Mass Spectrometric Characterization of a Protein−Ligand Interaction

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Received July 20, 1994[⊗]

Abstract: Src homology 2 (SH-2) domains are found in a variety of protein kinases and are believed to be recognition sites for specific phosphotyrosine-containing peptide sequences. We have investigated the deuterium exchange behavior of a recombinant SH-2 domain, using electrospray ionization mass spectrometry to monitor the incorporation of deuterium. In the presence of a tight-binding phosphopeptide, the exchange slows dramatically. Because the NMR and X-ray crystal structures of the protein do not indicate a large conformational shift upon binding, we interpret the ESI−MS results as an increase in conformational stability. Phosphoserine or phosphothreonine sequences do not show the effect; neither does an un-phosphorylated analogue or a shorter peptide containing phosphotyrosine. The protein can be titrated with ligand by monitoring the exchange behavior to give the stoichiometry of the complex. Ions from a noncovalent complex of SH-2 and the phosphopentapeptide exhibit the same exchange kinetics as those of the uncomplexed protein in the presence of ligand.

Introduction

The development of very gentle ionization methods, such as electrospray ionization[1] has allowed mass spectrometry (MS) to be applied recently to the study of secondary structure in proteins and peptides.[2] In particular, the combination of deuterium exchange with MS has been used to explore protein conformation[3] and the location and stability of α-helices and β-sheets.[4] Another important aspect of protein structure involves the interaction of a protein and its binding ligands. We here report on the deuterium exchange−MS of such a system: the interaction of a src homology 2 (SH-2) domain and a phosphotyrosine-containing peptide.

Phosphotyrosine recognition plays a vital role in cell signaling. A variety of protein kinases contain one or more SH-2 domains, whose function is believed to be the recognition of specific phosphotyrosine-containing proteins,[5] leading to subsequent changes in enzyme activity and signal propagation. We have been studying an important protein of this class, the SH-2 domain of src. The src protein is a cytosolic nonreceptor tyrosine kinase[6] and has been implicated in both colon and breast carcinoma. The SH-2 domain and its interaction with peptide ligands has been well-studied by X-ray crystallography[7] and NMR,[8] and thus serves as a good model to use in validation of mass spectrometric techniques.

Experimental Section

Recombinant SH-2 from pp60c-src (residues 144−259) and synthetic peptide ligands were prepared at Glaxo as reported elsewhere.[7b]

For standard mass spectra, a 5 μM aqueous solution of protein was infused into a P-E Sciex API-III (Thornhill, Ontario) mass spectrometer at 2 μL/min using a Harvard syringe pump (S. Natick, MA). The ionspray needle was maintained at 5300 V and the orifice was at 80 V unless otherwise noted. Scan time was 3 s/scan from *m/z* 700 to 2200; several scans were averaged to improve signal/noise.

For deuterium exchange experiments, samples of protein and protein mixed with ligand were dried to about 1 μL volume and reconstituted in D_2O to minimize exposure to protons. Protein concentrations were typically 5−10 μM and ligand concentrations were as noted in the text. The ionization chamber of the mass spectrometer was flushed with 4 L/min of nitrogen to prevent back-exchange. The combined data from several charge states in each spectrum were used to calculate the molecular mass, which was plotted against time. Kinetic analysis of the exchange was conducted as previously described.[4c]

Results and Discussion

We have produced a recombinant SH-2 protein that retains the binding properties of wild-type pp60c-src, but does not have the kinase activity associated with the carboxyl-terminus of the protein.[7b] The recombinant SH-2 consists of 107 amino acid residues and includes 213 exchangeable protons. Figure 1 shows the ESI mass spectrum of the free SH-2 protein, from which the molecular mass was readily calculated to be 12 286.3 ± 0.9, in excellent agreement with the calculated value of 12 285.9.

Deuterium exchange was conducted on the free protein and on the protein in the presence of a series of ligands with varying

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⊗ Abstract published in *Advance ACS Abstracts,* January 15, 1995.

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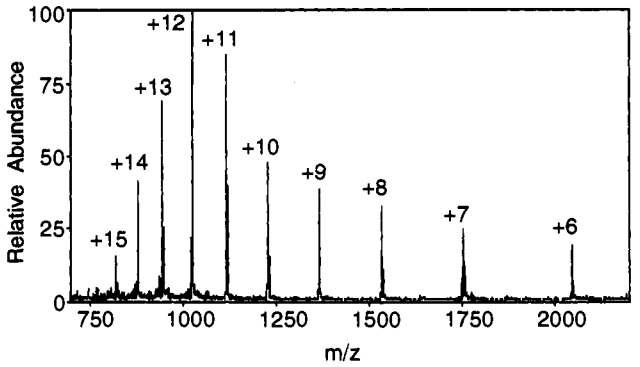

Figure 1. Electrospray ionization mass spectrum of recombinant SH-2 protein.

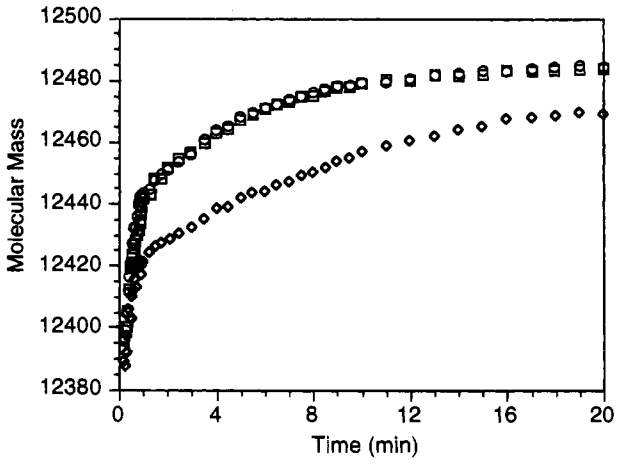

Figure 2. Change in molecular mass of SH-2 protein with time after exposure to D_2O: SH-2 alone (○), SH-2 in the presence of Ac-Y*EEIE (◇), SH-2 in the presence of Ac-YEEIE (□).

binding affinities. The deuterium exchange was monitored by electrospray ionization (ESI) MS as the time-dependent increase in molecular mass of the protein. Upon exposure to D_2O, the exchangeable protons rapidly begin to exchange, leading to a steady increase in the mass, as observed in Figure 2. The molecular mass would ultimately be expected to increase from 12 286 (fully protonated) to 12 499 (fully exchanged). Under the experimental conditions used, deuterium exchange occurs in the solution phase prior to ionization, and back-exchange of deuterium by hydrogen was negligible during ESI–MS analysis in the gas phase. It is important to note that at the first time point we can measure, about 12 s after exposure to deuterated solvent, 110 labile hydrogens have already exchanged. These fast exchanging protons are believed to be on side chains and on backbone amides at the surface of the protein. The remaining 103 exchangeable hydrogens are assumed to be buried or involved in intramolecular hydrogen bonding. The rate of exchange of these protons is sensitive to conformational stability (i.e., flexibility) because several unfolding steps of the protein may be necessary before the protons are accessible to solvent and can be exchanged.

Based on the work of Songyang, et al.,[9] we used the phosphopeptide acetyl-(phospho)Tyr-Glu-Glu-Ile-Glu (Ac-Y*EEIE) as a model of a tight-binding ligand for SH-2.[7b] When the deuterium exchange of SH-2 was repeated in the presence of a 2-fold molar excess of this phosphopeptide, a dramatic slowing of the exchange was observed (Figure 2). In contrast, the pentapeptide Ac-YEEIE, which is not phosphorylated on tyrosine and does not bind to SH-2,[7b] showed no effect on the deuterium exchange rate.

Table 1. Distribution of Exchangeable Hydrogens of SH-2 According to Their Measured Exchange Rates

	SH2 only	SH2 & Ac-Y*EEIE
very fast $k > 7.0$	98 ± 2	94 ± 1
fast $0.70 < k < 7.0$	48 ± 3	0
slow $0.07 < k < 0.70$	45 ± 3	56 ± 2
very slow $k < 0.07$	22 ± 3	63 ± 1

[a] Each category represents approximately one order of magnitude in exchange rate. Rates are expressed as min^{-1}.

The ESI–MS data for SH-2 suggest that approximately 25–30 protons have significantly slower exchange in the presence of the tight-binding peptide than in its absence (Figure 2). If the mass spectrometric data are analyzed more quantitatively,[4c] one can divide the exchangeable protons into a series of groups based on their exchange rates. In the case of the SH-2 data, a plot was constructed of the logarithm of the number of protons remaining unexchanged as a function of time. From regression analysis of the linear portions of this plot, four populations of exchangeable protons were identified, as shown in Table 1. Although the protein in the free and bound state start out with roughly the same number of "very fast"-exchanging protons, the free protein has some 48 protons in the "fast" category that move to the "slow" or "very slow" groups when the protein binds to the ligand.

There could be several explanations for the change in exchange behavior. A large conformational shift in the protein upon binding of the ligand could result in a slowing of the deuterium exchange rate. However, if there were a conformational change, one might expect that the number of very fast exchanging protons, presumably representing the protons on the surface of the protein, would be substantially different in the bound and free proteins. Table 1 suggests that this is not the case. Additionally, X-ray[7] and NMR[8] studies indicate that SH-2 does not undergo a major conformational shift upon binding AcY*EEIE.

A second possible explanation for the observed slowing of the deuterium exchange rate is that the ligand is covering a region of the protein's surface, protecting it from solvent and delaying its exchange. It is known that the phosphopeptide fits into SH-2 like a two-pronged plug into a two-holed socket,[7,8] with the phosphotyrosine fitting into one cavity on the SH-2 surface and the side chain of the Ile nestling into a nearby hydrophobic pocket. Once again, the similarity of the numbers of very fast-exchanging protons in the free and bound proteins (Table 1) suggests that surface coverage by the ligand interacting at the protein's surface is not a factor. Molecular modeling of the protein, based on the X-ray structure, allows the solvent accessibility of all atoms in the protein to be estimated. This calculation indicates that approximately 12 exchangeable protons are significantly less accessible to solvent in the ligand-bound protein than in the free protein (Mil Lambert, Glaxo, Inc., personal communication). This is insufficient to explain the large change in the deuterium exchange rate. As further evidence, the phosphopeptide itself exchanges very fast (less than 12 s), indicating that breaking the hydrogen bonds between peptide and protein is not rate-limiting in the exchange.

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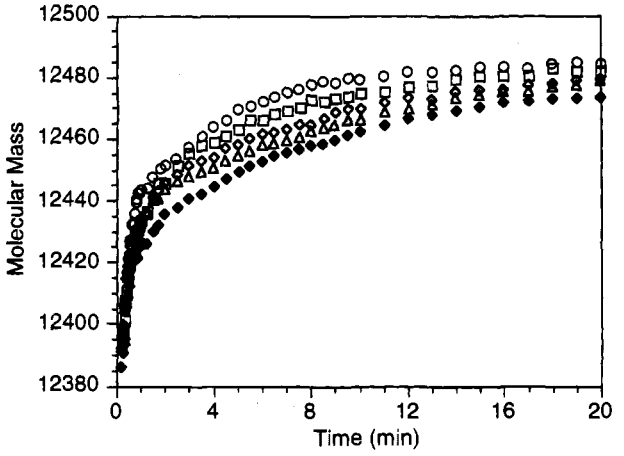

Figure 3. Time-dependent mass increase for the deuterium exchange of SH-2 in the presence of increasing molar ratio of tight-binding ligand, Ac-Y*EEIE. The curves represent ligand:protein ratios of 0:1 (○), 0.25:1 (□), 0.5:1 (◇), 0.75:1 (△), and 1:1 (◆). Experimental conditions are the same as in Figure 2.

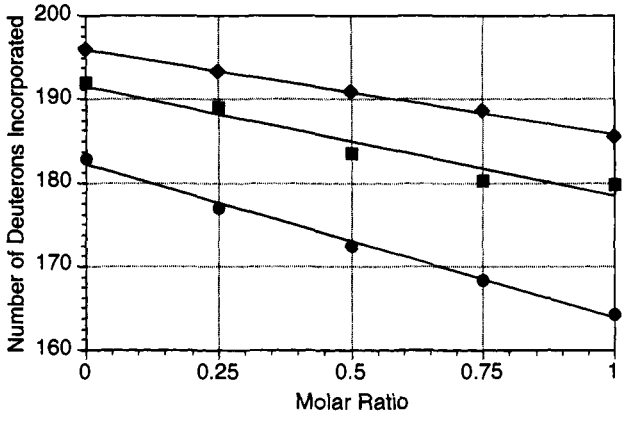

Figure 4. Change in extent of deuteration with increasing molar ratio of ligand. Curves represent different times of exposure [5 min (●), 10 min (■), and 15 min (◆)] to deuterated solvent. Data are from Figure 3.

The third, and we believe the most likely, possible explanation for the decrease in deuterium exchange rate is that ligand binding increases the conformational stability of SH-2, making local unfolding, and therefore the deuterium exchange, slower. In this model, the protein conformation remains largely the same in the bound and free states, but in the bound state, the conformation has less local flexibility because of the association of the ligand. Dissociation of the ligand must occur before the protein can unfold, and hence the deuterium exchange is slower than in the free protein. Of course, all three of these mechanisms could be contributing to some extent to cause the observed shift.

Both prongs of the ligand "plug" seem to be necessary to induce the deuterium exchange effect. The phosphodipeptide Ac-Y*E, lacking the hydrophobic prong, shows no effect on deuterium exchange rate up to an 8-fold molar excess over protein. Similarly, the nonphosphorylated pentapeptide Ac-YEEIE, lacking the phosphotyrosine prong, has no effect on deuterium exchange. In both cases, the mass vs time plot for SH-2 in the presence of the nonbinding peptide is superimposable with that of free SH-2. The dipeptide is known to be a much weaker binder of SH-2, with an IC_{50} about 73 times higher; the pentapeptide shows no binding at all.[7b] The effect of ligand on exchange rate is specific for phosphotyrosine. The peptides Ac-T*EEIE and Ac-S*EEIE, even at a 6-fold molar excess over SH-2, show no reduction of deuterium exchange rate; the mass vs time plots completely overlap that of free SH-2 (data not shown). These peptides show no measurable binding to SH-2 in an ELISA assay.[7b]

The deuterium exchange data can also be used to determine the stoichiometry of the ligand binding. If less than a stoichiometric amount of AcY*EEIE is added in the experiment, the observed change in exchange rate is intermediate between the rate of free SH-2 and fully bound SH-2. Figure 3 shows the mass vs time plots for exchange with increasing concentration of ligand. As the molar ratio increases from 0:1 to 1:1, the exchange rate gradually and linearly decreases, due to an increase in the proportion of protein bound.

In these experiments, the observed rate of exchange results from a time-averaged exchange of free protein and protein bound to ligand. If the ligand binds to the protein at a slow rate (relative to the exchange), one might expect to see the signal for the protein ions begin to split into two sets of signals as deuterium exchange proceeds: one relatively faster exchanging set of ions from the free protein, and a second *slower-exchanging* set of ions from the bound protein. That is not what is observed. Only one set of protein ions appears in the mass spectrum, but the exchange rate corresponds to an intermediate between those of the free and bound protein. (In some circumstances, another series of ions corresponding to a noncovalent complex of ligand and protein is also observed in the mass spectrum, but these were not used for deuterium exchange measurements. See below.)

If the extent of exchange at any given time point is plotted as a function of the molar ratio of ligand to protein, a linear decrease is observed (Figure 4). This linearity suggests that the dissociation constant for the complex is well below the concentration of protein used in these experiments (5 μM). As the molar ratio of the peptide to protein is increased from 1:1 to 4:1, the exchange curves overlap, indicating that no further increase in stability is achieved. From this evidence, one can conclude that the stoichiometry of the complex is 1:1, as has been demonstrated by other methods.[7,8] The ESI–MS experiment will likely only be useful in determining the stoichiometry of tight binding ligands (those with K_D < 100 times lower than the protein concentration), because weak binding compounds do not significantly alter the deuterium exchange rate.

An interesting observation in the ESI mass spectra of SH-2 and its ligands is the appearance of an ion series corresponding to a complex of SH-2 with ligand. Figure 5 shows the mass spectra derived from deuterated solutions of SH-2 and two pentapeptides, Ac-Y*EEIE and Ac-YEEIE. The spectrum of the protein and the tight-binding ligand (Figure 5B) contains an ion series for the free protein and a second series corresponding to a mass of the free protein plus 814 Da, exactly the mass of the fully deuterated phosphopeptide. The spectrum of the protein and Ac-YEEIE, a nonbonding peptide, shows only the ion series from the free protein (Figure 5A). We assume that the protein–ligand complex is noncovalent; the complex is easily dissociated by, for example, the HPLC solvent (water/acetonitrile/TFA). In all of the experiments described above, the deuterium exchange rate of the SH-2 was measured using the ions of the free protein, not those of the complex. Hence, the appearance of the complex ions was not at all essential for the exchange measurement.

Recently ESI–MS has shown potential for observing noncovalent macromolecular interactions.[10] Most of the reports to date have used relatively high concentrations of the protein and ligand (50–100 μM), and many questions remain about the nature and specificity of the interactions. We observe these

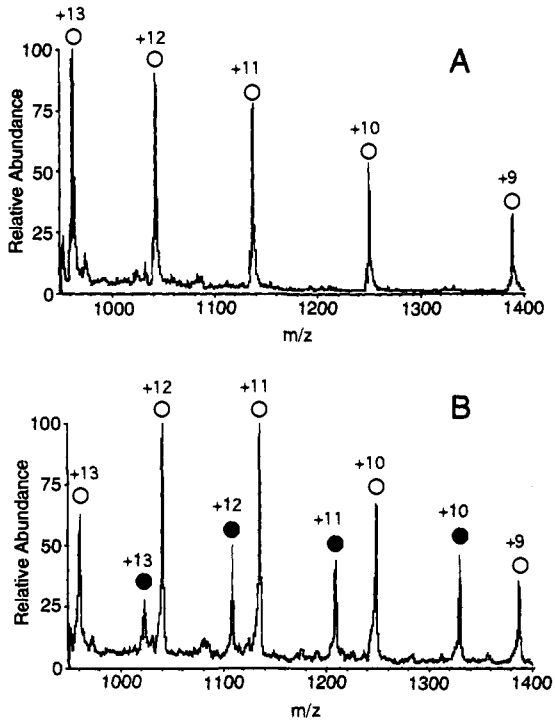

Figure 5. Electrospray ionization mass spectrum of SH-2 (5 μM) in the presence of Ac-YEEIE (A) and Ac-Y*EEIE (B). Peptide concentration was 10 μM. Free protein charge states are marked with open circles; noncovalent complex ions are marked with filled circles. Mass spectrometric conditions are as in Figure 1.

ions at a protein concentration of only 5 μM and a ligand concentration as low as 2 μM. The ability to observe the complexes at low concentration may be attributed in part to the stability of the SH-2/ligand complex in water without the requirement of high ionic strength that would be detrimental to the mass spectrometric analysis. In addition, the Coulombic interactions between the positively charged gas-phase protein ions and the negatively charged phosphate of the ligand may increase the stability of the gas-phase complex, allowing the ions to be observed at relatively low concentration.

The complex ions were also observed in the deuterium exchange experiments. The molecular mass of the complex could be calculated, along with that of the free protein, and the extent of deuteration of the complex was computed. The resulting mass vs time curves were identical, when correction was made for the mass of the ligand. The implication is that the protein, regardless of whether it appears in the mass spectrum as a free protein or as the complex, is exchanging at the same rate. Presumably, all the protein in solution is bound to the ligand (in these experiments, there was a 2-fold molar excess of ligand) and is exchanging at the bound rate. During the course of the mass spectrometric ionization, some of the complex dissociates, leading to the appearance of free protein ions in the mass spectrum.

At higher concentration of ligand relative to protein, other complexes appear that are probably nonspecific. For example, if the concentration of nonphosphorylated Ac-YEEIE is increased to 60 μM, a 12:1 molar ratio to protein, an ion series corresponding to the noncovalent complex is observed in the mass spectrum. We assume the complex is nonspecific because the peptide has no measurable solution affinity for SH-2.[7b] The ions in this series can be completely dissociated by raising the energy of the incoming ion beam, and hence the energy of collisions in the high-pressure region of the source. At an orifice voltage of 100 V, the ions corresponding to the complex (SH-2 and Ac-YEEIE) were less than 10% relative abundance compared to the ions of the free protein. At 200 V, no ions of the complex could be detected. In contrast, the ions for the complex of SH-2 and Ac-Y*EEIE persisted at an orifice voltage of 250 V, the maximum available on the instrument.

The dissociation of complex ions by increasing their energy has been reported for other noncovalent complexes, and is even used as proof that a complex is noncovalent.[11] It is noteworthy that the complex of SH-2 and Ac-Y*EEIE is not easily dissociated by this method. Using a solution of 5 μM SH-2 and 10 μM ligand, the ions corresponding to the complex could not be dissociated even at the highest energy available on our instrument. This result suggests that the mass spectrometric dissociation of ions may not be an appropriate indicator of whether or not complexes are noncovalent.

In summary, the deuterium exchange rate provides a sensitive measure of conformational stability of the SH-2 protein during ligand binding. The amount of time (30–60 min) and material (200 pmol/experiment) needed for the mass spectrometric experiments is small enough to be used with a range of proteins, although the electrospray process itself has a somewhat limited tolerance to biological buffers. A detailed analysis of the shape of the exchange curves can lead to kinetic information about populations of protons and their relative exchange rates.[4c] A comparison of that type of data might ultimately allow one to assess whether or not two different binding ligands were binding at the same site or in the same fashion. For tight-binding complexes, the ESI–MS data can indicate the stoichiometry of binding.

Acknowledgment. The authors gratefully acknowledge Steve Jordan, Lisa Shewchuk, Dan Kassel, Mike Luther, Mil Lambert, Robert Xu, and Peter Jeffs of Glaxo for helpful discussions; Derril Willard and Tom Consler for SH-2 protein; and Marc Rodriguez for peptide ligands.

JA9423678

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