The parsley plastocyanin–turnip cytochrome *f* complex: a structurally distorted but kinetically functional acidic patch

Peter B. CROWLEY*, David M. HUNTER†, Katsuko SATO†, William McFARLANE† and Christopher DENNISON†¹

*Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Av. Da República, Apartado 127, 2781 901 Oeiras, Portugal, and †School of Natural Sciences, Bedson Building, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

In general, inter-protein electron transfer proceeds via the formation of transient complexes. The initial stage of the interaction between plastocyanin (PCu) and cytochrome *f* (cyt *f*) from plants is mediated by complementary electrostatics. Given the diffuse nature of its acidic patch, parsley PCu is an atypical example of a plant PCu. The interaction of this PCu with turnip cyt *f* was investigated by stopped-flow kinetics, NMR spectroscopy and protein-docking simulations. We show that, despite the altered acidic patch, parsley PCu is as efficient as spinach PCu in accepting electrons from cyt *f*, over the

physiological range of ionic strength. At high ionic strength, the rate constant for the reaction of cyt *f* with parsley PCu is twice that of the spinach protein. This difference in reactivity is attributed to variations in the hydrophobic patch of parsley PCu. The results of NMR studies and protein-docking simulations indicate that parsley PCu and its spinach analogue adopt different orientations in their complexes with cyt *f*.

Key words: cytochrome *f*, docking, electron transfer, NMR, plastocyanin, protein interaction.

INTRODUCTION

Plastocyanin (PCu) functions as a one-electron transporter between the membrane-bound cytochrome *b₆f* complex and Photosystem I in photosynthetic organisms [1]. The redox activity of this molecule is controlled by a type 1 copper centre [2], located approx. 6 Å (1 Å = 0.1 nm) beneath the protein surface [3]. Electron transfer (ET) between PCu and its redox partners is mediated via short-lived protein interactions. Such transient complexes are required to maintain high-turnover conditions along the ET chain [4,5]. An essential feature of transient protein interactions is fast dissociation (rate of dissociation $k_{\text{off}} \geq 10^2 \text{ s}^{-1}$), which ensures a limited lifetime for the complex. The rate of association k_{on} is also high, in the range of 10^7 – $10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ [1,4–6]. Oppositely charged surfaces on the partner proteins are largely responsible for enhancing k_{on} , since Coulombic attraction provides long-range recognition and can pre-orient the diffusing proteins towards productive binding [4,7]. Whereas electrostatics can promote association, specificity and reactivity are determined by short-range forces [8]. Hydrophobic interactions, in particular, guide the proteins into reactive conformations [9,10]. In the absence of such favourable interactions, the partners have to explore a larger region of the protein surface to achieve reactivity [11,12].

Sequence and structure analysis reveals distinct features on the surface of PCus. A charged patch, which typically consists of six or seven acidic residues, is found in the proteins from plants [1,3,13] (see Figures 1 and 2). This region of the PCu surface is highly variable in different organisms, usually consisting of fewer acidic residues in green algae, and is completely absent from or slightly basic in cyanobacteria [14] (Figure 1). The strong ionic-strength dependence of reaction rates [6,15], covalent cross-linking studies [16,17] and the influence of mutations in this region on reactivity [1,6] support the role of this acidic patch in complex formation with plant cytochrome *f* (cyt *f*) and Photosystem I. A second surface feature, which is a characteristic of the cupredoxin family in general [18], is a cluster of hydro-

phobic side chains surrounding a partially exposed histidine ligand [3] (Figures 1 and 2). Mutagenesis studies have shown that the hydrophobic patch of PCu is also important for the interaction with cyt *f* [19,20]. The solution structure of the complex of PCu and cyt *f* from plants reveals that a combination of charged patches and hydrophobic surfaces defines the interface between the two proteins [21]. The complex of PCu and cyt *f* from the cyanobacterium *Phormidium laminosum*, on the other hand, does not utilize complementary electrostatics. In this case, the complex interface was found to involve only hydrophobic patches on both proteins [22].

Parsley PCu is an atypical example of a plant PCu, and a structural alignment with the spinach protein [13] indicates that residues 60 and 61 are deleted in parsley PCu, reducing the usual upper acidic patch to a single glutamic residue [23] (see Figure 1). This diminished acidic patch is flanked by two unusual substitutions, namely Ser-58 to Pro and Leu-62 to Tyr (Figure 1). The former replacement further contributes to the constriction of this region of the surface, which normally forms a conspicuous projection away from the hydrophobic patch. Although the classical acidic patch is diminished in parsley PCu, there are three additional glutamic residues in this region (Figure 1), resulting in a more diffuse distribution of negative charge. A recent NMR study of electron-self-exchange processes indicated that the reactivity of parsley PCu is changed as a consequence of its altered acidic patch [24]. To investigate further the effect of the acidic patch on protein–protein interactions, the reaction with cyt *f* has been investigated by stopped-flow kinetics, NMR spectroscopy and protein-docking simulations.

EXPERIMENTAL

Protein samples

Wild-type parsley PCu was isolated and purified as described previously [24]. The spinach PCu gene was overexpressed in

Abbreviations used: cyt *f*, cytochrome *f*; ET, electron transfer; PCu, plastocyanin; r.m.s.d., root mean square deviation.

¹ To whom correspondence should be addressed (e-mail christopher.dennison@ncl.ac.uk).

			20
Spinach	..VEVLGGG	DGS	LAFLPGD
Parsley	..AEVKLGSD	DGGLVFS	PSS
Chlamy	.DATVKLGAD	SGALEFV	PKT
Synecho	ANATVKMGSD	SGALVFEP	ST
			40
Spinach	FSVASGEEIV	FKNN	AGFP
Parsley	FTVAAGEKIT	FKNNAGF	PHN
Chlamy	LTIKSGETVN	FVNNAGF	PHN
Synecho	VTIKAGEEVK	WVNNKLSP	HN
			60
Spinach	VVFDEDEIPS	GVDAAKIS	MS
Parsley	IVFDEDEVPA	GVNAEKIS	QP
Chlamy	IVFDEDAIPS	GVNADAISR	D
Synecho	IVFAADGVDA	DT.AAKLSH	K

			80
Spinach	EED	LI	NAPGE
Parsley	E..Y	LN	GAGE
Chlamy	D..Y	LN	APGE
Synecho	G..LA	FAAGE	SFTSTFTEPG

			100
Spinach	TYKFYCS	EHQ	GAGMVGKVTV N
Parsley	TYKFYCE	PHA	GAGMKGEVTV N
Chlamy	EYGYCE	PHQ	GAGMVGKIIIV Q
Synecho	TYTYCE	PHR	GAGMVGKVVV D

Figure 1 Alignment (DcClustal [35]) of PCu amino acid sequences

The spinach and parsley sequences and a representative sequence of green algal (*Chlamydomonas reinhardtii*) and cyanobacterial (*Synechocystis* PCC6803) PCus are included in the alignment. The conserved acidic-patch residues of plant PCus (asterisks), the deletions at positions 60 and 61 (‘.’) and the unusual glutamic residues found in parsley (‘E’ in boldface) are indicated. Regions of the primary structure that contribute to the solvent-exposed hydrophobic patch in spinach PCu [13] are enclosed by boxes.

Figure 2 Ribbon diagram of the structure of spinach PCu [13]

The type 1 active-site residues and the acidic patch residues surrounding the conserved Tyr-83 are shown in ball-and-stick representation. The copper ion is represented as a black sphere.

Escherichia coli TG1, and the protein was isolated and purified as described recently [25]. Turnip cyt *f* was obtained from Sigma and further purified on a CM-Sephadex column. Protein concentrations were determined by UV/visible spectroscopy (PerkinElmer λ 35 spectrophotometer) using molar absorption

coefficients of $\epsilon_{597} = 4.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for cupric parsley and spinach PCus and $\epsilon_{556} = 31.5$ and $\epsilon_{421} = 195 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for ferrous cyt *f*.

Kinetic studies

Oxidation of cyt *f*(II) by spinach and parsley PCu(II)s was monitored at 421 nm on an Applied Photophysics SX.18MV stopped-flow reaction analyser at 25 °C. The proteins were in 20 mM Tris (pH 7.5). All rate constants were obtained under pseudo-first-order conditions [with > 10-fold excess PCu(II) using cyt *f*(II) concentrations of typically 0.1 μ M] and are an average of at least five determinations using the same solutions. To investigate the influence of ionic strength on reactivity, the appropriate amount of NaCl (AnalaR; BDH–Merck, Poole, Dorset, U.K.) was added to the buffer to give total ionic strengths (*I*) over the range of approx. 0.04–2.58 M.

NMR spectroscopy

Protein solutions were reduced using a solution of sodium ascorbate and exchanged into 99.9% deuterated 5 mM potassium phosphate (pH* 7.3, where pH* indicates a pH meter reading for which no correction was made for the deuterium isotope effect). Protein solutions were then concentrated by ultrafiltration (5 kDa molecular mass cut-off; Amicon equipment; Millipore, Watford, Herts., U.K.). Reducing conditions were maintained by the presence of 1.0 mM sodium ascorbate. Samples were placed in an NMR tube, flushed with nitrogen and sealed. To study the PCu–cyt *f* complex, turnip cyt *f* was added to a 1 mM parsley PCu sample in three successive steps to give 0.1, 0.2 and 0.3 M equivalents. The sample pH* was verified before and after each NMR experiment. Two-dimensional homonuclear TOCSY spectra were obtained at 500.16 MHz on a JEOL Lambda 500 spectrometer at 35 °C. Spectra were recorded with a mixing time of 40 ms and spectral widths of 6 kHz, with 2048 points for t_2 , 512 points for t_1 and 120 transients/increment. The residual HDO signal was suppressed by presaturation at its resonant frequency. Chemical shifts were calibrated relative to sodium 3-(trimethylsilyl)-2,2,3,3- d_4 -propionate. The previously published proton resonance assignments of parsley PCu [23] were used to assign the spectra. Chemical-shift changes ($\Delta\delta_{\text{bind}}$), arising from complex formation, were identified by comparing PCu spectra recorded in the presence and absence of cyt *f*.

Protein-docking simulations

Docking simulations between turnip cyt *f* (PDB accession code 1 hcz [26]) and PCu from both spinach (1 ag6 [13]) and parsley (1 pla [23]) were performed using the web-based suite of programs ClusPro, maintained by Camacho and co-workers [27,28]. The methodology has been described in detail previously [27] and is presented here in brief. Rigid-body docking was performed in DOT [29], yielding 20 000 candidate geometries ranked according to geometric complementarity. In a second step, the desolvation free energy and the electrostatic interaction energy were independently calculated for each orientation. The 500 structures with the lowest desolvation free energies and the 1500 structures with the lowest electrostatic energies were retained [27]. These filtered solutions were then subjected to a pairwise hierarchical clustering process, in which docking orientations were selected and ranked according to those having the highest number of neighbours within a cluster radius of $\leq 9 \text{ \AA}$ C α r.m.s.d. (root mean square deviation) [27]. Finally, to remove possible

Figure 3 Ionic-strength dependence of the oxidation of cyt *f* by PCu

The effect of ionic strength on $\ln(k_2)$ for the oxidation of turnip cyt *f* by spinach ($\diamond$) and parsley ($\bullet$) PCus is shown. The physiological range of ionic strength (100–150 mM) is marked by the broken lines.

side-chain clashes, a van der Waals energy minimization was performed using CHARMM [30].

RESULTS AND DISCUSSION

Kinetics of cyt *f* oxidation by PCu

In all of the kinetics studies, linear plots of first-order rate constants against [PCu(II)] were obtained. The slope of such plots provides the second-order rate constant k_2 for the oxidation of cyt *f*(II) by PCu(II). This rate constant has been defined [6,10] as shown in the following equation:

$$k_2 = k_{\text{on}}k_{\text{f}}/(k_{\text{off}} + k_{\text{f}}) \quad (1)$$

where k_{off} is the rate of dissociation before the ET reaction has taken place and k_{f} is related to k_{ET} . It has been demonstrated previously [10] that, for the reaction between PCu and cyt *f*, $k_{\text{f}} \gg k_{\text{off}}$ and thus $k_2 = k_{\text{on}}$. A consequence of this kinetic model is that variations in the reduction potentials of the PCus should not influence k_2 . However, the reduction potentials of spinach [6] and parsley PCus are almost identical [31]. In agreement with previously published results [6,10], k_2 for the oxidation of turnip cyt *f* by spinach PCu is approx. $4 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ at very low ionic strength. As the ionic strength is increased, electrostatic screening results in the loss of Coulombic attractions between complementary charged patches and, consequently, k_2 tends to decrease (Figure 3). This leads to an almost 200-fold decrease in the rate constant at high ionic strength. A similar behaviour was observed for the oxidation of turnip cyt *f* by parsley PCu(II). At low ionic strength, k_2 is slightly lower than the value for the spinach protein, indicating that the diffuse acidic patch of parsley PCu is less efficient at enhancing protein association. However, in the physiological range of ionic strength, the reactions are equally fast.

At high ionic strength, the rate constant for the reaction of parsley PCu with cyt *f* is approximately twice that for the spinach protein (Figure 3). It has been demonstrated that variations in the hydrophobic side chains surrounding the Tyr-1 ligand of cyt *f* results in appreciable differences in k_2 at high ionic strength [10]. Therefore the faster rate for parsley PCu implies that hydrophobic interactions are enhanced with respect to the spinach protein. The basis for this enhancement is discussed in a later section.

Figure 4 Overlay of a part of the TOCSY spectra of parsley PCu(I) in 5 mM phosphate buffer at pH 7.3 (35 °C)

The cross peak between the $\text{C}^{\alpha 2}\text{H}$ (7.06 p.p.m.) and $\text{C}^{\alpha 1}\text{H}$ (7.64 p.p.m.) resonances of His-87 is shown. The overlay of the spectra illustrates the influence of increasing molar equivalents of turnip cyt *f*(II) on the position of this cross peak.

The parsley PCu–turnip cyt *f* complex

Protein interactions were investigated by ^1H NMR spectroscopy, using a method similar to that described by Ejdebäck et al. [32] for spinach PCu and turnip cyt *f*. The addition of cyt *f* results in a perturbation of the chemical-shift values of certain ^1H resonances of parsley PCu (Figure 4). Line broadening is also observed, consistent with the longer rotational correlation time of the complex [33]. The magnitude of the $\Delta\delta_{\text{Bind}}$ was dependent on the amount of added cyt *f*, indicating that the free and bound forms of PCu were in fast exchange on the NMR time scale [33]. Owing to overcrowding of the spectra from cyt *f* resonances, the titration was not continued past 0.3 M equivalents and, therefore, a binding curve could not be determined.

Proton resonances from 25 amino acids in parsley PCu experienced a significant $|\Delta\delta_{\text{Bind}}|$ (≥ 0.02 p.p.m.) in the presence of 0.3 M equivalents of cyt *f* (see Table 1). The largest shift (0.19 p.p.m.) was observed for the C^{β}H resonance of the exposed copper ligand, His-87 (in this paper, the parsley PCu residues are numbered according to the spinach sequence; see Figure 1). Sixteen of the affected residues are hydrophobic, whereas seven are charged. Chemical-shift mapping on to a space-filling model of the parsley PCu structure [23] reveals a distinct binding patch involving the hydrophobic residues that surround His-87 (Figure 5A). Remote from the hydrophobic patch are several affected amino acids, diffusely distributed near the ‘eastern face’ of the molecule. This region of the binding map consists predominantly of glutamic residues (Figure 5A).

A comparison of the chemical-shift maps of parsley and spinach PCus in the presence of cyt *f* draws attention to the significant variation in the binding-site composition of these homologous proteins (Figure 5). Proton resonances of 40 spinach PCu residues experienced a $\Delta\delta_{\text{Bind}}$ in the complex [32], and the magnitude of these shifts was, on average, larger than those observed in parsley. However, only one resonance, arising from a methyl group of Leu-12, experiences a larger shift (0.31 p.p.m.) compared with the largest shift in parsley PCu. In addition to differences in the number of residues affected and the magnitude of shifts, the distribution of the affected residues is also different. In particular,

Figure 5 Chemical-shift maps of PCu in complex with cyt *f*

Parsley (**A**) and spinach (**B**) PCus in the presence of 0.3 M equivalents of turnip cyt *f*. The map for spinach is reproduced from Ejdebäck et al. [32]. Protein structures are represented as space-filling models and the residues are coloured according to the size of the largest chemical shift observed for any proton resonance in that residue. Blue, insignificant (< 0.02 or 0.03 p.p.m. in parsley and spinach PCus respectively); pink, 0.02–0.05 p.p.m. or 0.03–0.05 p.p.m. in parsley and spinach PCus respectively; orange, 0.05–0.10 p.p.m.; red, ≥ 0.10 p.p.m. The Figures were drawn with Molscript [41] and rendered using Raster3D [42].

the spinach PCu-binding map has a more compact appearance when compared with that found for parsley PCu (Figure 5).

There are a number of possible explanations for the altered appearance of the binding map for parsley PCu. One explanation relates to the percentage of PCu bound in the complex, which determines the magnitude of the observed shift (in the fast exchange limit). The overall smaller shifts in parsley PCu probably suggest that less complex is formed. The percentage of bound protein depends on both the binding affinity (K_a) and protein concentrations. Within experimental error, the two experiments were performed under identical conditions of ionic strength and protein concentrations. The k_2 value at low ionic strength is slightly smaller for the reaction of cyt *f* with parsley PCu when compared with the spinach protein (Figure 3). Assuming that the rates of ET k_{ET} and k_{off} are similar in both complexes, this suggests that K_a is slightly lower for parsley PCu. This could explain the overall difference in magnitude of the observed shifts in the two complexes. However, it does not rationalize the qualitative differences between the two maps. An alternative explanation is that parsley PCu adopts a different orientation in the complex with cyt *f*, thus giving rise to different chemical-shift perturbations. To test this hypothesis, parsley PCu was modelled into the structure of the plant PCu–cyt *f* complex [21] using Swiss-PDB Viewer [34]. An illustration of this model (Figure 6) supports the idea that parsley and spinach PCus interact differently with cyt *f*. In particular, severe steric clashes in the region of residues 59–61 and the lack of contacts at the edge of the hydrophobic patch suggest that parsley PCu must be rotated with respect to the orientation of spinach PCu in the complex.

Protein docking

To investigate alternative binding orientations in the parsley PCu–cyt *f* complex, protein-docking simulations were performed using the methodology developed by Camacho and co-workers [27,28]. Analysis of the spinach PCu–cyt *f* interaction was also performed using this approach.

Among the top ten docking configurations found for spinach PCu, five were positioned with the hydrophobic patch facing the haem region of cyt *f*. Three of the top ten solutions were calculated

Figure 6 Structure of parsley PCu superimposed on the co-ordinates of spinach PCu in complex with cyt *f* [21]

Parsley PCu is shown as space-filling models, and the residues identified in the chemical-shift map (Figure 5) are highlighted in dark grey. Cyt *f* is represented as a backbone trace. The second orientation is rotated by 180° with respect to the first. The Figures were drawn with Molscript [41] and rendered using Raster3D [42].

Figure 7 Superposition of the results from docking simulations [27,28] with the NMR structure of the PCu–cyt *f* complex [21]

Cyt *f* is represented as a ribbon diagram, with the α -helix in front of the haem removed for clarity. PCu structures are represented as $C\alpha$ backbone traces. Spinach PCu from the NMR structure of the complex (thin trace, black), the top-ranked docking orientation for spinach PCu (medium trace, grey line) and the third-ranked docking orientation for parsley PCu (heavy trace, light grey) are shown.

to have a more favourable interaction around the small domain of cyt *f*. The remaining two solutions were found in a similar region, but with the hydrophobic portion of PCu orientated away from the cyt *f* surface. The top-ranking orientation was found to be very similar to the NMR structure of the spinach PCu–turnip cyt *f* complex (see Figure 7), with an r.m.s.d. of 7.4 Å calculated

Table 1 Parsley PCu(I) resonances that experienced significant $\Delta\delta_{\text{bind}}$ (p.p.m.) in the presence of 0.3 M equivalents of turnip cyt *f* (II)

$\Delta\delta_{\text{bind}}$ values classified as significant are those ≥ 0.02 p.p.m. and are presented in boldface. Residues are represented by their one-letter amino acid codas.

Residue*	C α H	C β H	C γ H	C δ H	Other
L5	–†	–, n.d.‡	– 0.02	– 0.01, – 0.02	
G6	– 0.02 , –				
S7	– 0.01	– 0.02 , – 0.02			
D9	–	– 0.02 , – 0.03			
G10	– 0.03 , – 0.04				
G11	– 0.03 , – 0.02				
L12	– 0.03	– 0.04 , n.d.	– 0.06	– 0.06 , – 0.10	
V13	–	– 0.02	– 0.02 , – 0.02		
A33	– 0.02	– 0.02			
G34	– 0.02 , – 0.03				
P36	0.01	0.01, – 0.01	– 0.01, –	– 0.02 , – 0.01	
H37	– 0.01	– 0.01, –		– 0.02	C ϵ H: – 0.03
V40	–	0.03	–		
E43	0.01	– 0.02 , 0.01	–		
K54	0.02	– 0.01, – 0.01	0.01, –	0.01	C ϵ H: –
Q57	0.02	–, n.d.	–		
P58	0.01	0.02 , –	– 0.03 , 0.01	0.01, –	
E59	0.02	– 0.01, 0.01	0.01, 0.01		
E68	– 0.02	– 0.01, n.d.	0.01, –		
E85	0.01	0.01, 0.02	0.01, 0.01		
H87	– 0.02	– 0.01, n.d.		– 0.19	C ϵ H: – 0.04
G89	0.03 , 0.03				
A90	– 0.01	– 0.06			
M92	n.d.	0.01, –	n.d.		C ϵ H: – 0.02
E95	– 0.03	0.01, – 0.01	– 0.01, – 0.01		

* Parsley PCu residues are numbered according to the spinach sequence (see Figure 1).

† Resonances shifted by $< \pm 0.005$ p.p.m.

‡ Resonances not detected during the titration.

for C α atoms. When only the residues of the hydrophobic patch were included in the calculation, the r.m.s.d. was decreased to 5.7 Å. Analysis of the NMR structure of the PCu–cyt *f* complex [21] reveals that 39 residues make one or more atom contacts across the interface (< 5 Å from any atom on the partner protein). Fewer residues were calculated to make contacts in the top-ranking docking orientation. Nevertheless, 23 residues were found in common with the NMR structure. Furthermore, the active-site ligands His-87 and Tyr-1 make van der Waals contact, and the copper to iron distance of 13.0 Å compares favourably with that found in the NMR structure (10.9 Å [21]). These observations indicate that the docking procedure successfully predicts the general features of the interface between spinach PCu and turnip cyt *f*. The main difference between the docking results and the NMR structure is a decrease in the ‘incidence’ angle for PCu (Figure 7), corresponding to a decrease in interactions between the acidic patch of PCu and basic residues in the small domain of cyt *f*. This suggests that the electrostatic contribution is calculated to be less favourable in the docking simulation compared with the NMR structure.

The top ten docking solutions for parsley PCu with cyt *f* were more diverse than those found for the spinach protein. The diffuse nature of the acidic patch of parsley PCu is a probable explanation for this difference, since the electrostatic energy calculations used to filter the docking solutions are highly sensitive to the positions of charged atoms [27]. For parsley, four of the solutions were calculated to bind near the haem region of cyt *f*, and in three of these significant contacts are made via the hydrophobic patch of PCu. As with spinach PCu, favourable docking solutions were found for parsley PCu in the small domain of cyt *f*, whereas the lowest-ranked orientation was calculated to bind on the opposite side of the haem region. The highest-ranked docking orientation

for parsley has an r.m.s.d. of 14.5 Å from the NMR structure of spinach PCu in complex with cyt *f*. This large difference in orientation can be attributed to an increase in hydrophobic contacts and a significant decrease in the interaction between the complementary charged patches. The third highest-ranking solution for parsley PCu bears higher similarity to the spinach orientation with an r.m.s.d. of 8.8 Å. Considering the shorter copper to iron distance of 13.4 Å (compared with 15.2 Å for the top-ranking solution), it is probably representative of the ET complex in solution. As in the top-ranking solution, the number of hydrophobic contacts is increased in this orientation (see Figure 7) compared with the NMR structure of the spinach PCu–cyt *f* complex. Although it is difficult to assess which of the docking solutions for parsley best satisfies the NMR data, each appears more reasonable than the model in which the parsley PCu molecule is simply superimposed on to the NMR structure of the PCu–cyt *f* complex. Thus it can be concluded that parsley and spinach PCus adopt different orientations in their complexes with cyt *f*.

The distorted acidic patch

Two residues that contribute to the upper acidic patch in plant PCu are absent from the parsley protein (Figure 1). When the solution structure of parsley PCu was reported, Bagby et al. [23] proposed that the glutamate side chains at positions 53, 85 and 95 compensate for the deletions at positions 60 and 61. A sequence alignment (DbbClustal [35]) of the known PCu sequences indicates that residue 85, which is adjacent to Cys-84 (one of the copper ligands), is normally a serine residue in plant PCus and a glutamic residue in the green algal proteins (see Figure 1). Residue 95

is semi-conserved as a lysine residue and less frequently as a threonine residue, whereas the only other occurrence of glutamic acid at position 95 is in the carrot protein. Glu-85 of parsley PCu is positioned directly on the edge of the hydrophobic patch [23]. Our NMR investigations of the interaction of parsley PCu with cyt *f* provide evidence that Glu-85 and Glu-95 participate in complex formation (Table 1 and Figure 5). Values of $\Delta\delta_{\text{Bind}}$ also indicate that Glu-68 makes a contribution to the interface (Figure 5). Although structurally distorted and diffuse, the acidic recognition site of parsley PCu is competent for reactivity towards cyt *f*, as demonstrated by the similarity of the k_2 values to those of the spinach protein. Therefore the evidence suggests that the overall electrostatic potential is more important than the exact charge distribution. It can be concluded that the role of the acidic patch is limited to enhancing the k_{on} and it provides only a crude level of specificity [8,10]. Previously, it has been shown that the introduction of a positive charge in the upper acidic patch of spinach PCu leads to a dramatic decrease in the reaction rate and the ability to form a complex with cyt *f* [6,36]. Coulombic repulsions between this positive charge and the basic side chains in the small domain of cyt *f* diminish the rate of formation of the productive complex.

Hydrophobic interactions

NMR studies of PCu–cyt *f* interactions have identified the importance of the hydrophobic patch of PCu for complex formation [8,21,22,32,37]. Theoretical and experimental studies demonstrate that protein association rates are strongly influenced by desolvation of hydrophobic side chains when electrostatic interactions are absent [9,10]. Compared with spinach PCu, the parsley protein has three unusual substitutions in the region of the hydrophobic patch (Figure 1). Two polar side chains, Ser-11 and Gln-88 (typically conserved in plant and algal PCus), are replaced by Gly and Ala respectively in parsley PCu. Removal of polar groups from this region of the protein increases the hydrophobic content of the binding site and probably enhances protein interactions at high ionic strength. However, the most notable difference is the introduction of a tyrosine residue at position 62, which is normally a leucine (a feature in common with green algal PCu). The aromatic side chain of this residue is highly solvent-exposed [23] and most probably contributes to hydrophobic interactions with the haem region of cyt *f*. Therefore Tyr-62 is advantageous, since it increases the hydrophobic interaction area, favouring productive binding, resulting in the larger k_2 of parsley at high ionic strength (Figure 3). The importance of tyrosine in protein interfaces has been discussed in detail previously [38,39]. It has been shown that tyrosine is three times more abundant in protein interfaces [38] than on the protein surface and is frequently involved in interface hot spots [contributing > 2 kcal/mol (8.37 kJ/mol) to the K_a] [39].

Conclusion

Alterations in the upper acidic patch of parsley PCu do not impede the ET reaction with cyt *f*. The more diffuse arrangement of acidic residues is no less efficient at accelerating protein association. The NMR results and protein-docking studies suggest that the structure of the complex with parsley PCu is different from that of spinach PCu with cyt *f*. This supports the results of previous studies, which indicate that the nature of transient protein interactions can be different even for homologous proteins [8,15,22,40].

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