PRELIMINARY NOTES

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Indirect evidence for the production of superoxide anion radicals by pig kidney diamine oxidase

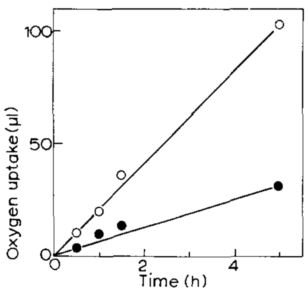
Recent work on the mechanism of action of pig kidney diamine oxidase (diamine:oxygen oxidoreductase (deaminating), EC 1.4.3.6) has indicated that a partial reduction of copper by substrate occurs in the absence of oxygen[1]; a molecule of aldehyde product per atom of enzyme-bound copper is released in anaerobiosis, and the reduced enzyme reacts with oxygen *via* a binary complex[2]. This preliminary note concerns the last step of the diamine oxidase reaction, *i.e.* the reaction with oxygen to give rise to H_2O_2. As a first approach to this problem, we inquired whether electron transfer to oxygen might occur *via* univalent steps involving superoxide anion free radicals ($O_2{}^{\cdot -}$) as intermediates. It is known that stimulation of sulfite oxidation and O_2-dependent reduction of cytochrome *c* by a functioning enzyme system are due to the production of $O_2{}^{\cdot -}$ during the catalytic activity[3-5]. In the present study we searched for such evidence in diamine oxidase and obtained results which suggestt hat $O_2{}^{\cdot -}$ free radicals are generated during the reduction of molecular oxygen by this enzyme.

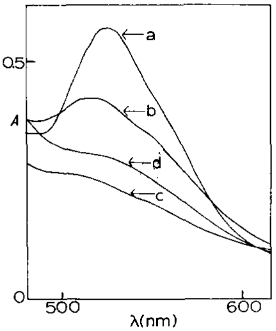
Diamine oxidase was purified according to MONDOVÌ *et al.*[6]. Enzyme units are defined as previously reported[7]. Cytochrome *c* (horse heart, 70% pure) was from Boehringer: its reduction was measured spectrophotometrically at 550 nm in a Beckman DK 2A Ratio Recording Spectrophotometer, using $\Delta\varepsilon$ (reduced — oxidized) of $2.10 \cdot 10^4$ cm²/mmole (see ref. 8).

Fig. 1 shows the autoxidation of $SO_3{}^{2-}$ in the absence and in the presence of diamine oxidase acting on cadaverine as substrate at pH 7.4. A significant increase of oxygen uptake is produced by the presence of the functioning enzyme system.

Figs. 2 and 3 show the results obtained testing the substrate–cytochrome *c* reductase activity of diamine oxidase. Experiments were made at pH 7.4 and pH 9.2, since it has been reported that with xanthine oxidase the pH optimum for aerobic cytochrome *c* reduction is higher that under anaerobiosis[9]. It is evident that at pH 7.4 (Fig. 2) denaturation of cytochrome *c* occurred, owing to H_2O_2 produced during diamine oxidase reaction. This fact could mask cytochrome reduction. At pH 9.2, however, it was possible to observe the reduction of 0.046 µmole of cytochrome *c* after aerobic incubation for 30 min in the presence of diamine oxidase and cadaverine (Fig. 3). The oxygen consumed in a parallel incubation carried out under the same conditions in a Warburg apparatus was 3 µmoles. Under anaerobic conditions only 0.017 µmole of cytochrome *c* was reduced after 30 min.

Direct demonstration of univalent reduction of molecular oxygen in the catalytic action of oxidases is based on the detection of the electron paramagnetic resonance spectrum of $O_2{}^{\cdot -}$ during the enzymatic reaction. However, many observations support the idea that the initiation of $SO_3{}^{2-}$ oxidation and the aerobic reduction of cytochrome *c* indicate the involvement of oxygen free radicals in enzymatic processes[4,5]. As yet, the proteins for which this mechanism has been proposed are metallo-flavoproteins, iron sulfur proteins and heme proteins[10]. Thus diamine oxidase





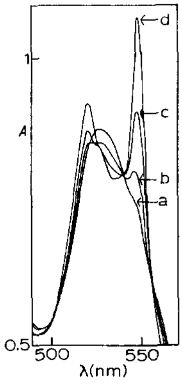


Fig. 1. Effect of diamine oxidase and cadaverine on the autoxidation of SO_3^{2-}. Oxygen consumption was followed in a conventional Warburg apparatus. Incubation mixtures (3 ml total volume) in 0.1 M phosphate buffer (pH 7.4) *plus* 0.05% EDTA. ●———●, Na_2SO_3 (100 µmoles); ○———○, Na_2SO_3 (100 µmoles), diamine oxidase (0.05 unit) and cadaverine (50 µmoles). The oxygen uptake in the absence of sulfite was substracted.

Fig. 2. Aerobic incubation of cytochrome *c* with diamine oxidase and cadaverine at 38° and pH 7.4. Reaction mixture (3 ml total volume) contained 50 µM cytochrome *c*, diamine oxidase (0.1 unit) and cadaverine (5 µmoles) in 0.1 M potassium phosphate buffer (pH 7.4). Spectra performed at room temperature. Light path: 1 cm. a, zero time; b, after 30 min; c, after 60 min; d, spectrum of 50 µM cytochrome *c* in the presence of H_2O_2 (5 µmoles).

Fig. 3. Aerobic incubation of cytochrome *c* with diamine oxidase and cadaverine at 38° and pH 9.2. Reaction mixture (3 ml total volume) contained 0.1 mM cytochrome *c*, diamine oxidase (0.1 unit) and cadaverine (5 µmoles) in 0.3 M carbonate–bicarbonate buffer (pH 9.2). Spectra performed at room temperature. Light path: 1 cm. a, zero time; b, after 6 min; c, after 15 min; d, after 30 min.

is the first copper protein which has been reported to show evidence for such a mechanism.

Reduction of cytochrome *c* by amine oxidase from *Aspergillus niger*, an enzyme very similar to the pig kidney one, has already been observed under anaerobic conditions with various amines as substrates[11]. These authors noted that with all amines more reduction of cytochrome *c* was obtained under aerobic conditions, but they did not give any explanation for that. This result can now be interpreted in view of a general capability of amine oxidases of generating oxygen free radicals.

As to the pH-dependence of the aerobic reduction of cytochrome *c* by diamine oxidase, it should be recalled that the stability of $O_2^{\cdot -}$ is increased in alkaline solution[10]. It is reasonable to think that under our conditions the steady-state ratio $H_2O_2/O_2^{\cdot -}$ strongly favored the peroxide at pH 7.4, whereas the contrary happens at the higher pH. It is interesting to notice that diamine oxidase itself is inactivated by the H_2O_2 produced in the reaction only at neutral pH, whereas at pH 9 it is not[12].

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1 B. MONDOVÌ, G. ROTILIO, A. FINAZZI AGRÒ, M. P. VALLOGINI, BO. G. MALMSTRÖM AND E. ANTONINI, *FEBS Letters*, 2 (1969) 182.

2 A. Finazzi Agrò, G. Rotilio, M. T. Costa and B. Mondovì, *FEBS Letters*, 4 (1969) 31.
3 I. Fridovich and P. Handler, *J. Biol. Chem.*, 236 (1961) 1836.
4 P. Handler, K. V. Rajagopalan and V. Aleman, *Federation Proc.*, 23 (1964) 30.
5 J. M. McCord and I. Fridovich, *J. Biol. Chem.*, 243 (1968) 5753.
6 B. Mondovì, M. T. Costa, A. Finazzi Agrò and G. Rotilio, *Arch. Biochem. Biophys.*, 119 (1967) 373.
7 B. Mondovì, G. Rotilio, M. T. Costa, A. Finazzi Agrò, E. Chiancone, R. E. Hansen and H. Beinert, *J. Biol. Chem.*, 242 (1967) 1160.
8 V. Massey, *Biochim. Biophys. Acta*, 34 (1959) 255.
9 S. Muraoka, H. Enomoto, M. Sugiyama and H. Yamasaki, *Biochim. Biophys. Acta*, 143 (1967) 408.
10 R. Nilsson, F. M. Pick and R. C. Bray, *Biochim. Biophys. Acta*, 192 (1969) 145.
11 S. Muraoka, A. Hoshika, H. Yamasaki, H. Yamada and O. Adachi, *Biochim. Biophys. Acta*, 122 (1966) 544.
12 B. Mondovì, G. Rotilio, A. Finazzi Agrò and M. T. Costa, *Biochim. Biophys. Acta*, 132 (1967) 521.

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