FOR THE RECORD

Surface interactions in the complex between cytochrome *f* and the E43Q/D44N and E59K/E60Q plastocyanin double mutants as determined by ¹H-NMR chemical shift analysis

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Abstract

A combination of site-directed mutagenesis and NMR chemical shift perturbation analysis of backbone and side-chain protons has been used to characterize the transient complex of the photosynthetic redox proteins plastocyanin and cytochrome *f*. To elucidate the importance of charged residues on complex formation, the complex of cytochrome *f* and E43Q/D44N or E59K/E60Q spinach plastocyanin double mutants was studied by full analysis of the ¹H chemical shifts by use of two-dimensional homonuclear NMR spectra. Both mutants show a significant overall decrease in chemical shift perturbations compared with wild-type plastocyanin, in agreement with a large decrease in binding affinity. Qualitatively, the E43Q/D44N mutant showed a similar interaction surface as wild-type plastocyanin. The interaction surface in the E59K/E60Q mutant was distinctly different from wild type. It is concluded that all four charged residues contribute to the affinity and that residues E59 and E60 have an additional role in fine tuning the orientation of the proteins in the complex.

Keywords: Plastocyanin; cytochrome *f*; chemical shift; protein–protein interaction; site-directed mutagenesis

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Transient protein complexes play a crucial role in electron transfer reactions such as photosynthesis and the respiratory chain. During photosynthesis, the electron transfer between the cytochrome *bf* complex and photosystem I in the thy-

lakoid membranes of the chloroplasts is mediated by a small soluble copper protein, plastocyanin (Kato and Takamiya 1965; Fromme et al. 1994; Cramer et al. 1996). Structural work on higher plant plastocyanin shows two conserved acidic patches (Guss and Freeman 1983; Moore et al. 1991; Bagby et al. 1994; Xue et al. 1998; Inoue et al. 1999; Sugawara et al. 1999). In spinach plastocyanin, the larger acidic patch comprises Asp 42, Glu 43, Asp 44, and Glu 45, whereas the smaller acidic patch involves Glu 59, Glu 60, and Asp 61. A growing body of evidence, including crystal structures of cytochrome *f* (Martinez et al. 1994; Chi et al. 2000) and site-directed mutagenesis studies (Young et al. 1997; Hippler et al. 1998; Olesen et al. 1999), suggests that the negatively charged acidic patches on plastocyanin interact with positively charged residues on its reaction partners,

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cytochrome *f* and the *psaF* subunit of photosystem I. The solution structure of the complex between plastocyanin and cytochrome *f* (Ubbink et al. 1998) also demonstrates this. The residues in both acidic patches make van der Waals contacts with positively charged residues on cytochrome *f*, and the metal ligand histidine 87 in plastocyanin is conveniently located on a plausible electron transfer pathway (Ubbink et al. 1998). Kinetic measurements on unmodified (Niwa et al. 1980; Takabe and Ishikawa 1989; Meyer et al. 1993; Qin and Kostic 1993), chemically modified (Takemura and Takabe 1984; Anderson et al. 1987; Adam and Malkin 1989; Gross et al. 1990; Gross and Curtiss 1991; Christensen et al. 1992), and genetically modified forms of plastocyanin (He et al. 1991; Modi et al. 1992; Lee et al. 1995; Kannt et al. 1996; Illerhaus et al. 2000) and cytochrome *f* (Soriano et al. 1998; Gong et al. 2000) show the highly electrostatic nature of the interaction, and show that both acidic patches are important for complex formation, at least in vitro (Soriano et al. 1996).

The overall reaction is very fast ($2 \cdot 10^8 \text{ M}^{-1} \text{ s}^{-1}$ at 25°C , 100 mM ionic strength, pH 6.0 (Gong et al. 2000)) and the complex lifetime is much less than 1 ms (Ejdeback et al. 2000). A two-stage interaction in complex formation was suggested (Qin and Kostic 1993; Bendall 1996), based on the finding that the cross-linked complex between plastocyanin Asp 44 and cytochrome *f* Lys 187 (Morand et al. 1989) was inactive in electron transfer (Qin and Kostic 1993). However, the role of the acidic patches in stabilization of the complex during electron transfer, or in the rapid complex dissociation following electron transfer, is not yet understood. In this work, the complex formed between plastocyanin mutated in either acidic patch and cytochrome *f* is studied in order to elucidate the role of the acidic patches and their residues.

Results and Discussion

We have used NMR techniques and genetically modified plastocyanin to study the role of the acidic patches in complex formation with cytochrome *f*. Two-dimensional ^1H -NMR spectra of the complexes were recorded and compared with that of free plastocyanin mutants. Addition of cytochrome *f* to the NMR sample caused changes in the proton chemical shifts. The magnitude of these changes increased with increasing amounts of added cytochrome *f*. The observed changes in the NMR spectra were all consistent with a fast exchange of bound and free plastocyanin on the NMR time scale. At ~ 0.3 molar equivalents cytochrome *f*, resonances that originated from the cytochrome started to become visible. However, the cytochrome *f* signals were too weak at these concentrations to interfere with the analysis of the plastocyanin resonances. A small general line broadening was observed upon complex formation due to the long rotation correlation time of the complex.

Chemical shift changes larger than 0.02 ppm were considered significant and attributed to a specific interaction between the proteins. The significant chemical shift changes are mapped onto a surface representation of plastocyanin in Figure 1.

For the E43Q/D44N mutant plastocyanin, in which two negative charges are neutralized, the largest chemical shift changes upon complex formation were observed in residues 10–12, 34, 87, and 88 (Table 1). In total, 10 protons were significantly shifted during titration. The largest shift change was observed for a proton in the side chain of His 87. The affected residues are located in, or immediately adjacent to, the hydrophobic surface of plastocyanin. The overall result is similar to what was found in the wild-type case (Ejdeback et al. 2000), although the magnitude of the

Fig. 1. Chemical-shift changes per residue mapped onto the surface of plastocyanin according to the color scale in the figure. Mutated residues are mapped in yellow. Pertinent residues (see Results and Discussion) are labeled on the mutant plastocyanin surface representations. The wild-type figure was adapted from Ejdeback et al. (2000). The chemical-shift changes were measured between plastocyanin peaks in homonuclear NMR spectra before and after addition of 0.3 (wild-type and E43Q/D44N) or 0.4 (E59K/E60Q) molar equivalents of cytochrome *f*.

chemical-shift changes decrease considerably in the mutant, reflecting the drop in binding constant due to a decrease in the electrostatic interaction.

The double-mutant E59K/E60Q results in an effective charge change of +3, essentially neutralizing the smaller acidic patch. The magnitude of chemical shift changes decreased further compared with the E43Q/D44N mutant, and only five protons, located in residues 68, 83, 85, and 88 were significantly shifted (Table 2). Residues 83, 85, and 88 form a continuous surface, partly overlapping the hydrophobic patch, and extending in the direction of the larger acidic patch. Interestingly, the largest shifts were observed in the side chain of Glu 68, which in the absence of the negative charges on 59/60, may serve as an attracting surface for the positive charges on cytochrome *f* together with Asp 61 on plastocyanin. The magnitudes of the shift changes are just above what we consider significant, but on the other hand, residues that were strongly affected in the wild-type and the E43Q/D44N mutant do not seem to be affected in this mutant (e.g., Gly 10, Ser 11, Leu 12, and Gly 34). There is no direct correlation between the magnitude of the chemical shift change and physical interaction, but our results suggest that the interface between the E59K/E60Q mutant plastocyanin and cytochrome *f* differs from that of the wild-type or E43Q/D44N mutant plastocyanins.

In the characterization of the electron transfer reaction from cytochrome *f* to mutants of plastocyanin the overall rate constant decreased a factor of 7 and 13 for the E43Q/D44N and E59K/E60Q mutants, respectively. In combination with the data on other charge mutants, it was concluded that the charges contribute equally to the reaction rate, independent of their location in either acidic patch (Kannt et al. 1996). Similar results were reported for the overall reaction between spinach plastocyanin acidic patch mutants and the intact cytochrome *bf* complex (Illerhaus et al. 2000). The kinetic results clearly indicate that in the charge mutants the association rate is decreased, resulting in a lower affinity. It is unclear whether the mutations also affect the dissociation rate or the electron transfer rate. Our results suggest that the mutations in the large acidic patch lower only the affinity and do not change the structure of the complex, whereas the mutations in the small acidic patch affect both the affinity and the structure of the complex. The latter could change the dissociation and electron transfer rates. Because the E59K/E60Q mutant is still able to perform fast electron transfer, it is implied that a complex with this non-native orientation of plastocyanin is also capable of electron transfer. Alternatively, the chemical-shift map can represent an average of multiple orientations, some of which are electron-transfer competent (Liang et al. 2000). The NMR analysis yields a time and population average, and we cannot rule out either of the two possibilities.

In the wild-type protein, the role of the large acidic patch (residues 42–45) would be mainly to increase the attraction

between the molecules, whereas the role of the small acidic patch (residues 59–61) both increases the attraction and orients the two molecules in the complex. Interestingly, the large patch is almost completely conserved in higher plant plastocyanins, whereas variations in the region of the small patch are more pronounced.

The importance of the hydrophobic surface in fine tuning the structure of the transient complex to allow for efficient electron transfer has been discussed (Ubbink et al. 1998; Morand et al. 1989; Gong et al. 2000; Illerhaus et al. 2000). We suggest that the small acidic patch may also be important in this respect.

Materials and methods

Spinach plastocyanin mutants were produced as described previously (Sigfridsson et al. 1996). A 2D NOESY and TOCSY spectra of 1 mM reduced E43Q/D44N or E59K/E60Q spinach plastocyanin mutants in 10 mM potassium phosphate (pH 6.0), 100 mM sodium ascorbate, 200 mM sodium 3-(trimethylsilyl)-2,2,3,3-d₄-propionate (TSP) and 10 % D₂O were recorded. The reduced form of the soluble domain of turnip cytochrome *f* was added in 0.1, 0.2, and 0.3 molar equivalents to the E43Q/D44N mutant plastocyanin, and in 0.1, 0.25, and 0.4 molar equivalents to the E59K/E60Q mutant plastocyanin. Protein concentrations were calculated from the optical absorbance using $\epsilon_{597} = 4.7 \text{ mM}^{-1}\text{cm}^{-1}$ for oxidized plastocyanin and $\epsilon_{554} = 31.5 \text{ mM}^{-1}\text{cm}^{-1}$ for reduced cytochrome *f*. Spectra were recorded on an Avance DMX 600 MHz Bruker NMR spectrometer. Spectral widths were 8992.81 Hz, with 2048 points for *t*₂, 400–700 points for *t*₁ and 32 transients per increment. The mixing times were 100 and 40 ms in the NOESY and TOCSY spectra, respectively. Spectra were recorded at 310 K.

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