New conformational properties induced by the replacement of Tyr-64 in *Desulfovibrio vulgaris* Hildenborough ferricytochrome c_{553} using isotopic exchanges monitored by mass spectrometry

Philippe Guy[a], Michel Jaquinod[a], Hervé Rémigy[a], Jean-Pierre Andrieu[a], Jean Gagnon[a], Beate Bersch[a], Alain Dolla[b], Laurence Blanchard[b], Françoise Guerlesquin[b], Eric Forest[a,*]

[a] *Institut de Biologie Structurale Jean-Pierre Ebel, CEA/CNRS, 41 Avenue des Martyrs, 38027 Grenoble Cedex 1, France*
[b] *Laboratoire de Bioénergétique et Ingénierie des Protéines CNRS, 31 Ch. J. Aiguier, 13402 Marseille Cedex, France*

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Abstract In order to study the conformational stability induced by the replacement of Tyr-64 in *Desulfovibrio vulgaris* Hildenborough (DvH) cytochrome c_{553}, fast peptic digestion of deuterated protein followed by separation and measurement of related peptides using liquid chromatography coupled to electrospray ionization mass spectrometry was performed. We show that the H-bonding and/or solvent accessibility properties were modified by the single-site mutation. The mutant proteins can be classified into two groups: the Y64F and Y64L mutants with nearly unchanged deuterium incorporation compared to the wild-type protein and the Y64S, Y64V and Y64A mutants with increased deuterium incorporation. The 70–74 peptide was the most affected by mutation of Tyr-64, the phenylalanine mutant inducing slight stabilization whereas the serine mutant was significantly destabilized. In addition, from the analysis of the overlapping 37–57 and 38–57 peptides we can conclude that the amide proton of Tyr-38 has been replaced by deuterium in all proteins.

Key words: Electrospray ionization mass spectrometry; Hydrogen/deuterium exchange; Peptic cleavage; Conformation; *Desulfovibrio vulgaris* Hildenborough ferricytochrome c_{553}

1. Introduction

Proteins are one of the most abundant and important biopolymers, and one aim of biochemistry is to understand the factors that control their stabilities as well as their activities. The relationship between activity and 3D structure can be studied by site-directed mutagenesis techniques followed by circular dichroism [1], calorimetry measurement [2], NMR [3], X-ray crystallography, and more recently by MS [4]. ESI-MS was first used to determine the molecular mass of proteins up to 100 kDa with an accuracy of 0.01% [5]. The charge-state distribution, resulting from the electrospray ionization process, can be used as a snapshot of the protein conformation in solution [6,7] and several authors have used it to probe the influence of parameters such as temperature [8], pH [9–11] or solvent [12]. However, the charge state distribution is not always sufficient, and therefore the introduction of isotopic exchanges monitored by ESI-MS seems to be a promising and complementary approach to study further the conformational properties of proteins [13–17]. The H/D exchanges in proteins are known to be dependent on the accessibility of labile hydrogens to the solvent. Hydrogens which are buried in the hydrophobic core or involved in H-bonding found in secondary structures, such as α-helices or β-sheets, are slowly exchanged [3,6,18]. To give a more precise description of the location of isotope exchange sites, we have developed a new procedure (summarized in Fig. 1) based on fast peptic cleavage of deuterated protein at 0°C, pH 2, followed by fast separation using liquid chromatography (LC) coupled to UV and MS detection [19].

In this paper, we describe the conformational study of a ferricytochrome c_{553} isolated from a sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough (DvH). The DvH cytochrome c_{553} is of great interest because of its very low redox potential (+20 mV), compared to the normal range of approx. 200–450 mV for the cytochrome *c* family. The three-dimensional structure of this protein has recently been determined [20]. Several studies have shown that aromatic residues are often involved in the preservation of the hydrophobic core around the active center and in hydrogen bonding networks [21,22]. These residues are also supposed to act during the electron-transfer process [23]. Tyr-64 is situated close to the N-terminal helix, the C-terminal helix and the heme [20]. It has been replaced by Phe, Leu, Val, Ala and Ser (Y64F, Y64L, Y64V, Y64A, Y64S) in order to study its possible role in electron transfer and redox potential regulation. These mutants were submitted to H/D exchanges, to determine modifications in solvent accessibility due to structural perturbations. The deuterated sites were located using peptic cleavages combined with mass spectrometry.

2. Materials and methods

2.1. Materials

Acetic acid-*d* and deuterium oxide were purchased from Sigma (St Louis, MO, USA). Poly(propylene) glycol was purchased from Aldrich (Aldrich-Chemie, Steinheim, Germany). DvH wild-type and mutants ferricytochrome c_{553} were obtained as previously described [24,25].

2.2. H/D exchange

For the analysis of deuterated peptides, 3 nmol of the ferricytochromes c_{553} were dissolved in deuterated water (p*D* 5.8) at a final concentration of 300 pmol/μl. Exchange reactions were carried out at

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

Abbreviations: ESI, electrospray ionization; MALDI, matrix-assisted laser desorption/ionization; H/D, hydrogen/deuterium; DvH, *Desulfovibrio vulgaris* Hildenborough; LC, liquid chromatography; MS, mass spectrometry.

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25°C for 3 h, then quenched by addition of 190 μl of an aqueous solution of HCl (pH 1.9).

2.3. Enzymatic hydrolysis

For the enzymatic hydrolysis reactions, 400 μl of immobilized pepsin (Pierce Chemical Co., Rockford, USA) were deposited into the filter cup of an ultra free Mc filter 10000 MWN (Millipore, Yonezawa, Japan) and washed 3 times with 190 μl of the aqueous solution of HCl (pH 1.9) to eliminate possible contamination. After quenching, the deuterated cytochromes c_{553} (3 nmol) were digested with immobilized pepsin for 40 s at 25°C under nitrogen, at pH 1.9 at a substrate-to-enzyme ratio 1:50 (w/w). The reaction was stopped by high-speed centrifugation (Jouan A14 Microfuge, Jouan, France) for 30 s at 12000×*g* and the released peptides were separated as described below.

2.4. ESI-MS

ESI mass measurements were performed on a Sciex API III+ triple quadrupole mass spectrometer (Perkin-Elmer Sciex Instruments, Thornhill, Ontario, Canada) equipped with a nebulizer-assisted electrospray (ionspray) source. Calibration was performed using poly(propylene) glycol 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 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* 400 to 1400 with 0.5 Da steps and 2 ms per step. MacBioSpec software (Perkin-Elmer Sciex) was used to calculate theoretical peptide molecular mass.

2.5. LC/ESI-MS of peptic digests

Liquid chromatography (LC) was directly coupled to ESI mass spectrometry using a 140B syringe pump system (Applied Biosystems, Foster City, USA). Digest mixtures were immediately injected onto a Brownlee reverse-phase C_{18} column (5 μm, 2.1 mm×150 mm, Applied Biosystems) at a flow rate of 170 μl/min. Using a Valco T, a split of 1/6 was applied such that approx. 30 μl/min was directed to the mass spectrometer. The remaining effluent was diverted to an Applied Biosystems 785 UV detector monitoring at 214 nm. Peptide separations were achieved using a linear gradient of 15–50% acetonitrile (0.1% TFA) for 15 min. Solvents and column were stored in a ice bath. Mass spectra (*m/z* 400–1400) were acquired with a 2 ms dwell time per step of 0.5 Da.

2.6. MALDI-MS

MALDI mass spectra were recorded on a Perseptive Voyager instrument (Perseptive Biosystems, Cambridge MA, USA). α-Cyano-4-hydroxycinnamic acid dissolved in 70% acetonitrile solution was used as matrix. 1 μl of the digests (15 pmol/μl), dissolved in an aqueous solution of methanol/water (50:50) containing 5% acetic acid, was mixed with 1 μl of the matrix solution. 1 μl of this mixture was deposited on the target and dried. Spectra were recorded from 30 laser shots (nitrogen laser, 337 nm). The instrument was calibrated using human insulin or ACTH/angiotensin I mixture as external standard. All ions were accelerated with an extraction voltage of 30 keV.

2.7. Amino acid sequencing

Amino acid sequences of the cytochrome c_{553} peptides were obtained using an Applied Biosystems sequenator (477A) coupled to an on-line PTH-amino acid analyzer (120A). All sequencing reagents were obtained from Applied Biosystems.

3. Results and discussion

3.1. Identification of peptic peptides

Comparison of the total ion current profiles of the peptic digests of wild-type and mutant proteins showed that similar retention times for peptic fragments were obtained (see Fig. 2), indicating that mutations of Tyr-64 had no influence on peptic cleavage. Note that the relative intensities of the total ion current profiles were not identical, mainly for the Y64A mutant. These variations were essentially due to the 58–69 peptide which contains the mutation. The ESI process which produced multiply charged ions complicated the mass attribution and was not suited for the unambiguous identification of peptic peptides. Therefore, an aliquot of each protein peptic mixture was measured by MALDI-MS, which gave essentially singly charged ions. The Y64A peptic mixture measured in the MALDI reflectron mode is shown in Fig. 3. Surprisingly, peptides present with poor yield of cleavage were observed by MALDI-MS. This phenomenon was probably due to better sensitivity and different desorption from the matrix with this ionization technique. However, peptides presenting lower masses were absent in the MALDI spectrum because of the matrix selectivity. In order to eliminate possible misinterpretation due to the non-specific cleavage of pepsin, peptides were also sequenced by Edman degradation. The analyzed peptides were shown to cover the whole protein sequence (Table 1).

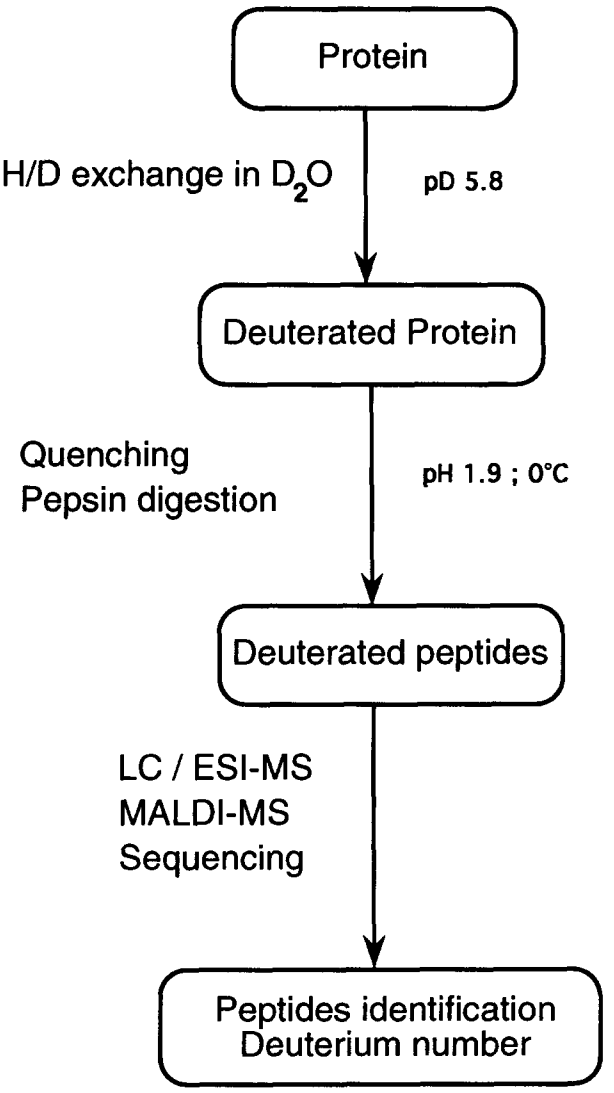

Fig. 1. Schematic representation of H/D exchange followed by peptic digestion and mass spectrometry analysis.

3.2. Analysis of the peptic peptides from the deuterated wild-type

For each peptide, deuterium incorporation was calculated

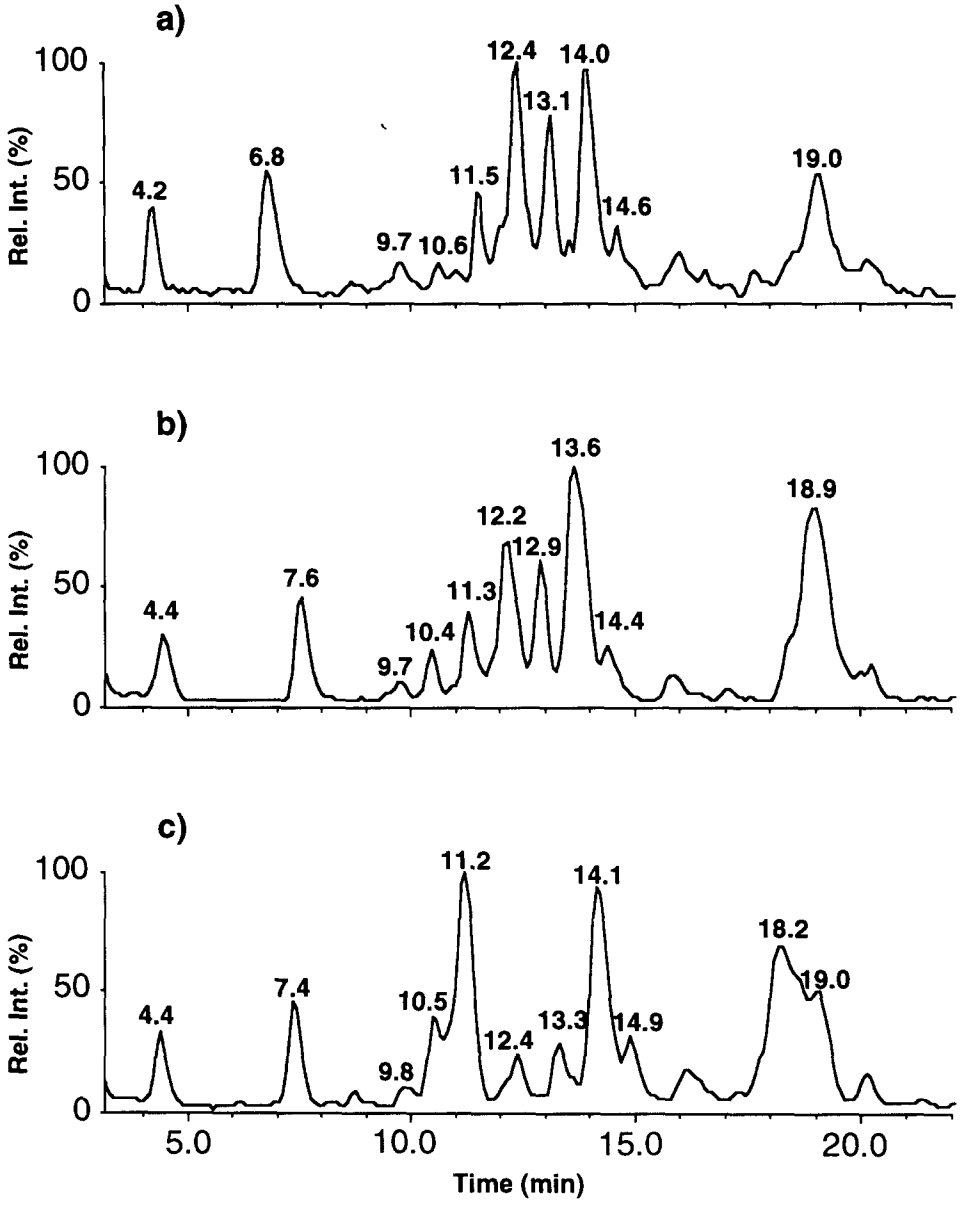

Fig. 2. Comparison of the total ion current profiles of the Y64L (a), Y64F (b) and Y64A (c) peptic digested mutants.

as a mass increase compared to the theoretical value. The accuracy of the mass measured for peptides was ±0.3 Da. Deuterium incorporation can be related to the stability of the corresponding peptide in the native protein. In the case of DvH wild-type c_{553} we can identify peptic peptides (Table 1) with relatively low deuterium incorporation (9 and 10% for the 7–37 and 70–74 peptides, respectively), and relatively high incorporation (18, 20 and 21% for the 75–79, 37–57 and 58–69 peptides, respectively). However, as only few amide protons were exchanged in each fragment and because the central fragments were quite long, we cannot draw conclusions on significantly stabilized or destabilized regions in this protein.

There are two possible reasons for the low number of exchanged amide protons: high stability of the native protein with few amide protons being accessible to solvent or back exchange of deuteriums during separation and analysis. Previous NMR measurements indicated very low exchange rates for amide protons belonging to two helices (residues 38–41 and 69–77) with lifetimes of more than a few weeks at room temperature [26]. On the other hand, the half-life of the reverse exchange reaction (i.e. replacement of hydrogen with deuterium) has been measured by mass spectrometry and corresponds to 1 h [14].

3.3. *Comparison between the peptic peptides from the deuterated mutants of cytochromes* c_{553}

As shown in Table 1, the proteins can easily be separated into two groups as the wild-type and the Y64F, Y64L mutants are found to incorporate less deuterium than the Y64S, Y64V and above all the Y64A mutants. This was particularly true for the central peptides where the exchange in the N- and C-terminal fragments (fragments 1–6 and 75–79) was comparable for all proteins. The most significant differences corresponded to the 7–37, 38–57 and 37–57 segments with an increase in deuterium incorporation of 2 or 3 for the Y64A, Y64S and Y64V mutants. The 58–69 peptide had almost the

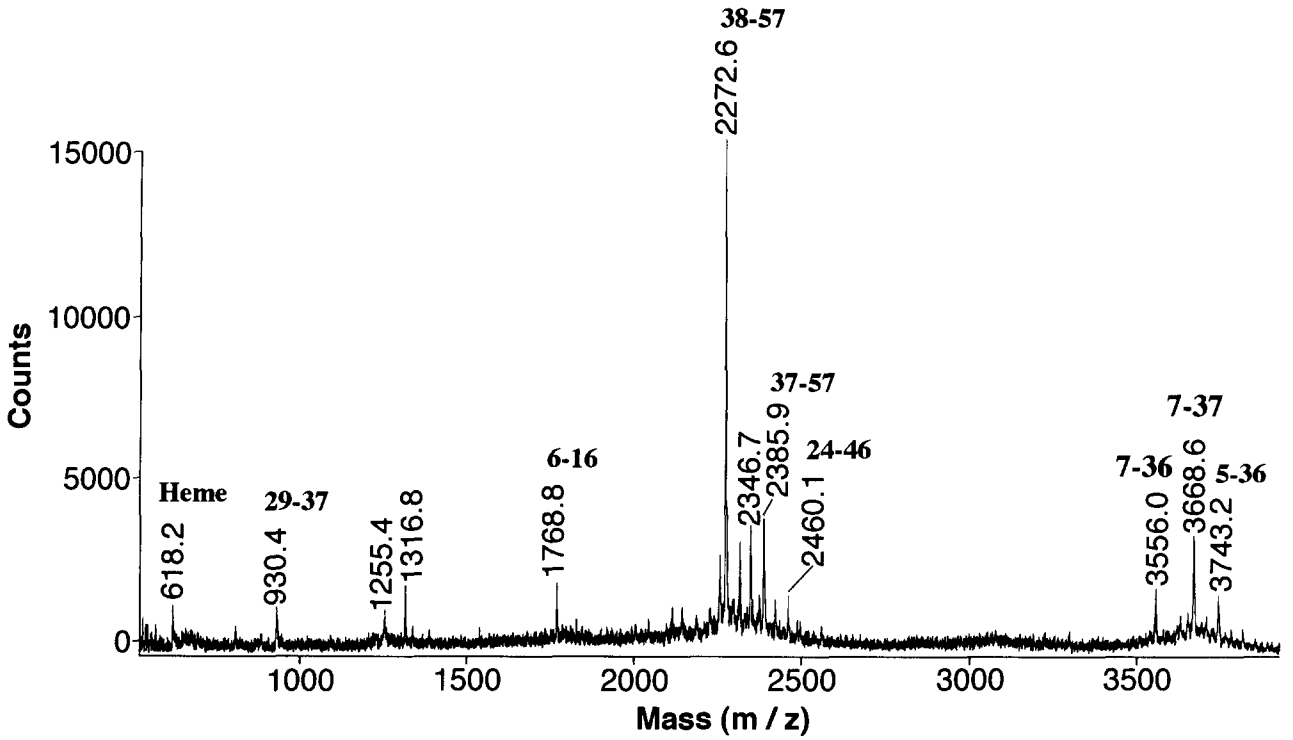

Fig. 3. MALDI spectrum acquired in the reflectron mode of the crude peptic digested Y64A mutant.

same values for the Y64A, Y64S and Y64V mutants with approximately one more deuterium in the backbone amide than for the wild-type, Y64F or Y64L mutants. The 70–74 peptide deuterium content was low for the Y64F mutant whereas two deuteriums were measured in the Y64S mutant. However, the amount of deuterium incorporation for the N- and C-terminal segments clearly showed that these peptides have the lowest mass increase, similar for all the mutants.

The overlapping 37–57 and 38–57 peptides displayed a mass difference for deuterium incorporation of approx. 1.0 Da for all proteins. This indicated that the tyrosine amide NH backbone in position 38 incorporates a deuterium. Thus, the presence of overlapping peptides increased the spatial resolution of the method. It is of note that this deuterium incorporation in Tyr-38 was not influenced by the mutation.

The Tyr-64 mutation resulted in local modifications of the protein conformation leading to different peptic peptide exchange rates. The replacement of the tyrosine with serine, alanine or valine had a destabilizing effect. This led to greater accessibility of the backbone amide proton to the deuterated

Table 1
Peptides obtained from the peptic digestion of deuterated wild-type and mutant DvH ferricytochrome c_{553}

Structure[a]	Loc.	M_c (Da)	N	ΔM					
				WT	Y64L	Y64F	Y64S	Y64V	Y64A
Helix 1	1–6	516.3	5	0.7	0.5	0.9	1.2	0.6	1.0
Helix 1 Ω-loop β-Turn	7–37	3667.5	29	2.6	3.1	2.9	5.0	5.2	5.9
Helix 2 Loop	38–57	2271.7	19	2.8	2.5	2.7	4.4	5.4	6.1
Helix 3	37–57	2384.8	20	4.0	3.6	4.1	5.6	6.4	7.1
Helix 3 Turn Helix 4	58–69	[b]	11	2.3	2.7	2.4	3.7	3.5	4.0
Helix 4	70–74	516.3	4	0.4	0.4	0.1	2.0	1.3	1.6
Helix 4	75–79	640.3	4	0.7	0.9	0.8	1.2	1.0	0.9

The accuracy of the ΔM values for the several peptides corresponds to ± 0.3 Da.
[a]The three-dimensional structure of the cytochrome c_{553} is reported for each peptide from NMR results [24]; Loc., localization of peptic peptides. M_c, calculated mass; ΔM, mass difference corresponding to deuterium incorporation; N, number of exchangeable backbone NH.
[b]58–69 peptide calculated mass is 1396.5 Da in the case of the wild-type, 1346.5 Da for Y64L, 1380.5 Da for Y64F, 1320.4 Da for Y64S, 1332.5 Da for Y64V, and 1304.4 Da for Y64A.

solvent. These results were in good agreement with a previous study using H/D exchanges of the whole protein monitored by ESI-MS which showed that the various cytochromes c_{553} can be classified by a stability ladder with the wild-type > Y64-F=Y64L > Y64V > Y64A [27]. In the case of Y64S mutant, Blanchard et al. [25] have shown that this particular mutant had a stability close to the Y64V.

In conclusion, we have shown that mass spectrometry can provide structural information based on deuterium incorporation in proteins. This new approach was used on the ferricytochrome c_{553} and several mutants in which the Tyr-64 was replaced by either phenylalanine, leucine, valine, alanine or serine. We have shown that regions involved in structural perturbation can be assigned. This method can be applied in a relatively short time, requires only nanomole quantities, and large proteins can be analyzed.

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References

[1] Hurley, J.K., Caffrey, M.S., Markley, J.L., Cheng, H., Xia, B., Chae, Y.K., Holden, H.M. and Tollin, G. (1995) Protein Sci. 4, 58–64.
[2] Linske-O'Connell, L.I., Sherman, F. and McLendon, G. (1995) Biochemistry 34, 7103–7112.
[3] Kim, K.S., Fuchs, J. and Woodward, C. (1993) Biochemistry 32, 9600–9608.
[4] Chowdury, S.K., Katta, V. and Chait, B.T. (1990) J. Am. Soc. Mass Spectrom. 112, 9012–9013.
[5] Mann, M. and Wilm, M. (1995) Trends Biochem. 20, 219–224.
[6] Suckau, D., Shi, Y., Beu, S.C., Senko, M.W., Quinn, J.P., Wampler III, F.M. and McLafferty, F.W. (1993) Proc. Natl. Acad. Sci. USA 90, 790–793.
[7] Ashton, D.S., Bedell, C.R., Cooper, D.J., Green, B.N. and Oliver, R.W.A. (1993) Org. Mass Spectrom. 28, 721–728.
[8] Mirza, U.A., Cohen, S.L. and Chait, B.T. (1993) Anal. Chem. 65, 1–6.
[9] LeBlanc, J.C.Y., Guevremont, R. and Siu, K.W.M. (1993) Int. J. Mass Spectrom. Ion Processes 125, 145–153.
[10] Ogorzalek-Loo, R.R., Loo, J.A., Udseth, H.R., Fulton, J.L. and Smith, R.D. (1992) Rapid Commun. Mass Spectrom. 6, 159–165.
[11] Feng, R. and Konishi, Y. (1993) J. Am. Soc. Mass Spectrom. 4, 638–645.
[12] Loo, J.A., Ogorzalek Loo, R.R., Udseth, H.R., Edmonds, C.G. and Smith, R.D. (1991) Rapid Commun. Mass Spectrom. 5, 101–105.
[13] Katta, V. and Chait, B.T. (1991) Rapid Commun. Mass Spectrom. 5, 214–217.
[14] Thevenon-Emeric, G., Kozlowski, J., Zhang, Z. and Smith, D.L. (1992) Anal. Chem. 64, 2456–2458.
[15] Stevenson, C.L., Anderegg, R.J. and Borchardt, R.T. (1993) J. Am. Soc. Mass Spectrom. 4, 646–651.
[16] Miranker, A., Robinson, C.V., Radford, S.E., Aplin, R.T. and Dobson, C.M. (1993) Science 262, 896–900.
[17] Anderegg, R.J. and Wagner, D.S. (1995) J. Am. Chem. Soc. 117, 1374–1377.
[18] Wagner, D.S., Melton, L.G., Yan, Y., Erickson, B.W. and Anderegg, R.J. (1994) Protein Sci. 3, 1305–1314.
[19] Jaquinod, M., Halgand, F., Caffrey, M., Saint-Pierre, C., Gagnon, J., Fitch, J., Cusanovich, M. and Forest, E. (1995) Rapid Commun. Mass Spectrom. 9, 1135–1140.
[20] Blackledge, M.J., Medvedeva, S., Poncin, M., Guerlesquin, F., Bruschi, M. and Marion, D. (1995) J. Mol. Biol. 245, 661–681.
[21] Kassner, R.J. (1972) Proc. Natl. Acad. Sci. USA 69, 2263–2267.
[22] Sandberg, W.S. and Terwilliger, T.C. (1989) Science 245, 54–57.
[23] Dolla, A., Guerlesquin, F., Bruschi, M. and Haser, R. (1991) J. Mol. Recognition 4, 27–33.
[24] Blanchard, L., Marion, D., Pollock, B., Voordouw, G., Wall, J., Bruschi, M. and Guerlesquin, F. (1993) Eur. J. Biochem. 218, 293–301.
[25] Blanchard, L., Dolla, A., Bersch, B., Forest, E., Bianco, P., Wall, J., Marion, D. and Guerlesquin, F. (1994) Eur. J. Biochem. 226, 423–432.
[26] Marion, D. and Guerlesquin, F. (1992) Biochem. 31, 35, 8171–8179.
[27] Guy, P., Remigy, H., Jaquinod, M., Bersch, B., Blanchard, L., Dolla, A. and Forest, E. (1996) Biophys. Biochem. Res. Commun. 218, 97–103.