THE REACTION OF NITRIC OXIDE WITH *RHUS VERNICIFERA* LACCASE

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Received 8 April 1975

1. Introduction

Among blue copper oxidases, laccases are the simplest, as each molecule of enzyme contains a single Type 1, Type 2 and Type 3 copper [1]. For this reason it seemed worthwhile to investigate the reaction of NO with a laccase, as this gas proved reactive with ceruloplasmin [2], which is a far more complex enzyme than laccase in terms of content and distribution of copper atoms. This communication deals with a set of experiments aimed to define the conditions of the reaction between NO and *Rhus vernicifera* laccase. The results obtained indicate that laccase and ceruloplasmin react with NO quite differently from each other.

2. Materials and methods

Rhus vernicifera laccase was purified and the two isoenzymes were isolated according to Reinhammar [3]. Argon was obtained from S.I.O. (Società Italiana Chimici), Milan; Nitric oxide was from B.D.H. (99% pure). Optical spectra were obtained with a DK 2A Beckman spectrophotometer and X-band EPR spectra with a V-4502 Varian Spectrometer. Both the optical cuvette and EPR tube were sealed to a Thunberg system in order to run optical and EPR spectra on the same sample after equilibration with NO to the pressure of 0.8 to 1 atm.

3. Results

Fig.1 reports optical spectra of the reaction of NO with laccase at pH 7.6. It is shown that equilibration of NO with the enzyme at room temperature leads to only a small decrease of the band around 600 nm, while a short incubation at 42°C results in almost total bleaching of the blue color. Substitution of NO with argon did not reverse significantly the bleaching, while a full recovery was obtained by re-admitting air into the sample.

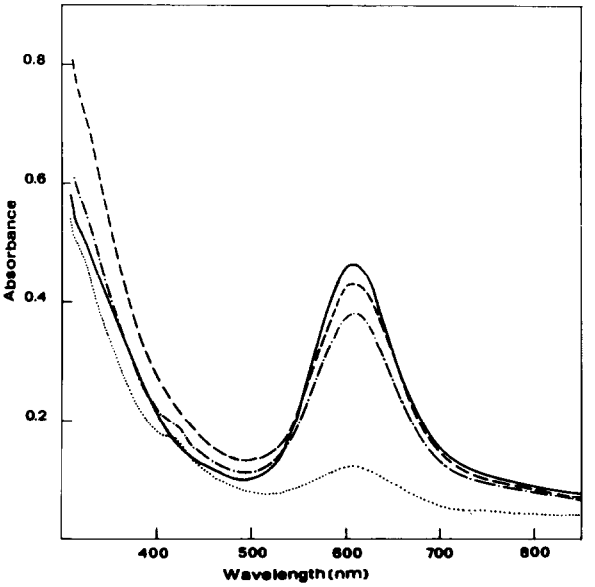

Fig.1. Optical spectra of the reaction of *Rhus* laccase with NO. The enzyme, 0.36 mM was dissolved in 0.1 M phosphate buffer, pH 7.6. Optical path: 0.2 cm. Temperature: 22°C. (———): the solution aereated and equilibrated with argon; (–.–.): the previous solution equilibrated with 1 atm NO at room temperature; (.....): the previous solution after 3 min at 42°C; (– – – – –), the previous solution after substitution of NO with argon (no effect) and then with air.

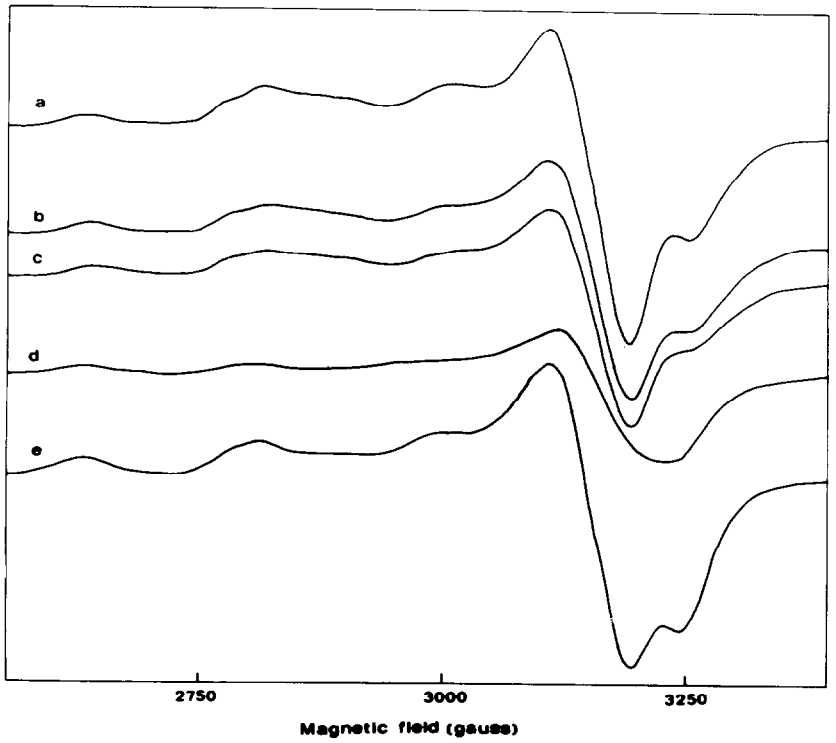

Fig.2. EPR spectra of the reaction of *Rhus* laccase with NO. The conditions are as in fig. 1. a) the enzyme deaerated and equilibrated with argon: b) equilibrated with NO at room temperature; c) kept 15 min at 0°C; d) 3 min at 42°C; e) after removal of NO and readmission of air. Temperature: −160°C. Modulation amplitude, 10 gauss. Microwave power: 20 mW.

Fig.2 shows the EPR spectra of the same experiments. Equilibration with NO at room temperature (fig.2,b) resulted in a significant, though small, modification of the spectrum, consisting of a relative decrease of Type 1 copper signal with respect to the Type 2 copper signal. Incubation of the same sample at ~ 0°C gave nearly the same spectrum (fig.2,c). On the other hand, the short incubation at 42°C resulted in the nearly complete disappearance of the Type 1 copper signal, and the sample showed only a Type 2 copper signal (fig.2,d). The last spectrum was practically unmodified by incubation at 0°C or substitution of NO with argon.

A significant recovery of signal intensity and line shape was obtained by readmission of air into the sample (fig.2,f). In fig.3 control EPR spectra are reported. It appears that samples treated as before, but without NO, show only slight modifications of the spectral line shape. However it is apparent by comparison of the last spectrum in the two series that the Type 2 copper signal is relatively more intense in the samples treated with NO.

Quantitative evaluation of the EPR detectable copper, after double integration of the spectra showed that EPR detectable copper was increased by about 20% after treatment with NO. On the other hand, the spectral changes observed in the control samples were not associated with an increase of the EPR detectable copper.

NO did not react with laccase at pH 5.4, and raising the temperature at this pH resulted in just irreversible changes of the spectrum of treated and untreated samples as well.

Similar experiments on the two isolated isoenzymes present in preparations of *Rhus* laccase [3] showed that they react identically with NO.

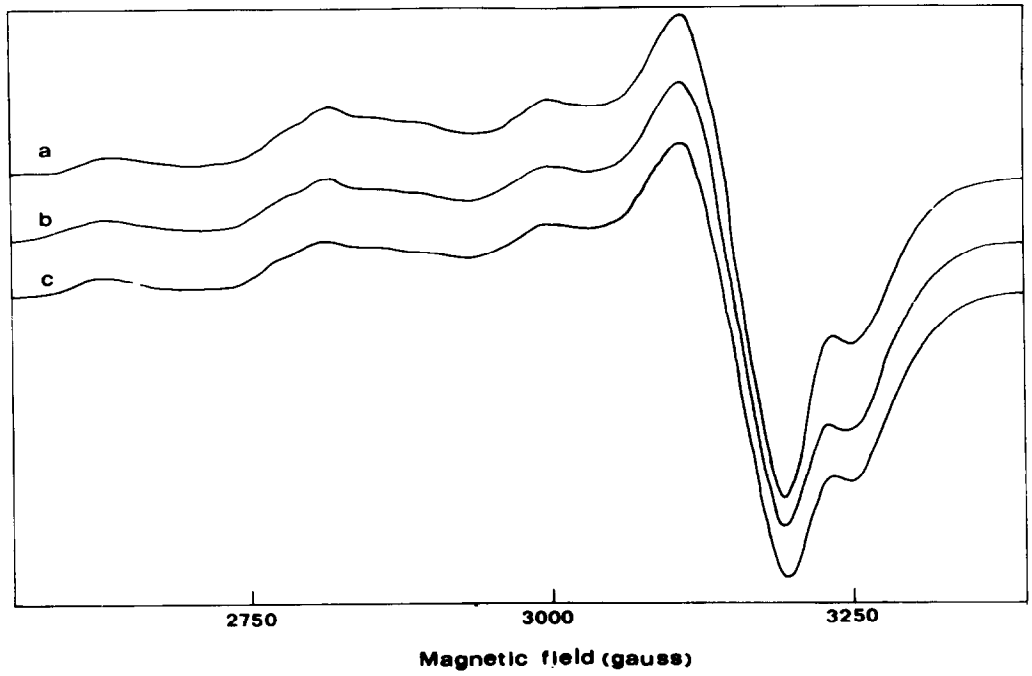

Fig.3. EPR spectra of *Rhus* laccase after incubation at different temperatures. The solution, 0.4 mM in enzyme and 0.1 M in phosphate buffer, pH 7.6, was treated as in fig.2, but without NO. a) as curve a of fig.2; b) as curve d of fig.2; c) the same as b, after removal of argon and re-admission of air at room temperature. Other conditions as in fig.2.

4. Discussion

The results described above show that *Rhus* laccase behaves towards NO quite differently from ceruloplasmin [2], though the general features of the reaction are the same, that is reversible disappearance of optical and EPR spectra of Type 1 copper. First of all, decreasing the temperature increased the reactivity of ceruloplasmin [2], while it did not modify the reactivity shown by laccase at room temperature (see fig.2,b and c). On the other hand nearly complete reaction was observed with laccase by raising the temperature to 42°C. The rise in temperature which brings about a complete reaction may affect the protein itself or the reaction of NO with the copper sites. Control experiments showed: (a) the increase in temperature by itself does not produce the changes observed in the presence of NO; (b) the dependence of reactivity on temperature is not due to molecular heterogeneity of the samples, since the two isolated isoenzymes showed the same behavior towards NO.

The reaction is clearly a charge transfer from NO to type 1 copper as demonstrated by the parallel disappearance of EPR spectrum and bleaching of the 600 nm band, but it is difficult to assess whether Type 1 copper is also a binding site for the ligand. The lack of reversibility on substitution of NO with argon, which furtherly differentiates *Rhus* laccase from ceruloplasmin [2], may be taken as an indication that the charge transfer between NO and Type 1 copper is not associated with a stable complex between NO and laccase and that the reaction cannot be described by a simple binding equilibrium, but NO decays in solution to irreversible products. However, the data available do not permit to discriminate between this possibility and kinetic effects which may be associated with a very slow dissociation velocity constant of the ligand from the complex.

Finally, it is important to notice that, while charge transfer to the blue copper is completely reversed by readmission of oxygen after removal of NO, some irreversible changes occur at other points of the mole-

cule. These lead to the appearance of some EPR-non detectable copper as additional EPR-detectable copper, which gives a signal almost superimposable to that of Type 2 copper. Work presently in progress will hopefully clarify the details of the reaction of NO with laccase.

References

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