BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 97–103 (1996)
Article No. 0018

Study of the New Stability Properties Induced by Amino Acid Replacement of Tyrosine 64 in Cytochrome c_{553} from *Desulfovibrio vulgaris* Hildenborough Using Electrospray Ionization Mass Spectrometry[1]

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Received November 17, 1995

Hydrogen / deuterium exchange as well as charge state distribution monitored by electrospray ionization mass spectrometry were demonstrated to be a powerful and effective new tool for probing conformational properties of proteins in solution. In this paper, the influence of single amino acid replacements on the global conformation of cytochrome c_{553} from *Desulfovibrio vulgaris* Hildenborough using isotopic exchange monitored by electrospray ionization mass spectrometry is reported. Based on their respective charge state distributions and isotopic exchanges, we have differentiated relative stability of mutants and a ladder classification with the order being wild-type > Y64F = Y64L > Y64V > Y64A, under specific conditions of pH, is proposed.

One objective of the protein chemistry is to understand the relationship existing between amino acid sequence and the protein conformation (i. e. three-dimensional structure). Number of interactions are known to stabilize protein conformations and the usual way to investigate this relationship is the use of oligonucleotide mutagenesis technique to produce mutants followed by the measurement of the new thermodynamic parameters using chemical or temperature denaturation, or by other techniques such as circular dichroism, X-ray crystallography or NMR. It has been demonstrated that mass spectrometry (MS) is a method of choice in protein characterization, particularly since the introduction, in the latest eighties, of a new gentle ionization technique for biomolecular analysis, the electrospray (ESI) [1, 2]. This new ionization technique enables the measurement of the molecular mass of peptides and proteins up to 100 kDa at picomole level with an accuracy of 0.01 to 0.02% [3, 4]. More recently, number of publications have shown that this method is a useful tool for the protein three-dimensional structure investigation. The CSD resulting in the net charge of the ionized protein, directly reflects the solvent accessibility of charged protein side chains and therefore depends on the protein structure. A number of authors have used these data to show the influence of various parameters such as temperature [5, 6], pH [7, 8] or solvent [9] on the protein conformation. This approach is limited by the relative small number of ionizable amino acids in a protein. A more global and complementary method is the determination of H/D exchange which is slow for amide protons implicated in hydrogen bonds or buried in the protein interior. Therefore proteins which have highly folded structure may reduce, by several orders of magnitude, the rates at which hydrogens undergo isotopic exchange [10, 11]. Until recently, H/D

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Abbreviations: H/D, hydrogen / deuterium; CSD, charge state distribution, ESI-MS, electrospray ionization mass spectrometry; DvH, *Desulfovibrio vulgaris* Hildenborough; acetic acid-*d*, deuterated acetic acid.

0006-291X/96 $12.00

exchange has mainly been studied by NMR spectroscopy for the determination of protein secondary structure [12] and unfolding [13]. In comparison to NMR, mass spectrometry offers the advantage of using very low amounts of protein material and the results are rapidly available with a high reliability. Actually, monitoring H/D exchange by ESI-MS seems to become a new and powerful standard procedure for probing protein structure and stability [14–18]. It has already been successfully applied to study conformation properties of horse cytochrome c and of cytochrome c_2 from *Rhodobacter capsulatus* [19–21]. A new type of cytochrome c was studied using this approach.

Single site-directed mutagenesis was designed to determine the role of the tyrosine 64 residue in the three-dimensional protein structure of the cytochrome c_{553} from the sulfate-reducing bacteria *Desulfovibrio vulgaris* Hildenborough [22]. This residue, which was unconserved when various cytochromes c were compared, was expected to play an important role in the structure/function relationships. Mutants (Tyr64Phe, Tyr64Leu, Tyr64Val, Tyr64Ala) were overexpressed in *Desulfovibrio desulfuricans* G200 [23]. It has been shown that Tyr64, located in the close vicinity of the N-terminal helix, the C-terminal helix and the heme [24], is not directly involved in the potential modulation but influences the internal mobility and the stability of the protein [23]. In this paper, we extend the stability study of the protein mutants by the combination of CSD measurements with the determination of H / D exchange by ESI-MS.

MATERIALS AND METHODS

Materials. H/D exchange reactions were carried out in Eppendorf vials (0.5 ml, 30 × 8 mm i. d.) from Sarstedt, Nümbrecht, Germany. Acetic acid, deuterium oxide, and acetic acid-*d* were purchased from Sigma (St Louis, Missouri, USA). Glycol polypropylene was purchased from Aldrich (Aldrich-Chemie, Steinheim, Germany). *Desulfovibrio vulgaris* Hildenborough wild-type and mutants cytochrome c_{553} were obtained as previously described [20, 21].

H/D exchange. For the H/D exchange experiments, 3 nmol of the ferricytochromes c_{553} were dissolved in deuterated water (pD 5.8) or in deuterated water containing various percentages of acetic acid-*d* at a final protein concentration of 20 μM. Exchange reactions were carried out at 25°C for 3 h. In the first 3 min, aliquots were taken every 15 sec and immediately measured by ESI-MS without further treatment. Subsequent aliquots were analyzed every 5 min during the first hour then once per hour.

ESI-MS. ESI-MS was performed using a Sciex API III+ triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Canada) equipped with a nebulizer-assisted electrospray (ionspray) source. Calibration was achieved using glycol polypropylene ions. Samples were infused into the source using a Harvard 22 syringe pump (Harvard Apparatus, Ealing, USA) at a flow rate of 5 μl/min. Full scan spectra were acquired in positive mode using a Quadra 950 data system (Apple Computer Inc., Cuppertino, USA) and several scans were averaged in order to improve the signal-to-noise ratio. The mass spectrometer was scanned from m / z 600 to 1600 with 0.5 Da steps and 2 ms per step.

RESULTS AND DISCUSSION

CSD and H/D exchange of cytochromes c_{553} monitored by ESI-MS were studied in the oxidized state of the molecule close to the physiological conditions at pH 5.8 and under mild denaturing conditions.

ESI-MS analyses of DvH cytochromes c_{553} at pH 5.8. ESI mass measurements performed on the four mutants indicated that the amino acid replacements were effective (Table 1). As shown in Figure 1, three peaks were observed centered on the +7 charge ion. In all cases, the maximum of charge was +8 indicating that 8 of the 15 basic sites are shielded from the solvent, involved in salt bridges or buried in the protein interior. Similar results were obtained with *Rhodobacter capsulatus* cytochrome c_2 where 11 of the 20 basic sites were not protonated at pH 5.8 [21]. What we can conclude from our experiments is, that these particular replacements do not introduce dramatic structural modifications, as the CSD are always centered around the +7 charge peak. To further complete the cytochromes study in water, we have proceeded to hydrogen / deuterium exchange.

To determine the extent of H/D exchange, the masses were calculated from the +6 and +7 ion charges after incubating the samples in D_2O at room temperature for three hours. Wild-type and Y64F or Y64L mutants were found to have approximately the same H/D exchange (80) which

TABLE 1
Hydrogen/Deuterium Exchange of Cytochromes c553 in Several Acidic Conditions after Incubation for 3 Hours (ΔM, mass increase)

protein	calculated mass (Da)	measured mass in H_2O (Da)	ΔM in D_2O (Da)	ΔM in 0.1% acetic acid-*d*	ΔM in 0.5% acetic acid-*d*
wild-type	8918	8919	80	83	86
Y64F	8902	8902	80	85	90
Y64L	8868	8868	82	87	90
Y64V	8854	8855	86	92	94*
Y64A	8826	8827	92	102	105*

* Measured mass from the +6 and +7 ions.

correspond to 60% of the total exchangeable hydrogens (136 in case of the wild-type). The Y64V and Y64A mutants present 86 and 92 H/D exchanges, respectively, which correspond to 64 and 68% of the total exchangeable hydrogens (Table 1). This confirms that the stability is decreased in these particular replacements.

The CSD as well as the H/D exchange experiments both measure the accessibility of either ionizable side chains or labile hydrogens to the protons of the solvent, as the only ionization which can occur under our experimental conditions is protonation. Side chain protonation and hydrogen

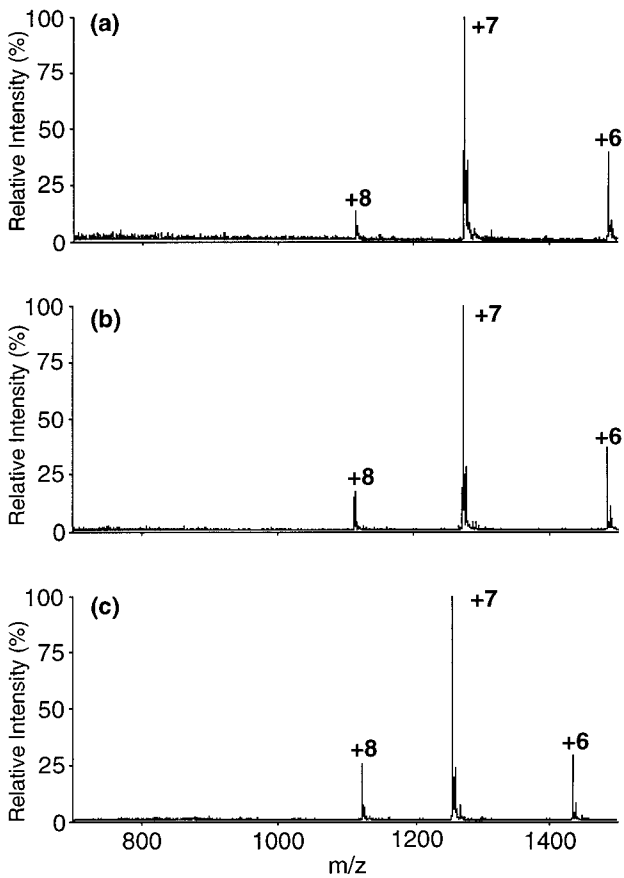

FIG. 1. CSD of the DvH wild-type (a) and Y64F (b), Y64A (c) mutants in pure water (pH 5.8).

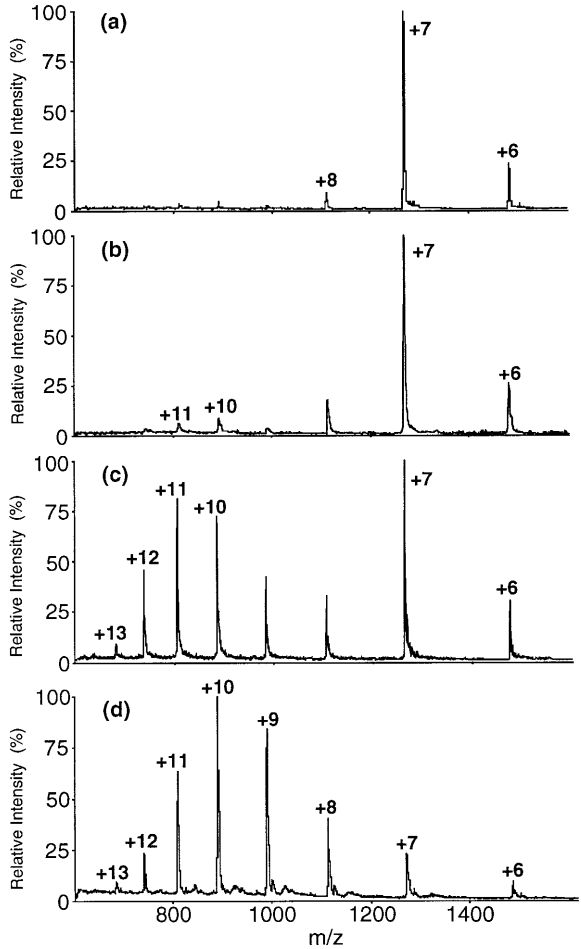

FIG. 2. CSD of the DvH Y64V in pure water (pH 5.8) (a), in water containing 0.1 % (pH 3.8) (b), 0.5% (pH 3.2) (c) or 2% (pH 1.9) (d) acetic acid.

exchange are both prevented by the localization of the corresponding group in the protein interior. In addition, hydrogen exchange also depends on the implication in a hydrogen bond whereas for charged side chains the pKa varies in function of the local environment. Favorable electrostatic interactions render deionization more difficult. Therefore, an increase in net positive charge and / or isotopic exchange indicates either a higher solvent exposure of the protein interior or a less favorable electrostatic or hydrogen bond energy. All these terms can be related to the flexibility of the protein molecule. The results presented here are in good agreement with the previous observation by NMR that the replacement of Y64 by Phe, Leu, Val or Ser increased the mobility of buried aromatic side chains [23]. Our isotopic exchange measurements in particular confirm the relative flexibilities, increasing with decreasing size of the side chain (wild-type, Phe, Leu, < Val < Ala).

ESI-MS analyses of DvH cytochromes c_{553} in presence of acetic acid. To further study the stability of mutants compared to the wild-type, samples were analyzed with various percentages of acetic acid. At lower pH, the appearance of new charge states was observed, which confirmed the destabilization of the ferricytochrome c_{553} mutants. The results obtained are in agreement with the relative stabilities deduced from the experiments at pH 5.8 (wild-type = Y64F = Y64L > Y64A > Y64V). For example, at 0.5% of acetic acid, the CSD of Y64V and Y64A were found to be

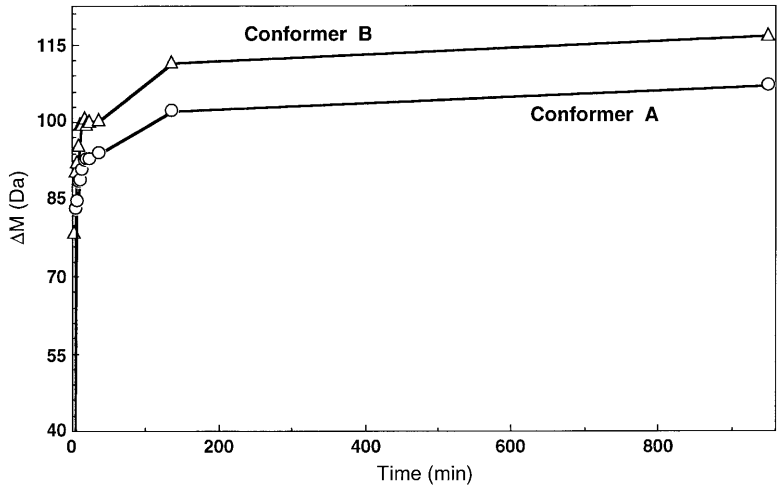

FIG. 3. H/D exchange curves of the DvH Y64A in water containing 0.5% acetic acid-*d* monitored by ESI-MS (pD 3.2).

significantly different from the wild-type or even the Y64F or Y64L mutants with the appearance of two distributions with a new CSD centered around the +10 charge peak. The change of the CSD spectrum of the Y64V mutant in function of the pH is shown in Figure 2. The apparition of a second gaussian distribution indicates a structural transition increasing the solvent exposure of further ionizable sites. The observed charge (+13) differs from the expected maximal charge of +16. This difference might be explained by an electrostatic effect generated by the close proximity of basic side chains (i. e. K39, K40 and K42) or by the persistence of salt bridges which are neutral. It is interesting to note that in spite of the lower pH (pH 3.2 at 0.5% acetic acid) the CSD centered on the +7 charge ion is still present. This might indicate that this form is stable with all ionizable side chains either buried in the protein interior or implicated in structurally important electrostatic interactions.

To further study this bimodal repartition, hydrogen exchange has been monitored by ESI-MS at various acetic acid-*d* concentration (see Table 1). Both CSD are observed at pD 3.2 (0.5% acetic acid-*d*) for Y64A and Y64V mutants. The hydrogen exchange kinetics in case of Y64A are reported in Figure 3 where the mass of the A form was calculated with +6 and +7 charge ions, and +11 and +12 charge ions for the B form. The difference in mass due to deuterium incorporation in the A and B forms of Y64A (105 and 117 H/D exchanges, respectively) 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 *Rhodobacter capsulatus* cytochrome c_2 when pH was used as denaturant [21]. The apparition of a second CSD under acidic denaturation has already been described by Goto *et al.* who have shown the presence of three possible conformers of horse cytochrome c in solution [25]. Similar effects of horse cytochrome c were observed by LeBlanc *et al.* in a study of heating induced conformational change of proteins [5]. In reference to the Ashton 'aqueous solution equilibrium' model [26], this bimodal repartition might be due to the presence of two conformations in solution with an abundance of each form corresponding to the relative intensity representing 45 / 55%, respectively. We propose that the appearance of the second CSD can be due to the acidic transition, a complex mechanism involving two or three protons. The dissociation of both iron ligands (histidine and methionine) gives rise to several less structured conformers in equilibrium [27, 28]. The apparition of a second conformer is known to be pH dependent and our results are in agreement with the pK_{ac} values determined for these proteins [23].

Figure 4 reports the H/D exchange kinetics of wild-type and mutants solubilized in deuterated water containing 0.5% acetic acid-*d* within a period of 10 hours. The calculated masses were

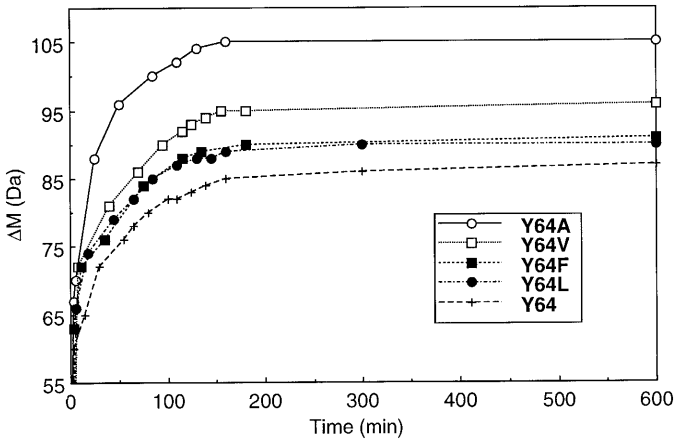

FIG. 4. H/D exchange curves of the DvH wild-type and mutants in pure water containing 0.5% acetic acid-*d* (pD 3.2).

obtained from +6 and +7 charged ions. Comparing the exchange behaviour, we confirm once more the relative order of stability for the 5 proteins. The maximum number of deuteriums incorporated during this time period is 86 for Y64, 90 for Y64L and for Y64F, 94 for Y64V, 105 for Y64A. The addition of higher concentrations of acid led to increased exchange rates. At 2% acetic acid (pH 1.9), the H/D exchange rates of the wild-type and mutants were equivalent (129 H/D exchanges), indicating that the structures were 'disrupted' or 'opened'.

Our results presented here confirm the previous observation that the replacement of Tyr64 in the *Desulfovibrio vulgaris* cytochrome c_{553} by Phe, Leu, Val or Ala has a destabilizing effect, increasing with decreasing side chain volume. The small difference in the behaviour of the wild-type protein and the Y64F and Leu mutants indicates that the contribution of Tyr64 to the stability of the protein is predominated by hydrophobic interactions rather than by the hydrogen bond with the Lys8 amide proton [24]. In this paper, we have shown that the isotopic exchanges study monitored by ESI-MS is a sensitive method probing the three-dimensional structure of proteins and the stability study of amino acid replacement in protein. Based on CSD and H/D exchange studies a stability ladder being wild-type > Y64F = Y64L > Y64V > Y64A is proposed. At last, we have demonstrated that these combined methods are useful tools to show the presence of conformers in solution and therefore this technique might be extended to other studies such as protein folding or interaction between protein / protein or protein / DNA complex by the masking effect.

ACKNOWLEDGMENTS

Cécile Vigouroux is acknowledged for her technical support. Perkin-Elmer Sciex and the C.E.A. are also acknowledged for the Philippe Guy fellowship.

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