Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Mass Spectrometry 240 (2005) 285–290
International Journal of Mass Spectrometry
www.elsevier.com/locate/ijms

Protein–protein interaction dynamics by amide H/^{2}H exchange mass spectrometry

Elizabeth A. Komives*

Department of Chemistry and Biochemistry, University of California San Diego, 9500 Gilman Dr., 9500 La Jolla, CA 92093-0378, USA

Received 3 August 2004; accepted 28 September 2004
Available online 5 November 2004

Abstract

Amide H/^{2}H exchange detected by mass spectrometry provides a powerful tool for observing changes that occur upon protein–protein interactions. In general, it is possible to observe protection of surface amides, they become less solvent exposed when they are buried at the interface. The information thus obtained about the location of the protein–protein interface is useful for building a correct docked structure of the protein–protein complex. Examples of protein–protein interfaces that were correctly identified by such methods include the thrombin–thrombomodulin interaction and the interaction between the regulatory and catalytic subunit of protein kinase A. Amide exchange also affords a view into the subtle changes in the ensemble of states that occur upon protein modification or protein–protein binding. Examples of proteins in which amide exchange has been used to observe phosphorylation-induced changes include ERK2 and CheB. Amide exchange showed the pathway of communication between the cAMP-binding site and the catalytic subunit site within the regulatory subunit of protein kinase A. Clues as to how thrombomodulin regulates the catalytic activity of thrombin were also obtained.

Keywords: Amide H/^{2}H exchange; MALDI-TOF mass spectrometry; Hydration; Protein interface

1. Introduction

We have recently reported amide exchange experiments detected by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) that probe for solvent accessibility changes in protein–protein interactions. Several different systems have been studied including an antibody–antigen interaction, the thrombin–thrombomodulin interaction, and the interaction between the regulatory and catalytic subunits of protein kinase A [1–5].

The analysis of protein–protein interactions by amide exchange requires fundamentally different experimental approaches than have traditionally been performed to understand protein folding and unfolding which probe the amide exchange rates related to hydrogen bond formation [6]. In the case of protein–protein interfaces, there are not usually hydrogen bonds forming across the interface, it is rather a case of decreased solvent accessibility which can arise because of a number of factors including side chain interactions, decreased loop mobility, etc. Since it is known that the amides in the interior of a protein exchange more slowly than those on the surface, it is nearly always the more rapidly exchanging surface amides that require monitoring for protein–protein interface studies. Typically, amides on the surface of a protein exchange within seconds to minutes. This exchange rate is slower than that for unstructured peptides but much faster than the exchange rates of amides in hydrogen-bonded structures within the core of the protein [7,8]. Mass spectrometry is an ideal method of detecting these intermediate exchange rates, which cannot often be probed by NMR exchange experiments. Typically, the exchange experiment is performed under physiological conditions for relatively short periods

Abbreviations: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; H/^{2}H, hydrogen/deuterium

* Tel.: +1 858 534 3058; fax: +1 858 534 6174.
E-mail address: ekomives@ucsd.edu.

1387-3806/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijms.2004.09.016

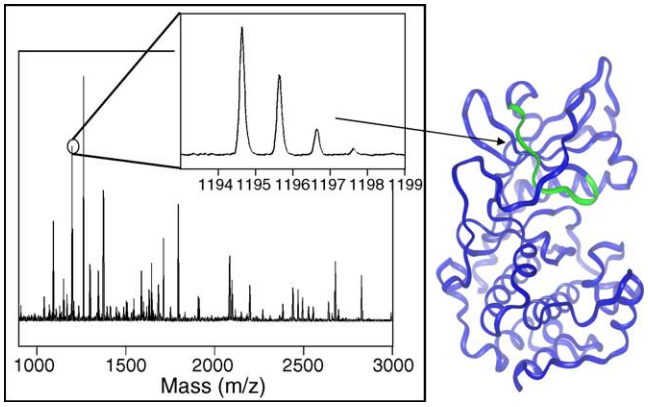

Fig. 1. The amide H/^{2}H exchange detected by MALDI-TOF mass spectrometry gives information on the solvent accessibility of regions of the protein. Prior to the amide exchange experiments, the protein is digested with pepsin, and each resulting peptide is sequenced so that its location within the protein structure can be determined. Although pepsin is a "non-specific" protease, meaning its cleavages cannot be predicted with high accuracy, it cleaves an individual protein the same way every time. Thus, the same peptides are produced from every pepsin digestion. Information about the entire protein is contained in a single MALDI-TOF mass spectrum, which contains many peptides that cover much of the sequence of the entire protein. The mass envelope of one of the peptides in the spectrum is shown in the inset.

of time. The protein–protein complex is analyzed in parallel with each of the individual proteins as controls. The exchange reaction is quenched by lowering the pH to 2.5 and the temperature to 0 °C and the proteins are digested with pepsin. Pepsin is a highly active protease that is relatively non-specific and is capable of completely digesting most proteins within minutes under the low pH and temperature conditions required [9]. Once the proteins are digested with pepsin, the peptides can be identified by standard sequencing techniques and localized on the protein structure (Fig. 1). In the MALDI-TOF experiment, all of the peptides are analyzed from a single mass spectrum. For the study of protein–protein interactions, either on-exchange or off-exchange experiments can be performed. The on-exchange experiment results in a lack of incorporation of deuterium at amide positions in the interface whereas the off-exchange experiment results in a retention of deuterium at the interface (Fig. 2).

2. Protein–protein interfaces

The analysis of solvent accessibility at protein–protein interfaces requires some prior knowledge of the protein–protein binding equilibrium constant. We have previously shown that if the binding constant is weak (>10 nM), then an excess of one binding partner must be used in order to observe interface protection of surface amides [3]. This is because if the complex is dissociating and re-associating rapidly, then the amides will have time to exchange when the proteins are unbound, and interface protection will not be observed. It is also possible that H_2O molecules will be able to access part of the interface albeit to a reduced degree, and it is difficult to understand how and when it is best to observe these partly excluded amides. Several different groups have observed amides at the protein–protein interface that are completely solvent excluded as long as the two proteins remain bound [3,10–12]. For these completely solvent excluded amides, the observed H/^{2}H exchange rate is controlled by the protein–protein dissociation and association rates as well as the intrinsic rate of hydrogen exchange (k_{ex}):

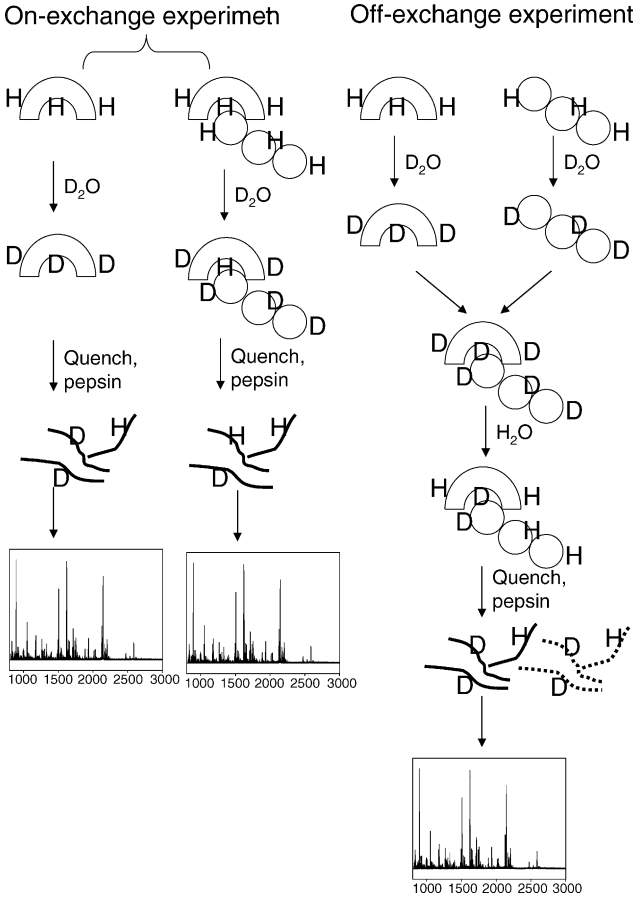

Fig. 2. Flow chart diagram of the two types of amide exchange experiments used to study protein–protein interfaces. In the on-exchange experiment, the protein–protein complex shows a region in which less deuterium is incorporated when compared to control experiments using each protein alone. In the off-exchange experiment, each protein is first allowed to incorporate deuterium, and then the complex is formed and deuteriums are off-exchanged by dilution back into H_2O. In the off-exchange experiment on the protein–protein complex, the presence of remaining deuterons after off-exchange compared with control experiments using each protein alone indicates the interface.

$$[R_D L] \underset{k_a}{\overset{k_d}{\rightleftarrows}} R_D + L \xrightarrow{k_{ex}} R_H \quad (1)$$

where R_H is protonated receptor, R_D is deuterated receptor, L is ligand, k_{ex} is the intrinsic amide exchange rate (min^{-1}) for amides in the uncomplexed receptor, k_d the rate of dissociation of the complex (min^{-1}), and k_a the rate of association of the proteins undergoing complexation ($M^{-1}\,min^{-1}$).

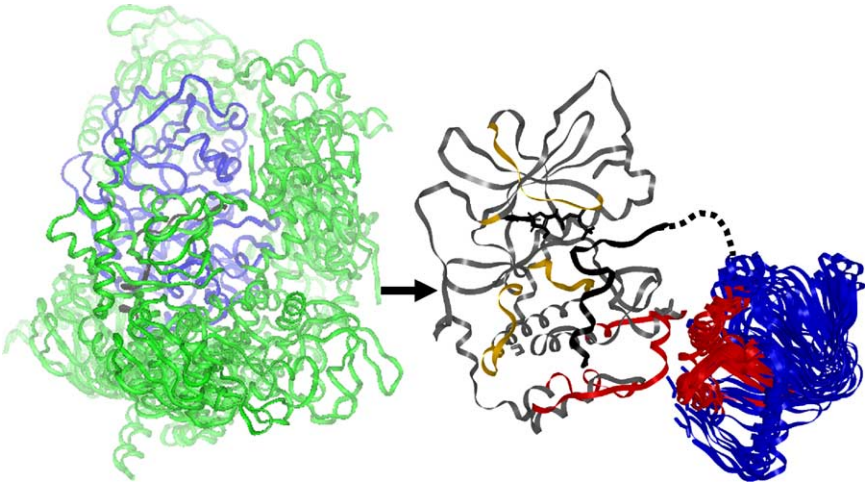

Fig. 3. Use of amide exchange data to filter results from computational docking can significantly improve the results. On the left is shown the top 50 results from computational docking of the cAMP-binding A-domain of the regulatory subunit of protein kinase A (green) with the catalytic subunit (blue). On the right is shown the final structures after the top 100,000 structures were filtered for consistency with the amide exchange data. The catalytic subunit is gray, the regulatory subunit cAMP-binding domain is blue, and the pseudosubstrate region is black. Within the catalytic subunit, the peptic peptides that were protected when the pseudosubstrate region was bound are colored yellow, and those that were only protected when the cAMP-binding domain of the regulatory subunit was bound are colored red. Within the regulatory subunit, the peptic peptides that were protected when the catalytic subunit was bound are also colored red.

The surface protection that is observed when data from the protein–protein complex is compared to data from each individual protein is especially useful. Often, structures of the individual proteins are known, but the structure of the complex is not, and amide exchange data can be used to localize the binding interface. For protein kinase A, computational docking studies failed to locate the interface between the catalytic and regulatory subunits (Fig. 3). This is probably because the binding is bipartite, involving a pseudosubstrate region (residues 92–99 of the regulatory subunit) and a region of the first cAMP-binding domain also in the regulatory subunit. The structure of this cAMP-binding domain was known, as was the structure of the catalytic subunit with the pseudosubstrate region bound at the active site, but the interface between the catalytic subunit and the cAMP-binding domain was not known. We were able to obtain pro-

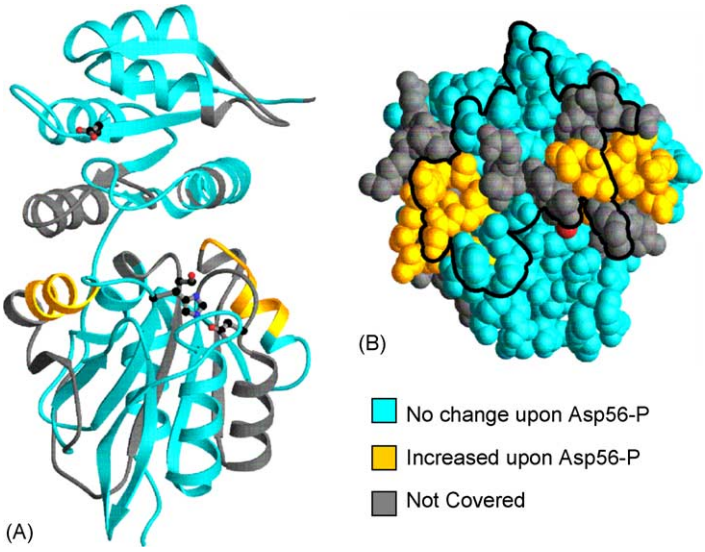

Fig. 4. Amide $H/^2H$ exchange experiments were used to analyze solvent accessibility changes in CheB upon phosphorylation of Asp56 in the regulatory domain with phosphoramidite. Two regions of the catalytic domain (colored gold) at the edges of the active site become more solvent exposed upon phosphorylation. The solvent accessibility of the rest of the protein remains unchanged upon phosphorylation (blue). Control experiments in which the catalytic domain alone was studied showed that the regulatory domain does not completely detach from the catalytic domain, as was previously suggested. (A) Side view of the CheB molecule showing the regulatory domain (top) and the catalytic domain (bottom) with the Asp56 in red and black in the regulatory domain. (B) Top–down view of the catalytic domain showing the footprint of the regulatory domain outlined in black.

Table 1
Peptides that showed protection upon complex formation for the protein kinase A holoenzyme

Peptide (*m*/*z*)	Amides	C-subunit alone	Holoenzyme	Full-length holoenzyme
Catalytic subunit				
212–221 (1167.58)	9	6.18 ± 0.25	3.38 ± 0.10	3.37 ± 0.00
247–261 (1793.97)	5	10.32 ± 0.15	7.53 ± 0.02	7.74 ± 0.42
278–289 (1347.75)	11	6.09 ± 0.01	NA	4.99 ± 0.05

Peptide (*m*/*z*)	Amides	R-subunit alone	Holoenzyme	Full-length holoenzyme
Regulatory subunit (cAMP-binding domain)				
136–148 (1594.73)	12	5.31 ± 0.41	3.88 ± 0.14	5.81 ± 0.17
222–229 (1011.46)	7	1.56 ± 0.19	0.91 ± 0.03	1.55 ± 0.05
230–238 (1046.61)	8	4.23 ± 0.2	3.38 ± 0.17	4.40 ± 0.13

tection data for both the catalytic and regulatory subunits (Table 1). The protection data was used to "filter" the computational docking solutions to find the correct interface [5] (Fig. 3).

The rates of off-exchange of deuterons from protein–protein interfaces can provide additional information about the solvent accessibility of the interface [3]. The experiment includes measurement of the on-exchange of deuterium into each of the proteins involved in the interaction. After on-exchange is measured, a suitable set of times of on-exchange are chosen and these samples are then allowed to complex, and are diluted into H_2O for the off-exchange part of the

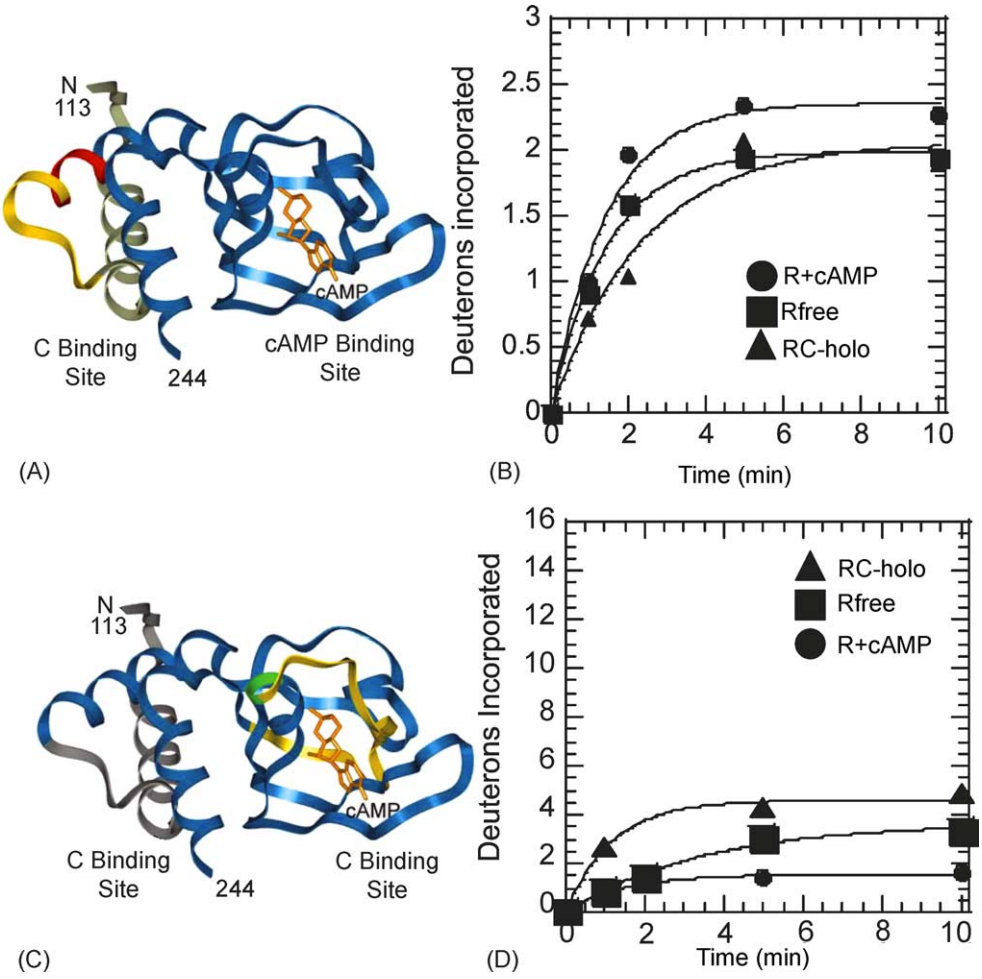

Fig. 5. Amide $H/^2H$ exchange experiments revealed conformational changes occurring in the regulatory subunit of protein kinase A. (A) Amide exchange in a fragment of the regulatory domain corresponding to the binding site for the catalytic subunit (gold). (B) The kinetics show that when cAMP binds, the binding site for the catalytic subunit becomes more solvent accessible. (C) Amide exchange in a fragment of the regulatory subunit corresponding to the cAMP-binding site (gold). (D) The kinetics show that when the catalytic subunit binds, the cAMP-binding site becomes more solvent accessible.

experiment. With both on-exchange and off-exchange properties of the interface available, it is possible to find the solvent-excluded part of the interface. Wells and colleagues used site-directed mutagenesis to demonstrate that although protein–protein interfaces appear large in structures of complexes, only a few of the residues at the interface are essential for the interaction (termed the "hot spot" of the interaction) [13]. We have found that the solvent excluded part of the thrombin–thrombomodulin interface is located at the same site as the "hot spot" as determined by mutagenesis [3].

3. Conformational changes upon protein–protein interactions

Several groups including our own have used amide exchange detected by mass spectrometry to discover subtle conformational changes that may not be detectable by X-ray crystallography. Natalie Ahn's group showed that upon phosphorylation near the active site cleft, the mitogen-activated protein kinase, ERK2, showed both regions of increased solvent accessibility and regions of decreased solvent accessibility. The residues undergoing these changes were located more than 10 Å from the site of phosphorylation [14]. In collaboration with Ann Stock, our lab showed that phosphorylation of the two-component regulatory protein CheB on an aspartic acid residue also causes conformational changes. The lifetime of the phosphoaspartate is only a few seconds, so amide exchange detected by mass spectrometry was an ideal way to capture the conformational changes occurring upon phosphorylation. Consistent with the observation that phosphorylation activates CheB, solvent accessibility increases were observed at the edges of the active site (Fig. 4). The changes were more subtle than expected, as the prevailing hypothesis was that phosphorylation opened the interface between the two domains completely [15]. These studies accentuate the fact that proteins are a dynamic ensemble of states and that phosphorylation can alter the equilibrium distribution of states.

Natalie Ahn and Judith Klinman have recently shown that amide exchange can give clues about the residues involved in catalysis of hydride transfer in the thermophilic alcohol dehydrogenase (htADH) [16]. This very interesting study capitalized on the prior knowledge that hydride tunneling rates were highly temperature dependent in this enzyme. Amide exchange studies carried out at different temperatures gave clues as to which residues were increasing in mobility (as indicated by their increased solvent accessibility) at the higher temperature.

In collaboration with Susan Taylor, we have used amide exchange to observe changes in the regulatory subunit of protein kinase A upon binding of cAMP or binding of the catalytic subunit. As expected, cAMP binding caused decreased solvent accessibility at the cAMP-binding site. It was completely unexpected that cAMP would also cause increased solvent accessibility at the catalytic subunit-binding

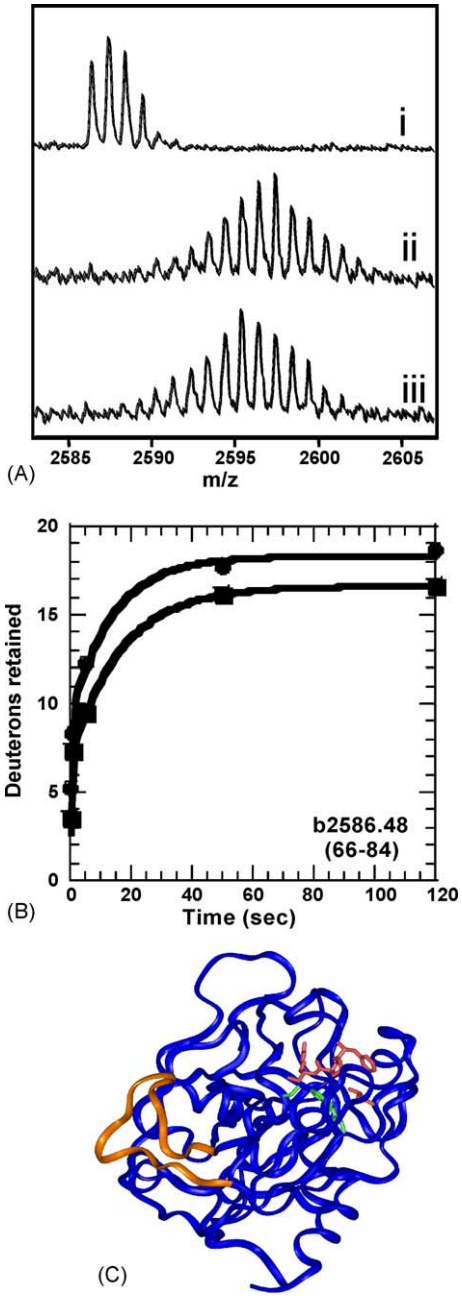

Fig. 6. Amide $H/^2H$ exchange experiments revealed conformational changes occurring in thrombin when the active site is occupied. (A) An expansion of the mass spectrum from thrombin showing the mass envelope for the peptide that corresponds to anion binding exosite 1: (i) before deuteration, (ii) after 2 min of deuteration with the active site open, (iii) after 2 min of deuteration with the active site occupied. (B) Kinetics of amide exchange of the peptide from (A) showing that up to 2 min, the anion binding exosite has significantly lower solvent accessibility when the active site is occupied. (C) Backbone structure of thrombin (blue) showing the active site residues (green), the D-Phe-Pro-Arg-chloromethylketone that occupies the active site (red), and the peptide corresponding to anion-binding exosite 1 (gold) that changes solvent accessibility when the active site is occupied.

site (Fig. 5). The converse was also true, that binding of the catalytic subunit caused increased solvent accessibility at the cAMP-binding site. These results provided the first clue as to how the regulatory subunit undergoes exclusive binding of only one or the other of its two ligands [4].

Our group has also studied conformational changes that occur upon substrate binding in thrombin. Thrombin is thought to be allosterically regulated by thrombomodulin, which binds to anion-binding exosite 1 [17]. Consistent with this, we had observed changes in solvent accessibility in loops surrounding the thrombin active site when thrombomodulin was bound [3]. We reasoned that if the changes were truly allosteric, we should be able to see solvent accessibility changes at anion-binding exosite 1 when substrate was bound to the active site, which causes the loops to close. Indeed, at short times (<2 min), the large surface loop that constitutes anion-binding exosite 1 showed decreased solvent accessibility when the active site was occupied (Fig. 6) [18]. No differences could be seen in thrombin comparing the crystal structures of thrombin alone with thrombin bound to thrombomodulin. However, preliminary amide exchange experiments with active vs. inactive thrombomodulin fragments show that only the active thrombomodulin fragments cause changes in solvent accessibility at the thrombin active site. These results again suggest that amide exchange may uniquely allow a view into the subtle changes in the distribution of the ensemble of states upon protein–protein interaction.

4. Concluding remarks

The use of amide $H/^2H$ exchange experiments detected by mass spectrometry for the study of protein–protein interactions is less than 10 years old. Much has been learned in this relatively short time, and much more stands to be gained from applying this experiment to a broad range of interactions. The experiment affords a unique view of subtle changes in the distribution of states in the conformational ensemble, but often the interpretation of the data requires integration with other experimental results including X-ray structures, kinetic, and other biophysical experiments. With improved mass spectrometric methods, it may also be possible to determine which amides within the peptide segment are showing differences in exchange, and this will afford higher resolution data that may be more readily interpreted.

References

[1] A. Baerga-Ortiz, C.A. Hughes, J.G. Mandell, E.A. Komives, Protein Sci. 11 (2002) 1300.
[2] J.G. Mandell, A.M. Falick, E.A. Komives, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 14705.
[3] J.G. Mandell, A. Baerga-Ortiz, S. Akashi, K. Takio, E.A. Komives, J. Mol. Biol. 306 (2001) 575.
[4] G.S. Anand, C.A. Hughes, J.M. Jones, S.S. Taylor, E.A. Komives, J. Mol. Biol. 323 (2002) 377.
[5] G.S. Anand, D. Law, J.G. Mandell, A.N. Snead, I. Tsigelny, S.S. Taylor, L. Ten Eyck, E.A. Komives, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 13264.
[6] S. Englander, L. Mayne, Y. Bai, T. Sosnick, Protein Sci. 6 (1997) 1101.
[7] Y. Bai, J.S. Milne, L. Mayne, S.W. Englander, Proteins 17 (1993) 75.
[8] K. Dharmasiri, D.L. Smith, Anal. Chem. 68 (1996) 2340.
[9] J.J. Rosa, F.M. Richards, J. Mol. Biol. 133 (1979) 399.
[10] Z. Zhang, D.L. Smith, Protein Sci. 5 (1996) 1282.
[11] F. Wang, R.W. Miles, G. Kicska, E. Nieves, V.L. Schramm, R.H. Angeletti, Protein Sci. 9 (2000) 1660.
[12] A. Baerga-Ortiz, S.P. Bergqvist, J.G. Mandell, E.A. Komives, Protein Sci. 13 (2004) 166.
[13] J.A. Wells, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 1.
[14] A.N. Hoofnagle, K.A. Resing, E.J. Goldsmith, N.G. Ahn, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 956.
[15] C.A. Hughes, J.G. Mandell, G.S. Anand, A.M. Stock, E.A. Komives, J. Mol. Biol. 307 (2001) 967.
[16] Z.X. Liang, T. Lee, K.A. Resing, N.G. Ahn, J.P. Klinman, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 9556.
[17] C.T. Esmon, Biochim. Biophys. Acta 1477 (2000) 349.
[18] C.H. Croy, J.A. Koeppe, S.P. Bergqvist, E.A. Komives, Biochemistry 43 (2004) 5346.