CROSSLINKING OF CYTOCHROME *c* TO PEROXIDASE: COVALENT COMPLEX CATALYZES OXIDATION OF CYTOCHROME c_1 BY H_2O_2

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1. Introduction

Yeast cytochrome *c* peroxidase (EC 1.11.1.5) is a hemoprotein from the intermembraneous space of mitochondria [1–3]. The enzyme catalyzes the oxidation of ferrocytochrome *c* by H_2O_2. The mode of interaction of cytochrome *c* with the peroxidase might be exemplary for other electron transfer reactions with cytochrome *c* since several hemoproteins are known to bind to the same 'active site' of the cytochrome *c* molecule (reviewed in [4]).

Here we report on the preparation of a covalent complex of cytochrome *c* from horse heart with yeast peroxidase. When compared to free peroxidase the covalent complex still has 10% peroxidase activity toward exogenous ferrocytochrome *c*. In addition, the complex also catalyzes the electron transfer from reduced cytochrome c_1 which by itself is not a substrate of the peroxidase, to H_2O_2. These findings are discussed with respect to the adequacy of the complex as a model for the non-covalent enzyme–substrate complex.

2. Materials and methods

Cytochrome *c* peroxidase, prepared as in [5], had a heme content of 29.5 nmol/mg protein and a E_{408}/E_{280} ratio above 1.2. Cytochrome c_1 was isolated from beef heart [6] and was further purified by affinity chromatography on cytochrome *c* coupled to Sepharose 4B (R. Bechtold and R. Rieder, unpublished). [^{3}H]Methylated cytochrome *c* (3.2 methyl groups per molecule, 5.7 mCi/μmol) was prepared as in [7]. Dithiobis(succinimidylpropionate) (DTSP) was from Pierce, cytochrome *c* (type III) from Sigma and DEAE-cellulose (DE 32) from Whatman. Concentrations of peroxidase and cytochrome c_1 and *c* were determined as in [4,5].

[^{3}H]Methylated cytochrome *c* (20 nmol), native cytochrome *c* (362 nmol) and peroxidase (255 nmol) in 5 ml of 5 mM Na-phosphate pH 7.0 were treated at 0°C with *N*-ethylmaleimide (27 μmol) for 5 min followed by DTSP (3.7 μmol) for 15 min (pretreatment with *N*-ethylmaleimide blocks the single SH-group of peroxidase and prevents disulfide exchange with DTSP. The free SH-group is not essential for peroxidase activity [1]). Excess of DTSP was destroyed with $(NH_4)HCO_3$ (300 μmol). The reac-

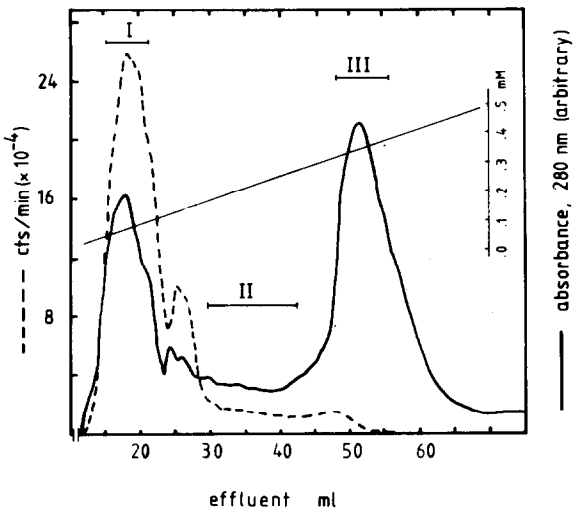

Fig.1. Purification of the covalent cytochrome *c*–peroxidase complex on DEAE-cellulose. The protein peak from the Sephadex G-25 column (12 ml) was applied on a column of DEAE-cellulose (11 cm × 0.9 cm) equilibrated with 20 mM Na-phosphate pH 6.0. Elution was with a linear gradient from 20 mM to 500 mM Na-phosphate, pH 6.0 at a flow-rate of 1.4 ml/h (molarities refer to the phosphate concentration). Aliquots of 5 μl of each fraction of 0.7 ml were tested for ^{3}H-radioactivity.

tion mixture was chromatographed on a column of Sephadex G-25 in 20 mM Na-phosphate pH 6.0. The protein peak was pooled and chromatographed on DEAE-cellulose (fig.1). Fractions from the DEAE-cellulose column were analyzed by electrophoresis on polyacrylamide gels [8].

Cytochrome *c* peroxidase activity was measured according to [5]. The assay mixture contained 25 μM ferrocytochrome *c*, 170 μM H_2O_2 and 0.5 nM to 1 nM peroxidase in 20 mM Tris/HCl pH 7.0. Cytochrome c_1 peroxidase activity was measured by following the decrease of absorbance at 553 nm. Further details are given in the legend to fig.3.

3. Results

Treatment of cytochrome *c* (containing some [^{3}H]methylated cytochrome *c* as a marker) and peroxidase with the cleavable crosslinking reagent DTSP [9] produced a small amount of a ^{3}H-labeled compound of apparent molecular weight 46 000 (fig.2, lanes A and A′). This compound was separated from cytochrome *c* (mol. wt 12 500) and peroxidase (approx. mol. wt 31 000) by chromatography on DEAE-cellulose (figs.1 and 2, lanes B–D). Some cytochrome *c* dimer was also produced by reaction with DTSP (fig.2, lanes B,B′).

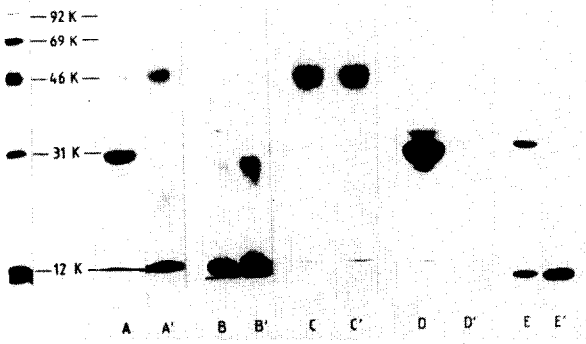

Fig.2. Analysis of products of crosslinking reaction on 12% polyacrylamide gels. Coomassie-stained gels (A to E) and corresponding autoradiograms (A′ to E′) are shown. Reaction mixture before DEAE-cellulose chromatography (A,A′), pool I (B,B′), pool II (C,C′) and pool III (D,D′) from DEAE-cellulose column (fig.1), products after treatment of pool II with dithioerythritol (E,E′). Molecular weight markers: cytochrome *c* (12 500), carboanhydrase (31 000), methylated ovalbumin (46 000), albumin (69 000) and phosphorylase B (92 000).

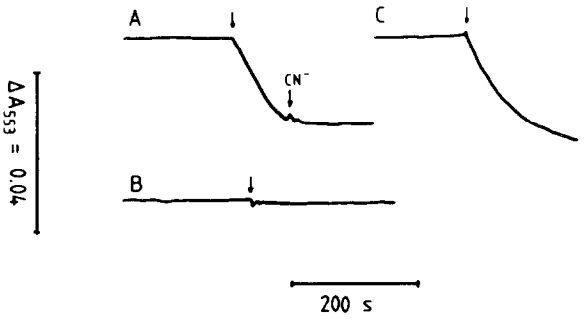

Fig.3. Oxidation of cytochrome c_1^{2+} (3 μM) by H_2O_2 (80 μM) catalyzed by 0.1 μM covalent complex (A) or by 0.1 μM free peroxidase (B). Cytochrome *c* (0.3 μM) had no effect on the oxidation of c_1 (3 μM) catalyzed by 0.1 μM covalent complex (C). Reactions were run at 5°C in 0.3 M Tris-acetate pH 7.5. Identical traces were obtained whether reactions were started by adding H_2O_2 (arrows) or covalent complex. The reaction in A was stopped by 20 mM KCN (arrow). The concentration of the covalent complex was estimated photometrically using ϵ_{408} = 200 mM^{-1} cm^{-1} (sum of ϵ_{408}'s of ferricytochrome *c* and peroxidase).

Incubation of the 46 000 compound with dithioerythritol (50 mM) produced ^{3}H-labeled cytochrome *c* and peroxidase (fig.2, lanes E,E′). Hence, the 46 000 compound consists of cytochrome *c* linked to peroxidase. The molecular weight is in accord with a 1:1 complex. Higher ratios of cytochrome *c* to peroxidase in the crosslinking reaction led to some 2:1 complex (data not shown).

The covalent complex still had about 10% cytochrome *c* peroxidase activity when compared with the parent enzyme (turnover numbers at 20°C of 60–80 s^{-1} and 800 s^{-1}, respectively). The activity was not due to contaminating peroxidase (fig.2, lane C). Contrary to the parent enzyme, the covalent complex also catalyzed the oxidation by H_2O_2 of reduced cytochrome c_1 (fig.3, traces A,B). The reaction had a turnover number of about 0.2 s^{-1} (5°C) and was inhibited by cyanide, or by low ionic strength (data not shown).

The covalent complex was slightly contaminated with free cytochrome *c* (fig.2, lane C). Therefore, the c_1-peroxidase activity might have been due to the following chain of redox reactions: c_1 → free *c* → covalent complex → H_2O_2. This possibility was ruled out by showing that additional cytochrome *c* did not increase the c_1-peroxidase activity (fig.3, trace C).

4. Discussion

One way of elucidating the binding site for cytochrome *c* on peroxidase is to prepare a covalent complex and to analyze the site of crosslinking. The difficulty with this approach is that crosslinking might also occur outside of the binding site proper. With a single binding site per molecule of peroxidase [1,10,13], the covalent 1:1 complex should be inactive. Hence, we may explain the remaining 10% of cytochrome *c* peroxidase activity of our complex by heterogeneity of the sites of attachment. Alternatively, if crosslinking occurred between residues peripheral to the actual site of inter-protein electron transfer, and given a length of 11 Å of the spacer [9], some covalently linked cytochrome *c* might be mobile enough to allow for productive binding of exogenous cytochrome *c*. The latter explanation could also hold for the cytochrome c_1-peroxidase activity, an exclusive property of the covalent complex (fig.3). Bound cytochrome *c* must be able to oscillate between the nearby electron acceptor site of the peroxidase and exogenous cytochrome c_1 for there is only a single binding site on cytochrome *c* for both, peroxidase and c_1 [6,11,12]. This interpretation is further strengthened by the observation that c_1-peroxidase activity disappears at low ionic strength where binding between *c* and c_1 and between *c* and peroxidase becomes very tight [6,1].

Analysis of the sites of crosslinking is now underway in our laboratory. The results, together with those already obtained from another covalent complex [13] and from model studies [14] should help to reveal the cytochrome *c* binding site on the peroxidase molecule.

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