Protein conformer selection by ligand binding observed with crystallography

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Abstract
A large-scale movement between "closed" and "open" conformations of a protein loop was observed directly with protein crystallography by trapping individual conformers through binding of an exogenous ligand and characterization with solution kinetics. The buried indole ring of Trp191 in cytochrome c peroxidase (CCP) was displaced by exogenous ligands, causing a conformational change of loop Pro190-Asn195 and exposing Trp191 to the protein surface. Kinetic measurements are consistent with a two-step binding mechanism in which the rate-limiting step is a transition of the protein to the open state, which then binds the ligand. This large-scale conformational change of a functionally important region of CCP is independent of ligand and indicates that about 4% of the wild-type protein is in the open form in solution at any given time.

Keywords: artificial cavity; cytochrome c peroxidase; kinetics; ligand binding; loop movement; protein crystallography; site-directed mutagenesis

Much evidence exists that proteins exhibit considerable flexibility. Ligands are often found in the center of proteins with no observable path to the surface, suggesting that the protein must transiently unfold in order for the ligand to enter. Examples include myoglobin and hemoglobin oxygen binding to the heme (Karplus & Petsko, 1990), fatty acid binding to fatty acid binding protein (LaLonde et al., 1994), retinol binding to retinol binding protein (Zanotti et al., 1993), and ligand binding to artificial cavities introduced into proteins (Eriksson et al., 1992; Fitzgerald et al., 1994, 1996; Feher et al., 1996). Flexible surface-loops in protein structure are thought to play an important role in substrate binding and molecular recognition. Crystal structures have shown that, for a number of enzymes and antibodies, substrate and/or antigen binding causes a conformational change of a surface-loop either from an "open" to a "closed" form to partially cover the bound substrate (Joseph et al., 1990; Lolis & Petsko, 1990; Derewenda et al., 1992; Jia et al., 1995), or from a "partially closed" to "open" form, resulting in an induced fit for the antigen or ligand binding (Rini et al., 1992; Concha et al., 1993). These conformational changes have been observed directly by NMR, and the relevance of these motions to dihydrofolate reductase function has been demonstrated (Epstein et al., 1995). All of these examples reveal that proteins switch between conformations and that these changes are often key to protein function.

Here we trap a large conformational change of a protein loop by binding an exogenous ligand to the protein and directly visualizing the structure by X-ray crystallography. The ligand displaces a buried tryptophan side chain and leaves the displaced loop on the surface. This loop displacement was first observed in the crystal structure of the F202G (i.e., Phe202 replaced by Gly) mutant of cytochrome c peroxidase, which is somewhat less stable relative to wild-type. The same loop displacement was observed subsequently in the wild-type protein. Kinetic measurements are consistent with a model in which the rate-limiting step of ligand binding is a first-order reaction of the protein, presumably, the protein unfolding from the closed to the open state, followed by relatively rapid ligand binding. In the absence of ligands, as calculated from solution kinetics, the ratio of the open to closed conformer is on the order of 1:28. In the F202G mutant, the protein is more flexible and the ratio of loop open to closed forms is 1:11.

Results

Crystal structures of ligand trapped conformational changes
A buried cavity was introduced into CCP by replacing Phe202 with glycine (F202G). The crystal structure (Table 1) showed that a cavity (cavity 202) was formed and, other than the fact that the B-values for many of the loops in the protein had increased, the structure was otherwise largely undisturbed. Several cationic ligands, such as the protonated forms of 1,2-dimethylimidazole and
Table 1. X-ray diffraction data collection and refinement statistics

<table>
<thead>
<tr>
<th>Data collection</th>
<th>F202G&lt;sup&gt;a&lt;/sup&gt; soaked in DMI&lt;sup&gt;c&lt;/sup&gt;</th>
<th>F202G&lt;sup&gt;b&lt;/sup&gt; soaked in 2a5mT&lt;sup&gt;d&lt;/sup&gt;</th>
<th>wt CCP&lt;sup&gt;b&lt;/sup&gt; soaked in 2a5mT&lt;sup&gt;d&lt;/sup&gt;</th>
<th>F202G&lt;sup&gt;b&lt;/sup&gt; soaked out 2a5mT&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Crystal form</td>
<td>Yeast</td>
<td>Yeast</td>
<td>MKT</td>
<td>Yeast</td>
</tr>
<tr>
<td>Unit cell</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
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<td>107.7</td>
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<td>107.5</td>
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<tr>
<td>b (Å)</td>
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<td>76.6</td>
<td>74.2</td>
<td>76.1</td>
</tr>
<tr>
<td>c (Å)</td>
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<td>51.7</td>
<td>45.2</td>
<td>51.6</td>
</tr>
<tr>
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<td>2.4</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>I/σ&lt;sub&gt;(av)&lt;/sub&gt;</td>
<td>20.6</td>
<td>6.5</td>
<td>30.9</td>
<td>22.3</td>
</tr>
<tr>
<td>I/σ&lt;sub&gt;(last shell)&lt;/sub&gt;</td>
<td>1.8</td>
<td>1.0</td>
<td>3.6</td>
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</tr>
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<td>19,829</td>
<td>27,395</td>
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<tr>
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<td>98</td>
<td>82</td>
<td>93</td>
</tr>
<tr>
<td>R&lt;sub&gt;liftm&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.054</td>
<td>0.097</td>
<td>0.049</td>
<td>0.068</td>
</tr>
<tr>
<td>R&lt;sub&gt;liftm&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.18</td>
<td>0.18</td>
<td>0.20</td>
<td>0.21</td>
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<tr>
<td>Resolution (Å)</td>
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<td>2.4</td>
<td>2.0</td>
<td>2.0</td>
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<tr>
<td>No. of waters</td>
<td>98</td>
<td>98</td>
<td>98</td>
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</tr>
</tbody>
</table>

<sup>a</sup>The crystal was grown by dialysis against distilled water (PDB code 1CCJ).
<sup>b</sup>Crystals were grown and preserved in MPD.
<sup>c</sup>Structure refined including DMI (PDB code 1CCI).
<sup>d</sup>Structures were refined with loop 190-195 omitted.
<sup>e</sup>R<sub>liftm</sub> represents the agreement between I<sub>o</sub> for symmetry equivalent reflections. I/σ<sub>(last shell)</sub> is the average I/σ<sub>o</sub> for the 10% of the data at highest resolution. R values give the crystallographic residuals between the observed and model-derived structure factor amplitudes. Both F202G and wtCCP were soaked in 50 mM DMI<sup>+</sup> or 50 mM 2a5mT<sup>+</sup>.

aniline, which were found previously to bind in the W191G mutant cavity (Fitzgerald et al., 1994, 1996; R.A. Musah, M.M. Fitzgerald, D.E. McRee, & D.B. Goodin, unpubl. results), also bind at the position of the Trp<sup>191</sup> side chain (named "191 cavity" or "191 position") of F202G. This binding is accompanied by rearrangement of loop 190-195 and displacement of the Trp<sup>191</sup> side chain. Similar to a rearrangement seen previously for W191C (Fitzgerald et al., 1996). After soaking a crystal of F202G in 50 mM

Fig. 1. Loop open conformer trapped by ligand binding to CCP F202G mutant. The 2F<sub>e</sub> − F<sub>c</sub> omit map (1.4σ, gray lines) at the loop region with residues 190-195 omitted shows that this loop in F202G protein is in an open conformer. The loop 190-195 open structure of F202G (thick lines, PDB code 1CCI) is superimposed on the loop closed structure (thin lines, PDB code 1CCJ). The position of DMI<sup>+</sup> density is indicated by a label, but a model is not shown. The F202G crystal was soaked in 50 mM of DMI<sup>+</sup> at pH 6.0. The structure factors of the F202G structure (Table 1, column 1), without adding any water or ligand molecules in the F202G cavity and with residues 189-195 omitted, were used to construct the 2F<sub>e</sub> − F<sub>c</sub> map.
DMI\textsuperscript{+}, the $2F_o - F_c$ map (Fig. 1) showed that there was no electron density at the position of the main chain between residues 190 and 195. Instead, a new, continuous electron density, beginning at Pro\textsuperscript{190} and ending at Asn\textsuperscript{195}, had appeared in what was formerly solvent. The electron density for the side chain of Trp\textsuperscript{191} at the protein surface was clear and could be well fitted. After refinement of the model fit to the new loop density and with DMI\textsuperscript{+} at the 191 cavity, the $R$-factor at 2.4 Å was 17.8\% (Table 1). The maximal displacement of this loop was 8.7 Å for the alpha carbon (Trp\textsuperscript{191,Ca}) and 12.6 Å for the side chain (Trp\textsuperscript{191,CD1}). Pro\textsuperscript{190} changes from trans to cis, forming one hinge point, and Asn\textsuperscript{195} interchanges with the side-chain and main-chain positions at the other end to form the second hinge. The value of the pseudo dihedral angle of Trp\textsuperscript{191}(Ca\textsuperscript{closed})-Gly\textsuperscript{189}(C\textsuperscript{closed})-Asn\textsuperscript{195}(C\textsuperscript{open})-Trp\textsuperscript{191}(Ca\textsuperscript{open}) is 107° from the closed to the open conformer about an axis passing through two hinge points, Gly\textsuperscript{189}-C and Asn\textsuperscript{195}-C. In the loop open form, the hydrophobic side chain of Trp\textsuperscript{191} on the protein surface was not stabilized by any crystal contact or hydrogen bond.

Loop opening was also observed in wild-type CCP crystals when they were soaked in 2-amino-5-methylthiazolium iodide (Table 1; Fig. 2). Of all ligands found thus far bound in the W191G cavity, 2a5mT\textsuperscript{+} has the highest affinity (R.A. Musah & D.B. Goodin, unpublished results). For this reason, 2a5mT\textsuperscript{+} was expected to strongly compete with the indole side chain, and the sulfur atom of 2a5mT\textsuperscript{+} would be expected to show a greater density difference over the C and N atoms of the Trp\textsuperscript{191} indole side chain. After soaking a wtCCP crystal in 50 mM 2a5mT\textsuperscript{+}, the position of the sulfur atom was indicated by a positive density peak at >5σ in the $F_o - F_c$ map (Fig. 2). By comparison to the earlier F202G loop open structure, it was observed that the electron density of loop 190–195 represents a mixture of open and closed forms. A series of extrapolated ($F_{soaked} - F_{unsoaked}$/partial fractional occupancy) difference maps assuming 0.10, 0.20, and 0.30 partial fractional occupancy were calculated to estimate the partial occupancy of the open form of the loop and to make a map that shows only the open form. An advantage of this method is that it is done in the absence of any model(s) and thus eliminates model bias. In the 0.20 partial occupancy map (i.e., with the differences scaled by a factor of 5), the map showed a fully occupied loop, no density at the main chain of the closed position, and strong density at the ligand binding site where the indole side chain had been displaced (Fig. 3). Using this method, the fractional occupancy of the closed and open forms of the loop were estimated to be 0.8 and 0.2, respectively.

In addition, we also refined the loop occupancy using a model containing both the loop in and loop out partial structures. The relative occupancies were refined in four groups. Group 1 contained the Pro\textsuperscript{190} hinge, group 2 contained the loop residues 191–194, except for the indole side chain of 191, which was placed in group 3, and group 4 contained the Asn\textsuperscript{195} hinge. The indole side chain was placed in a separate group because overlap of its density with that of 2a5mT\textsuperscript{+} would have resulted in an overestimation of the closed form. The refinement was done in two ways. In the first, the $B$-values were fixed at 20.0, and the relative occupancies of the two slit parts were allowed to refine. This led to an occupancy of 0.62 for the loop closed chain and a corresponding occupancy of 0.38 for the loop open chain. In the second, the $B$-values were freed and allowed to change. In this case, the occupancies refined to 0.61 and 0.39, respectively. These observations were further verified by the observation of convergence (albeit slow) after 100 cycles of refinement when the starting occupancy ratios were at 0.8 open and 0.5. Refinement from above or below in occupancy eventually gave essentially the same values. Thus, in this case, we found that the extrapolated difference method may not be entirely reliable for estimating the value of the partial structure occupancy of the loop. However, it is useful in map interpretation because it cleanly separates the density for the two interleaved structures from one other, and simplifies model-building and interpretation.

The opening of loop 190–195 is a reversible process in the CCP crystal. After soaking an F202G crystal in 50 mM 2a5mT\textsuperscript{+} at pH 5.2 for 15 min, the loop 190–195 was found partially open. Two 2a5mT\textsuperscript{+} molecules were found in the F202G structure: one at

![Fig. 2. Open loop conformer partially trapped by binding of ligand to wtCCP. $2F_o - F_c$ electron density map (1σ, gray lines) of wtCCP soaked with 2a5mT\textsuperscript{+} indicates that part of the loop 190–195 is in a mixture of open and closed conformers because there is density at both positions (closed model, thin lines, PDB code 1CCA; open model, thick lines, PDB code 1CCI). The $F_o - F_c$ difference map (black, thick contours) is contoured at >5σ and shows a peak at the position of the known 2a5mT\textsuperscript{+} binding site (R.A. Musah & D.B. Goodin, unpublished results). The position of 2a5mT\textsuperscript{+} density is indicated, but a model is not shown. The crystal was soaked in 50 mM 2a5mT\textsuperscript{+}, pH 5.2, for 20 min. The $F_o$ and $\alpha$ of the wtCCP (Fitzgerald et al., 1994) with residues 189–195 omitted were used to construct the $2F_o - F_c$ and $F_o - F_c$ map.](image-url)
Protein conformer selection by ligand binding

Fig. 3. Open loop conformer extracted from open/closed mixture in Figure 2 by extrapolation. Extrapolated $F$ map (gray lines) assuming 20% partial occupancy is shown superimposed on the structure of the open loop form (thick lines) and closed loop model (thin lines). The map shows only density at the open form and at the position of the $2a5mT^+$ ligand (labeled $2a5mt$ in figure) with no density at the closed position (thin lines). The phases of the wtCCP (Fitzgerald et al., 1994) with residues 189–195 omitted were used to construct the $2F_{\text{exposed}} - F_{\text{wtCCP}}$ map (see Materials and methods).

Fig. 4. Superposition of backbone structures of the loop 190–195 closed conformer (orange, with closed loop in pink) and the open conformer (yellow, with open loop in white) of CCP. In the loop closed conformer, the Trp$^{191}$ side chain (pink, ball-and-stick) is buried and forms an H-bond to Asp$^{235}$ at the active site. In the loop open conformer, Trp$^{191}$ (white, ball-and-stick) moves to the protein surface and becomes exposed to the solvent, leaving an empty binding pocket to which DMI$^+$ and $2a5mT^+$ bind.
both wtCCP and F202G with a high concentration of DMI+ 
by measuring the absorption changes in the Soret band. Titration of 
total structures. To determine whether the red shift was due to non-
W191G mutant with DMI+ a similar low-spin spectrum has been observed previously for the 
binding of DMI+ to F202G, and approximately 50 mM for the 
determined from a Scatchard plot (Fig. 
levich et al., 1991; Goodin & McRae, 1993). The kinetics of these 
spectral shifts were nearly exponential and extremely slow, with 
lifetimes of 10^4 for F202G and 10^3 for wtCCP at 20°C. Because 
a similar low-spin spectrum has been observed previously for the 
W191G mutant with DMI+ bound in the 191 cavity, the red shift 
determined during titration of F202G and wild-type with DMI+ was 
rationalized in terms of the displacement of Trp 191 with DMI+, 
accompanied by loop movement as had been observed in the crystal 
structures. To determine whether the red shift was due to non-
specific effects at high concentration, two neutral ligands, 
2-mercaptoimidazole and thiazole, which were not expected to 
binding in the 191 cavity, were added to the wild-type CCP solution 
to give a final concentration of 200 mM and 133 mM, respectively. 
After equilibration for 12 h at room temperature (~22°C), no red 
shift was observed. Figure 5 shows the transition of F202G from 
high spin to low spin upon addition of DMI+. The K_d at 20°C, as 
determined from a Scatchard plot (Fig. 5, inset), is 20 mM for 
binding of DMI+ to F202G, and approximately 50 mM for the 
binding of DMI+ to wtCCP.

The kinetics of ligand binding as a function of ligand concen-
tration l) under conditions in which the second step (ligand-binding) 
are in constant equilibrium in solution even in the absence of

\[ K_{obs} = k_1 + \frac{k_1}{([L]k_{-1})/(K_{d3}k_1)} + 1 \]  

\[ K_{d3} = 11.4 \pm 0.0003 \text{M} \]  

\[ K_{d2} = 28 \pm 0.0006 \text{M} \]  

\[ K_{d1} = 0.0000 \text{M} \]  

Discussion

Implications for protein flexibility

Our experiments show that the open and closed forms of wtCCP 
are in constant equilibrium in solution even in the absence of

In the mechanism in Equation 1, the observed induced-fit binding 
upper path, loop opening simultaneous with ligand binding) is 
broken into two steps along the lower path: (1) loop opening 
independent of ligand, and (2) lock-and-key binding in which the 
ligand binds to the loop open conformer.

Fig. 5. Dissociation constant determination for binding of DMI+ to F202G. 
UV-vis absorption spectra obtained by titration of F202G with DMI+, 
pH 6. Inset: Scatchard plot utilized to obtain K_d3 from the difference 
absorbance (ΔAbs) at 418 nm. The fraction bound (v) was determined by 
extrapolating 1/ΔAbs versus 1/[DMI+] to infinite DMI+ concentration 
assuming one binding site (Fitzgerald et al., 1994).

In the mechanism in Equation 1, the observed induced-fit binding 
upper path, loop opening simultaneous with ligand binding) is 
broken into two steps along the lower path: (1) loop opening 
independent of ligand, and (2) lock-and-key binding in which the 
ligand binds to the loop open conformer.

Fig. 6. Solution kinetics of DMI+ binding to wtCCP consistent with a 
pre-equilibrium of the protein. The apparent rate (k_{obs}) of binding of DMI+ 
to wtCCP is plotted as a function of DMI+ concentration [L]. The raw data 
circles) were fitted to a line using Equation 2 in Materials and methods. 
The shape of the curve is characteristic of a process with a pre-equilibrium 
first-order step followed by a relatively fast second-order binding of ligand 
where the apparent rate of binding decreases with increasing ligand 
concentration.

The kinetics of ligand binding can be rationalized in terms of 
Equation 1, where E_c and E_o represent the the closed and open 
conformers of the protein, respectively, and E_cL is ligand-bound 
protein. Both the apparent rate (k_{obs}) and the dissociation constant 
(K_d) of binding of DMI+ to F202G and wtCCP were determined 
by measuring the absorption changes in the Soret band. Titration of 
both wtCCP and F202G with a high concentration of DMI+ 
(>50 mM) resulted in a red shift of the heme Soret band from 
408 nm to 416 nm, which was accompanied by an increase in the intensities of the β and α bands at 530 and 560 nm, respectively. 
The observed spectral changes are consistent with a high-spin to 
the low-spin state transition of the ferric iron–heme complex (Smu-
leovich et al., 1991; Goodin & McRae, 1993). The kinetics of these 
spectral shifts were nearly exponential and extremely slow, with 
lifetimes of 10^4 for F202G and 10^3 for wtCCP at 20°C. Because 
a similar low-spin spectrum has been observed previously for the 
W191G mutant with DMI+ bound in the 191 cavity, the red shift 
determined during titration of F202G and wild-type with DMI+ was 
rationalized in terms of the displacement of Trp 191 with DMI+, 
accompanied by loop movement as had been observed in the crystal 
structures. To determine whether the red shift was due to non-
specific effects at high concentration, two neutral ligands, 
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high spin to low spin upon addition of DMI+. The K_d at 20°C, as 
determined from a Scatchard plot (Fig. 5, inset), is 20 mM for 
binding of DMI+ to F202G, and approximately 50 mM for the 
binding of DMI+ to wtCCP.

The kinetics of ligand binding as a function of ligand concen-
tration Fig. 6, are consistent with a pre-equilibrium model (Equa-
tion 1) under conditions in which the second step (ligand-binding) 
(K_d) is much faster than a first zero-order pre-equilibration step 
(Fersht, 1984). Fitting the data in Figure 6 to a pre-steady 
state kinetic model (Equation 2, see Materials and methods), gave 
estimates for K_d of approximately 11.4 (±0.3) for F202G and 28 
(±6) for wtCCP.

\[ K_{obs} = k_1 + \frac{k_1}{([L]k_{-1})/(K_{d3}k_1)} + 1 \]
ligand. The loop open conformer in the native form of CCP leaves an empty binding pocket (filled with water molecules) to which DMI' can bind. The binding of DMI' selects this open conformer and shifts the loop close-open equilibrium toward the loop open conformer. We estimate that, at any given time, about 4% of the wild-type protein and about 9% of the destabilized F202G mutant are in the loop open conformation. In the absence of ligand binding, it would be difficult to observe this motion because it represents such a small percentage of the total population. It can be surmised that other loops in the protein may also be in similar motions that are not detectable due to the lack of a means of trapping or observing them. The binding of ligand in our case does not "cause" the loop to open, but, rather, because the ligand binds to the open conformer, it selects this conformer and shifts the equilibrium to the open conformer. This binding only appears to be induced fit because of the equilibrium shift; before ligand addition, a small amount of the protein pre-exists in the open conformer as shown by the pre-equilibration kinetics.

This mechanism has also been observed for the motion of loop-6 in triosephosphate isomerase (Williams & McDemott, 1995). The loop motion is not ligand gated, but a natural motion of the protein as revealed by NMR studies, and the population ratio between loop open and closed forms, is shifted by the presence of ligand: it changes from 10:1 (without ligand bound) to 1:10 (with ligand bound). NMR data (Williams & McDemott, 1995) reveal that the loop open and closed conformers are in equilibrium in the native protein, and binding of the substrate shifts this equilibrium toward the loop closed conformer.

Is the loop opening involved in the electron transfer process?

CCP catalyzes the transfer of electrons from reduced c to peroxide, forming water. In the oxidized "ES" state, Trp19' is reversibly oxidized to a stable cation free radical, which is believed to play a major role in cyt c oxidation (Erman et al., 1989; Sivarama et al., 1989; Houseman et al., 1993; Huyett et al., 1995). Although the details of how electrons are transferred from reduced c to CCP are still unclear, the crystal structure of the CCP-cytochrome c complex does indicate that cyt c binds near the loop 190-195 region of CCP (Pelletier & Kraut, 1992). The significantly increased thermodynamic motion of loop 190-195 was not only found in the F202G mutant protein, but also in a previously reported structure of nitric oxide (NO) bound to the heme Fe of wtCPP, where a slight movement of Trp19' away from His175 and an increase in the B-factor of Trp19' from 18.7 Å² to 28.3 Å² was observed (Edwards et al., 1988; Edwards & Poulos, 1990). This experiment reveals that the binding of NO to the distal side of the heme iron, the same site where peroxide binds, can significantly affect the thermodynamic motion of loop 190-195. This raises the interesting question of whether or not the loop opening is involved in the electron transfer process. Because Trp19' is the site of the free radical in the ES complex, loop opening would effectively transport the electron hole on the side-chain radical to the surface, where it could interact directly with an electron donor. However, there are a number of experimental results that disfavor this idea. (1) The loop opening kinetics (calculated half-life > 10² s from Equation 2 in Materials and methods) appear to be too slow for the electron transfer. (2) There are no reported structures of the ES form (Edwards et al., 1987; Fulop et al., 1994; Miller et al., 1994), indicating that the B-factor of loop 190-195 is increased. (3) The structure of the CCP-cytochrome c complex (Pelletier & Kraut, 1992) does not show the B-factor of loop 190-195 to be significantly altered. There is, however, no direct evidence to exclude this possibility.

Materials and methods

Materials

The F202G mutant of CCP was constructed using site-directed mutagenesis as described previously, and both wild-type and F202G protein were expressed and purified as described previously ( Fitzgerald et al., 1994).

X-ray crystallography

Structural determination was done as described earlier ( Fitzgerald et al., 1994). Single crystals of X-ray diffraction quality were grown from 25% MPD by vapor diffusion ( Wang et al., 1990). F202G was crystallized in sitting drops with approximately 0.17 mM CCP, 70-110 mM potassium phosphate, pH 6, and 8.3% MPD against 25% MPD. Large (0.8 × 0.4 × 0.03 mm) F202G crystals were obtained by macroseeding small F202G crystal seeds (~0.2 mm in length) into freshly prepared protein solution (0.17 mM CCP in 80 mM KPO, 8.3% MPD). wtCPP and F202G crystals were soaked for about 45 min in the test compounds before mounting (50 mM DMI in water, pH adjusted to 6.0 with H3P04, or 50 mM 2a5mT in water, pH adjusted to 5.2 with H3P04). X-ray diffraction data were collected at 17°C using Cu Ka radiation from the rotating anode of Rigaku X-ray generator and a Siemens area detector. Data were analyzed by difference Fourier techniques using the XtalView software ( McRee, 1993). Different Fourier maps ([Fo,F202G] - [Fo,wtCPP], αwCCP or [Fo,unsoaked] - [Fo,wtCCP]) were used for detecting structural changes. Models were built using XtalView and refined with repeated cycles of positional and B-factor refinements using the program X-PLOR ( Brünger et al., 1987). Partial occupancy refinements of the loop closed/open model were done using SHELX-97 ( George Sheldrick, University of Göttingen, Germany).

Extrapolated difference maps were calculated using the equation $Fo_{extrap}_{F202G} = Fo_{wtCPP} + (Fo_{F202G} - Fo_{wtCPP})/(partial occupancy)$ ( Gerick et al., 1997). Outliers were rejected by not using reflections where $|Fo_{F202G} - Fo_{wtCPP}|/(Fo_{wtCPP}) > 0.4$. Phases with the loop 190-195 omitted from the wild-type model were used and the partial occupancy was set to 0.2 for the map used in Figure 3.

Kinetics

Substitution of DMI' for the indole ring of Trp19' and opening of loop 190-195 shifted the UV-vis absorption spectrum of CCP from high spin ($\lambda_{max} = 408$ nm) to low spin ($\lambda_{max} = 418$ nm). This $\lambda_{max}$ shift was used to measure the apparent rate of binding of DMI' to CCP ($k_{a}$) and the dissociation constant ($K_{d}$, Equation 1). For simplicity, because 2a5mT' binds to two sites in F202G, whereas DMI' binds to only one, only DMI' dissociation constants were measured and compared. Compounds were obtained from Sigma or Aldrich. UV-vis spectra were collected at 20°C using a Hewlett-Packard 8452A or 8453 diode-array spectrophotometer.

Binding assays were performed by difference absorption spectroscopy ( Fitzgerald et al., 1994). Stock solutions of 1.0 M dimethylimidazole were prepared with 100 mM Bis-tris propane/
MES, pH 6.0. Stock protein solutions were prepared by dissolving CCP crystals in 500 mM Bis-tris propane/MES, pH 6.0. This stock solution was then diluted into the stated buffer, so as to give an absorbance of 1.0 at the Soret maximum. The protein solution was allowed to equilibrate at 20°C for 20 min in the spectrophotometer with stirring, and the instrument was blanked. An aliquot of the DMI” stock solution (10 μL for F202G and 100 μL for wtCCP) was added to the cuvette, and the change in absorption spectrum was recorded after a 30-min equilibration time. Dissociation constants were determined from Scatchard plots based on the difference absorbance at the Soret maximum of the heme. Because of the extremely long equilibration time during the titration (30 min per addition for a total of 7 h for F202G, and 12 h for each addition for a total of 48 h for wtCCP) at 20°C, the protein was subject to significant unfolding. To account for the increased light scattering due to the protein unfolding, the ratio of 422 nm to 408 nm was used to calculate the concentration used in the Scatchard plot.

Apparent rates (k_{obs}) of binding of DMI” to F202G or wtCCP were measured at 418 nm. The data were fitted to a single exponential curve that gave the value of k_{obs}, and a straight line background drift. The equation $k_{obs} = k_1 + k_{-1}/[L]/K_{d2} + 1$ (Equation 4.52 in Fersht, 1984) describes a pre-equilibrium model (Equation 1) under conditions in which the second step ($K_{d2} = k_{-2}/k_2$) is much faster than the first ($K_{d1} = k_{-1}/k_1$). Because we have experimental data for $K_{d1} = k_{-1}/k_1$ in Equation 1, $K_{d2}$ in the above equation can be replaced by $K_{d2} = K_{d2}/K_{d1} = K_{d2} * k_{1}/k_{-1}$, which gives Equation 2:

$$k_{obs} = k_1 + k_{-1}/[(aL) + k_{1}]/(K_{d2} * k_{1}) + 1].$$ (2)

The experimental data were plotted as $k_{obs}$ versus $[L]$ in Figure 5. Fitting this plot using Equation 2 gives $k_1$ and $k_{-1}$, and, therefore, $K_{d1}$. This fitting indicates that step 1 in Equation 1 is rate limiting. The program Sigma Plot (Jandel Scientific) was used for fitting operations.

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