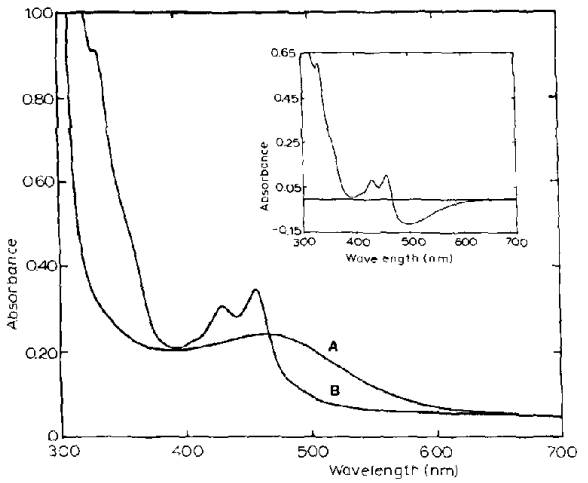


Fig.1. Optical absorption spectra of the cyanide complex of pig kidney diamine oxidase (PKAO) prior to (A) and following (B) the anaerobic addition of the substrate, 1,5-diaminopentane, in 0.1 M potassium phosphate buffer, pH 7.2, at 20°C. [PKAO] = 4.8×10^{-5} M; [NaCN] = 5.0×10^{-2} M; [substrate] = 3.3×10^{-4} M. The spectrum obtained prior to substrate addition exhibits a broad absorption near 480 nm. (Inset) Difference spectrum generated by digitally subtracting the spectrum of resting enzyme from the substrate-reduced spectrum. Wavelengths quoted in the text are taken from the difference spectrum.

Fig.2 presents the EPR spectrum obtained following the anaerobic addition of cadaverine to the cyanide complex of PKAO. The EPR signal attributed to Cu(II) [15] is markedly decreased in intensity (initially to about 45% of the original signal) (fig.2A) whereas a new signal appears at $g = 2$, which increases in intensity with time. The kinetics of the formation of the new optical species and of the free radical are comparable. When CN^- is absent from the reaction mixture, the Cu(II) EPR spectrum is only slightly perturbed by substrate addition [18], i.e. CN^- is required in order to observe the $g = 2$ signal.

An expansion of the $g = 2$ region of the EPR spectrum is shown in fig.3. Hyperfine structure is clearly resolved and this may represent proton or nitrogen coupling. Moreover, the signal is anisotropic and is saturated at low powers ($< 100\ \mu W$) at 77 K. Hence, this EPR signal is attributable to a protein-bound organic radical. It should be noted that the optical absorption and EPR spectral changes brought about by substrate

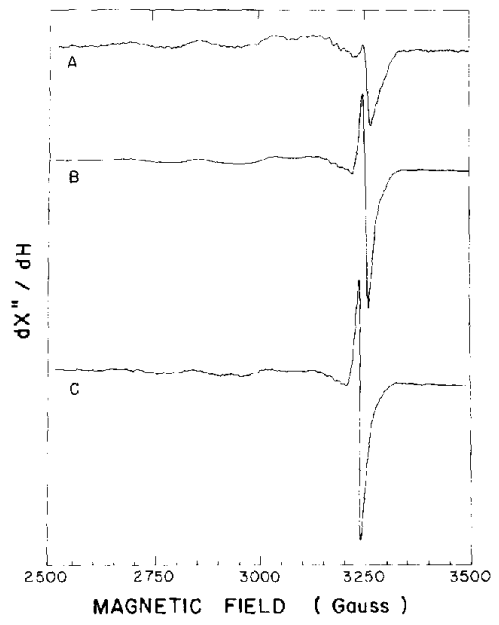


Fig.2. EPR spectra of the cyanide complex of PKAO following the anaerobic addition of 1,5-diaminopentane. Substrate was added at room temperature, allowed to react for the indicated times, and the sample was then rapidly frozen in liquid N_2. Spectrum A was obtained about 5 min after addition of substrate. Spectrum B was obtained by thawing the sample studied in A, incubation at room temperature for 4 min, and freezing for the EPR study. Spectrum C was obtained by thawing the same sample and incubation for an additional 6 min. Spectrometer conditions: frequency, 9.09 GHz; power, 10 mW, modulation amplitude, 6.3 G; temperature, 77 K.

addition to the cyanide complex of PKAO are reversible, and that copper is not lost from the enzyme under the conditions used, provided the CN^- is promptly removed by dialysis.

The spectrum shown in fig.3, obtained at 77 K, is as well-resolved as the room-temperature spectrum reported for the cyanide derivative of lentil seedling amine oxidase reacted with the substrate *p*-dimethylaminomethylbenzylamine, and can be satisfactorily analyzed with similar hyperfine splittings [14]. The fact that essentially identical EPR spectra can be generated from different amine oxidases and with different substrates strongly supports the suggestion that the radical whose spectrum is shown in fig.3 is derived from the bound organic cofactor, PQQ.

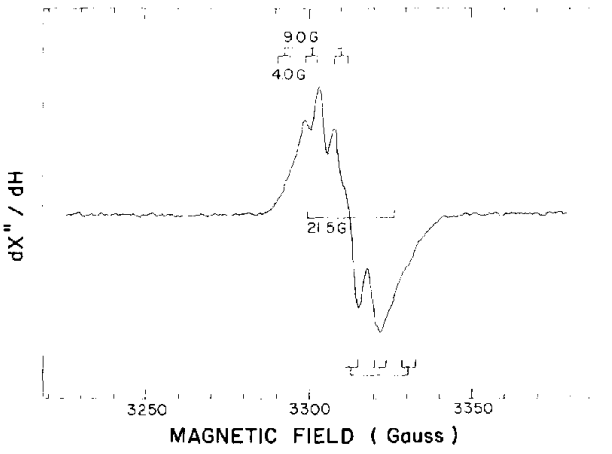
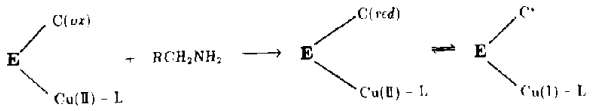


Fig.3. EPR spectrum of the organic free radical produced by substrate reduction of the cyanide complex of pig kidney amine oxidase. Spectrometer conditions: frequency, 9.24 GHz; power, 1 mW; modulation amplitude, 0.4 G; temperature, 77 K. Also shown is the hyperfine splitting scheme proposed in [14].

4. DISCUSSION

Recent work has established that PQQ, or a closely similar derivative, is the bound organic cofactor in mammalian copper-containing amine oxidases [3–9]. The optical absorption spectrum of the free PQQ semiquinone has been measured in solution [19]; intense bands occur at 460 and 360 nm. Semiquinone forms of enzymes known to contain PQQ (or a derivative) also display bands in this spectral region [20–22]. In addition, the EPR spectrum shown in fig.3 is similar in several respects to the spectrum of PQQ semiquinone [23,24] and, for that matter, semiquinones in general [25,26]. Most importantly, the zero-point *g* value of the EPR absorption derivative is 2.0058, which is significantly higher than the free electron *g* value, while the linewidth (peak-to-trough) is 20 G [23–26]. We suggest, then, that a detectable semiquinone form of the bound organic cofactor, PQQ, is generated by substrate interaction with the cyanide complex of PKAO.

The present results show that a substrate-reduced form of PKAO can be generated having optical and EPR properties similar to those of intermediates previously reported for the cyanide derivatives of plant amine oxidases but with dif-



Scheme 1. Possible mechanism for the formation of an organic radical (semiquinone) in copper-containing amine oxidases by substrate reduction. C, organic cofactor (PQQ); L, OH_2, CN^-.

ferent substrates. Our data are consistent with the assignment of the optical absorption and EPR spectra of this substrate-reduced form to a single species, a bound PQQ semiquinone. Although there is a large variation in substrate specificities among the copper-containing amine oxidases, these results suggest that similar intermediates are nevertheless formed. However, for PKAO, the spectral features observed upon substrate addition in the presence of cyanide have not been observed in either time-resolved or steady-state kinetics experiments [1,2]. It is therefore necessary to understand the role that cyanide may play in reactions leading to their formation.

Our working hypothesis is outlined in scheme 1. Substrate is thought to reduce PQQ in a two-electron reaction. This is followed by an internal redox equilibrium between the two-electron-reduced form of the organic cofactor and enzyme-bound Cu(II). As cyanide is known to stabilize Cu(I) [27], its binding to the metal ion is expected to shift the equilibrium in that direction, thereby leaving the organic cofactor in the one-electron-reduced form. The redox partition will depend on the magnitude of the equilibrium constant. One would predict that for certain plant amine oxidases [10–12] the one-electron-reduced form of the cofactor might even be detected in the absence of cyanide.

ACKNOWLEDGEMENTS

We thank Cheryl Cote for several helpful discussions and Cheryl Snow-Powers for her capable technical assistance. We are also grateful to Charles Dickinson for access to the EPR spectrometer at the University of Massachusetts. This research was supported by NIH grants GM-27659 (D.M.D.) and HL-13399 and RR-92583 (J.P.).

REFERENCES

[1] Knowles, P.F. and Yadav, K.D.S. (1984) in: Copper Proteins and Copper Enzymes (Lontie, R. ed.) vol.2, pp.103–129, CRC Press, Boca Raton, FL.
[2] Mondovi, B. (1985) Structure and Functions of Amine Oxidases, CRC Press, Boca Raton, FL.
[3] Lobenstein-Verbeek, C.L., Jongejan, J.A., Frank, J. and Duine, J.A. (1984) FEBS Lett. 170, 305–309.
[4] Ameyama, M., Hayashi, M., Matsushita, K., Shinagawa, E. and Adachi, O. (1984) Agric. Biol. Chem. 48, 561–565.
[5] Moog, R.S., McGuirl, M.A., Cote, C.E. and Dooley, D.M. (1986) Proc. Natl. Acad. Sci. USA 83, 8435–8439.
[6] Williamson, R.S., Moog, R.S., Dooley, D.M. and Kagan, H.M. (1986) J. Biol. Chem. 261, 16302–16305.
[7] Knowles, P.F., Pandeya, K.B., Rius, F.X., Spencer, C.M., Moog, R.S., McGuirl, M.A. and Dooley, D.M. (1987) Biochem. J. 241, 603–608.
[8] Van der Meer, R.A. and Duine, J.A. (1986) Biochem. J. 239, 789–791.
[9] Van der Meer, R.A., Jongejan, J.A., Frank, J. jzn and Duine, J.A. (1986) FEBS Lett. 206, 111–114.
[10] Hill, J.M. and Mann, P.J.G. (1964) Biochem. J. 91, 171–182.
[11] Kluetz, M.D., Adamsons, K. and Flynn, J.E. jr (1980) Biochemistry 19, 1617–1621.
[12] Bellelli, A., Brunori, M., Finazzi-Agrò, A., Floris, G., Giartosi, A. and Rinaldi, A. (1985) Biochem. J. 232, 923–926.
[13] Finazzi-Agrò, A., Guerrieri, P., Costa, M.T. and Mondovi, B. (1977) Eur. J. Biochem. 74, 435–440.
[14] Finazzi-Agrò, A., Rinaldi, A., Floris, G. and Rotilio, G. (1984) FEBS Lett. 176, 378–380.
[15] Dooley, D.M. and McGuirl, M.A. (1986) Inorg. Chim. Acta 124, 231–236.
[16] Bardsley, W.G., Crabbe, M.J.C. and Shindler, J.S. (1972) Biochem. J. 127, 875–879.
[17] Rinaldi, A., Giartosio, A., Floris, G., Medda, R. and Finazzi-Agrò, A. (1984) Biochem. Biophys. Res. Commun. 120, 242–249.
[18] Mondovi, B., Rotilio, G., Costa, M.T., Finazzi-Agrò, A., Chiancone, E., Hansen, R.E. and Beinert, H. (1967) J. Biol. Chem. 242, 1160–1167.
[19] Faraggi, M., Chandrasekar, R., McWhirter, R.B. and Klapper, M.H. (1986) Biochem. Biophys. Res. Commun. 139, 955–960.
[20] Kenney, W.C. and McIntire, W. (1983) Biochemistry 22, 3858–3868.
[21] Dekker, R.H., Duine, J.A., Frank, J., Verwiel, P.E.J. and Westerling, J. (1982) Eur. J. Biochem. 125, 69–73.

[22] Eckert, T.S. and Bruice, T.C. (1983) J. Am. Chem. Soc. 105, 4431–4441.
[23] Duine, J.A., Frank, J. and Westerling, J. (1978) Biochim. Biophys. Acta 524, 277–287.
[24] DeBeer, R., Duine, J.A., Frank, J. and Large, P.J. (1980) Biochim. Biophys. Acta 622, 370–374.
[25] Westerling, J., Frank, J. and Duine, J.A. (1979) Biochem. Biophys. Res. Commun. 87, 719–724.
[26] Hales, B.J. (1975) J. Am. Chem. Soc. 97, 5993–5997.
[27] Greenwood, N.N. and Earnshaw, A. (1984) in: Chemistry of the Elements, pp.1386–1387, Pergamon, Oxford.