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Stability study of *Rhodobacter capsulatus* ferrocytochrome c_2 wild-type and site-directed mutants using hydrogen/deuterium exchange monitored by electrospray ionization mass spectrometry**

Michel Jaquinod[a], Philippe Guy[a], Frederic Halgand[a], Michael Caffrey[a], John Fitch[b], Michael Cusanovich[b], Eric Forest[a,*]

[a]*Institut de Biologie Structurale Jean-Pierre Ebel, CEA / CNRS, 41 avenue des Martyrs, 38027 Grenoble Cedex 1, France*
[b]*Department of Biochemistry, University of Arizona, Tucson, AZ 85721, USA*

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Abstract To estimate the stability of *Rhodobacter capsulatus* ferrocytochrome c_2 wild-type and site-directed mutants, charge state distributions and hydrogen/deuterium exchange rates were monitored by electrospray ionization mass spectrometry. The relative stability of the mutants was observed with the order: V11 insert > Y75F > wild-type = K32E > K12D = K14E ≥ K52E > K14E/K32E > W67Y > P35A > I57N > G34S. (A preliminary account has been presented for mutants G34S and P35A [Jaquinod et al. (1995) Rapid Commun. Mass Spectrom. 9, 1135–1140].) This approach is shown to be a useful tool for rapid characterization of mutational effects on protein conformation.

Key words: Electrospray ionization mass spectrometry; Hydrogen/deuterium exchange; Conformational stability; *Rhodobacter capsulatus* cytochrome c_2

1. Introduction

The relationship between sequence and protein three-dimensional structure is not well understood. Generally highly conserved residues are chosen as mutation sites for investigating the role these residues play in the conformational properties of proteins. For site-directed mutagenesis studies, there are several factors to consider in the choice of substituting groups: charge, H-bonding capability, size and hydrophobicity [1]. The conformational stability of site-directed mutants is generally studied by chemical or temperature denaturation or H/D exchange monitored by NMR. Denaturation studies measure the resistance of secondary structure to chemical or temperature denaturation (generally by monitoring the 220 nm circular dichroism signal) and thus are limited to the mutational effects on secondary structure. On the other hand, NMR studies of H/D exchange can characterize all structural elements of the protein backbone but are time consuming and require relatively large amounts of protein. Recently, ESI-MS, which produces intact multiply charged gas-phase ions from solution, has shown the capability to analyze large biopolymers [2,3]. Consequently, a number of authors have used the CSD of proteins to measure conformational perturbations induced by numerous factors [4–7]. More recently, the rates of H/D exchange [8–15] have been monitored by ESI-MS to characterize structural perturbations in proteins. These studies have shown that H/D exchange rates are related to numerous factors including: the presence of H-bonds and/or solvent accessibility [9,10], the temperature [11], the CSD [12].

The class I cytochromes *c* are small soluble heme-proteins that are involved in electron transfer [16]. Site-directed mutants have been generated in a number of species including: *Rhodobacter capsulatus* cytochrome c_2 [17], *Thiobacillus versutus* cytochrome c_{550} [18], *Desulfovibrio vulgaris* cytochrome c_{553} [19], *Saccharomyces cerevisiae* (yeast) cytochrome *c* [20], *Drosophila melanogaster* (fruit fly) cytochrome *c* [21], and *Rattus norvegicus* (rat) cytochrome *c* [22]. In the present study, the stability properties of *Rb. capsulatus* ferrocytochrome c_2 wild-type and site-directed mutants are characterized by ESI-MS. The site-directed mutants include: V11 insert, K12D, K14E, K14E/K32E, K32E, G34S, P35A, K52E, I57N, W67Y and Y75F. We show that the combined use of ESI-MS and H/D exchange is particularly attractive for the initial characterization and classification of mutant stability because it requires relatively small amounts of protein and can be performed rapidly.

2. Materials and methods

2.1. Materials

Deuterium oxide and acetic acid-*d* were purchased from Sigma (St Louis, Missouri, USA). Poly(propylene glycol) was purchased from Aldrich (Aldrich-Chemie, Steinheim, Germany). *Rb. capsulatus* wild-type and mutant cytochromes c_2 were prepared as previously described [17,23]. Prior to analysis by MS, the wild-type and mutant cytochromes c_2 were reduced with an excess of sodium dithionite, which was subsequently removed by ultrafiltration. Control experiments demonstrated that there was no detectable change in redox state, as monitored by the absorbance at 550 nm, under the experimental conditions of the MS analyses (i.e. 3 hours at pH 3.0 or 5.8).

2.2. H/D exchange

For the H/D exchange experiments, 0.6 nmol of the cytochromes c_2 were dissolved in deuterated water (pD 5.8) or in deuterated water containing various percentages of acetic acid-*d*: 0.3% (pD 3.0), 0.5% (pD 2.9) or 1% (pD 2.7) to a final protein concentration of 15 pmol/μl. Exchange reactions were carried out at 25°C for 3 hours. For the kinetic studies, aliquots were taken every 15 seconds in the first 3 minutes and every minute until 15 minutes. Subsequent aliquots were analyzed every 10 minutes during the first hour and then every hour. In all cases, the samples were measured by ESI-MS without further treatment.

2.3. ESI-MS

ESI-MS was performed using a SCIEX API III+ triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill,

*Corresponding author. Fax (33) 76 88 53 58; e-mail: forest@ibs.fr

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Abbreviations: ESI-MS, electrospray ionization mass spectrometry; H/D, hydrogen/deuterium; *Rb. capsulatus*, *Rhodobacter capsulatus*; CSD, charge state distribution; Gdn-HCl, guanidine hydrochloride.

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Canada) equipped with a nebulizer-assisted electrospray (ionspray) source. Calibration was performed using Poly(propylene glycol) ions. The samples were infused using a syringe pump (Harvard 22, South Natic, USA) at a flow rate of 5 μl/min. The mass spectrometer was scanned from *m/z* 600 to 2000 with 0.9 Da steps and 2 ms per step. Mass spectra were analyzed using a Quadra 950 data system (Apple Computer Inc., Cuppertino, USA). MacBioSpec software (Perkin-Elmer Sciex) was used to calculate the mass of the proteins from their sequence

3. Results

3.1. Probing cytochrome c_2 conformation using CSD

It was suggested that the ion abundance profiles in ESI-MS might reflect the abundances of preformed, multiply charged species in aquous solution [24]. Therefore a 'snapshot' of the cytochrome c_2 wild-type and mutant conformations could be obtained by observing the CSD in the positive ESI mass spectrum, where the number of charges corresponds to the number of ionized basic residues. The *Rb. capsulatus* wild-type cytochrome c_2 consists of 116 amino acids, including 20 protonable basic sites (i.e. N-terminal amine, 1 histidine, 1 arginine and 17 lysines). In the case of the single charge mutants K12D, K14E, K32E and K52E there are 19 protonatable basic sites and in case of the double charge mutant K14E/K32E there are 18 protonatable sites. The ferrocytochrome c_2 samples were dissolved in pure water (pH 5.8) and their mass was measured by ESI-MS. With a mass difference between the calculated and the measured masses inferior or equal to one Da, these analyses indicated that the amino acid replacements were effective. No significant difference was observed between the CSD of the wild-type and mutants. Example of the mass spectrum of wild-type is given in Fig. 1. The maximum number of charges is +9, indicating that 11 (10 for K32E) of the total basic sites are shielded from solvent or not protonated under the conditions used for ESI-MS. This result is similar to that previously observed for bovine cytochrome *c*, in which the maximum number of basic sites was +8 at pH 6.6 [5]. Surprisingly, substitution of lysines 12, 14, 32 and 52 did not induce a shift in the CSD in spite of the decreased global charge of these mutants. One explanation is that the mutated lysines are not charged in the wild-type cytochrome c_2. An alternative explanation is that there is a competitive effect between lysines which are spatially close in the three-dimensional structure. Nonetheless, the wild-type and mutant ferrocytochromes c_2 are in a tightly folded native state and the ionizable groups may be close together or buried under these conditions, and therefore the protonatable groups are inaccessible, leading to fewer charges in the ESI mass spectrum.

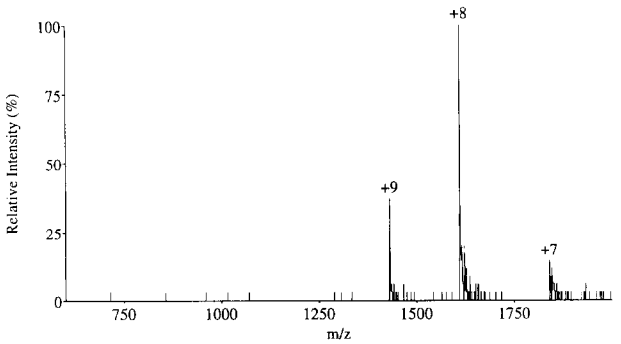

Fig. 1. CSD of the *Rb. capsulatus* wild-type ferrocytochrome c_2 in water (pH 5.8).

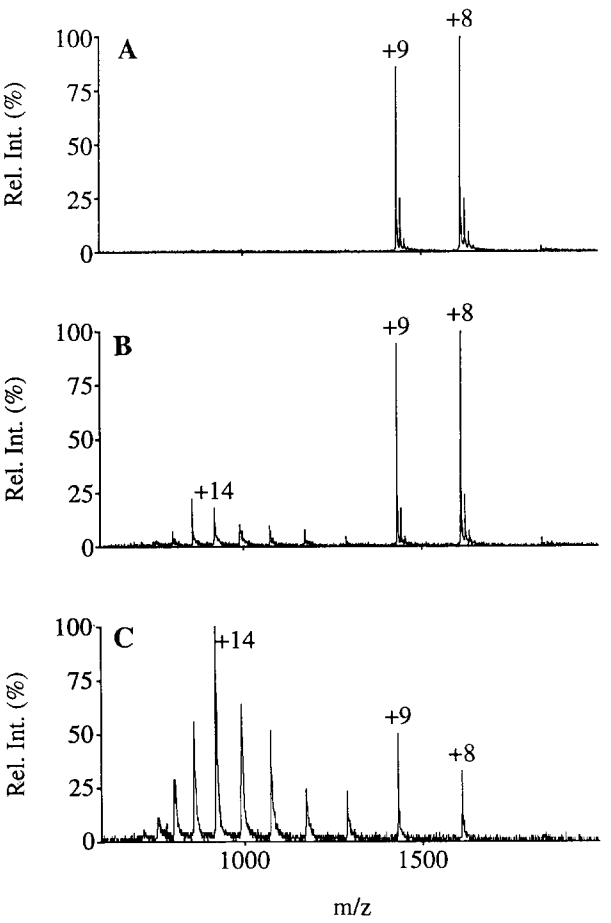

Fig. 2. CSD of the *Rb. capsulatus* wild-type (A), K52E (B) and I57N (C) ferrocytochromes c_2 in water containing 0.3% acetic acid (pH 3.0).

To further characterize the accessibility of the basic sites, the wild-type and mutant ferrocytochromes c_2 were analyzed in various percentages of acetic acid. No real difference in the CSD was observed until 0.3% acetic acid was reached. Under these conditions (pH 3.0), the CSD of the wild-type, V11 insert, K32E and Y75F remain unchanged (Fig. 2A). The CSD of K12D, K14E, K14E/K32E, P35A, K52E, and W67Y were found to be slightly different from that of the wild-type with the appearance of a second gaussian distribution of relatively low intensity (Fig. 2B). In contrast, the CSD of G34S and I57N were found to be significantly different from that of the wild-type (Fig. 2C) with the appearance of a second gaussian distribution from +11 to +18, centred at charge +14, which is more intense than the gaussian centred at +8. This indicates that G34S and I57N possess structural perturbations that increase the solvent exposure of some basic sites under these solvent conditions. In the presence of 0.5% acetic acid (pH 2.9), the second gaussian distribution of K12D, K14E, K14E/K32E, P35A, K52E, and W67Y, centred at charge +14, appears similar to those of G34S and I57N at 0.3% acetic acid (data not shown). At pH 2.9 the wild-type, V11 insert, K32E and Y75F are partially denaturated and present a CSD close to that observed for K14E at 0.3% acetic acid (Fig. 2B). At 1% acetic acid (pH 2.7) the CSD of K14E, G34S, P35A, I57N, W67Y and K14E/K32E were entirely shifted to higher charge states in contrast to the wild-type, V11 insert, K32E and Y75F which

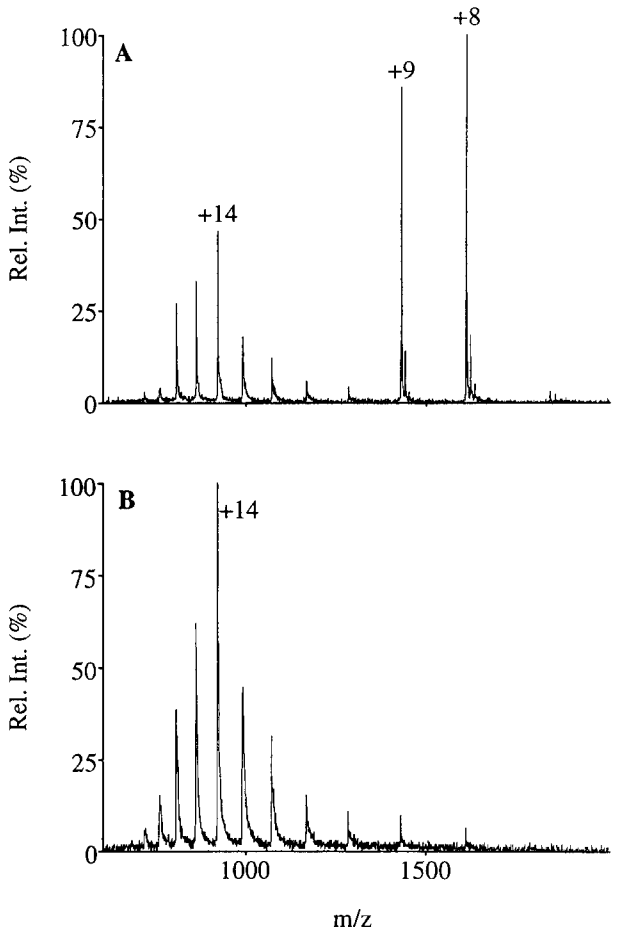

Fig. 3. CSD of the *Rb. capsulatus* wild-type (A) and I57N (B) ferrocytochromes c_2 in water containing 1% acetic acid (pH 2.7)

showed a bimodal distribution (Fig. 3). Thus, most of the mutants are more susceptible to acid denaturation than the wild-type. Notable exceptions are V11 insert, K32E and Y75F.

3.2. H/D exchange study

The wild-type and mutant ferrocytochromes c_2 were incubated in D_2O at 25°C and aliquots were sampled at different times and directly analyzed by ESI-MS. After three hours of incubation, an equilibrium was reached. The measured masses are presented in Table 1. Interestingly, significant differences are observed between the amount of H/D exchange observed for the wild-type and mutants at pD 5.8. For instance, after 3 hours the total number of H/D exchanges varies between 66 for the V11 insert to 109 for G34S. In contrast, no differences were observed for the CSD at pH 5.8 (discussed above). The unexchanged labile hydrogens (e.g. 121 in the wild-type) are presumably involved in H bonds or inaccessible to the solvent (i.e. buried and not at the protein surface). Mutations that exhibit an increased number of H/D exchanges with respect to the wild-type appear to either disrupt H-bonds and/or increase the solvent accessibility of exchangeable H. Mutants that exhibit significant increases in mass, with respect to the wild-type, include G34S, P35A, I57N and W67Y. On the other hand, the V11 insert, K12D, K14E, K14E/K32E, K32E, K52E, and Y75F exhibit similar amounts of H/D exchange as the wild-type and thus do not appear to introduce a significant disruption of H-bonds nor increase solvent accessibility at pD 5.8 (note that an alternative explanation is that compensating changes are occurring).

Table 1
Masses and mass increases (Δm) of *Rb. capsulatus* wild-type and mutant ferrocytochromes c_2 after 3 h of incubation in deuterated water (pD 5.8)

Sample	Calculated mass (Da)	Measured mass (Da)	Δm
V11 Insert	12982	13048	66
Y75F	12867	12934	67
Wild-type	***12883***	***12953***	***70***
K32E	12884	12954	70
K14E/K32E	12885	12956	71
K14E	12884	12956	72
K12D	12870	12942	72
K52E	12884	12902	73
W67Y	12860	12945	85
P35A	12857	12947	90
I57N	12884	12978	94
G34S	12913	13022	109

In order to increase the mass differences and further classify the mutant stabilities, we have performed a second set of experiments under more denaturing conditions at pD 3.0. Under these new conditions, the mutant stabilities are further resolved with the relative stabilities: V11 insert > Y75F > wild-type = K32E > K12D = K14E ≥ K52E > K14E/K32E (Table 2). Under acidic conditions, the substitution of the lysines 12, 14 and 52 by an aspartate or a glutamate appears to introduce conformational effects on the protein stability in comparison to the wild-type. In contrast, no significant difference was observed between the H/D exchange properties of the wild-type and K32E (116 and 118 H exchanges, respectively). Interestingly, the deuterium incorporation of the V11 insert and Y75F suggests that these mutants are more stable than the wild-type under acidic conditions.

Another interesting feature of H/D exchange at pD 3.0 is found by monitoring the two families of CSD observed for G34S and I57N. In both case, the charge distributions centred at +8 and +13 represented 25% and 75%, respectively. As shown in Fig. 4, the two forms of I57N exhibited different kinetic behaviours, suggesting the presence of two conformers

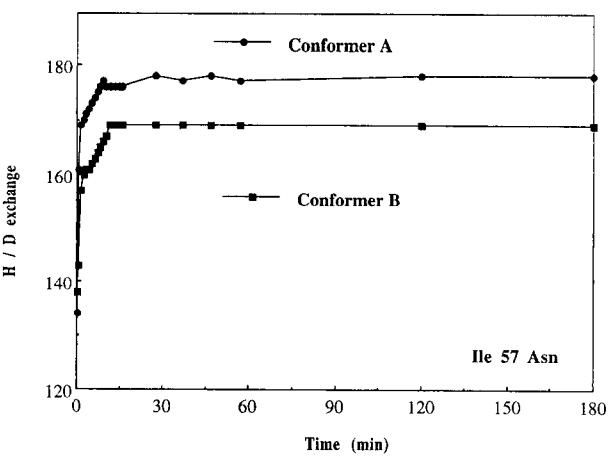

Fig. 4. Kinetics of H/D exchange for I57N ferrocytochrome c_2 in deuterated water containing 0.3% acetic acid-*d* (pD 3.0) monitored by ESI-MS.

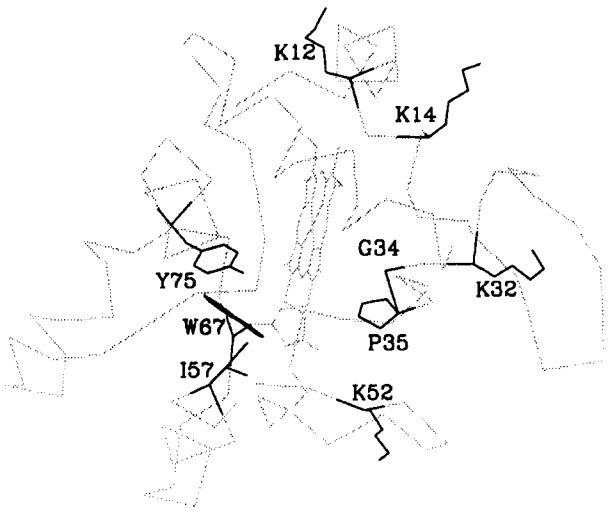

Fig. 5. Carbon alpha backbone of *Rb. capsulatus* ferrocytochrome c_2 crystal structure [27]. The side chains of mutated residues are presented in bold print.

in solution. The difference in the mass of the A and B forms of G34S and I57N was still detectable after 24 hours, indicating that the conformers are stable and do not exchange during this time period. Similar results were previously observed for bovine cytochrome *c* when temperature was used as the denaturant [11]. Moreover, the acid unfolded state of bovine cytochrome *c* has been studied and at last three conformational states were observed [25].

4. Discussion

Based on the CSD and H/D exchange rates at pH 5.8 and 3.0, the relative stabilities of the *Rb. capsulatus* ferrocytochromes c_2 are: V11 insert > Y75F > wild-type = K32E > K12D = K14E ≥ K52E > K14E/K32E > W67Y > P35A > I57N > G34S. To interpret the mutational effects on stability, it is of interest to consider the location of the mutated residues in the *Rb. capsulatus* cytochrome c_2 crystal structure (Fig. 5) [26]. *Rb. capsulatus* cytochrome c_2 contains a deletion at position 11 with respect to the eukaryotic cytochromes *c* [27]. The V11 insert was designed to stabilize the N-terminal helix of *Rb. capsulatus* cytochrome c_2, which had previously been shown by NMR studies to be less stable than horse cytochrome c [28]. The decreased H/D exchange of the V11 insert ferrocytochrome c_2 at pH 5.8 suggests that its stability is increased with respect to the wild-type and is consistent with the idea that this residue conveys stability to the N-terminal helix of cytochromes c. Residues K12, K14, K32, and K52 are basic residues found at the active site of cytochrome c_2 (i.e. the exposed heme edge) and are expected to form electrostatic contacts with the acidic residues of various redox partners [29,30]. Substitution by acidic residues tests the relative importance of specific electrostatic contacts in the electron transfer reactions of cytochrome c_2. However, for a full interpretation of mutational effects on electron transfer properties it is important to demonstrate that the charge substitutions do not introduce unanticipated structural changes which destabilize the protein [27]. The increased H/D exchanges of K12D, K14E, K14E/K32E and K52E ferrocytochromes c_2 at pH 3.0 suggest that these mutants are less stable than the wild-type. It was previously shown that K12D, K14E and K14E/K32E ferricytochromes c_2 are less stable to Gdn-HCl denaturation and this instability was explained to be due to an unfavorable interaction between the substituting acidic groups and the dipole of the N-terminal helix [31]. On the other hand, K52 is not found in a region of regular secondary structure, nor does it appear to be involved in a salt bridge (Fig. 5); thus, the instability of K52E ferrocytochrome c_2 is not easily explained at present and will require determination of the mutant structure by X-ray crystallography or NMR. In our study, mutant K32E ferrocytochrome c_2 exhibits exchanges similar to the wild-type and thus this mutation does not appear to introduce a destabilizing structural perturbation, an observation in agreement with Gdn-HCl denaturation studies which showed that the K32E ferricytochrome c_2 exhibited a stability similar to the wild-type [31]. Residues G34, P35, W67 and Y75 are highly conserved among the class I cytochromes c, located proximal to the heme, and are expected to play important roles in protein structure and stability [16,30]. The increased H/D exchange observed for G34S, P35A, and W67Y ferrocytochromes c_2 at pD 5.8 suggests that the mutations have introduced structural perturbations that destabilize the protein. The present results are in agreement with Gdn-HCl denaturation studies that have shown that the stabilities of G34S, P35A and W67Y ferricytochromes c_2 are decreased by 2.2 kcal/mol, 2.0 kcal/mol, and 2.5 kcal/mol, respectively (Caffrey and Cusanovich, unpublished results), [32,33]. Moreover, H/D exchange properties monitored by NMR have shown that P35A ferrocytochrome c_2 is less stable than the wild-type [34], an observation that is in agreement with the present study. Residue I57 of *Rb. capsulatus* cytochrome c_2 is located proximal to the heme and is a conserved asparagine residue in the eukaryotic cytochromes *c* [27]. The increased H/D exchange of I57N ferrocytochrome c_2 at pD 5.8 suggests that this mutant is less stable than the wild-type. Interestingly, a recent site-directed mutant study on yeast iso-1 cytochrome *c* has shown that an isoleucine at this position increases the stability of the ferric form of this mutant by 4.2 kcal/mol [35]. Moreover, yeast iso-1 cytochrome *c* mutants containing isoleucine at this position have been shown to be global suppressors of destabilizing substitutions at other positions [35,36]. Thus, the instability of the *Rb. capsulatus* I57N is in agreement with the idea that isoleucine at position 57 has a stabilizing effect on cytochromes *c*. The decreased H/D exchange of Y75F ferrocytochrome c_2 at pH 3.0 indicates that the mutation has a stabilizing effect. In agreement, Gdn-HCl de-

Table 2
Masses and mass increases (Δm) of *Rb. capsulatus* wild-type and mutant ferrocytochromes c_2 after three h of incubation in deuterated water containing 0.3% acetic acid-*d* (pD 3.0)

Sample	Calculated mass (Da)	Measured mass (Da) (deuterated)	Δm
V11 Insert	12982	13088	106
Y75F	12867	12978	111
Wild-type	***12883***	***12999***	***116***
K32E	12884	13004	118
K12D	12870	13001	131
K14E	12884	13016	132
K52E	12884	13019	135
K14E/K32E	12885	13027	142

Masses were calculated from the +7, +8 and +9 ions.

naturation of Y75F ferricytochrome c_2 suggests that the mutant is 0.4 kcal/mol more stable than the wild-type [23]. In contrast, the H/D exchange properties of Y75F ferrocytochrome c_2, monitored by NMR, showed that the mutant was less stable than the wild-type [34]. The reasons for the discrepancy between the various methods is not clear at present but could be due to differences in the experimental conditions, a possibility that is under investigation in our laboratories.

In conclusion, the present results demonstrate that ESI-MS studies of charge distribution and of H/D exchange are useful for the characterization of site-directed mutants. This approach is an especially useful tool for the initial characterization of mutant conformations since the analyses can be performed in a relatively short time period and require only picomole quantities of purified protein. Furthermore, we have shown recently [37] that this study on the global stability could be extended to local stability studies, using fast digestions and LC/ESI-MS. The effects on the local stability of each cytochrome c_2 mutant which were presented in the present study are currently under investigation.

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