Hydrogen exchange monitored by MALDI-TOF mass spectrometry for rapid characterization of the stability and conformation of proteins

Josep Villanueva, Francesc Canals, Virtudes Villegas, Enrique Querol, Francesc X. Avilés*

Institut de Biologia Fonamental and Departament de Bioquímica, Universitat Autònoma de Barcelona, 08193 Bellaterra (Barcelona), Spain

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Abstract Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used to monitor hydrogen exchange on entire proteins. Two alternative methods have been used to carry out the hydrogen exchange studies, exchanging deuteron (H to D experiments) or proton (D to H experiments). In the former case, the use of a deuterated matrix has made possible to overcome back-exchange problems and attain reproducible results. The methods presented have been used to determine the slow exchange core of the potato carboxypeptidase inhibitor in different folding states, and to differentially compare the activation domain of human procarboxypeptidase A2 versus three site-directed mutants of different conformational stability. In this work, we show that by using MALDI-TOF MS to monitor hydrogen exchange in entire proteins, it is possible to rapidly check the folding state of a protein and characterize mutational effects on protein conformation and stability, while requiring minimal amounts of sample.

Key words: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Hydrogen exchange; Conformational stability; Potato carboxypeptidase inhibitor; Activation domain of human procarboxypeptidase A2

1. Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has recently emerged as an effective tool for the mass measurements of biomolecules [1–3]. Simplicity, high accuracy (typically ±0.1%, and ±0.01% for polypeptides below 10 kDa), theoretically unlimited mass range and extreme sensitivity (pmol to subpmol range) have made of MALDI-TOF MS an excellent method for routine mass analysis.

Hydrogen exchange of amide backbone protons has been used for many years to study different aspects of protein structure [4–6]. A major problem for the measurements of hydrogen exchange results from the fact that during sample preparation and analysis the deuterium content of a protein or peptide sample can be drastically altered by back-exchange reaction. In the case of MALDI-TOF MS, the sample preparation procedure requires mixing the protein or peptide sample with a matrix. During this sample preparation process, back-exchange with protons from the matrix, solvent or the water vapor in the atmosphere [7,8] can occur.

Recently, a MALDI-TOF MS hydrogen exchange method for the study of amide proton solvent accessibility of a protein has recently been reported [9]. The method is based on the determination of amide hydrogen exchange rates of peptides obtained by digestion of the target protein. The referred work demonstrates that the deuterons in amide groups of peptides can be preserved with minimal loss through the sample preparation, thanks to the acidic conditions where H/D exchange rates are minimum. H/D exchange experiments monitored by MALDI-TOF have also been used to study the conformational stability of insulin adsorbed on to different solid surfaces [10] and to probe conformational changes of peptides in mixtures of organic solvents and water [11].

We report here the use of MALDI-TOF MS to measure hydrogen exchange to determine the number of slow exchanging hydrogens of entire proteins in order to assess their conformation and stability in solution. By using a deuterated matrix solution and carrying out the exchange solutions and sample preparation under an inert atmosphere, we have overcome the problem of back-exchange reactions. Under these conditions, MALDI-TOF MS measurements can provide accurate and reproducible determination of the number of slow exchange hydrogens of a protein. Alternatively, hydrogen exchange has been measured as D/H exchange starting from the fully deuterated proteins. In this case, no special precautions on sample preparation for MALDI-MS need to be taken. The results observed by this D/H exchange approach are in agreement with those observed for the 'direct' H/D measurements. To show the capability and feasibility of this methodology, we have chosen as model proteins potato carboxypeptidase inhibitor (PCI) [12,13], the activation domain of human procarboxypeptidase A2 (ADA2h) [14–16], and three site-directed mutants of this protein, rationally engineered to increase conformational stability [15]. A series of hydrogen exchange reactions have been carried out in order to determine the number of slow exchange hydrogens. In the case of the ADA2h proteins, the results obtained have been correlated to their conformational stability [15].

2. Materials and methods

2.1. Materials

D_2O and DCl were purchased from Aldrich Chemicals Company. Glycine, $NaHPO_4$, sinapic acid and dithiothreitol (DTT) were obtained from Fluka. Deuterated urea was obtained from Sigma. Acetonitrile was purchased from Romil. PCI, wild type ADA2h (Wt-ADA2h) and its site-directed mutants were obtained as previously described [13,15,16].

*Corresponding author. Fax: (34)-93-581 2011.
E-mail: fx.aviles@blues.uab.es

Abbreviations: MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PCI, potato carboxypeptidase inhibitor; ADA2h, activation domain of human procarboxypeptidase A2

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2.2. H/D exchange (H to D exchange)

All H/D exchange reactions were carried out at room temperature in 0.5 ml microcentrifuge capped tubes. The reaction mixtures were prepared under a soft N_2 flow and the reaction tubes were then sealed with parafilm. Values given in the text for pH and pD were taken directly from the pH meter and were not corrected for isotopic effects [17].

2.3. PCI

For hydrogen exchange experiments on PCI, lyophilized samples of the protein were dissolved in 20 mM deuterated glycine buffer, pD 3.0, to a concentration of 0.1 mg/ml. Aliquots were taken at different times and analyzed by MALDI-TOF MS until an exchange plateau was reached. To study the exchange of the unfolded state, lyophilized PCI was dissolved at 1 mg/ml in 50 mM deuterated Tris–HCl pD 8.5 containing 3 M deuterated urea and 50 mM deuterated DTT [18]. Aliquots of the sample were taken at different times, diluted 1/15 (by volume) in the matrix solution and analyzed by MALDI-TOF MS.

2.4. Wt-ADA2h and mutants

Lyophilized samples of Wt and mutant forms of ADA2h were dissolved in 20 mM Na_2DPO_4 (pD 7.0) to a concentration of 0.2 mg/ml. Aliquots were taken at different times and analyzed by MALDI-TOF MS until an exchange plateau was reached. The unfolded state of Wt-ADA2h was studied by dissolving lyophilized aliquots of the protein to a concentration of 2 mg/ml in 20 mM Na_2DPO_4 (pD 7.0) containing 5 M deuterated urea. Aliquots of the sample were taken at different times, diluted 1/25 (by volume) in the matrix solution and analyzed by MALDI-TOF MS.

2.5. D/H exchange (D to H exchange)

Lyophilized aliquots of the proteins were dissolved in 100 mM deuterated glycine at pD 3.0 to a concentration of 0.5 mg/ml (PCI) or in 100 mM Na_2DPO_4 (pD 7.0) to a concentration of 1 mg/ml (Wt-ADA2h and mutants). Samples were incubated at 90°C for 1 h and 30 min (PCI) or 3 h (Wt-ADA2h and mutants) to attain a complete exchange of the labile deuterons, and subsequently allowed to refold for 30 min at room temperature. The native deuterated proteins were then allowed to exchange by dilution with nine volumes of deionized water. Aliquots were taken at different times and analyzed by MALDI-TOF MS until an exchange plateau was reached.

2.6. Preparation of samples for MALDI-TOF MS

Exchange reactions and sample preparation were carried out at room temperature. The matrix used in H/D exchange studies was a solution containing 5 mg/ml of sinapic acid in 0.05% DCl (in D_2O)/acetonitrile 1:2 by volume (pD 2.5). The matrix was exchanged with D_2O overnight before use. Preparation of samples was always performed in the MALDI-TOF spectrometer room, under a soft N_2 flow. The parafilm-sealed 0.5 ml microcentrifuge tubes containing the samples were opened under a N_2 flow and 0.5 μl of sample was placed on a sample probe spot containing 0.5 μl of matrix solution. The mixture was mixed by pipetting and allowed to dry for 1–2 min under gentle warming with a tungsten lamp. All dried samples were washed once with 1 μl of D_2O in order to wash away the salts and to minimize the back-exchange that could occur during the sample drying. Once the samples were dried, the probe was immediately transferred into the mass spectrometer.

D/H exchange reaction samples were prepared with undeuterated matrix solution following standard procedures [1].

2.7. MALDI-TOF MS

All mass spectra were acquired on a Bruker Biflex TOF mass spectrometer equipped with a nitrogen laser with an emission wavelength of 337 nm. Spectra were obtained in the linear mode at an accelerating voltage of 19 kV. Deflection of the low mass ions was used to enhance the target protein signal. An external (next spot) calibration was performed for each measurement with the same native unlabelled protein (standard). Each mass determination was averaged from three sample–standard pairs. The accuracy in mass determination obtained for PCI experiments was ±0.9 U and for the Wt-ADA2h and site-directed mutants was ±1.9 U. Mass values given throughout the text correspond to *m/z* of the centroid of the envelope of the mass peaks for protonated molecular ions $[M+H]^+$.

3. Results and discussion

3.1. Measurement of hydrogen exchange (H/D) by MALDI-TOF MS

In order to develop a reliable method for monitoring proton/deuteron exchange by MALDI-TOF MS, a series of trials were initially performed to optimize the sample preparation procedure. The 11 amino acid peptide substance P, in which all the labile protons are expected to be accessible to the solvent, was chosen as a test compound for full exchange. This peptide (sequence RPKPQQFFGLM, molecular mass 1347.6 U) contains a total of 21 labile hydrogens. In a first set of hydrogen exchange experiments, the increase of mass in substance P due to deuteration was measured by MALDI-TOF MS using a standard procedure for sample preparation [1,2]. Preparation of the sample with non-deuterated matrix and no additional precautions resulted in a measured 40–60% deuteration, even after several hours of exchange. These observed low levels, in agreement with previous results [9], can be the consequence of back-exchange reaction due to the water vapor of the laboratory atmosphere [7,8] and to the quenching step [19]. In addition, when identical samples where analyzed in parallel using the same procedure, a very high variability in the measured deuterium contents was observed, as a result of the different extent of the back-exchange reaction. Similar low and highly variable levels of measured deuterium contents were observed on deuteration of model proteins (PCI and the Wt-ADA2h, see below), when analyzed by MALDI-TOF MS. This makes it very difficult obtaining meaningful H/D exchange data by this technique. To overcome the problems caused by the back-exchange reaction, we have assayed to introduce some modifications in the standard MALDI-TOF MS sample preparation procedure, in order to keep the deuteration of most of the faster exchanging protons through the sample preparation and analysis. The first modification was the use of a deuterated matrix (in this case sinapic acid), in a similar way to the procedure used by Buijs et al. [10]. The use of a deuterated matrix allowed us to achieve deuteration values of 80% on substance P. A second modification in the procedure consisted in performing both the exchange reaction and the sample preparation for MALDI-TOF MS under a dry N_2 atmosphere. Finally, D_2O was used to wash away the salts once the sample had been crystallized. This washing step could also contribute to the deuteration of the most labile protons that may back-exchange during the drying step of the sample.

The combination of all the above modifications resulted in much higher measured exchange levels. Fig. 1 shows the MALDI-TOF spectra of substance P unexchanged (Fig. 1A) and after 10 min incubation with D_2O (Fig. 1B). The measured average molecular mass of the exchanged species (1367.7 U) presents an increase of 19.5 U over the average molecular mass of unexchanged species (1348.2 U). This implies an exchange level of 93% (19.5 U/21 U). This result shows that under these conditions, the deuteration of most fast exchange protons can be kept through the MALDI-TOF MS analysis, leading to measured deuterium levels close to the theoretical values. At the same time, at the pH of the sample–matrix mixture (pH at which exchange rates are minimal) and during the relatively short time required for sample preparation and analysis, it is expected that exchange of the slowest exchanging protons is kept to a minimum, even if the

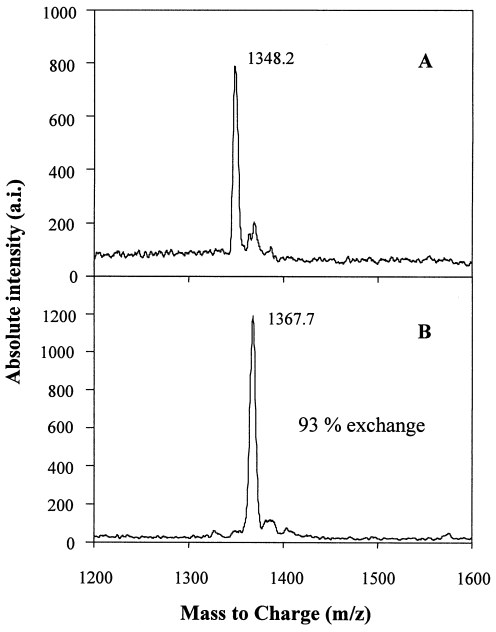

Fig. 1. MALDI-TOF mass spectra of substance P. The labels on the peaks indicate the measured average molecular mass. (A) Undeuterated peptide, substance P dissolved in deionized H_2O shows a dominant peak at *m/z* 1348.2 caused by naturally occurring isotopes. (B) Deuterated peptide, substance P dissolved in D_2O showing a dominant peak at *m/z* 1367.7, indicating a hydrogen exchange of 93% (see text). The peak at *m/z* M+206 corresponds to a matrix adduct.

presence of the matrix and of about 15% acetonitrile would affect the conformation of the protein [11]. The results presented below demonstrate that under these conditions, reliable measurements of the number of slow exchanging hydrogens for entire proteins by MALDI-TOF MS can be obtained.

Thus, with little modifications of the standard procedure for MALDI-TOF MS analysis on a stainless steel sample probe, this methodology allows to achieve accurate and reproducible measurements and requires very small amounts of protein, as only a few pmol are enough to carry out all experiments.

3.2. Hydrogen exchange in PCI

PCI is a small disulfide-bridged protein (molecular mass 4295.0 U) that contains a total of 65 labile hydrogens. Fig. 2A shows the MALDI-TOF mass spectrum of PCI 15 min after dissolving the protein in 20 mM deuterated glycine at pD 3.0. The molecular mass of the peak corresponding to the protein, with a mass/charge (*m/z*) of 4335.6, is higher than the molecular mass of PCI by 39.6 U. These results show that a partial hydrogen exchange of 61% occurred. As expected for a folded conformation, the exchange of PCI is not complete. Fig. 3A shows a kinetic hydrogen exchange experiment conducted on PCI and monitored by MALDI-TOF MS by measuring the *m/z* values at different times after dissolution of the protein in D_2O. After 2 h, an exchange plateau was reached where 83% of the labile protons had exchanged (Table 1). Despite of the low pD used, which is expected to keep hydrogen exchange at the minimum, the exchange reaction is considerably fast and reaches a value higher than what is the general case for a native state protein. However, these results agree with what is expected for this particular protein, since PCI has no regular secondary structure and is basically composed of loops linked by the disulfide bridges [12,20]. The subpopulation of 83% of the total labile protons that exchange rapidly in the native protein is expected to derive from side chain protons and solvent-exposed amide protons. Even though the percentage of exchanged protons is high for PCI in its native state, a number of protons still resist to exchange in native conditions. Incubation of PCI in a deuterated denaturing–reducing solvent (50 mM Tris–DCl, pD 8.5, containing 3 M deuterated urea and 50 mM deuterated DTT) renders a protein with a *m/z* of 4359.6, representing 91% of labile protons exchanged after only 15 min of incubation (Fig. 2B and Table 1). Thus, denaturation renders the majority of the labile protons accessible to the solvent.

We also monitored the hydrogen exchange reaction by an alternative procedure consisting in a D to H exchange (D/H exchange) experiment. All labile protons were first exchanged by deuterons in the denatured state of the protein and then, the protein was allowed to return to the native state. After subsequent dilution of the protein in a proton containing buffer, the reverse exchange to proton was monitored (see Section 2). Obviously, this procedure can only be applied to proteins with reversible denaturation. PCI was temperature-destabilized for 1 h at 90°C in D_2O, pD 3.0. Then the protein solution was changed to room temperature for 30 min. At this point, the protein solution was diluted 10-fold into proton buffer and the exchange was followed by MALDI-TOF MS as a function of time after diluting the sample (see Fig. 3B). An equilibrium was reached after 20 h, showing that approximately seven amide deuterons (10.7% of labile hydrogens) seem to be protected from exchange. This result agrees with the above H to D exchange (H/D exchange) experiment, in which the number of amide protons protected, measured by

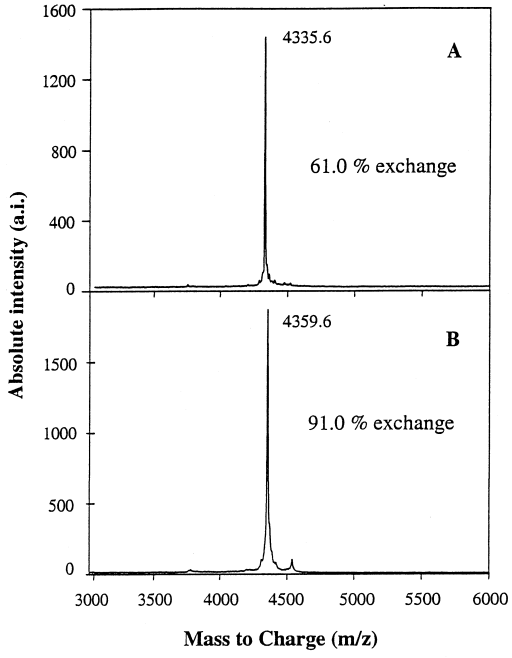

Fig. 2. MALDI-TOF mass spectra of exchanged PCI. The labels on the peaks give the measured molecular mass. (A) Mass spectrum of PCI 15 min after dissolving the protein in 20 mM deuterated glycine (pD 3.0). (B) Mass spectrum of PCI 15 min after dissolving the protein in 50 mM Tris–DCl containing 3 M deuterated urea and 50 mM deuterated DTT (pD 7.0). The peak at *m/z* M+206 corresponds to a matrix adduct.

the difference between the exchanged native and the complete exchanged denatured species, is about nine. The small difference observed between the two experiments is probably due to back-exchange.

The D/H exchange reaction was also performed on the reduced–denatured protein to demonstrate that no hydrogen protection is present in the PCI denatured state (data not shown). Since the final sample is diluted in a 1:20 H:D solvent, it could be expected that a residual 5% deuterons could be retained. However, since in the denatured state all hydrogens will have fast exchange rates, it is expected that they all can undergo exchange with ambient proton during sample preparation and this would explain that the observed number of deuterons retained is zero. Accordingly, the 10% of deuterons retained observed in the native state would correspond mainly to slow exchanging deuterons.

Interestingly, results obtained in both the H/D exchange (Fig. 3A) and the D/H exchange (Fig. 3B) experiments carried out on PCI agree with NMR results, that gave a number of about six amide hydrogens protected from exchange at similar pH [20]. This D/H exchange procedure, however equivalent to the H/D exchange procedure, has the important advantage of being independent from the back-exchange reaction. The good agreement between the results obtained by direct H/D exchange, the reverse D/H exchange measurements and the NMR data demonstrates the validity of the sample preparation procedure using deuterated matrix to determine the number of slow exchange protons in proteins. For proteins to which the D/H exchange procedure is possible, this could also be used for protein fragmentation/mass spectrometry studies [21].

It has been hypothesized that the last few NH hydrogens to exchange in a protein folded conformation are in the most rigid part of its structure and constitute a three dimensional cluster called the slow exchange core, which is expected to be formed by segments of secondary structure that are tightly packed and collapse early during folding [22,23]. This model is applicable to a number of proteins [23–26], although some exceptions have been reported [27,28]. Since the data on the slow exchange core obtained here from MALDI-TOF analysis do not provide information on the accurate location of the protected hydrogens, this hypothesis cannot be addressed by this methodology. Instead, the determination of the number of slow exchanging hydrogens is intended as to provide a protein stability parameter in order to discriminate between mutant forms or different folding states of a protein.

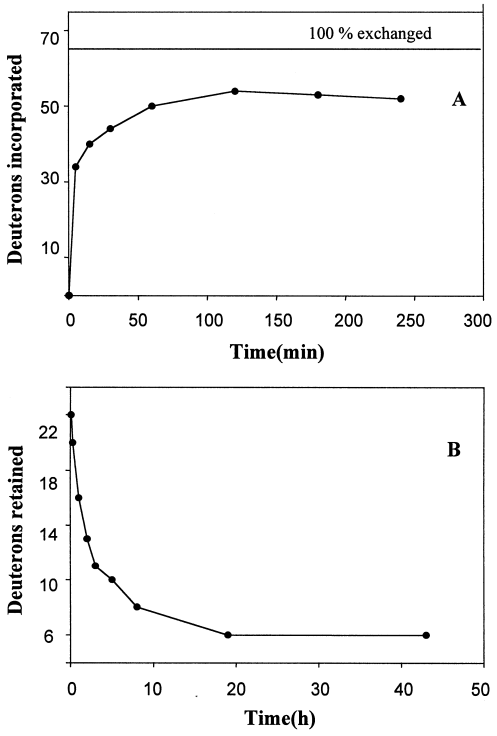

Fig. 3. Kinetic plots of the H/D exchange data obtained for PCI monitored by MALDI-TOF MS. (A) Increase in deuteration of PCI as a function of time of incubation in deuterated buffer (pD 3.0). The solid line above the plot marks the maximum expectable deuteration for PCI. (B) Decrease in deuteration of PCI as a function of time after dilution (1/10 by volume) of the complete deuterated protein in proton buffer. The number of deuterons was determined by calculating the centroid of each peak and subtracting the centroid of the undeuterated sample.

3.3. Hydrogen exchange in Wt-ADA2h and site-directed mutants

The Wt-ADA2h is a small α+β monomeric protein (81 residues) [14,29]. Recently, three site-directed mutants de-

Table 1
Comparison of the percentage of labile hydrogens exchanged at different times for the proteins studied in this work

Protein	Solvent	Labile hydrogens[a]	Exchanged in 15 min (%)	Exchanged in 2 h (%)	Exchanged in 45 h (%)	State
PCI	20 mM glycine in D_2O, pD: 3.0	65	61 ± 1.4[b]	83 ± 1.4	nd[c]	native
PCI	20 mM Tris–DCl, 3 M urea, 50 mM DTT in D_2O, pD: 8.5	71	91 ± 1.3	91 ± 1.3	nd[c]	denatured
Wt-ADA2h	50 mM sodium phosphate in D_2O, pD: 7.0	143	66.4 ± 1.3	70.6 ± 1.3	88.7 ± 1.3	native
Wt-ADA2h	50 mM sodium phosphate, 5 M urea in D_2O, pD: 7.0	143	91.2 ± 1.3	91.2 ± 1.3	nd[c]	denatured
M1-ADA2h	50 mM sodium phosphate in D_2O, pD: 7.0	146	51.3 ± 1.3	56.1 ± 1.3	71.2 ± 1.3	native[d]
M2-ADA2h	50 mM sodium phosphate in D_2O, pD: 7.0	140	52.8 ± 1.4	57.1 ± 1.4	71.5 ± 1.4	native[d]
DM-ADA2h	50 mM sodium phosphate in D_2O, pD: 7.0	143	50.3 ± 1.3	55.9 ± 1.3	68.5 ± 1.3	native[d]

[a]Labile hydrogens at pH 2.5. Masses of all mutants were corrected for amino acids changed.
[b]Error in percentage of deuteration calculated from triplicate measurements.
[c]Not determined.
[d]Mutants are native-like [15,16].

signed to increase the stability of the overall Wt-ADA2h fold have been obtained [15,16]. Each of its two α-helices was redesigned to introduce native-like local interactions. Mutations designed to stabilize the helix 1 of Wt-ADA2h were introduced in the mutant protein M1-ADA2h, and mutations to stabilize the helix 2 were introduced in M2-ADA2h [15]. Finally, a protein combining the two redesigned helices, DM-ADA2h, was produced [16].

Fig. 4A shows a H/D exchange reaction performed on Wt-ADA2h and their stabilized mutants by our MALDI-TOF MS approach. For each of the four proteins, a subpopulation of protons (approximately 50%) exchange in few minutes. These are expected to consist of side chain and solvent-exposed amide protons, and probably are the same for the Wt and mutant proteins. Interestingly, 15 min after starting the H/D exchange reaction, the exchange level of the Wt protein rises up to 66% while the mutant forms keep the exchange levels around 50% (see Table 1). After the first hour of exchange, a slow rate increase was observed for each of the four proteins. The reactions were further followed for 2 days, after which the Wt protein reached 88% deuteration while the mutants reached around 70%. This slow exchange behavior confirms that these proteins have a compact folded conformation in the conditions assayed, as it was previously suggested by urea denaturation experiments [15]. Only after following the

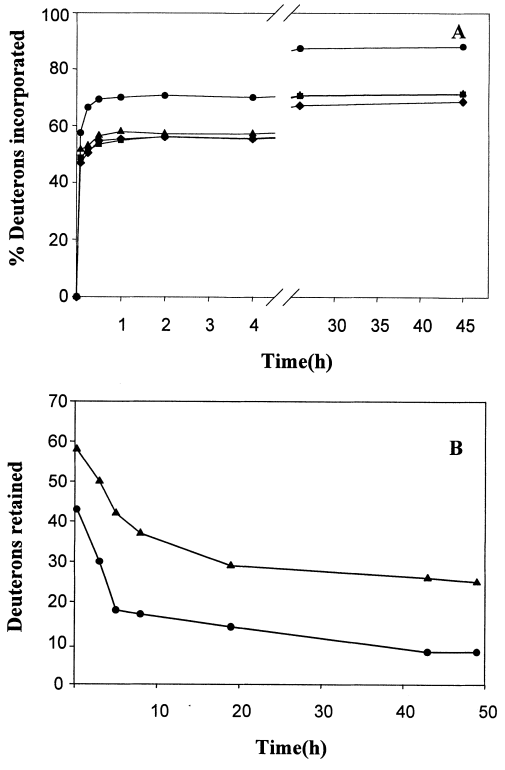

Fig. 4. Kinetic plots of the H/D exchange data obtained for Wt-ADA2h (●) and its mutants M1-ADA2h (■), M2-ADA2h (▲), DM-ADA2h (♦) monitored by MALDI-TOF MS. (A) Deuteration increase expressed as the percentage of their respective maximum expectable deuteration as a function of time of incubation in deuterated buffer. (B) Decrease in deuteration of Wt-ADA2h (●) and M2-ADA2h (▲) as a function of time after dilution (1/10 by volume) of the complete deuterated protein in proton buffer. The number of deuterons was determined by calculating the centroid of each peak and subtracting the centroid of the corresponding undeuterated protein.

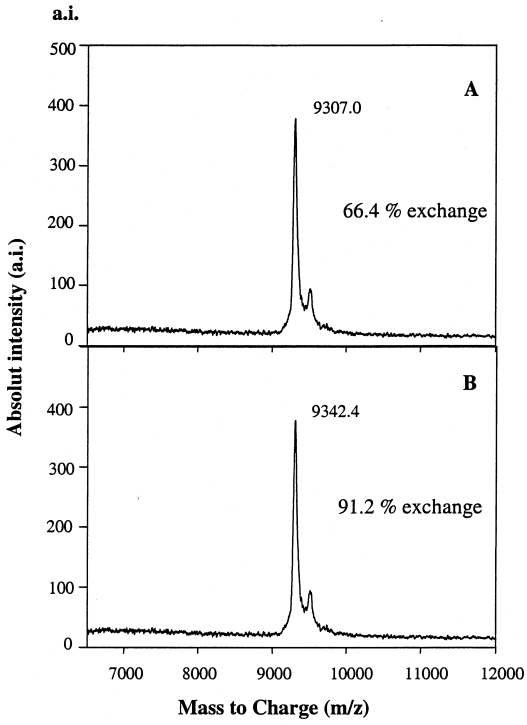

Fig. 5. MALDI-TOF mass spectra of exchanged Wt-ADA2h. The labels on the peaks give the measured molecular mass. (A) Mass spectrum of Wt-ADA2h 15 min after dissolving the protein in 20 mM Na_2DPO_4 (pD 7.0). (B) Mass spectrum of Wt-ADA2h 15 min after dissolving the protein in 5 M deuterated urea. The peak at *m/z* M+206 corresponds to a matrix adduct.

H/D reaction for 20–40 h, a difference in deuteration between the mutant forms was observed: 71% for the M1-ADA2h and M2-ADA2h proteins and 68% for the DM-ADA2h. This small difference is significant because the error in the measurement of the percentage of deuteration is about 1.3% (see Table 1). This difference agrees with previous results obtained by urea denaturation curves of Wt and mutant-stabilized forms of ADA2h, which showed that DM-ADA2h protein is the most stable of the three mutant forms [15,16]. The mass spectrum shown in Fig. 5A was obtained 15 min after dissolving the Wt-ADA2h in 20 mM Na_2DPO_4. Fig. 5B shows the spectrum of the same protein at the same time of exchange but under conditions which had previously been demonstrated to be denaturing (5 M urea) [30]. Both spectra differ in 35.4 U, indicating an exchange of 91.2% of the total of 143 labile protons in the denatured form.

In this case, hydrogen exchange has also been followed by the D/H exchange reaction as applied to PCI (see Section 3.2). Wt-ADA2h shows a fully reversible denaturation behavior at pH 7, showing a two state folding transition [16,30], allowing us to perform this experiment. The four proteins were heated at 90°C for 3 h. After 30 min at room temperature, the proteins were diluted 10-fold in proton containing buffer and the reaction was followed by MALDI-TOF MS as a function of time. Fig. 4B shows the D/H exchange reaction only for the Wt protein and for the M2-ADA2h protein, since the other two mutants failed to denature. After 2 days of reaction, the Wt protein retains eight deuterons protected from exchange and M2-ADA2h retains 32 deuterons. This difference in protected hydrogens (24) is similar to that in the H/D reaction

(26), although the absolute values obtained along the experiment are different (the measured deuteration was always smaller for the H/D exchange reaction), probably due to the back-exchange reaction. The same reaction was performed on the denatured protein in 5 M urea observing no protection of deuterons after a few minutes (data not shown). The difference in the percentage of deuteration between the Wt and M2-ADA2h is similar to the observed in the H/D exchange reaction. This experiment seems to indicate that the Wt protein has reached an exchange equilibrium, with eight hydrogens protected from exchange after 2 days of reaction. These eight hydrogens could constitute the slow exchange core of the protein. After 2 days of exchange, the M2-ADA2h protein still retains more than 30 deuterons. In this case, this high number of protected hydrogens probably includes amide hydrogens other than those in the slow exchange core, as suggested also by the fact that the exchange for M2-ADA2h (Fig. 4B) does not reach an evident plateau. Therefore, Wt-ADA2h and its mutants seem to have three subpopulations of labile hydrogens: a group belonging to flexible and solvent-exposed regions located mainly in external loops that exchange rapidly, a group of hydrogens in secondary structure not in the slow exchange core that exchange slowly at intermediate pH, and a group of a few hydrogens that belong to secondary–tertiary structure elements involved in the slow exchange core of the protein.

The results observed for the deuteration of ADA2h Wt and mutant forms are in good agreement with their known relative stabilities as measured by urea denaturation curves and kinetics [15,16]. In fact, the approximately 20% of deuteration difference between the ADA2h Wt and single mutant forms (Table 1) coincides with the previously described increase in stabilization energy of the mutants [15,16]. The double mutant, DM-ADA2h, shows further increase in deuteration difference (Fig. 4A), in agreement with the additional stabilization energy previously described [16], although in this case the reported additivity in stabilization energy [16] is not observed. It seems clear that a qualitative correlation between differences in the number of slow exchanging hydrogens and stabilization energy can be expected, suggesting that these different experimental techniques may allow similar conclusions. Further experimental measurements in other proteins are needed to confirm this point and evaluate its quantitative aspects, which could make MALDI-TOF MS hydrogen exchange an alternative approach to discriminate between mutant forms of a protein with different thermodynamic properties.

3.4. Conclusions

We have shown that MALDI-TOF MS can be used to monitor hydrogen exchange of entire proteins to assess their conformation and stability in solution. The measurement of H/D exchange by using a deuterated MALDI matrix and an inert atmosphere to carry out the exchange reactions allows reproducible and precise measurements, overcoming back-exchange problems. Alternatively, when the protein can be reversibly denatured, D/H exchange can be monitored after complete deuteration of the protein. In this case, standard conditions of sample preparation for MALDI-TOF MS can be used since there is no back-exchange problem. The method is fast, needs no modification of the current instrumental and only small amounts of protein are needed (typically few pmol). The current accuracy on the MALDI-TOF MS measurements would presumably allow the study of proteins up to around 30 kDa. The described results indicate that this method is suitable for the initial characterization of site-directed mutants. Our results are similar to those obtained by electrospray ionization mass spectrometry [31] but have the advantages of simplicity of operation and much higher tolerance to the presence of salts and chaotropes in the samples. Moreover, the back-exchange problem is more easily avoided [32]. Furthermore, if the similarity of results observed between the values obtained in urea denaturation curves and hydrogen exchange reactions are confirmed for other proteins, this approach could be used to evaluate and compare thermodynamic stabilities of proteins. Finally, this procedure could be used to evaluate the conformational state of a protein under different conditions, and help to characterize folding intermediates, or other folding species.

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