Electrochemical Study of Biological Functions of Particular Evolutionary Conserved Amino Acid Residues Using Mutated Molecules of Maize Ferredoxin

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Electrochemical study of mutated maize ferredoxin molecules clearly demonstrated, for the first time, that particular evolutionary conserved amino acid residues have distinguished roles in biological functions such as controlling the redox potential and the complex formation with an enzyme.

Plant ferredoxins are important electron-transfer proteins in the photosynthetic process having a simple [2Fe-2S] iron-sulfur cluster as the redox center, and are distributed widely in nature. Ferredoxin has also been received much attention as a key protein to understand the evolutional process, and amino-acid sequences of about 60 plant ferredoxins from different origins have so far been established, where some amino acid residues are conserved.[1] By understanding a function of an evolutionary conserved amino acid residue, if any, one can have deeper insights how ferredoxin molecules control the pathways to deliver electrons in nature. However, it is not yet clear how are the functions of ferredoxins controlled by the amino acid residues. On the other hand, we have recently succeeded to prepare polypeptide[2] and aminosilane[3] modified electrodes for rapid direct electron transfer of ferredoxins of various origins.[4] In the present study, by using ferredoxin molecules mutated at several evolutionary conserved amino acid residues, roles of amino acid residues of ferredoxins in biological functions have been clearly shown electrochemically, for the first time, where amino acid residues for controlling the redox potential and for binding with an enzyme are distinguished.

Polypeptide and aminosilane modified electrodes were obtained as described previously.[2,3] Cyclic voltammetry was carried out by using a BAS-50W Electrochemical Analyzer. Maize ferredoxin from photosynthetic (FdI) and non-photosynthetic (FdIII) organs and those of which particular amino acid residues had been modified by site-directed mutagenesis[5] were used. Since the 13th amino acid residue from the N-terminal is missing for FdI, Ser-46 of FdIII and Ser-45 of FdI, for example, is actually the same position in the structure of ferredoxin. Figure 1 shows the schematic representation of the structure of ferredoxin (FdIII) with numbers of mutated positions of amino acid residues. All mutated ferredoxins prepared in the present study showed very similar circular dichroism (CD) spectra to that of native ferredoxins (wild type: WT), and showed well-defined cyclic voltammograms at the modified electrodes[2,3] used. The voltammetric responses were diffusion-controlled in all cases, meaning the observed redox potentials were due to the properties of mutated ferredoxin but not any artifact due to the modified electrodes used.

For WT maize ferredoxins, the formal redox potential ($E^{0'}$), diffusion coefficient (D), and the heterogeneous electron transfer rate constant ($k^{0'}$) were obtained to be - 542 (± 3) mV for FdIII and - 609 (± 3) mV (vs. Ag/AgCl/ Sat. KCl) for FdI, 7.0 (± 0.5) x 10^{-7} cm^{-2} s^{-1}, and 2.0 (± 0.5) x 10^{-3} cm s^{-1}, respectively, at the poly-L-lysine and/or aminosilane modified In_2O_3 electrodes in a 50 mM tris-HCl buffer solution containing 0.33 M NaCl (pH 7.5) at 10 °C.

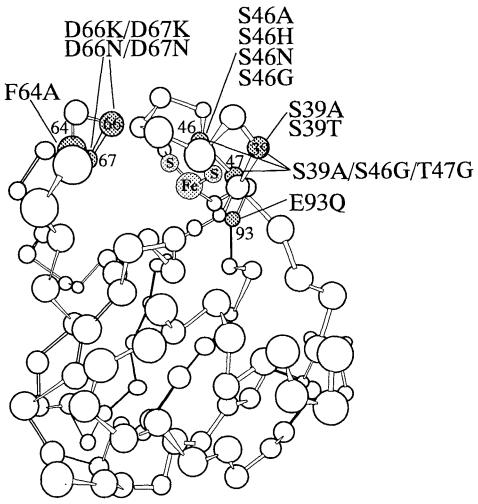

Figure 1. Structure of ferredoxin (FdIII) with indicating mutated positions of amino acids and mutated molecules used.

Some mutated ferredoxins, of which an evolutionary invariant residue such as Serine (Ser or S)-39 or Ser-46 near the [2Fe-2S] cluster of FdIII was changed to Alanine (Ala or A), Threonine (Thr or T), Histidine (His or H) or Asparagine (Asn or N), gave a similar redox potential to that of WT (see Table 1 for some results). On the other hand, very interestingly, when Ser-46 of FdIII was modified to be Glycine (Gly or G) (S46G), the redox potential showed a large positive shift by ca. 180 mV compared with that of WT. Similar large positive shift in redox potential was also observed for the S45G mutant of FdI. This is the first observation that mutation of only one amino acid residue caused so much shift in redox potential. When Ser-39, Ser-46 and Thr-47 of FdIII were modified at the same time to Ala-39, Gly-46 and Gly-47 (S39A/S46G/T47G), the shift in redox potential did not increase (see Table 1), suggesting Ser-46, but not Thr-47, is important for this large shift in redox potential. For FdIII, some other mutated ferredoxins, such as E93Q (E: Glutamic acid or Glu; Q: Glutamine or Gln) and F64A (F: Phenylalanine or Phe) also showed a positive shift in redox potential by ca. 55 and 95 mV, respectively.

Preliminary study of the computer modeling of mutated ferredoxins[6] showed that when Ser-46 of FdIII or Ser-45 of FdI was changed to Gly, a rather large distortion of the [2Fe-2S] cluster was suggested to be introduced. The Ser-46 or Ser-45 in

Table 1. The formal redox potentials of native and mutated molecules of maize ferredoxin (FdIII)

Ferredoxin[1)]	FdIII (WT)	S39A	S46A	S46N	S46G	S39A/S46G/T47G	E93Q	F64A	D66K/D67K	D66N/D67N
$E^{o'}$ / mV[2)]	-542	-548	-505	-542	-364	-371	-485	-448	-548	-547

1) S: Serine, A: Alanine, N: Asparagine, G: Glycine, T: Threonine, E: Glutamic acid, Q: Glutamine, F: Phenylalanine, D: Aspartic acid, and K: Lysine. For example, S46A means a mutated molecule obtained by site-directed mutagenesis of FdIII, where Serine-46 is modified to Alanine.
2) The $E^{o'}$ values are given against an Ag/AgCl /Sat. KCl reference electrode at 10 °C in a 50 mM Tris-HCl buffer solution containing 0.33 M NaCl (pH 7.5). The average values of more than three independent measurements are shown. The reproducibility of the $E^{o'}$ value was within 3 mV, independent of the modified electrodes used.

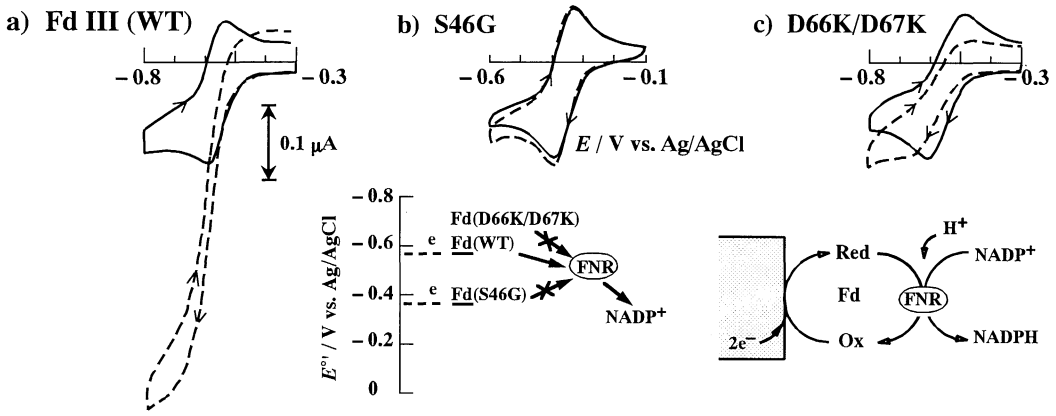

Figure 2. Cyclic voltammograms of 50 μM (a: WT) native and (b: S46G and c: D66K/D67K) mutated ferredoxins in the absence (solid curves) and presence (broken curves) of 0.05 U/ml FNR and 0.25 mM $NADP^+$. Scan rate: 2 mV/s. Reaction scheme and energy diagram are also shown schematically.

WT would be hydrogen bonding with Glu-93 of FdIII or Glu-92 of FdI, respectively, to keep the structure of ferredoxin stable. When Ser-46 was changed to His (S46H) or Asn (S46N), where again the hydrogen bonding would be expected, no remarkable change in the redox potential was observed. However, the hydrogen bonding does not seem to be essential to keep the structure similar to that of WT, because S46A of FdIII, where no hydrogen bonding is expected with Glu-93, gave a similar structure to that of WT with no structural distortion at the cluster, giving a small (ca. 35 mV) positive shift in redox potential. Since CD spectra of S46G of FdIII and S45G of FdI were almost the same as that of WT (not shown), electrochemical measurement is much sensitive for such small and local changes in structure at the redox center of protein.

By using WT ferredoxin as an electron transfer mediator, in the presence of ferredoxin-$NADP^+$-reductase (FNR, E.C. 1.18.1.2), $NADP^+$ (nicotinamide adenine dinucleotide phosphate) was effectively reduced to give NADPH through the reduction of ferredoxin at the electrode. Typical catalytic current was observed (Figure 2a), and during electrolysis the formation of NADPH was clearly monitored by using an optically transparent thin layer electrochemical cell[7] as an increase in absorbance at 340 nm. However, when the S46G of FdIII and S45G of FdI were used, no catalytic current due to the enzyme reaction was observed (Figure 2b). This is reasonably explained in terms of the positive shift in redox potential, where no electron transfer can be conducted from S46G of FdIII (or S45G of FdI) to FNR in the ferredoxin/ FNR/ $NADP^+$ system (see Figure 2). These results indicate Ser-46 of FdIII or Ser-45 of FdI is very important for controlling its redox potential.

On the other hand, the redox potentials of D66K/D67K and D66N/D67N of FdIII, where negatively charged Aspartic acid (Asp or D) was converted to positively charged Lysine (Lys or K) or neutral Asparagine (Asn or N), did not change at all (see Table 1), but again the very little catalytic current for the reduction of $NADP^+$ was observed (Figure 2c). Gel chromatography also showed that D66K/D67K and D66N/D67N did not bind significantly with FNR. These results suggest that Asp-66 and/or Asp-67 are the binding sites with FNR to form the ferredoxin-FNR complex.

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