Chiral discrimination in the oxidation of ferrocenes by cytochrome c peroxidase

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Cytochrome c peroxidase shows a two-fold difference in its reaction rate with enantiomers of a chiral ferrocene; the enantiomeric selectivity is reversed by a single site specific change in the surface charge of the enzyme.

Chiral recognition is a fundamental property of many biological molecules and many examples have been reported in the literature, including enzyme–substrate and ligand–receptor interactions.[1–3] Previous reports of chiral discrimination in electron transfer reactions between small redox proteins and chiral metal complexes have described small but significant differences in kinetic enantioselectivity (10–20%) which has been used to probe the pathway(s) of electron transfer.[4–6] Electron transfer between redox enzymes and small molecules has also been extensively studied in the context of electrochemical sensors[7–9] and ferrocenes have been particularly exploited in the role of mediators between enzymes and solid electrodes. Although differences in electron transfer rates are seen with different ferrocenes there does not appear to be a simple relationship between the structure of the mediator and its reactivity.[10] There is a clear dependence of the rate of protein-mediator electron transfer on both the redox potential difference and the nature of the group(s) substituting the cyclopentadienyl rings. One particular feature of ferrocenes that has not been explored with respect to the kinetics of electron transfer is their property of planar chirality when substituted with two different groups on the same cyclopentadienyl ring. Previously it has been shown that lipases can distinguish between two enantiomers in the esterification of ferrocenyl alcohols.[11] We report here the significant chiral discrimination shown by the enzyme cytochrome c peroxidase (CCP) with two ferrocene enantiomers and the reversal of this chiral specificity by a site specific change in the surface charge on the protein.

The yeast CCP used in this work was expressed in *E. coli*, purified by a previously published method[12] and had a turnover number (horse heart cytochrome as the substrate) of 200 s^{-1}. The mutation of a surface aspartate residue to a lysine residue employed the polymerase chain reaction and was confirmed by DNA sequencing. The synthesis of both (*S*)- and (*R*)-1-hydroxymethyl-2-dimethylaminomethylferrocene (HMDMF) was as described by Nicolosi *et al.*[13] Rates of reaction between the two enantiomers of HMDMF and CCP were estimated by cyclic voltammetry. Both enantiomeric ferrocenes exhibited quasi-reversible one-electron voltammograms with mid-point potentials of 390 mV (*vs.* SCE), a peak separation of 61 mV, peak currents proportional to the square root of the potential sweep rate. The latter observation confirming that the mass transport of mediator is occurring under conditions of planar diffusion. Upon addition of hydrogen peroxide to a solution of the mediator there was no change in the observed voltammograms showing that hydrogen peroxide does not give rise to significant oxidation currents on glassy carbon in the operating potential region. However, on addition of the CCP to a solution of mediator containing hydrogen peroxide, a characteristic catalytic wave with an enhanced reduction current and no oxidation peak was seen. Both the change in the appearance of the voltammogram and the increased current are consistent with catalytic regeneration of oxidised mediator in the diffusion layer as observed previously with non-chiral ferrocenes.[14,15] The absence of diffusional peaks in the cyclic voltammograms under conditions of enzyme turnover are consistent with the cathodic currents arising from enzymatically generated, rather than electrochemically generated, ferrocenium ions.

Fig. 1 shows the catalytic currents obtained in the system containing CCP and either (*S*)- or (*R*)-HMDMF as diffusional electron transfer mediators in the presence of hydrogen peroxide. It can be seen clearly from Fig. 1 that the *R*-enantiomer acts as a faster electron transfer mediator than the *S*. The catalytic current for (*R*)-HMDMF is about two-fold higher than that of (*S*)-HMDMF.

When the limiting currents in the cyclic voltammograms are plotted against the square root of the enzyme concentration a linear relationship is observed, consistent with the expected behaviour in an enzyme coupled system.[10] For each enantiomer the overall second order rate constant for the reaction between reduced mediator and oxidised enzyme was determined from the scan rate dependence of the catalytic currents at five different enzyme concentrations.[16] The values for the rate constants showed a significant ($P < 0.001$) difference between the two enantiomers with (*R*)-HMDMF reacting approximately twice as fast as (*S*)-HMDMF (Table 1). This difference in rate constants is comparable to that observed by Marx-Tibbon *et al.*[17] for the reaction of *N,N*-dimethyl-1-ferrocenylethylamine

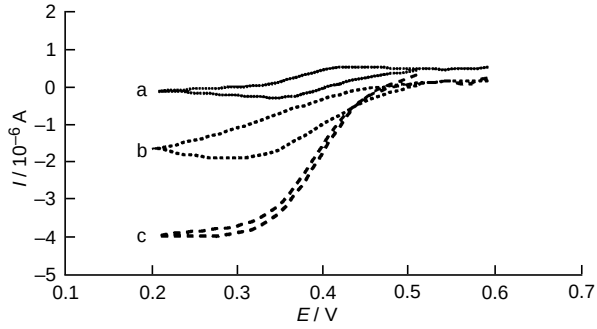

Fig. 1 Cyclic voltammogram of (*a*) 0.5 mm (*R*)-HMDMF in phosphate buffer (50 mm, pH 6.5) containing 2 mm hydrogen peroxide; (*b*) 0.5 mm (*S*)-HMDMF together with 2 mm hydrogen peroxide after addition of 0.7 μm CCP; and (*c*) 0.5 mm (*R*)-HMDMF together with 2 mm hydrogen peroxide after addition of 0.7 μm CCP. All voltammograms were recorded at 5 mV s^{-1} on a glassy carbon electrode with an area of 0.125 cm^2.

Table 1 Rate constants for the reaction of cytochrome c peroxidase wild type and mutant enzymes with both enantiomers of 1-hydroxymethyl-2-dimethylaminomethylferrocene

	R-Enantiomer	*S*-Enantiomer
Wild Type CCP	$(2.9 \pm 0.4) \times 10^6$ dm^3 mol^{-1} s^{-1}	$(1.6 \pm 0.2) \times 10^6$ dm^3 mol^{-1} s^{-1}
D34K CCP	$(5.9 \pm 0.5) \times 10^6$ dm^3 mol^{-1} s^{-1}	$(14.8 \pm 1) \times 10^6$ dm^3 mol^{-1} s^{-1}

(a ferrocene with a chiral side chain) with glucose oxidase, although this work has recently been the subject of a reinterpretation by Boudillon *et al*.[18]

The rates of reaction of the enantiomers of HMDMF with a mutant of CCP (Aspartate 34 to Lysine) were also determined. Aspartate 34 is a negatively charged residue, situated on the surface of CCP and has been implicated in its reaction with its physiological partner, cytochrome c on the basis of the crystal structure of the complex.[19] When the rate constants for this mutant were evaluated with the artifical ferrocene donor two striking observations were made, firstly the values for both enantiomers were increased, showing that this mutant turned over the ferrocene substrates more rapidly than the wild type enzyme. More interestingly the chiral preference of the enzyme was reversed with the *S*-enantiomer now being the faster substrate. In fact in this mutant the rate of reaction with the *S*-enantiomer is increased by an order of magnitude compared with the wild type. The corresponding rate constants for (*R*)- and (*S*)-HMDMF are also reported in Table 1. We have also investigated the reaction of this mutant with non-chiral ferrocenes[20] and, as might be anticipated, differences in rate are observed reflecting the different interactions of the two forms with the mediator. If however substrate chirality was not implicated in the reaction we should observe that both enantiomers would exhibit the same change in rate, in contrast to the observations here where quite different increases are seen.

These data clearly show that not only is there a specific molecular recognition event occurring between the chiral ferrocenes and CCP but also that this interaction is exquisitely sensitive to a specific surface charge on the CCP. Calculated potential maps were obtained for wild type and D34K CCP using the finite difference method introduced by Sharp and Honig[21] and show large changes in the proteins' electrostatic fields around the site of mutation the analysis of which will be published in detail elsewhere.

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Footnote

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