Mutations of surface residues in *Anabaena* vegetative and heterocyst ferredoxin that affect thermodynamic stability as determined by guanidine hydrochloride denaturation

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Abstract

The stability properties of oxidized wild-type (wt) and site-directed mutants in surface residues of vegetative (Vfd) and heterocyst (Hfd) ferredoxins from *Anabaena* 7120 have been characterized by guanidine hydrochloride (Gdn-HCl) denaturation. For Vfd it was found that mutants E95K, E94Q, F65Y, F65W, and T48A are quite similar to wt in stability. E94K is somewhat less stable, whereas E94D, F65A, F65I, R42A, and R42H are substantially less stable than wt. R42H is a substitution found in all Hfds, and NMR comparison of the *Anabaena* 7120 Vfd and Hfd showed the latter to be much less stable on the basis of hydrogen exchange rates (Chae YK, Abildgaard F, Mooberry ES, Markley JL, 1994, *Biochemistry* 33:3287–3295); we also find this to be true with respect to Gdn-HCl denaturation. Strikingly, the Hfd mutant H42R is more stable than the wt Hfd by precisely the amount of stability lost in Vfd upon mutating R42 to H (2.0 kcal/mol). On the basis of comparison of the X-ray crystal structures of wt *Anabaena* Vfd and Hfd, the decreased stabilities of F65A and F65I can be ascribed to increased solvent exposure of interior hydrophobic groups. In the case of Vfd mutants E94K and E94D, the decreased stabilities may result from disruption of a hydrogen bond between the E94 and S47 side chains. The instability of the R42 mutants is also most probably due to decreased hydrogen bonding capabilities. Those F65 mutants showing diminished stability (i.e., F65A and F65I) have previously been shown (Hurley JK, et al., 1993b, *Biochemistry* 32:9346–9354) to be severely impaired kinetically in their electron transfer (ET) reaction with ferredoxin:NADP⁺ reductase (FNR), a physiological reaction partner of Vfd. Mutants F65W and F65Y, which, as noted, are like wt in stability, also functioned like wt in the ET reaction with FNR (Hurley JK, et al., 1993a, *J Am Chem Soc* 115:11698–11701). Possible reasons for this correlation between ET properties of the F65 mutants and their conformational stabilities are discussed.

Keywords: electron transfer kinetics; ferredoxin:NADP⁺ reductase; hydrogen bonding; protein stability; protein unfolding; site-specific mutagenesis

The iron-sulfur-containing ferredoxins are small, soluble, electron-transfer proteins found in a large variety of organisms. The “plant-type” [2Fe-2S] fds, such as that from the cyanobacterium *Anabaena*, transfer electrons between the membrane-

bound Photosystem I and the soluble FNR (Lovenberg, 1973, 1974, 1977; Knaff & Hirasawa, 1991). The X-ray crystal structure of *Anabaena* 7120 vegetative fd has been recently determined to 2.5 Å resolution (Rypniewski et al., 1991) and refined to 1.9 Å (Holden et al., 1994) to reveal a [2Fe-2S] cluster located near the protein surface and secondary structure consisting of five β -sheet strands and three α -helices. The X-ray structure of *Anabaena* 7120 heterocyst fd has also been solved (Jacobson et al., 1992) and refined to 1.7 Å resolution (Jacobson et al., 1993). In this report, we describe effects of mutations of surface residues on the stability of the two wild-type proteins (Vfd and Hfd) toward Gdn-HCl denaturation. Three classes of mutations were investigated. (1) Previous studies (Hurley et al.,

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Abbreviations: wt, wild-type; fd, ferredoxin; Vfd, *Anabaena* 7120 vegetative ferredoxin; Hfd, *Anabaena* 7120 heterocyst ferredoxin; FNR, ferredoxin:NADP⁺ reductase; ET, electron transfer; Gdn-HCl, guanidine hydrochloride; F65A, phenylalanine 65 substituted by alanine; E94K/E95K, double mutant having glutamates 94 and 95 substituted by lysines; other mutants abbreviated in similar fashion.

1993a, 1993b) had demonstrated that an aromatic residue at position 65 in Vfd is critical for ET to FNR; mutations at this position were designed to probe the importance of the volume and aromatic nature of the side chain on protein stability. The present results show that mutants that preserve an aromatic residue at position 65 (F65Y and F65W) support wt stability, but that mutations to aliphatic amino acids (F65A, F65I) lead to decreased stability even if they preserve the wt residue volume. (2) Earlier studies also established that a negative charge at residue 94 is critical for ET to FNR: E94K and E94Q have diminished activity, whereas E94D and E95K have activities similar to wt (Hurley et al., 1993a, 1993b). Because the side-chain carboxyl of E94 is seen in the X-ray structure to hydrogen bond to the hydroxyl group of S47, the effects of these mutations on the stability of the protein are of interest. The results reported here demonstrate that mutants E94K and E94D have decreased stability, but that E94Q and E94K have stabilities similar to wt. Thus, mutations at this site do not show the correlation between stability and ET activity seen at position 65. (3) The third class of mutants was constructed in order to evaluate the role of residue 42, which is conserved as H in the HfDs that transfer electrons to the iron protein of nitrogenase during N_2 fixation, but is R in the VfDs, which are much less efficient in this reaction. (Vfd and Hfd have similar activities in photosynthetic ET to

FNR.) The Vfd mutation R42H was found to be destabilizing by 2.0 kcal/mol, whereas the inverse mutation of Hfd (H42R) was found to be stabilizing to the same degree (2.0 kcal/mol).

Results and discussion

Figure 1 presents UV-visible spectra of wt Vfd and its F65I mutant in the absence of Gdn-HCl and at two time points after addition of the protein to a 6 M Gdn-HCl solution. Note that the absorbance maxima in the visible region indicate that, for both proteins, the [2Fe-2S] cluster is still present after about 20 min in 6 M Gdn-HCl solution, although its environment is somewhat altered. After a longer time, the [2Fe-2S] cluster is lost by the denatured protein, as judged by the disappearance of the UV-visible spectral bands (Fig. 1). In contrast, the large decrease in the amplitude of the 220-nm CD signal (see below) for the wt and mutant proteins in 6 M Gdn-HCl (data not shown) clearly indicates a much faster loss of structure. In fact, at high Gdn-HCl concentrations, the loss of the CD signal at 220 nm is complete within the dead time of the instrument (approximately 1 s). At lower concentrations of Gdn-HCl (2.0–2.5 M), the majority of the change in CD intensity at 220 nm is completed in approximately 4 min, followed by a much smaller change (2–3 mdeg) over the course of more than 3 h.

The conformational stabilities of the wt and mutant fds in their oxidized states were quantified by monitoring the titration of the 220-nm CD signal with Gdn-HCl (Pace, 1975, 1986); this CD band reflects both α -helix and β -sheet secondary structures. When the fraction of unfolded protein (f_u) is plotted as a function of [Gdn-HCl] (Fig. 2A,B), it is evident that several Vfd mutants behave similarly to wt, whereas a number of others are clearly less stable. Two-state transitions between the native and denatured states prior to loss of the [2Fe-2S] cluster were assumed in deriving the thermodynamic parameters listed in Table 1. These parameters were obtained by linear regression analysis of data points in the midpoint regions of the transitions (Fig. 3). In the case of the wt Vfd, the midpoint of denaturation (C_m) occurs at 2.8 M, and the free energy of unfolding in the absence of denaturant (ΔG_u^*) is 6.3 kcal/mol. This latter value involves a large extrapolation (not shown) to 0 M Gdn-HCl and is, therefore, extremely sensitive to minor uncertainties in slope (m) within the region where the data were obtained. This problem has been pointed out previously (Greene & Pace, 1974; Ahmad & Bigelow, 1982; Pace, 1986; Kellis et al., 1988; Caffrey & Cusanovich, 1991; Caffrey et al., 1991; Jackson et al., 1993), and it was noted that a far better method for determining relative stabilities is to measure the differences within the range over which the data were obtained (Pace, 1986; Kellis et al., 1988; Caffrey & Cusanovich, 1991; Caffrey et al., 1991; Jackson et al., 1993). Thus, the value $\Delta\Delta G_u'$, calculated as described in the Materials and methods section (Caffrey & Cusanovich, 1991; Caffrey et al., 1991; Jackson et al., 1993), is preferred as a means of comparing stabilities of mutants relative to wt (Tables 1, 2). This method fits the data to the same equation used for the linear extrapolation method ($\Delta G_u = \Delta G_u^* - m[\text{Gdn-HCl}]$) but uses the differences in ΔG ($\Delta\Delta G_u'$) values at a point midway between the C_m values (where $\Delta G = 0$) for the mutant and wt proteins (see Materials and methods). The measurement of $\Delta\Delta G_u'$ is taken from the plots shown in Figure 3 at this midpoint (C_m' , see Materials and methods). It is important to note that because the $\Delta\Delta G_u'$ values are determined at relatively high ionic

Fig. 1. UV-visible spectra of native and Gdn-HCl-denatured wt (A) and F65I mutant (B) VfDs from *Anabaena* 7120. The sample pH was 7.5; the temperature was $25 \pm 0.2^\circ\text{C}$. The half-times for loss of the [2Fe-2S] cluster in the wt and F65I mutants were approximately 50 and 80 min, respectively. In contrast, half-times for loss of secondary structure in Gdn-HCl solution were ≤ 2 min for both wt and mutant fds in 6 M Gdn-HCl and slightly longer at lower Gdn-HCl concentrations (e.g., ≤ 4 min at 2.0–2.5 M Gdn-HCl).

Fig. 2. Fraction of unfolding (f_u) as a function of [Gdn-HCl] for wt and mutant Vfds. **A:** Curves are drawn through data points for wt Vfd and F65A. **B:** Curves are drawn through data points for E94D, E94K, and wt Vfd. All curves reflect the C_m values listed in Table 1. For the sake of clarity, curves are not drawn through all data sets.

strength, electrostatic interactions contributing to stability would tend to be minimized (Knapp & Pace, 1974; Ahmad & Bigelow, 1982).

Mutations at E94 and E95 in Vfd

Mutant E94K, whose rate constant for ET to FNR is four orders of magnitude smaller than that of wt Vfd (Hurley et al., 1993b), showed a decrease in stability of 0.5 kcal/mol and the mutant E95K, whose ET rate constant is equivalent to that of wt Vfd, showed wt stability (Table 1). By contrast, the effects of the other mutations at residue 94 on stability did not parallel their effects on their ET rate constants with FNR (Hurley et al., 1993b, 1994). Whereas the conservative mutation E94D preserved wt ET activity, it led to a large decrease in stability (lower by 0.8 kcal/mol). In addition, the mutation E94Q, which decreases the rate constant for ET to FNR by four orders of magnitude, showed wt stability.

The above thermodynamic results can be interpreted in terms of the X-ray crystal structures of wt and mutant Vfds. In Figure 4, a stereo view of the region near the [2Fe-2S] cluster is presented, which includes the mutated residues (see also Kinemage 1). The E94 side chain is partially exposed to solvent (Table 2).

Table 1. Stability properties of *Anabaena* Vfd and Hfd^a

Ferredoxin	C_m^b	m^c	ΔG_u^{*d}	$\Delta\Delta G_u^e$
wt Vfd	2.8 ± 0.1	2.3 ± 0.1	6.3 ± 0.4	—
E95K Vfd	2.9 ± 0.1	2.6 ± 0.1	7.3 ± 0.3	0.2 ± 0.2
E94K Vfd	2.6 ± 0.1	2.3 ± 0.1	5.9 ± 0.2	-0.5 ± 0.2
E94D Vfd	2.4 ± 0.1	2.1 ± 0.1	5.2 ± 0.2	-0.8 ± 0.2
E94Q Vfd	2.7 ± 0.2	2.6 ± 0.2	6.9 ± 0.5	-0.3 ± 0.3
F65A Vfd	2.2 ± 0.1	2.4 ± 0.1	5.3 ± 0.3	-1.4 ± 0.2
F65I Vfd	2.1 ± 0.1	2.6 ± 0.1	5.4 ± 0.3	-1.7 ± 0.2
F65Y Vfd	2.7 ± 0.1	2.2 ± 0.1	5.8 ± 0.3	-0.2 ± 0.2
F65W Vfd	2.8 ± 0.1	2.0 ± 0.2	5.7 ± 0.2	0.1 ± 0.2
R42A Vfd	2.1 ± 0.1	1.8 ± 0.1	3.9 ± 0.2	-1.3 ± 0.2
R42H Vfd	2.0 ± 0.1	2.5 ± 0.1	4.9 ± 0.2	-2.0 ± 0.2
T48A Vfd	2.8 ± 0.1	2.3 ± 0.1	6.4 ± 0.3	0.1 ± 0.2
wt Hfd	2.3 ± 0.1	2.0 ± 0.1	4.5 ± 0.2	-1.1 ± 0.2
H42R Hfd	3.2 ± 0.1	2.7 ± 0.1	8.5 ± 0.2	0.9 ± 0.2

^a Experimental conditions were pH 7.5 at 25 °C.

^b Concentration of denaturant required to unfold half of the protein. Units are mol/L. Error limits represent the standard deviations of the fit to the data in Figure 3, from which C_m was obtained at $\Delta G_u = 0$.

^c Units are kcal L/mol². Values obtained from linear least-squares fit to data of Figure 3 and error limits represent standard deviations of the fit.

^d Free energy of unfolding in the absence of denaturant. Units are kcal/mol. Values obtained from linear least-squares fit to data of Figure 3 and error limits represent standard deviations of the fit.

^e $\Delta\Delta G_u' = \text{mut } \Delta G_u - \text{wt } \Delta G_u$ at the concentration of Gdn-HCl midway between the C_m values of the mutant and wt proteins. Error limits represent the sum of the standard deviation of the linear least-squares fit to the wt data and that of the mutant data.

Fig. 3. Free energy of unfolding (ΔG_u) versus [Gdn-HCl] for wt and mutant Vfds.

Table 2. Hydrogen bonding characteristics and solvent-exposed surface areas^a of residues that have been mutated in wt *Anabaena Vfd*

Mutated residue	Side-chain H bond(s)	H bond length (Å)	Solvent-exposed surface area of residue (Å ²)	Fraction of the residue surface area that is exposed (Å ²) ^b
E94	OE1 to solvent ^c OE2 to OG of S47	2.7 ^d 2.8 ^d	51	0.28
E95	? ^{e,f}	—	138 ^g	0.75
F65	None	—	81	0.37
R42	NH1 to OE2 of E31 NH2 to OE1 of E31 NH2 to OD1 of D28 NE to solvent	2.8 ^d 2.7 ^d ≤3 ^f ≤3 ^f	80	0.33
T48	OG1 to solvent ^c	≤3 ^f	36	0.25

^a Calculated using the Lee and Richards (1971) algorithm and a search probe of 1.4 Å.^b This calculation used the total accessible surface of the amino acid residues as reported by Miller et al. (1987).^c This solvent molecule is H bonded to both T48 and E94.^d Taken from Holden et al. (1994).^e The E95 side chain is too disordered to establish H bonding.^f H.M. Holden (unpubl. results).^g This value is somewhat uncertain due to the disordered nature of the side chain.

From the crystal structure it is seen that the side-chain carboxyl group of this residue forms H bonds (Table 2) to S47 and to a water molecule. These H bonds are part of an evolutionarily conserved hydrogen bonding pattern believed to stabilize the [2Fe-2S] binding loop by tethering it to the short C-terminal α -helix (Holden et al., 1994). Preliminary X-ray crystallographic data on the E94K mutant (B.L. Jacobson & H.M. Holden, unpubl. data) suggest that the side chain in this mutant has a high degree of mobility, which might be expected due to disruption of this H bond pattern. E94Q can probably hydrogen bond to S47 as does the wt residue. E94D on the other hand, being one

methylene group shorter, may not be able to hydrogen bond as well as the wt protein, and this may account for its lowered stability. Further structural information is needed to test these hypotheses.

The wt stability of Vfd mutant E95K can be rationalized in terms of what is known about the environment of this residue. Because the electron density from the E95 side chain is at the protein surface and is not well defined in the electron density map derived from X-ray analysis (Holden et al., 1994), this residue is solvent exposed and appears not to participate in specific interactions with the rest of the protein.

Fig. 4. Stereo view of *Anabaena* 7120 Vfd showing sites of mutations.

Mutations at F65 and T48 in Vfd

The F65A and F65I mutants of Vfd, which replace the aromatic group at position 65 with aliphatic side chains, are destabilized by 1.4 and 1.7 kcal/mol, respectively, relative to wt, according to the parameter $\Delta\Delta G_u$ (Table 1). Both of these mutations also resulted in dramatically decreased (by approximately four orders of magnitude) rate constants for ET to FNR (Hurley et al., 1993b). In an attempt to assess the role of aromaticity in the protein-protein ET process, the mutants F65W and F65Y were made and shown to behave like wt Vfd in their ET reactivity with FNR (Hurley et al., 1993a). Strikingly, wt-like conformational stability was also retained in these mutants, as measured by $\Delta\Delta G_u$ (Table 1).

The conversion of T48 to A in Vfd has been shown previously to have no measurable effect on the ET kinetics with FNR (Hurley et al., 1993b), and this mutant is like wt in its stability toward Gdn-HCl denaturation (Table 1).

Again, it is useful to interpret the above thermodynamic results in terms of the X-ray crystal structure of wt Vfd (Holden et al., 1994). F65 is partially buried (Table 2), and substitution by the smaller side chains of alanine or isoleucine⁴ can be expected to result in decreased (energetically favorable) packing interactions or increased (energetically unfavorable) solvent exposure of previously shielded residues. This expectation is consistent with the fact that the F65W and F65Y mutants, which have aromatic side chains similar in size and shape to that in the wt protein, also have wt stability properties, whereas F65A and F65I, which have side chains that are smaller and have dissimilar shape, have lower stability. However, based on the relative sizes of the mutant residues (A and I), one might expect F65I to leave less underlying surface area exposed than F65A, thus resulting in less destabilization. This correlation does not hold, however. As noted above, F65 is partially buried, and it may be that the mutation F65A leads to the creation of a cavity in the protein interior. It is known that such cavity-creating mutations can lead to small movements of side chains in the vicinity of the mutated residue that tend to "compensate" for the cavity (Eriksson et al., 1992; Jackson et al., 1993); these side-chain movements result in a change in overall conformational energy of the protein that can be either stabilizing or destabilizing. Whether or not such side-chain movements occur in the present case must await further structural information.

The striking correlation that is observed in these F65 mutants between conformational stability and ET reactivity suggests that similar structural factors may be involved in determining both properties. However, it is important to note that such a correlation is not seen with the E94 or R42 mutants (see below). Thus, it is possible that those structural elements that are altered in the F65A and F65I mutants (e.g., hydrophobic surface characteristics, altered conformations in this region, etc.) and that influence conformational stability may also play a role in the docking mechanism leading to ET to FNR.

Mutations at R42 in Vfd and at H42 in Hfd

R42 is a highly conserved residue, being present in 22 of 24 non-halophilic plant-type vegetative fds (Matsubara & Hase, 1983).

⁴ The molecular volume of alanine is 103.4 Å³ less than phenylalanine and the molecular volume of isoleucine is 28.6 Å³ less than phenylalanine (Harpaz et al., 1994).

Fig. 5. Fraction of unfolding (f_u) versus [Gdn-HCl] for wt Vfd, wt Hfd, and mutants of each of these at position 42.

H42 is conserved in all heterocyst fds. Vfd mutants R42A and R42H, both of which are wt-like in their ET reactivity toward FNR, are 1.3 and 2.0 kcal/mol, respectively, less stable than wt Vfd. Hfd, found in specialized N₂-fixing cells of cyanobacteria such as *Anabaena* and which has H at position 42, as well as a variety of other substitutions (overall sequence identity of 51%; Böhme & Haselkorn, 1988), also shows wt-like ET reactivity toward FNR (unpubl. data) and is less stable than wt Vfd by 1.1 kcal (Table 1). In sharp contrast, the Hfd mutant H42R is 2.0 kcal/mol (Table 1) more stable than wt Hfd. This increase in stability is precisely equal in magnitude to the stability lost upon making the R42H mutant of Vfd (Table 1). Figure 5 compares the denaturation curves for Vfd, its R42H mutant, Hfd, and its H42R mutant.

Reference to the X-ray crystal structures of the wt Vfd and Hfd proteins lends insight into these stability changes. The R42 side chain of Vfd donates two hydrogen bonds to E31 and one to D28 (Fig. 6A; Kinemage 1; Table 2), which add to the stability of the polypeptide chain in the vicinity of the [2Fe-2S] cluster (Jacobson et al., 1993; Holden et al., 1994). The structural importance of multiple hydrogen bonds involving arginine and backbone carbonyl oxygens has been discussed recently (Borders et al., 1994). In R42A, this H bonding is certainly disrupted, and in R42H, the bonding may be disrupted enough to account for the observed decrease in stability. Consistent with this is the fact that in Hfd, H42 makes only one hydrogen bond (Fig. 6B; Kinemage 1). Also consistent with this, as noted above, the Hfd mutant H42R is 2.0 kcal/mol more stable than wt Hfd. It is of interest that an NMR comparison (Chae et al., 1994) of the *Anabaena* 7120 vegetative and heterocyst fds showed the latter to be much less stable on the basis of hydrogen exchange rates (Roder, 1989). Again, the validity of the correlations between changes in hydrogen bonding capabilities and thermodynamic stabilities of the proteins examined in the present study must await further structural information on the mutant proteins. Efforts to obtain this are currently underway in the laboratories of two of the authors (H.M.H. and J.L.M.).⁵

⁵ In addition to the structure of the E94K mutant, which has been solved to 1.8 Å resolution, crystals have been obtained for the F65A and F65Y mutants (B.L. Jacobson, J. Vanhooke, & H.M. Holden, unpubl. results).

A

Fig. 6. Structural comparison of the Vfds and Hfds in the vicinity of residue 42. A: Vfd; residue 42 is an R, which participates in three H bonds, to E32 and D28, as indicated by the dashed lines. B: Hfd; H42 participates in only one H bond, to D28. This figure has been adapted from Holden et al. (1994).

B

Materials and methods

Mutagenesis procedures and protein isolation and purification methods have been described elsewhere (Hurley et al., 1993b). Recombinant Hfd was prepared as described previously (Jacobson et al., 1992). Those mutants made at the University of Wisconsin (F65A, F65I, F65W, F65Y, R42A, R42H, T48A Vfd, and H42R Hfd), were reconstituted⁶ from apoprotein by using a

⁶ No significant difference in stability toward Gdn-HCl denaturation was observed for wt Vfd, which had been subjected to this reconstitution procedure, relative to nonreconstituted wt Vfd. Consistent with this, reconstituted wt Vfd is identical to nonreconstituted Vfd by NMR criteria (H. Cheng & J.L. Markley, unpubl. data), as well as by reactivity toward FNR (J.K. Hurley & G. Tollin, unpubl. data).

modification of the method of Coghlan and Vickery (1991). Mutants made at the University of Arizona (E94K, E95K, E94D, and E94Q Vfd) were isolated as the holoprotein and were not subjected to the reconstitution procedure. Gdn-HCl (U.S. Biochemical, Cleveland, OH; ultrapure) denaturations were performed as previously described (Caffrey & Cusanovich, 1991), except that protein concentrations were 3–5 μ M and samples were pre-equilibrated for 20 min. Samples were buffered at pH 7.5 with 20 mM Tris-HCl (Aldrich, Milwaukee, Wisconsin; ultrapure) containing 40 mM NaCl (Fisher, Fair Lawn, Tennessee; A.C.S. reagent grade). CD spectra were taken on an Aviv CD spectropolarimeter model 60 DS at 25 ± 0.2 °C. Data analysis procedures were also as previously described (Caffrey & Cusanovich, 1991). Briefly, estimates of ΔG_u^* (free energy of

unfolding in the absence of denaturant), m (cooperativity of unfolding), and C_m (concentration of denaturant required to denature half the protein) were made according to the methods described previously (Caffrey & Cusanovich, 1991; Caffrey et al., 1991). A two-state equilibrium was assumed in the current analysis, with K_u , the equilibrium constant for unfolding, being defined as $(S_{obs} - S_i)/(S_f - S_{obs})$, where S_{obs} , S_i , and S_f represent the observed, initial ([Gdn-HCl] = 0 M), and final CD signals ([Gdn-HCl] = 6 M), respectively, at 220 nm. The equations $\Delta G_u = -RT \ln K_u$ and $\Delta G_u = \Delta G_u^* - m[\text{Gdn-HCl}]$ (Pace, 1975, 1986) were used in linear regression analysis of ΔG_u versus [Gdn-HCl] to determine the parameters m and ΔG_u^* . The parameter $\Delta\Delta G_u'$ is the difference between the wt and mutant ΔG_u values at the point midway between their C_m values, i.e., $\Delta\Delta G_u' = \text{mutant } \Delta G_u \text{ (at } C_m') - \text{wt } \Delta G_u \text{ (at } C_m')$, where $C_m' = (\text{mutant } C_m + \text{wt } C_m)/2$ (Caffrey & Cusanovich, 1991; Caffrey et al., 1991).

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