Pergamon

Tetrahedron 56 (2000) 9471–9475

TETRAHEDRON

Loop Stability in the Engineered Potassium Binding Site of Cytochrome *c* Peroxidase

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Received 24 April 2000; accepted 26 June 2000

Abstract—The Trp^{191} containing flexible loop of cytochrome *c* peroxidase (CcP) exists in equilibrium between *open* and *closed* conformers. The *open* conformer creates a cavity in the loop, which enables it to bind protonated forms of imidazole derivatives such 1,2-dimethylimidazolium (DMI). In the present study we have engineered the K^+ binding site into CcP and find the equilibrium of the conformer shifted in favor of the *open* form probably due to electrostatic destabilization. Subsequent changing of a hinge residue in the loop, Asn^{195}, to Pro stabilizes the loop in the presence of the bound K^+.

Introduction

In nature proteins are known to exhibit considerable conformational flexibility. The literature is replete with instances of proteins changing conformation upon binding of ligands so as to favor a more specific interaction. Such changes in conformation have been known to bring about increased substrate and reaction specificities of enzymes[1,2] ligand induced inhibition and in some cases enhance thermal stability. Examples of such proteins are a legion viz. myoglobin, hemoglobin oxygen binding to heme,[3] substrate induced conformational change in cytochrome P450 BM3,[4] binding of substrate and substrate analogs in case of the pyridoxal-5′-phosphate (PLP) containing enzymes: aspartate aminotransferase and tryptophan synthase,[5,6] retinol binding to retinol binding protein (RBP)[7] and ligands binding to artificial cavities introduced into proteins.[8–11]

Flexibility in conformation is a characteristic feature in substrate binding and molecular recognition of some proteins. It has been shown by way of crystal structures that ligand binding promotes a conformational change of a surface loop from an *open* to a *closed* form to cover the bound substrate in serine hydroxymethyltransferase[2] and in triosephosphate isomerase[12,13] or from a *partially closed* to an *open* form, resulting in an induced fit for ligand binding.[14] This behavior also has been observed in cytochrome *c* peroxidase (CcP). Here we show, by a combination of protein engineering and biochemistry, that the proximal cation binding site introduced into CcP which made the surface loop unstable, can be stabilized by replacing a key residue, Asn^{195}, with Pro.

Results and Discussion

Yeast cytochrome *c* peroxidase (CcP), a biological redox partner of ferrocytochrome *c* (cyt. *c*) for which high-resolution crystal structures are available,[15,16] catalyzes the following reactions.

$$Fe^{3+}Trp + H_2O_2 \rightarrow \underset{\text{Compound I}}{Fe^{4+}{=}O\ Trp^{\cdot}} + H_2O$$

$$Fe^{4+} = O\ Trp^{\cdot} + \text{cyt. } c(Fe^{2+}) \rightarrow \underset{\text{Compound II}}{Fe^{4+}{=}O\ Trp} + \text{cyt. } c(Fe^{3+})$$

$$\underset{\text{Compound II}}{Fe^{4+}{=}O\ Trp} + \text{cyt. } c(Fe^{2+}) \rightarrow Fe^{3+}Trp + \text{cyt. } c(Fe^{3+}) + H_2O$$

CcP first reacts with peroxide to give *compound I*, an intermediate where the two oxidizing equivalents of peroxide are stored on the iron as $Fe^{4+}{=}O$ and as an amino acid free radical located on Trp^{191}.[17–20] *Compound I* then is reduced by cyt. *c* (Fe^{2+}) back to the resting state in two successive one-electron transfer reactions.

Although the biological function of CcP remains largely obscure, the complex formed between CcP and cyt. *c* has served as an important model system for understanding interprotein electron transfer reactions. Although the details of how electrons are transferred from reduced cyt. *c* to CcP

Keywords: cytochrome *c* peroxidase; *open*/*close* conformer; loop movement; ligand binding.

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0040–4020/00/$ - see front matter
PII: S0040-4020(00)00831-0

Table 1. Dissociation constants for binding of DMI to WTCcP and mutant CcP at pH 6.0

Protein	K_d (mM)
WTCcP alone	172.40
WTCcP+5 mM KCl	195.90
CcPK2 alone	54.47
CcPK2+5 mM KCl	54.88
N195PK2 alone	53.94
N195PK2+10 μM KCl	ND[a]

[a] Not determined.

are not clear, the crystal structure of CcP–cyt. *c* complex does indicate that cyt. *c* binds near the 190–195 loop region of CcP.[21] The CcP–cyt. *c* crystal structure[21] together with a wealth of biochemical data indicate that each electron delivered from ferrocyt. *c* is accepted by the Trp^{191} cationic radical[18,20] which explains why Trp^{191} is essential for activity.[22] A related heme peroxidase, ascorbate peroxidase (APX), contains a Trp residue, which is homologous to the redox active Trp^{191} in CcP.[23] Trp^{179} in APX sits directly adjacent to the proximal His ligand and donates an H-bond to a conserved buried Asp residue exactly the same as Trp^{191} in CcP.[23] Therefore, it was expected that APX would form a stable Trp radical, but instead, APX forms a porphyrin π-cation radical much like the other peroxidases.[24] This difference was attributed to the presence of K^+ ion bound within ~8 Å distance of the Trp^{179} of APX, which was absent in CcP. The proximal cation-binding loop (containing K^+ or Ca^{2+}) is another characteristic feature of all peroxidases whose structures are known, except CcP. The presence of this cation has been postulated to electrostatically destabilize the cationic Trp^+ radical in the APX.[24]

To test this hypothesis, the K^+ binding site of APX was engineered into CcP by changing 5 crucial amino acid residues into corresponding residues in APX responsible for binding K^+ viz. A176T, G192T, A194N, T199D and E201S.[25] This K^+-binding mutant of CcP has been termed CcPK2. As predicted, the engineered K^+ site in CcP destabilized the Trp^+ radical and led to an almost complete loss in activity.[25] However, mutations alone even in the absence of added cations destabilized the Trp^{191} radical, which contributed to a 10-fold loss in activity. One of the plausible explanations given is based on the work of Cao et al.[26] who showed, based on crystal structures, that CcP exists in an *open* and *closed* conformation and that the two forms are in equilibrium. The *closed* form has Trp^{191}

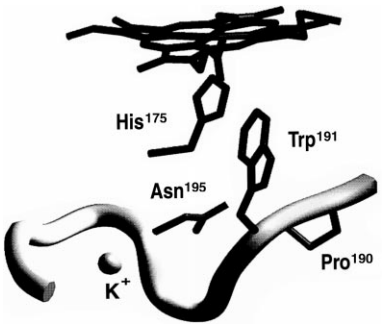

Figure 1. *Schematic model of conformation of the Trp^{191} containing proximal loop.* The model shows the two hinge residues in the loop Pro^{190} and Asn^{195}.

WTCcP	**A_{176}**	.....	**G_{192}**	A	A	N	N	V	F	T
APX	**T_{164}**	.....	**T_{180}**	S	N	P	L	I	F	**D**
LIP	**S_{177}**	.....	**D_{194}**	S	**T**	P	G	I	F	**D**
CcPK2	**T_{176}**	.....	**T_{192}**	A	**N**	N	N	V	F	**D**
CcPCA1	**T_{176}**	.....	**D_{192}**	A	**N**	N	N	V	F	**D**
CcPCA2	**T_{176}**	.....	**D_{192}**	A	**T**	N	N	V	F	**D**
N195PK2	**T_{176}**	.....	**T_{192}**	A	**N**	**P**	N	V	F	**D**

Figure 2. *Sequence alignments of the cation-binding loop in various peroxidases.* Side chain ligands are in **bold** face. The remaining ligands are provided by peptide carbonyl oxygen atoms. *WT*, wild-type; *APX*, ascorbate peroxidase; *LIP*, lignin peroxidase; *CcPK2*, *CcPCa1*, *CcPCa2*, and *N195PK2*, mutants of WTCcP

in its native position observed in the crystal structures, while in the *open* form, Trp^{191} moves ~10 Å to the molecular surface owing to a large movement of the surface loop consisting of residues 190–195. The cavity created in the proximal pocket in the *open* conformation enables it to bind small cationic imidazole derivatives like 1,2-dimethylimidazolium (DMI). The binding of DMI to Trp^{191} site results in an easily detectable spectral shift to a low-spin signal, which provides a simple method for following the open/closed equilibrium.

$$\text{close} \leftrightarrow \text{open} \overset{\text{DMI}}{\leftrightarrow} \text{open} - \text{DMI}$$

At any given time some fraction of the protein is in the *open* conformation, which represents a small percentage of the population. DMI selects this conformer and shifts the loop *close*/*open* equilibrium towards the *open* conformer. The reason for optimal binding is postulated to be due to the fact that methyl substituted imidazoles bind better than unsubstituted imidazoles due to higher pK_a, as well as efficiency of filling the cavity with a more appropriately sized molecule.[9]

Since the mutations required to form the cation site in CcPK2 involve the same loop responsible for the *open*/*close* equilibrium described by Cao et al.,[26] it was reasoned that the 190–195 segment of the loop may be less stable in CcPK2 than in WTCcP. If so, then DMI should bind in the Trp^{191} pocket more readily in the CcPK2 mutant than WTCcP. Indeed, CcPK2 could be readily titrated with DMI similar to what was observed by Cao et al.,[26] while WTCcP was more stable to the addition of DMI. As shown in Table 1, the estimated K_d from Scatchard plots were 54.47 mM for CcPK2 and 172.4 mM for WTCcP. Also consistent with the results of Cao et al.,[26] the rate of DMI binding is extremely slow. For WTCcP, hours were required to achieve equilibrium between additions of DMI while only 30 min was required for mutant CcP. Binding of DMI to either WTCcP or CcPK2 was not affected by the presence of the cation (K^+) as shown by similar K_d (Table 1). The results are consistent with the scheme proposed above. It is clear that mutations alone in CcPK2 resulted in the destabilization of Trp^{191} containing loop such that it can readily adopt the *open* conformation near the surface. This is very likely the source of activity loss and a decrease in the EPR signal of Trp^+ radical in CcPK2. Further addition of K^+ to the *closed* conformation in CcPK2 is the source of additional loss in activity due to electrostatic destabilization of the Trp^{191} cationic radical.[27]

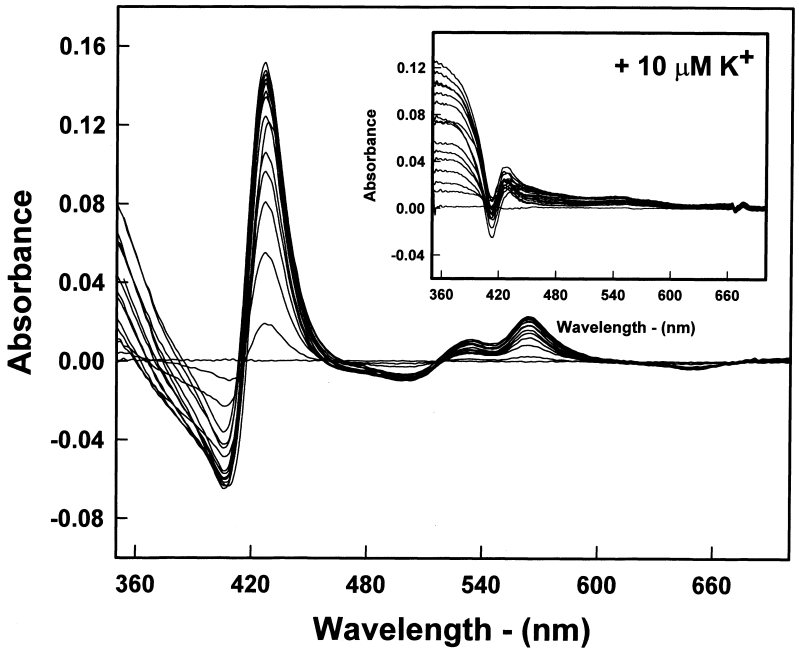

Figure 3. *Optical difference spectra obtained by titration of N195PK2 with 1,2-dimethylimidazolium (DMI).* A typical spectral titration curve for the N195PK2 is shown. Scatchard plots were used to determine the dissociation constant (K_d) by utilizing the difference absorbance (ΔAbs) at 412 nm. The fraction of DMI bound, $[DMI]_b$, was determined by extrapolating $1/\Delta A_{412}$ vs $1/[DMI]_f$ to infinite [DMI] assuming one binding site.[9] *Inset*: Difference spectra of N195PK2 titrated with DMI in the presence of stoichiometric concentrations of potassium.

The structural changes in going from the *closed* to *open* conformation involves two key hinge residues, Pro^{190} and Asn^{195}, as shown in Fig. 1. Pro^{190} goes from *trans* to *cis* and Asn^{195} side chain switches positions with main chain atoms.[26] Our next goal in this engineering saga was to stabilize this cation binding loop (residues 190–195) in CcPK2.

Initially, we were conservative with CcPK2 and changed only the cation ligand residues (Fig. 2). The next step was to introduce the entire APX cation loop into CcP. APX has Pro^{168}, which is Asn^{195} in CcP, at the hinge region. Since Asn^{195} is a key residue in the *closed* to *open* conformational switch, we reasoned that by changing Asn^{195} to Pro, the cationic loop may well be rigidified which would prevent the switch to the *open* conformation. The N195P mutation was introduced into the CcPK2 template by site-directed mutagenesis using appropriate primers and the mutant protein N195PK2 was purified as before. The spectral titration method with DMI was again used to determine the conformational states of Trp^{191} containing loop. To our surprise the newly engineered N195PK2 bound DMI with the similar affinity as CcPK2 as shown in Fig. 3 with an apparent K_d of 53.94 mM (Table 1) suggesting that the loop more readily adopts the *open* conformation. However, when N195PK2 was titrated with DMI in the presence of potassium, there was very little change in the difference spectrum as shown in Fig. 3. Since DMI binding reached saturation very early in the titration a very reliable estimate of K_d could not be obtained. Nevertheless, from these observations we can conclude that in the absence of bound K^+ in the cation-binding loop, Trp^{191} more readily adopts the *open* conformation while in the presence of K^+ the *closed* conformation is favored just as in WTCcP. This effect is specific for K^+ since DMI binding to N195PK2 was unaffected by the presence of either Na^+ or Ca^{2+} as indicated by difference absorption spectroscopy and similar K_d values. This points to the fact that the cation-binding loop of the engineered CcP cannot be stabilized without the cation being present. Our next approach will be to change the other hinge residue Pro^{190} involved in the conformational change to a residue, which may prevent *trans* to *cis* isomerization and hence may stabilize the loop.

In summary, these results show that cation loop in CcP designed to bind K^+ is unstable in the absence of K^+ and that even with a rigid Pro residue introduced into the cation loop, the loop remains unstable. In the presence of K^+, however, the stability of the loop reverts to that of WTCcP. This is not the case for the CcPK2 mutant, which also binds K^+ but retains the Asn at position 195. This means that Pro195 adds sufficient rigidity to the loop that the *closed* conformation is favored but only in the presence of K^+. We had anticipated that the N195PK2 cation loop might also be stable in the absence of K^+, which would enable us to differentiate the effects of loop stability and K^+ binding on enzyme activity. However, it appears that destabilization owing to electrostatic repulsion between cation ligands in the absence of cations cannot be overcome by having a Pro at position 195. We perhaps should have anticipated this result. We also have attempted to carry out the *reverse* engineering experiment by removing the cation binding ligands from APX. This mutant, however, is totally inactive and exhibits spectral properties consistent with a hexacoordinate low-spin complex which indicates substantial changes in active site structure.[28] Since the loop in N195PK2 more closely resembles that of APX than any of the other cation-binding mutants of CcP, in hindsight it is

not surprising that the loop requires a cation to maintain stability.

Materials and Methods

Materials

Enzymes and reagents for site-directed mutagenesis were purchased from Roche Molecular Biochemicals and New England Biolabs Inc. (Beverly, MA). Chromatography columns and media were purchased from Amersham-Pharmacia Biotech. 1,2-Dimethylimidazolium (DMI) was purchased from Aldrich. All other chemicals were molecular biology grade or better and were purchased from Sigma or Fisher.

Protein expression and purification

The cation mutants of CcP were designated CcPK2 and N195PK2. CcPK2 has 5 amino acid substitutions corresponding to the cation-binding loop in APX. CcPK2 is altered at the following residue positions: A176T, G192T, A194N, T199D and G201S as previously reported.[25,27] N195PK2 has the substitution N195P on CcPK2 protein. All mutagenesis experiments were performed according to the method of Kunkel et al.[29] as described earlier[25] using appropriate primers to get the desired mutations. All mutations were confirmed by DNA sequencing.

Both wild type CcP (WTCcP) and mutant CcP (CcPK2 and N195PK2) proteins were expressed using a T7 promoter in *Escherichia coli* BL21(DE3) cells induced at A_{600} of 1.2–1.5 with 750 μM IPTG. Proteins were purified as previously described by Fishel et al.[30] and Choudhury et al.[31] with the exception of an FPLC anion exchange step introduced using similar gradient parameters. After gel filtration on a Sephadex G-75 column and heme incorporation step by standard pH shift, CcPK2 was loaded on a 5 mL HiTrap-Q anion exchange column in 50 mM potassium phosphate buffer (KPB), pH 6.0, using an Amersham-Pharmacia Biotech FPLC. Protein was eluted by stepping the gradient first to 80 mM and then 130 mM KPB, pH 6.0. After heme incorporation and anion exchange chromatography CcP was crystallized by dialysis against Milli-Q water before being stored as crystals at −80°C in water. N195PK2 was produced in *E. coli* as a holo-protein and hence did not need heme incorporation step. CcP concentrations were estimated spectrophotometrically using an extinction coefficient at 408 nm (ϵ_{408}) of 96 mM^{-1} cm^{-1}.

Spectral titrations with 1,2-dimethylimidazolium (DMI)

Binding assays were performed by difference absorption spectroscopy using a Cary 3E UV–Visible spectrophotometer at 20°C.[9] Stock solutions of DMI were prepared with 100 mM bis-tris-propane/MES, pH 6.0. Stock protein solutions of CcP were also prepared in 100 mM bis-tris-propane/MES, pH 6.0 so as to give an absorbance of 1.0 at the Soret maximum. Protein solutions were allowed to equilibrate at 20°C for 30 min in the spectrophotometer and the instrument blanked. An aliquot of DMI stock solution (10 μL of 1 M DMI for mutant CcP and 5 M DMI for WTCcP) was added to the cuvette, allowed to equilibrate, and the difference spectrum recorded. Dissociation constants (K_d) were determined from the Scatchard plots of the data based on measuring the difference absorbance at the Soret maximum of the heme.

Acknowledgements

This research was supported by the National Institutes of Health.

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