

# Probing the reactivity of nucleophile residues in human 2,3-diphosphoglycerate/deoxy-hemoglobin complex by aspecific chemical modifications

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**Abstract** The use of aspecific methylation reaction in combination with MS procedures has been employed for the characterization of the nucleophilic residues present on the molecular surface of the human 2,3-diphosphoglycerate/deoxy-hemoglobin complex. In particular, direct molecular weight determinations by ESMS allowed to control the reaction conditions, limiting the number of methyl groups introduced in the modified globin chains. A combined LCESMS-Edman degradation approach for the analysis of the tryptic peptide mixtures yielded to the exact identification of methylation sites together with the quantitative estimation of their degree of modification. The reactivities observed were directly correlated with the  $pK_a$  and the relative surface accessibility of the nucleophilic residues, calculated from the X-ray crystallographic structure of the protein. The results here described indicate that this methodology can be efficiently used in aspecific modification experiments directed to the molecular characterization of the surface topology in proteins and protein complexes.

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**Key words:** Aspecific chemical modification; Mass spectrometry; Surface topology; Methyl bromide; Hemoglobin adduct

## 1. Introduction

Chemical modification reactions on proteins have long been employed in structure function studies aimed at identifying active site residues, studying chemical reactivity of amino acids, or modifying catalytic properties and proteolytic specific-

ity [1]. Different reagents have been utilized for the covalent derivatization of a specific functional group (i.e. primary amine, imidazole ring, sulphhydryl, guanidino or phenolic hydroxyl group) without any demonstrable effect on other functionalities [2]. These methodologies were greatly improved when mass spectrometric techniques were used to determine both the precise number and location of the derived amino acid residues.

More recently, site-directed modifications of lysine, arginine, tyrosine and histidine residues have been used to probe the surface topology of proteins or to investigate interacting regions in protein complexes. In fact, the careful selection of experimental conditions suitable to maintain the native conformation of the macromolecules and to limit the extent of modification to mono- or di-modified species allowed to infer information about the identity of the amino acid residues exposed on the molecular surface of proteins [3–5]. The simultaneous introduction of a multiple number of modifying groups was strongly avoided in order to limit local unfolding of the polypeptide chain resulting in a possible perturbation of the subtle network of interactions stabilizing the native conformation of the protein and in the accumulation of misleading data. Similarly, this approach was extended to localize functional regions of the proteins exposed to the solvent that became protected upon binding to other macromolecules or ligands [6–8].

Most of the reagents for chemical modification described in the literature are site specific; thus, the experimental conditions reported have been selected with the aim to avoid their simultaneous reaction with amino acids presenting different functionality. In this respect, the employment of the largest possible number of modifying agents was encouraged in order to obtain the largest number of information regarding the amino acids exposed on the molecular surface of proteins.

We recently reported an analytical methodology for the identification and quantitative evaluation of the modifications produced in hemoglobin as result of the in vitro exposition of human red blood cells to carcinogens as methyl bromide [9,10]. The possibility to detect even low level of methylation (0.5–5%) for cysteine, lysine, histidine, tyrosine and aspartic acid residues, result of the simultaneous alkylation of their side chains, led us to investigate the possible use of this reagent as a probe for the study of the reactivity of the nucleophile residues in proteins and protein complexes.

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**Abbreviations:** DPG, 2,3-diphosphoglycerate; ES, electrospray; GCEIMS, gas chromatography electron ionization mass spectrometry; h-deoxy-Hb, human deoxy-hemoglobin; LC, liquid chromatography; MeBr, methyl bromide; MS, mass spectrometry; PTH, phenylthiohydantoin; RP-HPLC, reversed phase high performance liquid chromatography; TBDMS, tert-butyl-dimethyl-silyl

## 2. Materials and methods

### 2.1. Alkylation with methyl bromide

Bubbling gaseous MeBr in cold pure ethanol prepared MeBr solution; its concentration was determined from the increase in the weight. Ten moles of human deoxy-hemoglobin were incubated with a 1–10-fold molar excess of MeBr in 10 ml of 10 mM sodium phosphate, 10 mM DPG, pH 7.5, at 37°C, under N<sub>2</sub> for different times. The reactions were stopped by acidification and the mixtures were then injected for chromatographic analysis. Due to the toxic action of this reagent we strongly recommend special care in the experiment settings.

### 2.2. Globin chains separation and LC-ESMS analysis of the tryptic peptide mixtures

Globin chains were purified by RP-HPLC using a 214TP54 Vydac column following the procedure previously reported [10]. Fractions eluted were manually collected and lyophilized. Trypsin hydrolysis was carried out in 50 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 8.5, at 37°C, overnight using a substrate:enzyme ratio of 50:1 (w/w). Tryptic digests were fractionated using a 218TP52 Vydac column eluted with a linear gradient (5–70%) of acetonitrile in 0.03% trifluoroacetic acid, at a flow rate of 200 µl/min. The column effluent was split 1:25 with a tee, to afford a flow rate of 8 µl/min into the ES nebulizer; the remaining effluent was run through an UV detector for peak collection. ES mass spectra were recorded using a PLATFORM single quadrupole instrument (Micromass, Manchester, UK) as previously reported [8,10,11].

### 2.3. Amino acid analysis and peptide sequencing

Ten nmoles of globins were hydrolyzed in 6 N HCl at 110°C for 24 h, under vacuum. Quantitative GCEIMS amino acid analysis of the protein hydrolysates was performed using a TRIO 2000 GCEIMS apparatus (Micromass, Manchester, UK) equipped with a 5890 series II gas chromatograph (Hewlett-Packard, Palo Alto, USA) as previously reported [12]. Peptide sequencing of intact Hb chains and peptides was performed by using a Perkin Elmer-Applied Biosystems 477A protein sequencer equipped with a model 120A PTH-amino acid analyzer as previously described [10].

### 2.4. Computer analysis

The structure of human deoxy-hemoglobin (Brookhaven Laboratory PDB file 1A3N) was examined on a Silicon Graphics workstation by using the Biosym Insight II/Discover software package. Static solvent accessible areas on the protein were calculated with the program CCP4 [13] by using a probe radius of 1.4 Å.

## 3. Results and discussion

The chemical reactivity of nucleophilic residues present on the 2,3-diphosphoglycerate/h-deoxy-Hb complex was investigated by incubation of the protein in the presence of the ligand with different molar excesses of MeBr, at neutral pH and room temperature.

Fig. 1A shows the chromatographic profile obtained using a 1:1 reagent/hemoglobin molar ratio; individual peaks were collected and directly submitted to ESMS analysis. Measurement of the accurate molecular mass of each component indicated that the extent of reaction was limited only to yield the mono-modified species. In fact, peak 1 exhibited the presence of two molecular components corresponding to the unmodified β-globin (theoretical value 15867.2 Da) and mono-methylated β-globin (theoretical value 15881.2 Da) (Fig. 1B). Similarly, peak 2 showed the presence of two species identified as the unmodified α-globin (theoretical value 15126.4 Da) and mono-methylated α-globin (theoretical value 15140.4 Da) (Fig. 1C). These modified globins consisted of a population of molecular species carrying a single modifying group but located at different sites. In fact, GCEIMS analysis of the purified species after acid hydrolysis demonstrated that methyl-

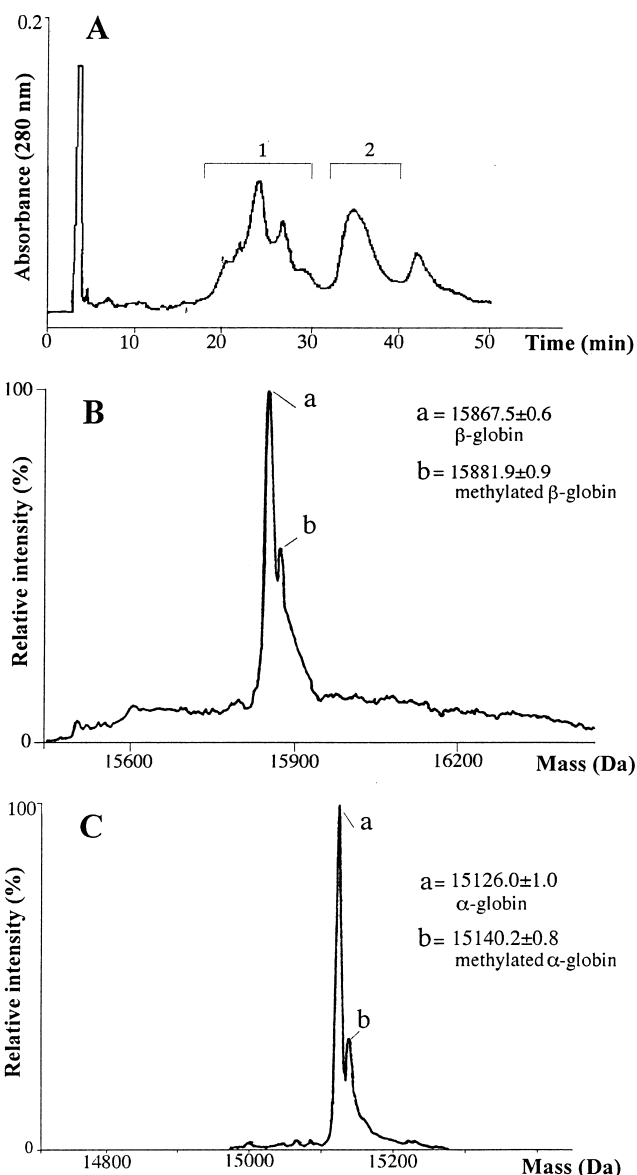


Fig. 1. Chromatographic separation and ESMS analysis of the globin chains generated from the reaction of the DPG/h-deoxy-Hb complex with 1 mM MeBr. The globins were resolved as described in Section 2 (A). Transformed ESMS spectra of the resulting β-globin species (peak 1 in panel A) (B) and α-globin species (peak 2 in panel A) (C), respectively.

ation occurred on N-terminal valine, cysteine and histidine residues, respectively.

In order to identify the nature of the amino acids alkylated, aliquots of peak 1 and 2 were individually digested with trypsin and the resulting peptide mixtures analyzed by LCESMS as previously described [10]. In most cases native and alkylated peptides eluted together as verified from the presence of a satellite signal corresponding to a mass increase of 14 Da with respect to the native peptide. The degree of methylation for each species was calculated from the relative intensities of their respective mass signals, assuming similar response factors in ESMS analysis [10]. The identification of the site of methylation was obtained by direct sequencing of the intact globins or their tryptic peptides by automated Edman degradation. Moreover, the possibility to analyze simultaneously in

the sequencer the methylated and unmethylated species and to determine at each cycle the relative abundance of the PTH-methyl amino acid with respect to the PTH-amino acid allowed to have an independent measure of the degree of modification for each peptide. A good correlation between the values determined by the different approaches was obtained as previously described [10]; the complete identification of the methylation sites in both globin chains and their percentage of modification is reported in Table 1. These data demonstrated that methylation occurred at Val-1( $\alpha$ ), His-20( $\alpha$ ), His-72( $\alpha$ ), His-89( $\alpha$ ), His-103( $\alpha$ ), Cys-104( $\alpha$ ), Val-1( $\beta$ ), Cys-93( $\beta$ ), His-97( $\beta$ ), Cys-112( $\beta$ ), His-116( $\beta$ ), His-117( $\beta$ ) and His-146( $\beta$ ), respectively.

Similarly, this methodology allowed to characterize the reaction products obtained by using higher reagent/hemoglobin ratios. In all cases, protein's solution behavior did not change, indicating that the reaction did not affect the Hb solubility. As expected, in the case of a 10:1 molar ratio, ESMS measurement of the  $\alpha$ - and  $\beta$ -globin fractions indicated that the reaction yielded a high percentage of multi-modified species; in fact, a mixture of the penta-, esa- and epta-modified globins was observed. Accordingly, GCEIMS analysis of the globin hydrolysates revealed a strong increase of the percentage of methylated amino acids. The peaks corresponding to the  $\alpha$ - and  $\beta$ -globins were pooled and analyzed as reported above, affording the identification of the methylation sites and their percentage of modification. Thus, in addition to the residues modified by using a 1:1 molar ratio, in this case methylation occurred at Lys-7( $\alpha$ ), Lys-11( $\alpha$ ), Lys-40( $\alpha$ ), His-45( $\alpha$ ), His-50( $\alpha$ ), Lys-56( $\alpha$ ), His-112( $\alpha$ ), His-122( $\alpha$ ), Lys-139( $\alpha$ ), Lys-17( $\beta$ ), His-77( $\beta$ ), His-92( $\beta$ ), His-143( $\beta$ ) and Lys-144( $\beta$ ), respectively.

The results obtained with this approach demonstrated that modification in native conditions was mainly concentrated at specific sulphydryl groups, primary amines and imidazole rings, respectively. Only in the case of the 10:1 reagent/hemoglobin molar ratio, a very small amount of selected N $\epsilon$ -methyl-lysine residues was also detected. On the contrary, peptide mapping analysis of the methylated globins resulting from the reaction of MeBr with irreversibly denatured DPG/*h*-deoxy-Hb complex showed that methylation was not concentrated at specific amino acids but diffused everywhere all over the nucleophilic residues present in the polypeptide chains.

In addition, these data were consistent with the *h*-deoxy-Hb structure determined by X-ray crystallography and reported in Fig. 2, where the methylated residues obtained using a 1:1 molar ratio are reported in red. In fact, modification occurred only at amino acid side chains exposed on the protein surface, whereas those present into the inner core resulted inaccessible to the reagent. Similarly, all the residues methylated using a 10:1 molar ratio were present on regions accessible to the reagent. However, the presence of a high number of modifying groups on each polypeptide chain should result in local unfolding of the Hb tetramer, determining a possible perturbation of the native conformation of the complex and in the accumulation of misleading data. For this reason, although these results suggested that the DPG/multi-methylated deoxy-Hb complex still presented the native conformation, we did not consider them for further interpretations.

The degree of methylation measured for the reactive amino acids of the  $\alpha$ - and  $\beta$ -globins by using both reagent/hemoglobin molar ratios is reported in Fig. 3, together with the static

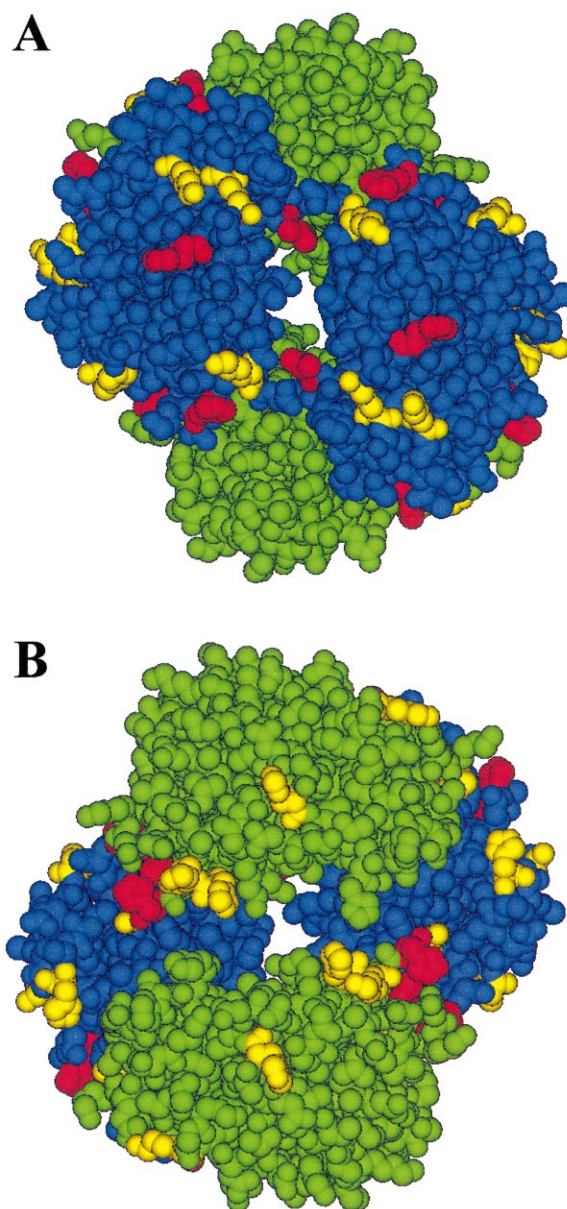
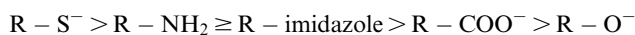


Fig. 2. Space-filling representation of the human deoxy-hemoglobin structure.  $\alpha$ -globins are shown in blue and  $\beta$ -globins in green. The amino acids modified with 1 mM MeBr are shown in red; those methylated with 10 mM MeBr are represented in yellow. Two Hb tetramer orientations rotated by roughly 180° are illustrated.

surface accessibility for the nucleophilic atoms present on N-terminal valine, cysteine and histidine residues, calculated on the basis of the deoxy-hemoglobin crystal structure. Quantitative evaluation of the relative modification observed for the different residues was in good agreement with the nucleophilicity order reported for the major groups in biological molecules [14]



with the exception of the N $\epsilon$ -amine of lysine side chains that resulted poorly methylated. In addition, these results provided evidence that the reactivity toward methylation of amino acids having the same nature generally well correlated with their

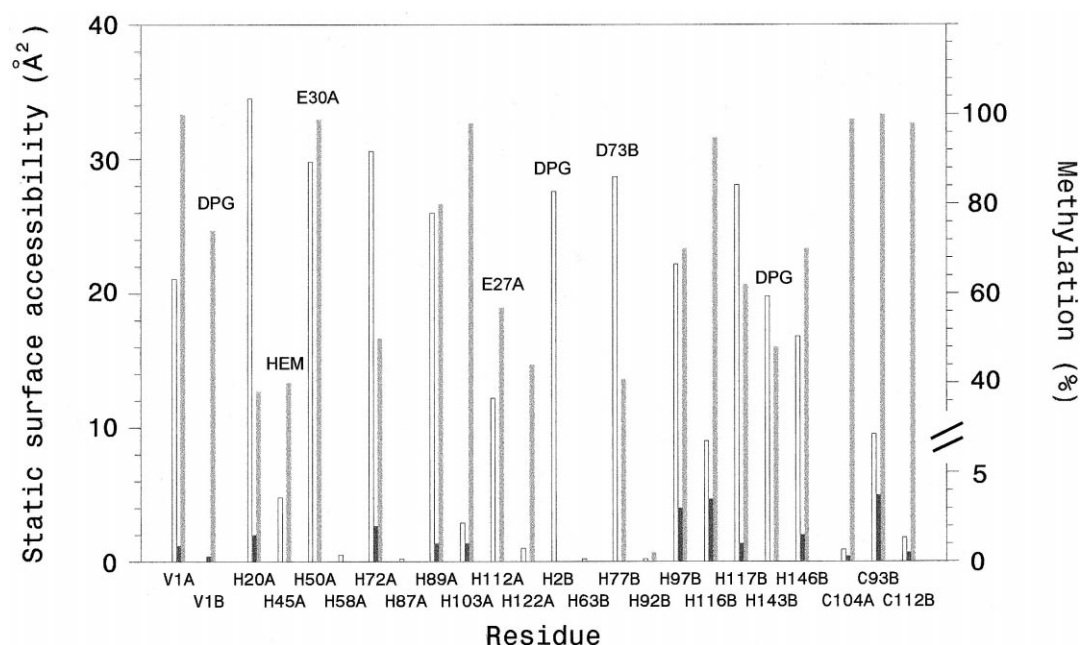


Fig. 3. Representation of the reactivity and the static solvent accessibility of the nucleophilic residues present in DPG/h-deoxy-Hb complex. The percentage of methylation obtained with 1 mM MeBr and 10 mM MeBr (determined by Edman degradation and ESMS analysis) are shown in black and gray, respectively. The static solvent accessible areas on the protein were calculated with the program CCP4 [12] using a probe radius of 1.4 Å and are shown in white.

Table 1  
ESMS and Edman degradation analysis of the tryptic peptides obtained from the reaction of DPG/h-deoxy-Hb complex with 1 mM MeBr.

| $\alpha$ -globin |   |                                |                                |                          | $\beta$ -globin |   |                                      |                                |  |
|------------------|---|--------------------------------|--------------------------------|--------------------------|-----------------|---|--------------------------------------|--------------------------------|--|
| Peak             | Meas. mass  | Peptide                        | ESMS                           | Edman deg.               | Peak            | Meas. mass  | Peptide                              | ESMS                           | Edman deg.   |
| 1                | 287.3 $\pm$ 0.1<br>337.5 $\pm$ 0.1<br>460.2 $\pm$ 0.1 | (91–92)<br>(140–141)<br>(8–11) |                                |                          | 1               | 317.8 $\pm$ 0.1<br>331.8 $\pm$ 0.1<br>411.4 $\pm$ 0.1 | (145–146)<br>(145–146)+Me<br>(62–65) | 1.4%                           | H146 (1.7%)  |
| 2                | 531.9 $\pm$ 0.3                                       | (12–16)                        |                                |                          | 2               | 951.3 $\pm$ 0.1                                       | (1–8)                                |                                |  |
| 3                | 728.4 $\pm$ 0.2                                       | (1–7)                          |                                |                          | 3               | 965.2 $\pm$ 0.2                                       | (1–8)+Me                             | 0.2% $\alpha$ -NH <sub>2</sub> | n.d.   |
| 4                | 742.9 $\pm$ 0.3                                       | (1–7)+Me                       | 0.8% $\alpha$ -NH <sub>2</sub> | n.d.                     | 4               | 1377.9 $\pm$ 0.3                                      | (121–132)                            |                                |  |
| 5                | 1170.6 $\pm$ 0.1                                      | (1–11)                         |                                |                          | 5               | 1149.2 $\pm$ 0.1<br>1314.0 $\pm$ 0.1                  | (133–144)<br>(18–30)                 |                                |  |
| 6                | 1184.7 $\pm$ 0.2                                      | (1–11)+Me                      | 0.7% $\alpha$ -NH <sub>2</sub> | n.d.                     | 6               | 1125.4 $\pm$ 0.4<br>1139.6 $\pm$ 0.2                  | (96–104)<br>(96–104)+Me              | 2.8%                           | H97 (3%)   |
| 7                | 1078.3 $\pm$ 0.2                                      | (81–90)                        |                                |                          | 7               | 1122.5 $\pm$ 0.4<br>1449.8 $\pm$ 0.3                  | (121–130)<br>(133–146)               |                                |  |
| 8                | 817.5 $\pm$ 0.1                                       | (93–99)                        |                                |                          | 8               | 1463.4 $\pm$ 0.1                                      | (133–146)+Me                         | 1.5%                           | H146 (1.9%)  |
| 9                | 1529.4 $\pm$ 0.2                                      | (17–31)                        |                                |                          | 9               | 1793.2 $\pm$ 0.2                                      | (66–82)                              |                                |  |
| 10               | 1543.3 $\pm$ 0.1                                      | (17–31)+Me                     | 1.7%                           | H20 (1.9%)               | 10              | 931.2 $\pm$ 0.1                                       | (9–17)                               |                                |  |
| 11               | 1086.6 $\pm$ 0.1                                      | (91–99)                        |                                |                          | 11              | 1148.4 $\pm$ 0.1                                      | (133–144)                            |                                |  |
| 12               | 1833.8 $\pm$ 0.2                                      | (41–56)                        |                                |                          | 12              | 1420.7 $\pm$ 0.4                                      | (83–95)                              |                                |  |
| 13               | 1252.2 $\pm$ 0.1                                      | (128–139)                      |                                |                          | 13              | 1434.5 $\pm$ 0.2                                      | (83–95)+Me                           | 3.7%                           | C93 (4.0%)   |
| 14               | 1071.6 $\pm$ 0.2                                      | (32–40)                        |                                |                          | 14              | 1718.9 $\pm$ 0.3                                      | (105–120)                            |                                |  |
| 15               | 1936.7 $\pm$ 0.2                                      | (62–80)                        |                                |                          | 15              | 1733.7 $\pm$ 0.4                                      | (105–120)+Me                         | 4.8%                           | C112 (0.6%), H116 (3.7%), H117 (0.9%)                        |
| 16               | 2063.2 $\pm$ 0.2                                      | (61–80)                        |                                |                          | 16              | 1274.3 $\pm$ 0.3                                      | (31–40)                              |                                |  |
| 17               | 3125.0 $\pm$ 0.3                                      | (61–90)                        |                                |                          | 17              | 1669.4 $\pm$ 0.6                                      | (67–82)                              |                                |  |
|                  | 3140.1 $\pm$ 0.1                                      | (61–90)+Me                     | 2.6%                           | H72 (1.6%), H89 (0.7%)   | 18              | 1797.8 $\pm$ 0.1                                      | (66–82)                              |                                |  |
|                  | 2996.5 $\pm$ 0.3                                      | (62–90)                        |                                |                          | 19              | 2527.2 $\pm$ 0.7                                      | (83–104)                             |                                |  |
|                  | 3010.4 $\pm$ 0.3                                      | (62–90)+Me                     | 2.4%                           | H72 (1.8%), H89 (0.9%)   | 20              | 2059.0 $\pm$ 0.2                                      | (41–59)                              |                                |  |
|                  | 2967.7 $\pm$ 0.3                                      | (100–127)                      |                                |                          | 21              | 889.2 $\pm$ 0.2                                       | (31–37)                              |                                |  |
|                  | 2982.9 $\pm$ 0.2                                      | (100–127)+Me                   | 1.3%                           | H103 (0.9%), C104 (0.4%) | 22              | 4231.7 $\pm$ 0.5                                      | (83–120)                             |                                |  |
|                  |   |                                |                                |                          | 23              | 4245.1 $\pm$ 0.6                                      | (83–120)+Me                          | 12.5%                          | C93 (3.8%), H97(3.0%), C112 (0.7%), H116 (3.5%), H117 (1.0%) |

relative static surface accessibility. As an example, the percentage of PTH-methyl-Cys recovered for cysteine residues using a 1:1 molar ratio was higher for Cys-93( $\beta$ ), than Cys-112( $\beta$ ) and Cys-104( $\alpha$ ) and proportional to the exposition of their sulphur atoms on the protein surface.

A significant deviation was found only in the case of some histidine residues; in particular, His-45( $\alpha$ ), His-50( $\alpha$ ), His-112( $\alpha$ ), His-2( $\beta$ ), His-77( $\beta$ ) and His-143( $\beta$ ) were not modified by using the 1:1 reagent/hemoglobin molar ratio with respect to their high accessibility on protein surface. The low reactivity of His-2( $\beta$ ) and His-143( $\beta$ ) was expected on the basis of the masking effect due to the 2,3-diphosphoglycerate. In fact, it has been previously reported that these amino acids are essential in binding to this ligand [15]. Quantitative experiments on deoxy-hemoglobin in the absence of DPG showed a methylation pattern almost identical to that reported in Fig. 3 and to the results previously published in the case of the *in vitro* exposed human red blood cells [10]. An increased reactivity was observed only for His-2( $\beta$ ) and His-143( $\beta$ ), whose percentage of methylation resulted 2.1 and 1.5, respectively. These results were in perfect agreement with the reported masking effect exerted from the ligand and with the crystallographic data indicating that binding of DPG results in poor changes in the tertiary structure of the  $\alpha$ - and  $\beta$ -subunits [15].

Similarly, the poor methylation observed for His-45( $\alpha$ ), His-50( $\alpha$ ), His-112( $\alpha$ ) and His-77( $\beta$ ) was easily explained in terms of changes in their  $pK_a$  due to microenvironmental effects experienced by an acid functionality close to these residues within the three-dimensional structure of the complex. In fact, a careful analysis of the structure of the deoxy-hemoglobin tetramer around these amino acids revealed that the Ne2 of His-45( $\alpha$ ) is in close proximity (3.5 Å) to the carboxylate of the heme; similarly, the N $\delta$ 1 of His-50( $\alpha$ ) is able to give an ionic interaction with Glu-30( $\alpha$ ). The side chain of Asp-73( $\beta$ ) introduces a negative charge within a few angstroms of His-77( $\beta$ ), whilst His-112( $\alpha$ ) presents a double hydrogen bond with the carboxylate of Glu-27( $\alpha$ ) (N $\delta$ 1) and the phenolic hydroxylate of Tyr-24( $\alpha$ ) (Ne2), respectively. These interactions would be expected to produce an electrostatic stabilization of the protonated form of the imidazole rings, with a consequent reduced nucleophilicity of these histidine residues [16].

The results obtained on human deoxy-hemoglobin clearly demonstrate that aspecific chemical modification experiments can be designed to probe simultaneously the accessibility of amino acids having different nature on the surface of proteins and to gain information on protein tertiary and quaternary structure. In fact, our experiments demonstrated that all the residues labeled by MeBr were found exposed on the molecular surface. However, in these studies local factors affecting the normal  $pK_a$  of the reacting side chains and, hence, their nucleophilicity values according to the Brønsted relationship, have to be carefully considered. In fact, as described above, amino acids not modified by MeBr can be anyhow present on the molecular surface of the system under investigation. Furthermore, this methodology was usefully extended to localize functional regions of the macromolecule protected upon binding to ligands as DPG, showing its applicability to the study of protein complexes.

We would like to emphasize again in these studies the importance of the experimental conditions, which have to be selected in order to ensure the maintenance of the protein native conformation or the stability of the complex. There-

fore, in the case of proteins whose three-dimensional structures have not yet been determined, the use of reagent concentrations able to afford only the mono- or di-modified products is strongly recommended in order to obtain data on the surface topology of the system under investigation, avoiding the acquisition of erroneous information. On the contrary, additional experiments using high protein/reagent molar ratios could be suggested for the study of the reactivity of amino acids in proteins whose structures have yet been elucidated. In this respect, a reagent as MeBr, able to introduce a modifying group with low steric hindrance, could be considered as a choice of excellence. In these cases, the effect of the simultaneous presence of an increased number of methyl groups on protein conformation has to be carefully evaluated.

Therefore, this approach can be considered as an additional tool to the selective chemical modification and limited proteolysis methodologies previously reported for studies directed to the identification of residues exposed on the molecular surface [5,8,11,17–19]. Thus, the use of these mass spectrometric procedures will provide preliminary or complementary data on protein and protein complexes to be completed with more traditional techniques as circular dichroism, X-ray crystallography, nuclear magnetic resonance and fluorescence spectroscopies.

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## References

- [1] Glazer, A.N. (1976) in: H. Neurath and R.L. Hill (Eds.), *The Proteins*, 3rd Edn., Vol. 2, Academic Press, New York, pp. 1–103.
- [2] Lundblad, R.L. (1991) *Chemical Reagents for Protein Modification*, 2nd Edn., CRC Press, Boca Raton, FL.
- [3] Suckau, D., Mak, M. and Przybylski, M. (1992) *Proc. Natl. Acad. Sci. USA* 89, 5630–5634.
- [4] Glockner, M., Borchers, C., Fiedler, W., Suckau, D. and Przybylski, M. (1994) *Bioconjug. Chem.* 5, 583–590.
- [5] Zappacosta, F., Ingallinella, P., Scaloni, A., Pessi, A., Bianchi, E., Sollazzo, M., Tramontano, A., Marino, G. and Pucci, P. (1997) *Protein Sci.* 6, 1901–1909.
- [6] Ohguro, H., Palczewski, K., Walsh, K.A. and Johnson, R.S. (1994) *Protein Sci.* 3, 2428–2434.
- [7] Ploug, M., Rahbek-Nielsen, H., Ellis, V., Roepstorff, P. and Dano, K. (1995) *Biochemistry* 34, 12524–12534.
- [8] Scaloni, A., Monti, M., Acquaviva, R., Tell, G., Damante, G., Formisano, S. and Pucci, P. (1999) *Biochemistry* 38, 64–72.
- [9] Ferranti, P., Sannolo, N., Mamone, G., Fiume, I., Carbone, V., Tornqvist, M., Bergman, A. and Malorni, A. (1996) *Carcinogenesis* 17, 2661–2671.
- [10] Mamone, G., Malorni, A., Scaloni, A., Sannolo, N., Basile, A., Pòcsfalvi, G. and Ferranti, P. (1998) *Rapid Comm. Mass Spectrom.* 12, 1–10.
- [11] Scaloni, A., Miraglia, N., Orrù, S., Amodeo, P., Motta, A., Marino, G. and Pucci, P. (1998) *J. Mol. Biol.* 277, 945–958.
- [12] Chavas das Neves, H.J. and Vasconcellos, A.M.P. (1987) *J. Chromatogr.* 392, 249–258.
- [13] CCP4 (1994) *Acta Crystallogr.* D50, 760–767.
- [14] Hermanson, G. (1996) *Bioconjugate Techniques*, Academic Press, San Diego, pp. 4–16.
- [15] Arnone, A. (1972) *Nature* 237, 146–149.
- [16] Bycroft, M. and Fersht, A. (1988) *Biochemistry* 27, 7390–7394.
- [17] Cohen, S.L., Ferre-D'Amare, A.R., Burley, S.K. and Chait, B.T. (1995) *Protein Sci.* 4, 1088–1099.
- [18] Kriwacki, R.W. and Siuzdak, G. (1998) *J. Biomol. Tech.* 9, 5–15.
- [19] Winston, R.L. and Fitzgerald, M.C. (1997) *Mass Spectrom. Rev.* 16, 165–179.