A MASS SPECTROMETRIC INVESTIGATION OF THE REACTION BETWEEN $^{18}O_2$ AND REDUCED TREE LACCASE

A differentiation between the two water molecules formed

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1. Introduction

Of the 4 copper ions in a molecule of laccase 2 are EPR detectable (type 1 Cu^{2+} and type 2 Cu^{2+}) while the other 2 constitute an EPR-nondetectable copper pair (type 3 Cu^{2+}) which is associated with an A_{330} band [1]. There is evidence that all the copper ions take part in the catalytic reduction of dioxygen to water [2].

When 4 e^- equiv. reducing substrate are added anaerobically to the oxidized tree laccase (fig.1B,C) the EPR signal and the A_{330} band disappear [3]. If O_2 is added subsequently, the type 1 copper and the type 3 copper pair are reoxidized in the ms range [4] and an EPR signal is formed, which by the use of ^{17}O-enriched O_2 was shown to be a true oxygen intermediate (fig.1D), probably O^- [5,6]. The decay of the O^- (fig.1E) has a half-time of 13 s at pH 7.4 and is correlated to the reoxidation of the type 2 copper [7].

Water formed in the catalytic reduction of O_2 by lacquer tree laccase is bound to the type 2 Cu^{2+} in the fully-oxidized enzyme [8]. This water is so slowly exchanged with the bulk solution that after 30 min more than half of the water is still bound to the protein. A model for the dioxygen reducing site has been proposed, including a cavity which can be open or closed. This could be important in stabilizing reactive oxygen intermediates [7,8].

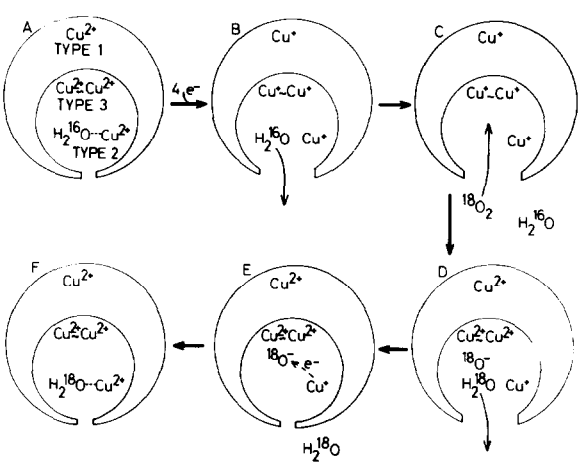

Fig.1. A model for the function of the dioxygen reducing site in laccases when the anaerobically-reduced enzyme reacts with O_2. A and F represent fully oxidized enzyme. B and C represent enzyme anaerobically reduced with 4 e^- equiv. reducing substrate. D and E represent intermediate forms of the enzyme in the reoxidation process.

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Two H_2O are formed in a complete reduction of O_2. From EPR studies it was not possible to state if both H_2O stay bound to the dioxygen reducing site or if one is rapidly released into the bulk solution. This knowledge is, however, required for a detailed description of the reaction.

The slow exchange of the H_2O bound to the type 2 Cu^{2+} made it possible to use a new technique. With a sensitive mass spectrometric method we can detect the increase in the natural abundance of $H_2{}^{18}O$ in the bulk solution upon reoxidation with ^{18}O-enriched O_2. One of the H_2O formed in the reduction of O_2 by tree laccase was found in the water phase.

2. Materials and methods

2.1. *Chemicals*

The ^{18}O-enriched O_2 (99%) was purchased from Miles Labs, Slough SL2 4LY. All other chemicals were of analytical grade. Sodium acetate buffer (0.1 M) at pH 4.0 was prepared from deionized distilled water and used in all experiments.

2.2. *Protein*

Rhus vernicifera laccase was prepared as in [9]. Protein concentrations were determined on the basis of $E^{1\%}_{1cm}$ 5.7 $mM^{-1}cm^{-1}$ at 615 nm. The enzyme was reduced with 4.0 e^- equiv. ascorbic acid. The reduced enzyme, about 0.7 mM, was transferred anaerobically into a syringe and mixed with O_2 enriched in ^{18}O. After 1 min, 2 ml reoxidized protein was transferred into another vessel. About 1 ml water was distilled under reduced pressure at 0°C and trapped at liquid nitrogen temperature. The total procedure, from mixing with O_2 to the separation of the water took about 5 min. Reference water was prepared in the same way from a solution containing no protein. Some reoxidation experiments were made in presence of an excess of ascorbic acid (20 e^- equiv.).

2.3. *Mass spectrometry*

The oxygen atom in the H_2O was converted into CO_2 by the usual equilibration technique [10]. The CO_2 samples were analysed on a micromass 602-C double collector mass spectrometer. The increase in the ratio of $^{18}O/^{16}O$ was determined relative to the reference water.

3. Result and discussion

Table 1 shows the result from 7 independent experiments with 3 different enzyme preparations.

Table 1
The increase in the relative amounts of ^{18}O in H_2O upon reoxidation of tree laccase with ^{18}O-enriched dioxygen

Sample	Prep.	Enzyme (mM)	^{18}O increase (%)	$H_2{}^{18}O$/molecule tree laccase[a]
A.				
1	I	0.71	1.01	1.62
2	I	0.71	0.83	1.33
3	II	0.74	0.85	1.30
4	II	0.74	0.77	1.17
5	III	0.77	0.83	1.12
6	III	0.77	0.87	1.28
			mean value	1.31
B.				
7	I	0.71	1.35	2.16

[a] The initial concentration of $H_2{}^{18}O$ is calculated as 113 mM from the 0.204% natural abundance [12]

The reaction was quenched after (A) 5 min at 0°C and (B) 24 h at 25°C

The mean value of the increase in the $H_2{}^{18}O$ found in the water phase after 5 min is 1.3 per reoxidized molecule of tree laccase. Sample 7 was kept at room temperature for 24 h before distillation and shows that two $H_2{}^{18}O$ are 'free' in the bulk solution as expected. From the loss in blue colour, the amount of protein which had been denatured during this treatment was estimated to be less than 5%. In presence of an excess of ascorbic acid 2 $H_2{}^{18}O$ were formed per 4 e^- equiv. ascorbic acid consumed in the catalytic turnover of the enzyme.

The present results mean that 1 of the 2 H_2O produced when 1 O_2 is reduced by the enzyme is present in the water phase at the shortest time possible for this kind of experiment. The other H_2O stays firmly bound to the type 2 Cu^{2+} [8]. The initial step in the reduction of O_2 to water is supposed to be a 2 e^- transfer from the reduced type 3 copper pair with the formation of $O_2{}^{2-}$ [1,11]. Immediately afterwards a 1 e^- transfer follows by which the type 1 copper is reoxidized and O^- and one H_2O is rapidly formed. We suggest that this water is released to the bulk solution from the dioxygen reducing site when the cavity was still open (fig.1D). The change in redox state of the enzyme probably induces a conformational change which closes the cavity thereby trapping the oxygen radical which in this way becomes stabilized by the enzyme (fig.1E).

A reduction of the time resolution to 10 s or less for the kind of experiment presented here would give a more firm evidence for the sequence of reactions seen in fig.1. If $H_2{}^{18}O$ is found in the water phase when O^- is bound to the protein, this must be the water formed after the initial transfer of 3 e^-, while the second water formed after the final slow transfer of the fourth electron to the oxygen intermediate becomes bound to the Type 2 Cu^{2+}.

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