Supporting Information

Footprinting of Protein Interactions by Tritium Labeling

Guillaume Mousseau[a], Quentin Raffy[b], Olivier P. Thomas[a], Morgane Agez[a], Robert Thai[a], Jean Philippe Renault[b], Serge Pin[b,c], Françoise Ochsenbein[a,d], Jean-Christophe Cintrat* [a] and Bernard Rousseau* [a]

a) IBITECS, b) IRAMIS, c) URA 331, Laboratoire Claude Fréjacques, CNRS d) URA 2096, Systèmes membranaires, photobiologie, stress et détoxication, CNRS

CEA

F-91191 Gif-sur-Yvette, France

EXPERIMENTAL SECTION

Reagents. Unless indicated otherwise, all reagents were used as purchased without further purification. The peptide $H3_{122-135}$, spanning the 122-135 sequence of histone H3 (KDIQLARRIRGERA) with the N-terminal Tag sequence GAMG was purchased from Epytop, France (Chemical synthesis). $hAsf1_{1-156}$ was prepared according to a published method (*1*). The protein was ultrafiltrated against phosphate buffer 10 mM pH 7.9 and stored at a concentration of 1 mM at -20 °C. The protein concentration was precisely determined by amino acids analysis.

Apomyoglobin was prepared from lyophilized horse heart metmyoglobin (Sigma M-1882) following known methods (*2*) and lyophilized. Apomyoglobin samples were prepared by dissolving the protein in pure water. The solution was then dialyzed against water and phosphate buffer 10 mM pH 7.9. The concentration was determined by spectrophotometry (λ_{280nm} = 13500 $M^{-1}cm^{-1}$) (*3*) and adjusted to 1 mM. The protein concentration was precisely determined by amino acids analysis.

Hydroxyl radical was generated by water radiolysis using an electron linear accelerator. The energy deposited in the samples (dose expressed in Gy or $J.Kg^{-1}$) was determined with a chemical dosimeter commonly used in radiation chemistry: the Fricke dosimeter (*4*). The 10 MeV-electron beam was provided by the ALIENOR facility in Saclay (Laboratoire de Radiolyse) (*5*). Pulse durations were 10 ns (20 $Gy.pulse^{-1}$), with a repetition rate of 0.4 Hz. In our gas saturated conditions, the energy deposited in the samples by electron beam corresponds approximately to an OH radical concentration of 11 $\mu mole.dm^{-3}.pulse^{-1}$.

$H3_{122-135}$ was purified after labeling on analytical HPLC (Agilent Zorbax C_{18}, 300 Å, 250*4.6 mm) with a Merck Hitachi Pump L-7100 using degassing system Degazys DG-1310 Uni*f*Lows, a SPD-10A VP Shimadzu UV-Vis detector and a Radioflow Detector LB509 with Quickszint flow 302 scintillation cocktail. Data were acquired via Borwin chromatography software.

Sequencing was done on an ABI 492 HT/ Procise peptide sequencer from Applied Biosystems (Foster City, CA, USA) with acquisition on Procise 1.1 and Model 610A 1.1 software. All products are commercially available (Perkin Elmer) and PTH-amino acids were collected using a Gilson FC 203B fraction collector. The collected samples were lyophilized and solubilized in 100 µL of ethanol / distilled water (50 / 50). Concentrations were determined by ultraviolet absorption between 240 and 310 nm. Molar extinction coefficients of PTH amino-acids used were published by Fraenkel-Conrat *et al* (*6*). Each

sample was diluted in 4 mL of scintillation cocktail Zinsser Analytic Unisafe 1 and radioactive counting was carried out with a Wallac 1409 liquid scintillation counter using the 1414 WinSpectral program. Specific activities were calculated in $Bq.nmol^{-1}$.

Protocol for $H3_{122-135}$ -based radical identification. In a septum-capped 4 mL vial, 1 mL of a 10 mM pH 7.8 phosphate buffer solution was prepared with ^{3}H-BPASS (140 µM; 72 MBq), the peptide $H3_{122-135}$ (140 µM), and either the protein $hAsf1_{1-156}$ (280 µM) or apomyoglobin (280 µM). The resulting solution was degassed under a flow of N_2O. It was then submitted to an electronic bombardment (100 pulses of 20 Gy at a frequency of 0.4 Hz). The remaining reaction mixture was passed through a SepPack light tC_2 and eluted with 5*1 mL of water to remove about 95% of ^{3}H-BPASS. Then 600 µL of 20/80/0.1 ACN/H_2O/TFA mixture were added, followed by 600 µL of 30/70/0.1 mixture to obtain the $H3_{122-135}$ peptide. The fractions containing the peptide were concentrated to dryness and then were treated with acid (TFA 10% v/v) for 14 hours to remove labile tritium and then lyophilized. The peptide $H3_{122-135}$ was then purified by HPLC on a column Agilent Zorbax C_{18}, 300 Å, 4.6*250 mm, eluant A: H_2O/TFA 0.1%, eluant B: ACN/TFA 0.1% using a gradient from t=0 A/B (98/2) to t = 20 min A/B (80/20). The purity of the obtained fractions was checked using the same conditions; Tr = 18.6 min. 5 nmoles of the purified peptide were submitted to automatic sequencing. The PTH amino acids were automatically collected and the specific activity of each amino acid was measured using known methods (*7*). No hydroxylated residues were detected during the sequencing. Each experiment was repeated three times, to estimate the error bars. In the experiments described above, we have chosen to use rather high concentrations of peptide and protein, only to avoid unnecessary complications. Tritium can be quantified with femtomolar concentrations, and our method is mainly limited by the sensibility of the peptide sequencer, which is around the picomole. This allows studies with much lower concentrations of sample than NMR.

CALCULATED SPECIFIC ACTIVITIES (Figure 3)

To calculate specific activities of H3 residues, their accessibility and reactivity toward ^{3}H-BPASS have been taken into account, and calculated as follows:

Accessibility:

The ^{3}H-BPASS accessibility of each $H3_{122-135}$ residue in presence or in absence of $hAsf1_{1-156}$ was calculated using the WHAT IF software with the 20 structures of the pdb file 2IIJ. A sphere of 3.5 Å of radius was taken to mime ^{3}H-BPASS. Accessibilities of the C-H of all residues are presented in Table 1.

Table 1: ^{3}H-BPASS accessibility of each $H3_{122-135}$ residue in presence or in absence of $hAsf1_{1-156}$.

H3 Residues	Accessibility of C-H H3 without $hAsf1_{1-156}$	Accessibility of C-H H3 with $hAsf1_{1-156}$
G	9.1	5.7
A	14.9	2.0
M	21.0	16.5
G	7.3	2.6
K	20.4	1.1
D	9.6	4.8
I	32.3	10.7
Q	9.8	0.0
L	31.6	0.1
A	17.4	1.1
R	11.4	1.0
R	13.2	0.0
I	31.5	0.0
R	11.9	4.2
G	5.4	0.8
E	10.2	0.6
R	12.7	0.5
A	16.4	2.5

Reactivity toward ^{3}H-BPASS:

The specific activities after irradiation in the presence of ^{3}H-BPASS have been measured for every amino acid (Table 2, (*7*)). These values were used to estimate the relative reactivity of each H3 residue toward ^{3}H-BPASS.

Table 2: Specific activity measured for each amino acid after irradiation with ^{3}H-BPASS (*7*).

Amino acid	**Specific activity (Bq /nmol)**
Ser	93
Gly	108
Asn	196
Thr	366
Pro	369
Gln	416
Ala	416
Tyr	525
Asp	536
His	629
Glu	694
met	833
Cys	1024
Val	1204
Phe	1330
Trp	1460
Ile	1548
Arg	1563
Leu	1903
Lys	2490

Calculated specific activities:

The specific activities of Figure 3 have been calculated as the product of the calculated accessibility of each residue and the specific activity measured for the corresponding amino acid. All values were normalized to the specific activity of L_{126} measured without $hAsf1_{1-156}$ (123.3 $Bq.nmol^{-1}$), to facilitate comparison between Figures 2 and 3 of the report. The specific activities calculated with $hAsf1_{1-156}$ have been estimated taking into account the 20% of unbound H3 in the conditions of the experiments (Table 3).

Table 3: Specific activities calculated for each $H3_{122-135}$ residue in presence or in absence of $hAsf1_{1-156}$.

H3 Residue	Specific activity H3 without $hAsf1_{1-156}$	Specific activity H3 with $hAsf1_{1-156}$
G	**2.0**	**1.4**
A	**12.7**	**3.9**
M	**35.8**	**29.6**
G	**1.6**	**0.8**
K	**104.0**	**25.3**
D	**10.6**	**6.3**
I	**102.4**	**47.5**
Q	**8.3**	**1.7**
L	**123.3**	**24.9**
A	**14.9**	**3.7**
R	**36.6**	**9.8**
R	**42.2**	**8.5**
I	**100.0**	**20.0**
R	**38.2**	**18.3**
G	**1.2**	**0.4**

E	**14.6**	**3.5**
R	**40.7**	**9.3**
A	**14.0**	**4.5**

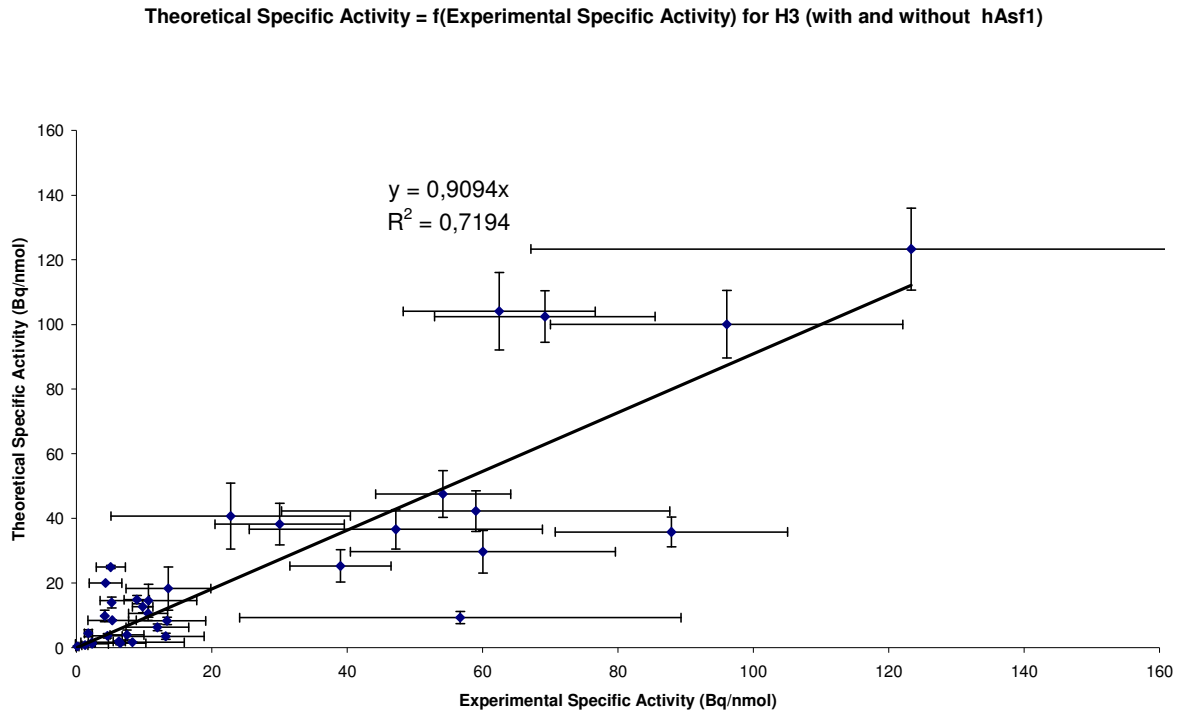

Figure 1 : Plot of the calculated specific activities of the residues of $H3_{122\text{-}135}$ versus the experimental ones, with and without $hAs1f1_{1\text{-}156}$. The line is the linear regression of the data.

REFERENCES

1. Mousson, F., Couprie, J., Thuret, J. Y., Neumann, J. M., Mann, C., Ochsenbein, F. (2004) *J. Biomol. NMR 29*, 413-414.
2. Rossi-Fanelli, A., Antonini, E., Caputo, A.(1958) *Biochim. Biophys. Acta 30*, 608-615.
3. Colonna, G., Irace, G., Parlato, G., Aloj, S. M., Balestrieri, C. (1978) *Biochim. Biophys. Acta 532*, 354-367.
4. Fricke, H. and Hart E. J. In *Radiation Dosimetry*; Vol. 2, Attix, F. H.; Roesch Eds, W. C., *Academic press,* New York and London, 1966; pp 167-232.
5. Mialocq, J.-C., Hickel, B., Baldacchino, G., Juillard, M. (1999) *J. Chim. Phys. 96*, 35-43.

6. Fraenkel-Conrat, H., Harris, J. I., Levy, A. L. (1955) *Methods Biochem. Anal. 2*, 389.
7. Mousseau, G., Thomas, O. P., Oppilliart, S., Coirier, A., Salcedo-Serna, A., Thai, R., Beau, F., Renault, J. P., Pin, S., Cintrat, J.-C., Rousseau, B. (2007) *Anal. Chem. 79*, 5444-5448.