BIOCHEMISTRY
including biophysical chemistry & molecular biology
Rapid Report

Biochemistry **2010,** *49,* 4297–4299 4297
DOI: 10.1021/bi100031a

Footprinting of Protein Interactions by Tritium Labeling

Guillaume Mousseau,[‡] Quentin Raffy,[§] Olivier P. Thomas,[‡] Morgane Agez,[‡] Robert Thai,[‡] Jean Philippe Renault,[§] Serge Pin,[§,∥] Françoise Ochsenbein,[‡,⊥] Jean-Christophe Cintrat,*[,‡] and Bernard Rousseau*[,‡]

[‡]*IBITECS,* [§]*IRAMIS,* [∥]*URA 331, Laboratoire Claude Fréjacques, CNRS, and* [⊥]*URA 2096, Systèmes membranaires, photobiologie, stress et détoxication, CNRS CEA, F-91191 Gif-sur-Yvette, France*

Received January 11, 2010; Revised Manuscript Received April 23, 2010

ABSTRACT: A new footprinting method for mapping protein interactions has been developed, using tritium as a radioactive label. As residues involved in an interaction are less labeled when the complex is formed, they can be identified via comparison of the tritium incorporation of each residue of the bound protein with that of the unbound one. Application of this footprinting method to the complex formed by the histone H3 fragment $H3_{122-135}$ and the protein $hAsf1A_{1-156}$ afforded data in good agreement with NMR results.

Protein interactions are increasingly recognized as main contributors to the diversity of action of proteins and play critical roles in many biological phenomena. Understanding the roles and consequences of protein interactions is thus fundamental for the development of systems biology as well as the development of novel therapeutics (*1*). Protein footprinting methods can examine the structures and dynamic processes of biological complexes by using reagents able to modify a protein surface regardless of its chemical nature. One of these techniques is $^1H-^2H$ exchange coupled with mass spectrometry (*2*). This approach is conservative because the hydrogen of backbone amides is replaced with deuterium. However, only information about the protein backbone is obtained, and the labeling is labile. Other footprinting techniques allow nonselective covalent modifications and provide information about protein side chains, which are the main participants in the interaction. This is the case of oxidation with hydroxyl radicals under aerobic conditions (*3*). Although products are generally stable, some are difficult to analyze by mass spectrometry at the residue level, and above all, surface oxidation can lead to protein unfolding (*4*). Structural alterations may also be observed with another method using highly reactive carbene generated by photolysis of diazirine (*5*).

We have developed an alternative footprinting approach to map protein interfaces using covalent labeling that avoids structural modifications and allows information about side chains to be obtained. This approach is based on the recent methodology used to detect carbon-centered radicals within proteins using a tritium atom as a reporter (*6*). Like deuterium, the tritium atom has the same chemical and physical properties as hydrogen and so does not perturb protein structures. This method allowed for the first time the localization and quantification of carbon-centered radicals on all the residues of a protein. In this study, we demonstrate that the method can be used to map protein−protein interactions. The general principle is described in Figure 1.

Briefly, the method consists of exposing a protein to the hydroxyl radical generated by water radiolysis under anaerobic conditions. The •OH, located close to the protein, abstracts hydrogen atoms from the side chain of solvent-exposed amino acids. Carbon-centered radicals formed are then repaired by tritium atoms given by a tritium donor, the benzene phosphinic acid sodium salt [[^{3}H]-BPASS (see the Supporting Information)]. This reaction thus leads to a proton−tritium exchange, and a stable C−^{3}H bond is obtained instead of the initial C−^{1}H bond. The amount of tritium incorporated for each C−^{1}H bond is related to its accessibility, but also to the reactivity of each amino acid toward both •OH and [^{3}H]-BPASS (*6*).

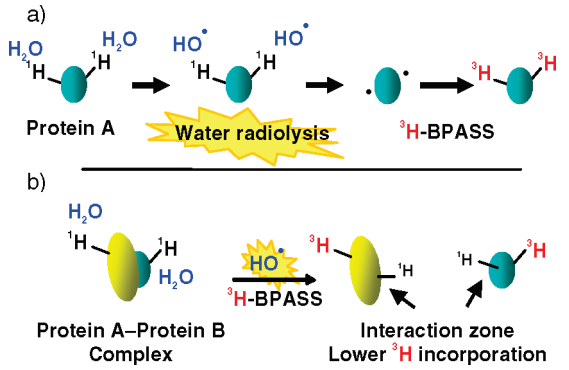

FIGURE 1: Principle of the footprinting method. (a) The surface residues of the protein A of interest react with the hydroxyl radicals produced by water radiolysis to yield carbon-centered radicals. These react with the tritium donor [^{3}H]-BPASS, which leads to tritium labeling of the solvent-exposed residues. (b) The same procedure is applied to the protein A−protein B complex. The residues involved in the interaction are not accessible to the solvent and therefore do not react with hydroxyl radicals. The interaction zone of protein A is identified by residues with a level of tritium incorporation lower than that in the free protein.

Thus, residues involved in the interaction are less exposed to solvent in the complex than in the free proteins and will exhibit a lower level of incorporation of tritium. Direct sequencing of purified tritiated peptides (if necessary produced from a proteolytic digest) allows measurement of the level of incorporation of tritium into amino acids.

The efficiency of the method in mapping protein surfaces buried upon complex assembly was probed for the complex associating the C-terminal helix of histone H3 ($H3_{122-135}$ peptide) with the conserved domain of human antisilencing function ($hAsf1A_{1-156}$ protein). The high-resolution structure of this complex was recently determined by NMR (*7*) and will allow the comparison of the structural and dynamic properties of the complex with the results recorded with our method. The protein Asf1 is a highly conserved histone chaperone found in the nucleus associated with histones H3 and H4. It is involved in the nucleosome assembly and disassembly associated with numerous

*To whom correspondence should be addressed. E-mail: jean-christophe.cintrat@cea.fr or bernard.rousseau@cea.fr. Telephone: +33 1 69 08 21 07. Fax: +33 1 69 08 79 91.

Published on Web 04/23/2010

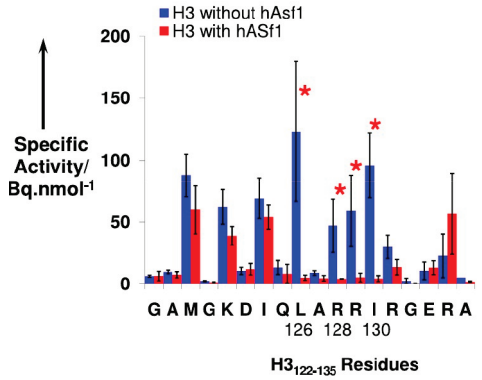

FIGURE 2: Tritium incorporation of the GAMG-$H3_{122-135}$ residues [peptide $H3_{122-135}$ (the same as in the NMR study) including an N-terminal tag (GAMG) added for cloning reasons] of the unbound form of the peptide[1] (blue histogram) and of the $hAsf1A_{1-156}$-bound form (red histogram) exposed to the same amount of $^{\bullet}OH$ with [^{3}H]-BPASS. The error bars are based on three replicas of the experiments. Asterisks indicate the residues for which the level of tritium incorporation decreases the most upon binding to $hAsf1_{1-156}$.

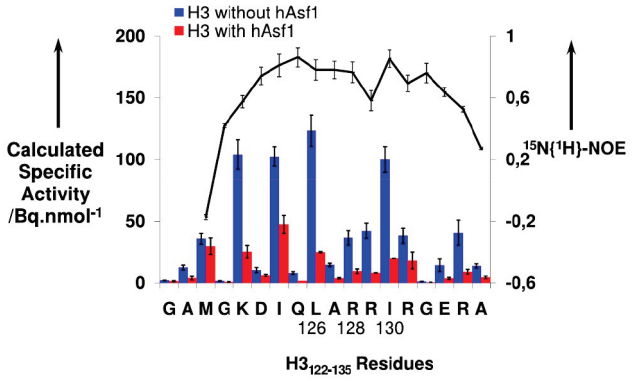

FIGURE 3: Estimation of the level of tritium incorporation of GAMG-$H3_{122-135}$ residues based on their accessibility and their measured relative reactivity toward the tritium donor.[2] The black curve shows the heteronuclear $^{15}N-^{1}H$ nuclear Overhauser effect measurements of the MG-$H3_{122-135}$ fragment *(7a)*.

crucial processes, including DNA replication, gene transcription, and DNA damage signaling and repair (*8*).

To validate our method, we probed the binding interface of the histone H3 peptide upon $hAsf1A_{1-156}$ binding. The proton−tritium exchange was performed (i) without $hAsf1A_{1-156}$ (unbound form)[1] and (ii) in a complex with $hAsf1A_{1-156}$ [80% bound form; $K_D = 50\,\mu M$ (*7b*)]. In both cases, tritium footprinting experiments were conducted with an equimolar amount of [^{3}H]-BPASS (based on H3 concentration). Experiments were performed under anaerobic conditions, under a nitrous oxide atmosphere to prevent reaction of the carbon-centered radicals formed with oxygen (*9*). After being labeled, the peptide was purified by reverse-phase HPLC and cleaved sequentially from N-terminal to C-terminal extremities by an automatic Edman sequencer. The resulting derivatized amino acids were collected at each sequencing cycle, and their level of tritium incorporation was measured. Results obtained for the free and bound forms of H3 are presented as a histogram in Figure 2.

Although glycine and alanine residues are not sufficiently labeled to afford accurate data, all other residues offer reliable tritium incorporation (Figure 2). Comparing the tritium incorporation of $H3_{122-135}$ residues in the presence and absence of $hAsf1A_{1-156}$ reveals differences along the peptide sequence. Two main regions can be distinguished: (a) the central region of the peptide, which presents a large decrease in the level of tritium incorporation upon $hAsf1A_{1-156}$ binding, and (b) both edges of the peptide, whose tritium incorporation remains unchanged.

The residues that display the most significant decreases in the level of tritium incorporation upon binding to the protein are L_{126}, R_{128}, R_{129}, and I_{130} (asterisks in Figure 2). These results correlate fully with previous NMR studies of the $H3_{122-135}$−$hAsf1A_{1-156}$ complex showing these four residues as the main residues in contact with the protein (*7*). Likewise, residues D_{123}, I_{124}, and R_{131}, for which no interaction with the protein has been detected by NMR, show the same tritium incorporation with and without $hAsf1_{1-156}$. This confirms the relevance of our method for highlighting residues involved in an interaction.

To assess the relationship between tritium incorporation and accessibility, the specific activities estimated from the static accessibility alone were calculated (Figure 3), taking into account the reactivity of each residue toward [^{3}H]-BPASS.[2] To do so, the mean accessibilities of all the C−H bonds of each peptide residue were calculated over the 20 best NMR structures obtained (Protein Data Bank entry 2IIJ), using WHATIF. The specific activities of Figure 3 were then calculated via combination of the mean accessibilities with the relative reactivity of each residue toward [^{3}H]-BPASS. The histograms of Figure 3 then represent the estimated levels of tritium incorporation based only on NMR static structures, with and without $hAsf1_{1-156}$.

Comparison of results (Figure 2) and calculations (Figure 3) shows that the measured level of tritium incorporation is globally consistent with NMR data.[3] The discrepancies correspond to residues located at both ends of the peptide (from G_{118} to I_{124} at the N-terminus and R_{134} and A_{135} at the C-terminus) that show similar tritium incorporation upon binding to the protein, whereas the calculated specific activities predict some protection. This result can be explained by the dynamics of these regions of the peptide, revealed by the low values of the $^{15}N-^{1}H$ nuclear Overhauser effect (NOE) measurements (*7a*) (black curve in Figure 3), usually considered as helix fraying ends. This flexibility likely reflects a local unfolding of the bound conformation which temporarily exposes the side chain of these residues to tritium incorporation. The tritium diminution concerns only the residues steadily bound to the protein in the complex, which makes this footprinting method exquisitely sensitive to the key residues of the protein interaction.

In conclusion, we have developed a new footprinting method for characterizing protein−protein interaction regions based on stable ^{3}H labeling, which can be applied to any kind of protein complex. Indeed, as opposed to NMR, our footprinting method is not limited by the size of the complex studied, provided we have a digest pattern of the partners,[4] and affords data about the side

[1] In the absence of $hAsf1A_{1-156}$, H3 was irradiated together with apomyoglobin, with which it has no particular interaction, to mimic the $^{\bullet}OH$ dose absorbed by $hAsf1A_{1-156}$.

[2] See the Supporting Information for more details about the calculations.

[3] As suggested by a referee, we drew some linear correlation between the experimental and theoretical tritium incorporations. The plot reveals a correlation coefficient of 0.72 and a slope of 0.9 (see the Supporting Information).

[4] We also must mention that histone H3 alone does not bind to hAsf1; it needs to be associated in a dimer with H4. The size of the trimeric complex formed does not allow its study by NMR. As mentioned, it could be studied with our tritium labeling method, after purification and proteolysis of the partners.

chains involved in the interaction that are not furnished by MS. The detection of the interaction key residues requires both structural information and dynamic information (*10*), which are naturally combined with our method. Water radiolysis is a technique broadly used for footprinting (*11*), and the tritium labeling agent used can be supplied by any specialized company.

In the future, the ability to identify the regioselectivity of hydrogen abstraction by $^{\bullet}$OH from C−H bonds should allow our method to be applied to other types of macromolecular complexes like protein−lipid or protein−DNA complexes.

SUPPORTING INFORMATION AVAILABLE

Reagents, protocol for $H3_{122-135}$-based radical identification, calculated specific activities, and a plot of calculated accessibilities versus labeling data. This material is available free of charge via the Internet at http://pubs.acs.org.

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