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Control of Reduction Thermodynamics in [2Fe-2S] Ferredoxins. Entropy-Enthalpy Compensation and the Influence of Surface Mutations

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Abstract

The reaction thermodynamics for the one-electron reduction of the [2Fe-2S] cluster of both human ferredoxin and various surface point mutants, in which each of the negatively charged residues Asp72, Glu73, Asp76, and Asp79 were converted to Ala, have been determined by variable temperature spectroelectrochemical measurements. The above are conserved residues that have been implicated in interactions between the vertebrate-type ferredoxins and their redox partners. In all cases, and similar to other 2Fe-ferredoxins, the reduction potentials are negative as a result of both an enthalpic and entropic stabilization of the oxidized state. Although all *Hs* Fd mutants, with the exception of Asp72Ala, show slightly higher $E^{\circ'}$ values than that of wild type *Hs* Fd, according to expectations for a purely electrostatic model, they exhibit changes in the $\Delta H^{\circ'}_{rc}$ values that are electrostatically counter-intuitive. The observation of enthalpy-entropy compensation within the protein series indicates that the mutation-induced changes in $\Delta H^{\circ'}_{rc}$ and $\Delta S^{\circ'}_{rc}$ are dominated by reduction-induced solvent reorganization effects. Protein-based entropic effects are likely to be responsible for the low $E^{\circ'}$ value of D72A.

Keywords

Ferredoxin; Homo sapiens; Electrochemistry; Enthalpy; Entropy; Compensation

1. Introduction

The aim of this work is to address the question of how and to what extent solvation controls reduction thermodynamics in [2Fe-2S] ferredoxins, in particular focusing on solvent

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reorganization effects and consideration of enthalpy/entropy compensation phenomena. Several models for the hydration of nonpolar solutes and biopolymers indicate that the structural reorganization of the hydrogen-bonding network in the hydration sphere of the biomolecule generally induces largely compensating enthalpy and entropy changes [1–10]. These effects lie at the origin of the compensation phenomena observed for the thermodynamic and/or kinetic parameters underlying a series of closely related solution reactions in several chemical contexts [10–15]. They are particularly common in processes involving biological macromolecules, such as protein unfolding, nucleic acid denaturation, enzyme kinetics and binding [12,13], and were previously detected for the reduction thermodynamics of cytochromes c and blue copper proteins [1,15,16].

The [2Fe-2S] ferredoxins are a family of small (80–130 residues) redox-active proteins that function as electron transfer agents in many biological redox processes, including photosynthesis, respiration, and oxidative metabolism. They act as single electron shuttles, cycling between Fe(III)/Fe(II) oxidation states in the metal cluster, with a potential range that varies from -0.230 to -0.420 V [17–22], revealing the highly reducing nature of the [2Fe-2S] ferredoxins. Members of these proteins are found in virtually every living organism and are divided into three classes: namely, plant-type, vertebrate-type, and bacterial-type ferredoxins.

The [2Fe-2S] site (Figure 1) consists of two tetrahedra that are doubly-bridged through a pair of sulfide ions, with the tetrahedral coordination of each Fe completed by two cysteine thiolates. The orientation of the cluster is such that Fe₁ is more solvent exposed than Fe₂, which is buried in the protein interior, and the difference in the chemical environment of the iron atoms is consistent with localization of the additional electron on Fe₁ following protein reduction, as demonstrated by NMR [23,24]. In the oxidized state, the two Fe³⁺ sites are antiferromagnetically coupled; i.e., the spins of the five electrons on the two iron atoms are oppositely aligned, producing an effective $S=0$, diamagnetic ground state. In the reduced form, a single unpaired electron is present, because Fe³⁺ and Fe²⁺ sites are again antiferromagnetically coupled, leaving one net unpaired spin and an $S = 1/2$ ground state.

Most of the [2Fe-2S] ferredoxins that have been structurally characterized display a similar folding topology. These structures are conveniently considered as consisting of two “lobes” with most of the N-terminal half of the polypeptide sequence concentrated in one lobe and the C-terminal half in the other [25]. The iron-sulfur cluster binding site is situated at one end of the molecule between the N- and C-terminal lobes (Figure 2). The N-terminal lobe contains the conserved hydrophobic core and adopts approximately the same structure in all of the [2Fe-2S] ferredoxins that have been characterized to date, consisting of a four-stranded β -sheet and a long α -helix that crosses the β -strands approximately perpendicularly. This is the so-called β -grasp motif [26]. The number of residues making up the conserved N-terminal structural core is fairly constant at around 36 amino acids, not including the iron-sulfur binding loop (which contains three of the four cysteinyl ligands of the metal cluster).

Unlike the N-terminal lobes, the C-terminal lobes show considerable variability, both in polypeptide length and in tertiary structure. The plant-type ferredoxins generally have shorter C-terminal peptides (35–45 residues) and show relatively little well-defined secondary structure in the C-terminal lobe. The C-terminal lobes of vertebrate-type ferredoxins typically contain more residues than the plant-type proteins (between 50 and 70 residues) and a more extensive regular secondary structure [25].

Human ferredoxin falls into the class of vertebrate-type ferredoxins that are small (13–14 kDa) acidic proteins with a single [2Fe-2S] cluster and are found in the mitochondria of steroid-metabolizing tissues. They transfer reducing equivalents from NADPH-dependent ferredoxin oxidoreductase (Fd-reductase) to cytochrome P450 enzymes involved in the biogenesis of

steroid hormones, the formation of vitamin D metabolites, and the production of bile acids. Human mitochondrial ferredoxin has been found to be involved in the initial step of progesterone biosynthesis, in which the side chain of cholesterol is cleaved to yield pregnenolone [27]. The amino acid sequences of six known vertebrate-type ferredoxins, from human, bovine, porcine, avian (chick), ovine and rat, exhibit a high degree of sequence identity (Figure 3).

The four cysteines that bind to the iron atoms of the cluster are strictly conserved at residue positions 46, 52, 55, and 92, while histidines at positions 10, 56, and 62 in the sequences of human, bovine, porcine, avian, and rat ferredoxins are also conserved. One of the conserved histidines (His56) is immediately adjacent to one of the cysteines that ligates the [2Fe-2S] cluster (Cys55). This feature distinguishes these ferredoxins from plant-type [2Fe-2S] ferredoxins, which have an alanine at this position [28].

All of the residues that have been implicated in interactions between the vertebrate-type ferredoxins and their redox partners are located either on the [2Fe-2S] binding loop or in the C-terminal lobe. These surface acidic residues include Asp72, Glu73, Asp76, and Asp79, where both Asp76 and Asp79 are important for the interaction with complementary charged residues in Fd-reductase. The same residues are also important for binding cytochrome P450, but in this case Asp79 need not necessarily be charged and is most likely involved in a hydrogen bonding interaction. Residues Asp72 and Glu73 appear to play only a minor role in Fd-reductase association, but contribute significantly to cytochrome P450 binding [18].

Herein, the reduction thermodynamics have been evaluated by temperature-dependent spectroelectrochemical measurements for human ferredoxin, or from literature sources for wild type [2Fe-2S] ferredoxins [17], and the ΔH°_{rc} and ΔS°_{rc} values (where the subscript rc stands for the Fe(III)/Fe(II) “redox couple”) and compensation plots evaluated. The changes in reduction thermodynamics due to point substitutions of surface residues in human ferredoxin, where Asp72, Glu73, Asp76, and Asp79 were replaced with the uncharged amino acid alanine have also been investigated. The selection of these residues was dictated by their crucial role in human ferredoxin activity since they have been implicated in the interaction between ferredoxins and redox partners [18].

2. Materials and Methods

2.1 General Materials

Homo sapiens (Hs) ferredoxin (Fd) and mutants of Hs Fd were obtained as described in supplementary information. Methyl viologen, indigodisulfonate, phenazine methosulfate, and methylene blue were purchased from Sigma-Aldrich Co. Lumiflavin 3-acetate was purchased from Fluka Co. All chemicals were reagent grade and used without further purification. Nanopure water was used throughout.

2.2 Spectroelectrochemical Measurements

All UV-Vis spectroelectrochemical experiments were carried out in a homemade OTTE (Optically Transparent Thin Layer Electrochemical) cell, and potentials were applied with an Amel mod. 2053 potentiostat/galvanostat. UV-visible (UV-Vis) spectra were recorded using a Cary C50 spectrophotometer. All experiments were carried out under argon using 1 ml samples containing between 0.05 and 0.08 mM protein made up in 40 mM phosphate buffer plus 0.1 M NaCl at pH 7, in the presence of methyl viologen (1:15 molar ratio with the protein) and lumiflavine-3-acetate, indigo disulfonate, phenazine methosulfate and methylene blue (each 1:515 molar ratio with the protein) used as mediators [29,30].

All reduction potentials reported in this paper are referred to the standard hydrogen electrode (SHE) and were found to be reproducible within ± 3 mV. Nernst plots consisted of at least five points and were invariably linear, with a slope consistent with a one electron reduction process [29–31]. For each species, the experiments were performed at least two times.

The temperature dependence of the reduction potential was determined using a “non-isothermal” cell configuration [32] and the temperature was varied from 5 to 35 °C with *Hs* Fd and surface point mutants. The nonisothermal behavior of the cell was carefully checked by determining the ΔH°_{rc} and ΔS°_{rc} values of the ferricyanide/ferrocyanide couple [32,33].

3. Results

3.1 Electronic Spectra

Oxidized *Hs* Fd exhibited an electronic spectrum (Figure 4) that was identical to prior reports [34], supporting the presence of a [2Fe-2S] cluster with absorption maxima observed in the 300–600 nm range associated with sulfur to iron charge transfer transitions [35]. The spectra of oxidized *Hs* Fd and its mutants (Figure 4) display absorption maxima at 455, 415 nm and a broad peak around 320 nm. In all cases reduction of the proteins by applying a potential leads to a broad band around 542 nm (Figure 4, dashed line). The identity of the UV/Vis spectra of oxidized and reduced wild type *Hs* Fd with those of the variants indicates that mutations do not exert any effect on the coordination of the iron-sulfur cluster.

3.2 Redox Potentials and Reduction Thermodynamic Parameters

The electronic spectra of *Hs* Fd recorded at various applied potentials at pH 7 in an OTTLE cell are reported in Figure 5. The electronic spectra of *Hs* Fd mutants (Figure 4) are analogous to those of the wild type species. The corresponding Nernst plot (Figure 5, insert) invariably shows linear behaviour with a slope close to the theoretical value of RT/F (0.059V), expected for the one-electron reduction of the iron-sulphur cluster from Fe(III)/Fe(III) to Fe(III)/Fe(II) [29–31].

The E° values for wild type and mutant *Hs* Fd measured at 25 °C and pH 7 are listed in Table 1, and E° values obtained from the absorbance maxima at 415 nm were found to be similar to those obtained from the absorbance maxima at 455 nm. The redox potentials for vertebrate-type ferredoxins typically lie in the range from -0.235 to -0.275 mV, whereas those for plant-type ferredoxins are generally lower, namely from -0.325 to -0.425 V [36].

The temperature dependences of the E° values and the E°/T vs $1/T$ plots for all studied ferredoxins at pH 7 are reported in Figure 6. In all cases, the reduction potentials were found to decrease linearly with increasing temperature from 5 to 30–35 °C. No experiments were carried out below 5 °C, because of the need of an excessively long time for the establishment of the equilibrium conditions that reflects the lower overall rate of electron transfer between the protein and the electrode. At temperatures above 35 °C for *Hs* Fd and its mutants, protein denaturation prevented any successful spectroelectrochemical experiments.

The thermodynamic parameters for cluster reduction from these nonisothermal experiments are reported in Table 1. Each of the studied ferredoxins display positive ΔH°_{rc} values and negative ΔS°_{rc} values, in agreement with reduction thermodynamic parameters previously determined for several plant-type ferredoxins [17].

4. Discussion

The reduction potential of human ferredoxin (-0.380 V) is significantly more negative than that of bovine adrenodoxin (-0.250 V) [37], although the two proteins are highly homologous.

It has been proposed that the higher reduction potential of bovine adrenodoxin, relative to values obtained for plant-type ferredoxins arises from a hydrogen bond formed between the imidazole of His56 and a labile sulfide of the iron–sulfur cluster [38,39]. Plant-type ferredoxins, which do not have a histidine residue near the iron-sulfur cluster, show a much more negative reduction potential (between -0.325 and -0.425 V). Prior ^1H NMR studies of human ferredoxin indicate that His56 does not interact directly with the iron-sulfur cluster [28]; but rather, it appears to point away from the cluster and interact directly with Ser88 [40]. Such a feature is consistent with the distinct values of the redox potential between human and bovine ferredoxin.

In this study the surface residues Asp72, Asp76, Asp79 and Glu73 have each been individually replaced with the neutral amino acid alanine. Suppression of these solvent-exposed charged side chains should not appreciably alter protein folding and the coordination features of the $[2\text{Fe-2S}]$ center. Consistent with this expectation, no appreciable mutation-induced changes in the electronic spectra were detected, and so these mutations leave the geometric and electronic properties of the iron-sulfur site essentially unchanged (Figure 4).

Simple electrostatic considerations predict that suppression of a negative charge in the surroundings of the metal cluster would selectively stabilize the more negatively charged reduced state, inducing a decrease in reduction enthalpy and a consequent increase in reduction potential (less negative E° values). Experimental mutation-induced enthalpy changes do not, however, conform to these predictions. In fact all *Hs* Fd mutants feature $\Delta H^\circ_{\text{rc}}$ values that are slightly more positive than that of wild type *Hs* Fd ($+2 < \Delta\Delta H^\circ_{\text{rc}} < +7$) (Table 1). This behavior can be interpreted by considering the experimental $\Delta H^\circ_{\text{rc}}$ values to include both an intrinsic protein-based term, which in the present case corresponds to electrostatic changes that arise from suppression of the surface negative charge, and also a solvent-based environmental term. The latter is related to the fact that a change of a net charge on the protein surface induces a more or less localized modification of the H-bonding network in the hydration shell of the protein. A number of models for the hydration of nonpolar solutes and biopolymers recognize that these solvent reorganization effects induce largely compensating enthalpy and entropy changes [2,15,41,42]. In particular, according to Grunwald [4,9] the free energy change for a process of protein reduction in solution is given by:

$$\Delta G^\circ_{\text{obs}}^{\text{red}} = \Delta G^\circ_{\text{int}} + \Delta G^\circ_{\text{env}} \quad (1)$$

The intrinsic (nominal) term ($\Delta G^\circ_{\text{int}}$) represents the free energy change for reduction of the solvated oxidized protein and consists of the term for reduction in the gas phase plus the difference in free energy of solvation between the reduced and oxidized protein. The environmental term ($\Delta G^\circ_{\text{env}}$) is due to solvent rearrangement processes that originate from the transfer of solvent molecules from the bulk solvent to the hydration sphere of the molecule in the two redox states. It can be demonstrated thermodynamically that the latter term does not contribute to the free energy of the overall process, because the corresponding nonzero entropy and enthalpy changes offset each other exactly [4,10,13]. These conclusions have a general validity, which is independent of the solution properties. However, in hydrogen bonding solvents, such as water, the effects of solvent reorganization are more pronounced [4,10], thus the compensation patterns can be observed more clearly. In fact, the more these contributions prevail on those accompanying the nominal process, the greater the chance to observe a compensation effect [4,10,13]. Compensation in the reaction thermodynamics for the reduction of a series of related proteins in solution implies that the variation of the reaction free energy within the series ($\Delta G^\circ_{\text{obs}}^{\text{red}}$) is much lower than that in the individual enthalpy and entropy changes. In other words, the latter are related by a linear relationship

$$\Delta H_i = a + b (T_i \Delta S_i) \quad (2)$$

where a and b are constants, the latter being a measure of the extent of H–S compensation. An exact compensation is realized when $b = 1$. In that case, a would correspond to the invariant $\Delta G_{\text{obs}}^{\circ \text{red}}$ value. When the enthalpic and entropic contributions arising from intrinsic and solvent reorganization processes become more similar, deviations of the slope from unity and larger scattering of the data points are expected [4,10].

When the entropic contributions to $E^{\circ'}$ at 298 K ($T\Delta S^{\circ'}_{\text{rc}}/F$) are plotted against the enthalpic terms ($-\Delta H^{\circ'}_{\text{rc}}/F$) for several type of wild type [2Fe-2S] ferredoxins (Table 1), and for *Hs* Fd mutants, they are observed to be linearly correlated (Figure 7). Least-squares fitting yields a slope (b) of -1.3 ± 0.1 and a regression coefficient (r) of 0.97. The observed slope of approximately 1.3 indicates that the entropic and enthalpic contributions to $E^{\circ'}$ at 298 K do indeed compensate, although not perfectly (in fact, the enthalpic term varies within the series to a lesser extent than the entropic term). To prevent any possible false interpretation of a compensation pattern that might arise from a statistical origin, a knowledge of the experimental uncertainties is essential, since it has been shown that a compensation pattern can be considered valid only if the confidence limits for each data point (indicated by the error bars) is much smaller than the overall range covered by the data [4,10,15,43]. It is apparent that the data of Figure 7 cover a range that is much greater than the experimental uncertainties, indicating that the resulting compensation patterns are significant [4,10,15,43].

The $T_{298}\Delta S^{\circ'}_{\text{rc}}/F$ vs. $-\Delta H^{\circ'}_{\text{rc}}/F$ plot is also found to be linear, when limited to wild type *Hs* Fd and its mutants, excluding D72A. Least-squares fitting yields a slope (b) of -1.2 ± 0.1 and a regression coefficient (r) of 0.94 (Figure 8). It is apparent that also within this series the entropic and enthalpic contributions to $E^{\circ'}$ at 298 K show a compensatory relationship. Derivative D72A *Hs* Fd lies outside of the compensation line: a fact that will be discussed below.

As noted above, observation of compensatory behavior for the experimental $\Delta H^{\circ'}_{\text{rc}}$ and $\Delta S^{\circ'}_{\text{rc}}$ values means that the reduction thermodynamics is dominated by solvent-based effects. However, since the latter are exactly compensatory, the free energy change of the reaction would correspond only to the protein-based free energy change. By virtue of the fact that the protein-based entropy changes are conceivably small, the $\Delta G^{\circ'}_{\text{rc}}$ values can be assumed to first approximation to correspond to the $\Delta H^{\circ'}_{\text{rc}}$ due to protein effects, ($\Delta H^{\circ'}_{\text{rc int}}$). This term is dictated by the coordination features of the metal cluster and the electrostatic influence of its immediate protein environment on the selective stabilization of the two redox states [17].

Table 1 shows that the $\Delta G^{\circ'}_{\text{rc}}$ ($=\Delta H^{\circ'}_{\text{rc int}}$) values for the various mutants are very similar to that of wild type *Hs* Fd. The small electrostatic effect that follows from the point mutations can be justified if we consider that the amino acid residues that have been substituted with Ala are distant from iron-sulfur cluster and exposed to solvent. The high dielectric constant of water weakens the Coulombic interaction between the surface charge and the metal attenuating the effect of the mutation. Interestingly, while the effect of the mutation for D79A and D76A is consistent with expectations based on electrostatic interactions (i.e. it determines a small and negative $\Delta\Delta G^{\circ'}_{\text{rc}}$ values, see Table 1), and is almost null for E73A, an opposite effect is observed for the D72A mutant. Therefore, it can be conceived that additional protein-based entropic and/or enthalpic changes are operative for this mutant. The former are most likely to be contributed by reduction-induced changes in protein flexibility, in more or less localized protein regions. The latter might be the result of a structural change of unknown origin. However, it is a fact that the deviations of data points from linear behavior in such compensation plots most likely indicate the occurrence in the corresponding species of an important protein-

based structural effect in addition to the dominant solvent-based effects as effectors of the reduction thermodynamics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Table of abbreviations

ΔH°_{rc} and ΔS°_{rc}	enthalpy and entropy changes for Fe(III) to Fe(II) reduction within the ferredoxin cluster (the subscript rc stands for the iron “redox couple”)
Fd	ferredoxin
SHE	standard hydrogen electrode
<i>Hs</i>	Homo sapiens.

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Fig. 1.

Schematic illustration of a ferredoxin-bound [2Fe-2S] cluster. The tetrahedral coordination of each iron atom is defined by a pair of bridging sulfide ions and two pairs of terminal cysteine thiolates. Fe₁ lies toward the surface of the protein, while Fe₂ is buried in the protein interior.

Fig. 2.

Ribbon representation of the crystallographic structure of spinach ferredoxin, showing the two structural domains common to ferredoxins. The hydrophobic core (N-terminal lobe) and the interaction domain (C-terminal lobe) are shown, with the [2Fe-2S] cluster (represented by the four grey spheres) positioned between them. This figure is based on coordinates from PDB code 1A70.

	10	20	30	40	50	60	70
Human	SSSEDKITVH	FINRDGETLT	TKGKVGDSLL	DVVVENNLDI	DGFGACEGTL	ACSTCHLIFE	DHIYEKLDAL
Bovine	-----	-----	---I---	---Q---	-----	-----	Q--F-----
Porcine	-----	---K---	-Q-----	---I---	-----	-----	---F-----
Avian	-----	---DK---	A---P---	-----	-----	-----	---F-----
Ovine	-----V--N	---K---	-----	-----	-----	-----	-----
Rat	-----V---	---K---	-----	---I---	-----	-----	-----
	80	90	100	110	120		
Human	TDEENDMLDL	AYGLTDRSRL	GCQICLTKSM	DNMTVRVPET	VADARQSIDV	GKTS	
Bovine	-----	-----	-----A-	-----DA	-S---E---M	-MN-SKIE	
Porcine	-----	-----	-----A-	-----A	-----E-		
Avian	---M-----	-----	-----K---	-----A	-----V-L	S-N-	
Ovine	-----	-----	-----	-----	-----E---M	-MN-SKIE	
Rat	-----	-F---N----	---V---A-	-----A	---V---V-M	S-N-	

Fig. 3.

Amino acid sequences of six vertebrate-type ferredoxins. The rat ferredoxin sequence is a partial sequence. Residues conserved in all ferredoxins are indicated by –.

Fig. 4. UV-Vis spectra of oxidized *Hs* Fd and D72A, D76A, D79A, E73A mutants (solid line). Data are offset for ease of presentation and the dashed line shows the spectrum for the reduced wild-type protein.

Fig. 5. Electronic spectra of *Hs* Fd obtained at various applied potentials (from -0.36 to -0.22 V vs. SHE) in spectroelectrochemical experiments carried out with an OTTLE cell. Spectra were recorded at 20 °C. The corresponding Nernst plot is reported in the insert Figure, where X represents $[A_{\lambda_{ox}}/(A_{\lambda_{ox}}^{\text{Max}} - A_{\lambda_{ox}})]$, and $\lambda_{ox}=415$ nm.

Fig. 6.

Temperature dependence of the reduction potentials for all of the ferredoxins studied in this work. The slope of the plots (A) yields the $\Delta S^\circ_{\text{rc}}$ values; and the slope of the plots (B) yields the $\Delta H^\circ_{\text{rc}}$ values. (●) wild type human ferredoxin; (▽) D72A *Hs* Fd; (△) D76A *Hs* Fd; (□) D79A *Hs* Fd; (◇) E73A *Hs* Fd. Solid lines are least-squares fits to the data points obtained for mutants, while the dotted line corresponds to wild type *Hs* Fd.

Fig. 7. Compensation plot for the reduction thermodynamics determined for [2Fe-2S] ferredoxins. $T=298$ K. Filled symbols correspond to data obtained in this work. Open symbols indicate literature data [17]. Solid lines are least-squares fits to the data points.

Fig. 8. Compensation plots for the reduction thermodynamics determined for wild type and *Hs* Fd mutants. $T=298$ K. Solid lines are least-squares fits to the data points excluding the D72A mutant, which is indicated by (▲).

Table 1

Thermodynamic parameters for reduction of [2Fe-2S] ferredoxins ^a.

Protein	E ^o , ^b (V)	ΔH ^o , _{rc} (kJ mol ⁻¹)	ΔS ^o , _{rc} (J mol ⁻¹ K ⁻¹)	ΔG ^o , _{rc} (kJ mol ⁻¹)	-ΔH ^o , _{rc} /F (V)	TΔS ^o , _{rc} /F (V)	ΔΔG ^o , _{rc} ^d (kJ mol ⁻¹)
Wild Type <i>Hs</i> Fd	-0.380	+26	-37	+36.7	-0.269	-0.114	
D72A <i>Hs</i> Fd	-0.414	+33	-22	+40.0	-0.342	-0.068	+3.3
D76A <i>Hs</i> Fd	-0.368	+29	-24	+35.5	-0.300	-0.074	-1.2
D79A <i>Hs</i> Fd	-0.370	+28	-24	+35.7	-0.290	-0.074	-1.0
E73A <i>Hs</i> Fd	-0.382	+30	-21	+36.8	-0.316	-0.065	+0.1
<i>S. platensis</i> Fd ^c	-0.454	+15	-97	+44	-0.155	-0.299	
<i>P. umbilicalis</i> Fd ^c	-0.444	+8	-118	+43	-0.083	-0.365	
<i>S. oleracea</i> Fd ^c	-0.459	+25	-63	+44	-0.259	-0.195	

^a Average errors for E^o, ΔH^o, _{rc}, ΔS^o, _{rc} and ΔG^o, _{rc} values are ±3 (mV), ±4 (kJ mol⁻¹), ±8 (J mol⁻¹K⁻¹) and ±0.3 (kJ mol⁻¹), respectively (expressed as the upper values of the standard deviations for the data points). The sum -ΔH^o, _{rc}/F + TΔS^o, _{rc}/F often does not closely match the experimental E^o, as a result of the experimental errors in the ΔH^o, _{rc} and ΔS^o, _{rc} values, which are rounded to the closest integer.

^b The reduction potentials are measured at 25 °C and are referenced to SHE.

^c From ref. 17.

^d ΔΔG^o, _{rc} = [ΔG^o, _{rc}(mutant *Hs* Fd) - ΔG^o, _{rc}(WT *Hs* Fd)].