COPPER REDUCTION BY SUBSTRATE IN DIAMINE OXIDASE

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1. Introduction

Pig kidney diamine oxidase (diamine: oxygen oxidoreductase, EC 1.4.3.6) similarly to other amine oxidases [1,2], contains cupric copper and pyridoxal phosphate [3–6]. The copper content of pig kidney diamine oxidase is 1 g atom of metal per 90,000 g of protein. All the chemically determined copper can be accounted for by the electron paramagnetic resonance (EPR) spectra of the enzyme. The function of copper in these enzymes is not yet clear. Cupric copper restores the activity of copper-free diamine oxidase from pea seedlings [7], of bovine plasma amine oxidase [1] and of pig kidney diamine oxidase [3]. However, no reduction of copper has been observed by EPR spectra of these enzymes [3,5,6,8]. In the case of pig kidney diamine oxidase, although no valence change of copper after addition of excess substrate in the absence of oxygen was observed, changes which were specific for each individual substrate were demonstrated in the EPR spectrum. A direct binding between substrate and copper was not evident [3].

In this communication, we report evidence for reduction of copper in diamine oxidase in the presence of substrate.

2. Materials and methods

Preparation of homogeneous pig kidney diamine oxidase, enzyme assay and other technical details were the same as in previous studies [3,4].

Stopped-flow measurements were made with a Gibson-Durrum apparatus following the techniques routinely used in our laboratory for anaerobic work [9].

EPR measurements were made at 123°K in a Varian V-4502-14 spectrometer at 9.15 GHz.

Reagent grade chemicals were used.

3. Results

3.1. *Kinetic experiments*

Pig kidney diamine oxidase shows a broad absorption in the visible region. On addition of substrate in the absence of oxygen there is a decrease of optical density near 500 mμ. This optical density change is very small, but certainly significant and is reversed in the presence of oxygen [3].

Fig. 1 shows the time course of the optical density change at 500 mμ after mixing the enzyme with various substrate concentrations in the absence of oxygen. Two distinct phases are present in the progress curves: a fast one, with half times in the range of tenths of a second, followed by a plateau lasting seconds and by an even slower further optical density decrease. Although no detailed kinetic analysis has been attempted at this stage, the data in fig. 1 show an obvious dependence of various phases of the reaction on substrate concentration, other conditions being equal. These also depend on oxygen concentration. When the enzyme was mixed simultaneously with oxygen and substrate (experiments not shown) the plateau region tended to last longer. At high enough oxygen concentrations the slow phase tended

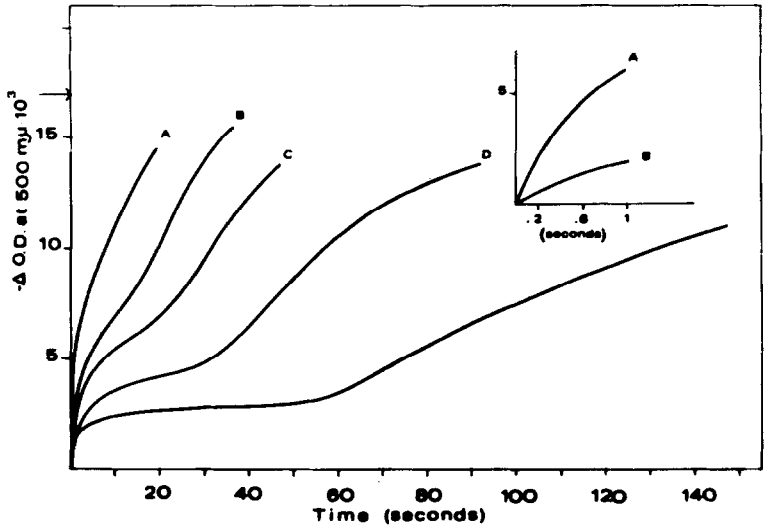

Fig. 1. Time course of the reaction of pig kidney diamine oxidase (1.83×10^{-5} M in 0.1 M potassium phosphate buffer pH 7.4) with various amounts of substrate under anaerobic conditions. A = 5×10^{-4} M cadaverine, B = 1.25×10^{-4} M cadaverine, C = 6.25×10^{-5} M cadaverine, D = 3.13×10^{-5} M cadaverine, E = 1.57×10^{-5} M cadaverine. The arrow indicates the asymptotes for curves A, B, C and D. The asymptote for curve E corresponds to an optical density of 13×10^{-3}. Insert shows details of the fast phase of the curves A and B.

to disappear and was replaced by a slow change in the opposite direction which restored the normal oxidized spectrum of the enzyme.

3.2. *EPR spectra*

Fig. 2 shows the EPR spectra of diamine oxidase performed under conditions comparable to those of the kinetic experiment of fig. 1D, i.e., after addition of about a twofold excess substrate in the absence of oxygen. It is clearly evident that the amplitude of the copper signal of the enzyme is reduced at about three minutes after the anaerobic addition of a slight excess of substrate, and that the initial amplitude is restored after a suitably long exposure to air. When, however, the solution was frozen at a time after mixing corresponding to the end of the first fast phase shown in the stopped-flow experiments (after about 10 sec), no reduction of the copper signal was observed, but changes were detected in the shape of the spectrum similar to those already described [3]: that is a slight increase of the signal amplitude and a change in the superhyperfine pattern around g ⊥.

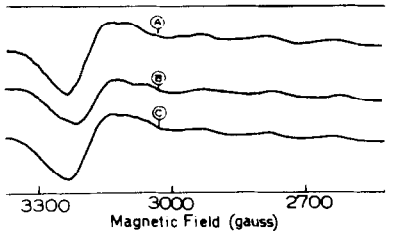

Fig. 2. EPR spectra of pig kidney diamine oxidase (1 mM in 0.1 M potassium phosphate buffer, pH 7.4). A: Enzyme as such. B: Enzyme with 2 mM neutralized cadaverine hydrochloride under anaerobic conditions, and frozen after 170 sec at room temperature. C: Reoxygenated enzyme (the solution was allowed to stand in air at room temperature for 15 min, with gentle occasional shaking).

The reduction of the intensity of the copper signal at a few minutes after mixing with substrate was calculated (by double integration) to be about 40% of that corresponding to the oxidized enzyme.

4. Discussion

The stopped-flow experiments show that the decrease in absorption of the enzyme at 500 mμ occurs in two distinct phases, the rates of both of which

depend on substrate concentration. The rapid one might reflect the formation of an enzyme substrate complex. The slow one, on the other hand, corresponds to the reduction of the enzyme. This is indicated by the fact that reduction of copper as seen in EPR experiments parallels in time the slow optical density change. When the enzyme is mixed anaerobically with the substrate and frozen as soon as possible (a few seconds), reduction of copper is not observed. The latter result is in accord with previous ones [3] and now leads to the conclusion that the early changes in EPR spectra were probably due to the formation of an enzyme substrate complex.

It should be pointed out that the enzyme maintains its full catalytic activity after the treatment involved in the stopped-flow and EPR experiments.

The finding that at least partial reduction of copper by substrate occurs in a time range comparable to that required for the changes in visible spectrum suggests that the question of a valence change in the catalytic action of the enzyme may have to be re-evaluated. However, the present results are complicated by evidence for heterogeneity of the copper in the enzyme preparation used (fig. 2). It is hoped that further studies, relating the rate of reduction and reoxidation to the velocity of the overall reaction, will reveal if the valence change is part of the catalytic mechanism or not.

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