Direct electrochemical oxidation of horseradish peroxidase: cyclic voltammetric and spectroelectrochemical studies

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The direct, unmediated electrochemistry of horseradish peroxidase (HRP) has been studied using a tin doped indium oxide coated glass working electrode at ambient pH. A well defined quasi-reversible cyclic voltammogram was obtained at high positive potentials corresponding to an $E_{1/2}$ of +700 mV *vs.* NHE. The cyanide complex of HRP also showed similar values of $E_{1/2}$, indicating formation of a pophyrin centred cation radical upon oxidation of the enzyme.

Horseradish peroxidase (HRP, E.C.1.11.1.7) is one of the most studied members of the peroxidase family,[1] a heme enzyme which catalyzes the oxidation of a large number of reductants by hydrogen peroxide. The resting state of the enzyme contains a ferric heme center at the active site. The enzymatic cycle of HRP has been shown to involve two short lived intermediates[2,3] called compound I and compound II. Various spectroscopic results including electronic absorption,[4] Mössbauer,[5] NMR,[6] ENDOR,[7] EXAFS[8] *etc.*, showed that compound I contains an oxoferryl iron weakly spin coupled to a porphyrin π cation radical ($[PFe^{IV}{=}O]^{\cdot}$, P = porphyrin dianion in HRP). Compound I is thus a two-electron oxidised species with respect to the resting state enzyme. Compound I, upon one-electron reduction, gives compound II which contains only an oxoferryl heme centre[2] ($[PFe^{IV}{-}O]$). Compound II is a one-electron oxidised species with respect to the resting enzyme.

The oxidation potentials for the formation of these intermediates have been estimated by mediated electrochemical methods using strong oxidising agents such as potassium hexachloroiridate[9] (K_3IrCl_6). Although several model hemes have been proposed to be oxidised at the electrode surface to give high-valent iron porphyrin species,[10] the direct electrochemical response of HRP has not been achieved so far. Recently, direct electrochemical studies on cytochrome c peroxidase (CCP) have been reported.[11] Compound I in cytochrome c peroxidase is largely different from that of HRP and it consists of a free radical at an amino acid (Trp) site instead of on the porphyrin ring.[12]

Direct electrochemistry of metalloenzymes and proteins has become one subject of extensive interest in the past ten years[13] mainly because of its potential application in the design of biosensors.[14] Direct, unmediated electrochemical methods can also help to determine the redox potential of the metalloenzyme unambiguously. Direct electrochemistry of HRP is difficult possibly because of the presence of the glycosidic chain attached to the protein.[1] The redox potential for the Fe^{3+}–Fe^{2+} couple in HRP had earlier been estimated,[15] using the redox titration method, to be −270 mV. Razumas *et al.*[16] have reported the electrochemistry of HRP at a methyl viologen modified gold electrode which however, showed a potential of −254 mV. Nevertheless no direct measurement of the catalytic intermediates has been reported so far.

By using potassium hexachloiridate as mediator titrant, the reduction potential of compound II–ferric centre (+869.1 mV) and compound I–compound II (+897 mV) was measured by thin-layer spectroelectrochemistry.[9] However, reversible voltammetric responses for either of the oxidation processes were not obtained. The reduction potential of the catalytically active redox intermediate of cytochrome c peroxidase was found to be +740 mV on a pyrolytic graphite electrode.[11] Although there are several reports[17] on the use of HRP in the design of amperometric sensors of H_2O_2, a systematic study on the electrode interaction with the enzyme has not been reported.

The present work reports on the direct electrochemical studies of HRP and for the first time, one well defined quasi-reversible cyclic voltammetric response for the oxidation of the ferric heme active center of the enzyme.†

Cyclic voltammetric experiments were carried out using a three-electrode assembly with a tin doped indium oxide coated glass working electrode (ITO), a platinum grid counter electrode and a Ag–AgCl reference electrode. A stable cyclic voltammogram was obtained after several cycles and after holding the potential at 900 mV for a few minutes, with 1 μM HRP in 50 mM potassium phosphate buffer (pH 7.4) containing 200 mM KCl solution. A control experiment with the electrode assembly under the same conditions in the absence of the enzyme was also carried out to ensure that the electrochemical response was indeed from the enzyme. Fig. 1 shows a well defined quasi-reversible cyclic voltammogram with almost equal cathodic and anodic peak currents. The two peaks were separated by (ΔE_p) 150 mV. The $E_{1/2}$ calculated

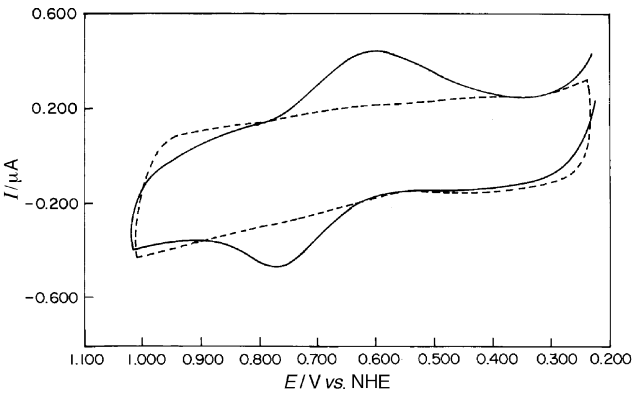

Fig. 1 DC cyclic voltammogram of horseradish peroxidase on pretreated ITO glass electrode (solid line). [HRP] = 1 μM in 50 mM potassium phosphate buffer at pH 7.4 containing 200 mM KCl. The dotted curve shows the background under the same solution conditions minus the enzyme. A similar background was also observed when apoHRP (heme depleted enzyme) was used under the same solution conditions. Scan rate 20 mV s^{-1} at 25 °C.

from the midpoint between the cathodic and anodic waves was found to be 700 mV *vs.* NHE. Cyclic voltammetry was carried out at different scan rates. Both the cathodic and anodic peak currents were found to vary linearly with the square root of scan rates ($v^{1/2}$) in the v region of 2 to 200 mV s^{-1}. The same results were obtained on changing the supporting electrolyte to $NaClO_4$ (in place of KCl) indicating that the supporting anion does not play any role in the electrochemical response or effect the midpoint potential of the enzyme.

The 'apparent diffusion coefficient' (D_{app}) of the enzyme calculated using the Randles Sevcik equation[18] was found to be 0.4 ($\pm$0.1) $\times$ 10^{-6} cm^2 s^{-1} at 25 °C which is close to the diffusion coefficient ($\approx 1 \times 10^{-6}$ cm^2 s^{-1}) of the enzyme estimated from the Stokes–Einstein equation assuming a spherical molecular structure[19] of radius 26 Å. An effective electrode area of 0.01 cm^2 estimated from the known diffusion coefficient of ferrocyanide was used for the calculation of the diffusion coefficient (D_{app}). The value of the midpoint potential ($E_{1/2}$) obtained in our study was however, smaller than that obtained by spectroelectrochemistry[9] for the formation of compounds II or I (see later).

In order to identify the species obtained at high positive potential, we have carried out *in situ* optical difference spectral studies on the system. Fig. 2 shows the optical difference spectra of HRP in the presence of an ITO electrode at an applied potential of 800 mV (*vs.* NHE) at different time intervals. A gradual increase in the absorbance at $\approx$420 nm with a consequent decrease at 400 nm in the difference spectra at a potential of 800 mV (*vs.* NHE) suggests the formation of HRP compound II (which has a characteristic Soret absorbance at 420 nm).[1] Thus at 800 mV (*vs.* NHE), the oxidised HRP species can form compound II which otherwise would require a much higher potential in order to be formed directly from the native enzyme as reported earlier.[9] The redox catalytic activity of HRP was checked before and after the experiment using an optical spectral method[20] from the oxidation of ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] by H_2O_2, which ensured that the enzyme was not denatured on the electrode surface and that the application of a high positive potential did not have any adverse effect on the redox catalytic activity of the enzyme. Thus, the observed redox potential ($E_{1/2}$) indeed corresponds to the reversible oxidation of the intact active enzyme.

We also carried out direct cyclic voltammetry of cyanide inhibited HRP using a similar method as discussed above. Cyanide binds to the iron of the heme active site of the enzyme and thus inhibits formation of the Fe^{IV}=O species. However, we observed that the cyanide bound HRP also gave a similar quasi-reversible cyclic voltammogram with an $E_{1/2}$

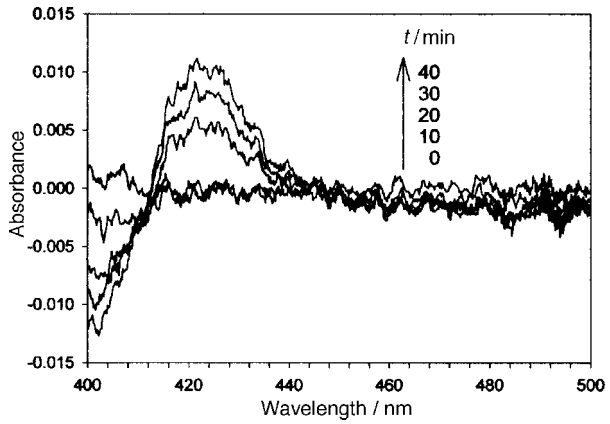

Fig. 2 Optical difference spectra of HRP in the presence of ITO electrode at an applied potential of 800 mV (*vs.* NHE) at different time intervals. Solution conditions are the same as in Fig. 1. A baseline correction was automatically made with respect to the same solution in the absence of an applied potential.

of +740 mV (*vs.* NHE). This indicated that the oxidation of HRP observed during the voltammetric studies in the present case probably corresponds to the formation of a cation radical at the porphyrin ring. Earlier studies[21] on the oxidative titration of zinc porphyrin reconstituted HRP (ZnHRP) by K_3IrCl_6 estimated an apparent redox potential of +740 mV (*vs.* NHE) for the formation of a cation radical at the porphyrin ring. The potential required for oxidation of the porphyrin ring is expected[9,21] to be smaller than that for the oxidation of iron from Fe(III) to Fe(IV). Moreover, since formation of compound I as well as compound II requires covalent attachment of an oxygen atom to give the oxoferryl iron, it is expected to be a slow process and one would not expect to observe a reversible elctrochemical oxidation in such a case. On the other hand, formation of a $[PFe^{III}]^{\cdot}$ (P = porphyrin–protein moeity) from the ferric heme would be fast and can be reversible. A recent report by Berglund *et al.*[22] suggested from photoinduced oxidation studies on HRP that the precursor for compound II can be a ferric porphyrin cation radical. Our results support the mechanism suggested by Berglund *et al.*[22] The initial event in the oxidation of the ferric state of HRP to compound II, as proposed by Berglund *et al.*,[22] is possibly an electron transfer from the porphyrin ring, thereby generating the radical intermediate which subsequently converts to the ferryl form. The UV-vis difference spectra shown in Fig. 2 thus detect the formation of compound II at 800 mV applied potential. The radical intermediate however, could not be detected by ESR at room temperature, probably because of spin-coupling with the ferric center of heme which is ESR silent at room temperature.[23]

Measurement of the redox potential of the Fe^{3+}–Fe^{2+} couple in the enzyme was also carried out by cyclic voltammetry with 1 μM HRP in 50 mM phosphate buffer (pH 7.4) in the presence of 100 mM sodium perchlorate as the counter ion. Before each experiment, care was taken to remove oxygen by careful and slow purging with argon gas. A cyclic voltammogram of this couple on pretreated ITO showed a cathodic peak at about −460 mV and the corresponding anodic peak at about −80 mV with a midpoint potential ($E_{1/2}$) of −270 mV *vs.* NHE. The cyclic voltammogram however, showed a large peak separation (ΔE_p 280 mV), but was stable and reproducible indicating that the electron transfer between the electrode and the ferroperoxidase is probably very slow. The midpoint potential for the formation of ferroperoxidase agreed well with the value reported earlier by Harbury.[15] In order to check whether the enzyme is functional or not, the catalytic current–potential response was checked at different H_2O_2 concentrations. A linear increase of the catalytic current was observed with increasing H_2O_2 concentration similar to that reported earlier.[11,17]

The results in the present study thus showed for the first time direct electrochemical oxidation of horseradish peroxidase. The one-electron oxidation obtained in the cyclic voltammetry of HRP at high positive potential (+700 mV *vs.* NHE) was assigned to the formation of a ferric porphyrin radical cation from the resting ferric prophyrin. The results suggest that this ferric porphyrin radical cation is the precursor of compound II of HRP which is an oxoferryl porphyrin species. The formation of compound II, as proposed by Berglund *et al.*[22] might follow the following mechanism:

$$[PFe^{III}], H_2O \overset{E_{1/2} = +700\ mV}{\rightleftharpoons} [PFe^{III}]^{\cdot}, H_2O$$

$$[PFe^{III}]^{\cdot}, H_2O \rightleftharpoons [PFe^{III}\text{–}H_2O]^{\cdot} \rightleftharpoons [PFe^{IV}{=}O] + 2H^+$$

It may be conjectured that the biochemical pathway of formation of the short lived intermediates formed by treatment of peroxides on peroxidase or models (*e.g.* myoglobin) might also involve the formation of a porphyrin centered radical cation as the precursor to the oxoferryl porphyrin species. The stability of the porphyrin radical cation is thus important for the

stability and catalytic activity of the peroxidase. The porphyrin ring in myoglobin and other models is vulnerable to decomposition by H_2O_2, as the radical formed in these cases tends to attach the *meso* carbon instead of the ferric center. The ferrous form of HRP was observed at -270 mV (*vs.* NHE) as estimated earlier.[15] Detailed studies on the formation of catalytic intermediates of HRP and other peroxidases and their catalytic activity are now underway.

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Notes and references

† Horseradish peroxidase (HRP) grade VI was purchased from Sigma Chemical Company. The enzyme was further purified using a reported method.[24] The enzyme was dissolved in 5 mM acetate buffer at pH 4.4 and applied to a CM52 column equilibrated with the same buffer. The bound protein was washed thoroughly with 5 mM acetate buffer at pH 4.4 and eluted by 100 mM acetate buffer at pH 4.4. The purified enzyme solution at pH 4.4 was dialysed against 50 mM sodium phosphate buffer (pH 7.4) prior to electrochemical experiments. The R_z (ratio of A_{403nm}/A_{280nm}) of the protein solution used for the experiment was above 3.2 to ensure purity of the enzyme.

Cyclic voltammetric experiments were carried out using an EG & G PAR potentiostat–galvanostat (Model M273) instrument. Voltammetric experiments were conducted in a three-electrode assembly with Ag–AgCl as the reference electrode and a platinum grid as the counter electrode. A glass plate with tin doped indium oxide (ITO) film on one side of it was used as working electrode. This ITO coated plate was pre-treated before every experiment using a reported method.[25] The electrode was ultrasonicated in detergent solution for 10 min followed by ultrasonication for 10 min once in ethyl alcohol and then twice in water. The electrode was used immediately after the pre-treatment.

Spectroelectrochemistry experiments were carried out using a Shimatzu 2100 UV-vis spectrophotometer. The electrochemical experiments were carried out in a rectangular quartz cell containing the working electrode (ITO) and other electrodes, to simultaneously observe spectral changes at different applied potentials. All experiments were carried out at room temperature (25 °C).

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