Identification of the sites of deoxyhaemoglobin PEGylation

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INTRODUCTION

The 2-iminothiolane reaction with protein amino groups adds a spacer arm ending with a thiol group, which can be further treated with molecules carrying a maleimido ring. This approach is currently used for the preparation of a candidate ‘blood substitute’ in which human Hb (haemoglobin) is conjugated with long chains of PEG [poly(ethylene glycol)]. To identify the thiolation sites by MS, we have carried out the reaction using deoxyHb bound to inositol hexaphosphate to protect some of the residues crucial for function and NEM (N-ethylmaleimide) to block and stabilize the thiol groups prior to enzymatic digestion by trypsin and pepsin. Under the conditions for the attachment of 5–8 PEG chains per tetramer, the thiolated residues were Lys139 and, with lower accessibility, Lys90, Lys99 and Lys60 of the α-chain and Lys8, Lys17, Lys59, Lys61 and Lys66 and, with lower accessibility, Lys65, Lys95 and Lys144 of the β-chain. The α-amino groups of α- and β-chains were not modified and the reaction of the Cys209 residues with NEM was minor or absent. After the modification with thiolane and NEM of up to five to eight lysine residues per tetramer, the products retained a large proportion of the properties of native Hb, such as low oxygen affinity, cooperativity, effect of the modulators and stability to autoxidation. Under identical anaerobic conditions, the conjugation of the thiolated Hb tetramer with five or six chains of the maleimido derivative of 6 kDa PEG yielded products with diminished cooperation, Hill coefficient h = 1.3–1.5, still retaining a significant proportion of the effects of the modulators of oxygen affinity and stability to autoxidation. Co-operativity was apparently independent of the topological distribution of the PEGylated sites as obtained by treating partly the thiolated protein with NEM prior to PEGylation [poly(ethylene glycol)].

Key words: blood substitute, haemoglobin (Hb)-based oxygen carrier, N-ethylmaleimide (NEM), protein mass spectroscopy (protein MS), protein poly(ethylene glycol)ation (protein PEGylation), protein thiolation.

Abbreviations used: α-CHC, α-cyano-4-hydroxycinnamate; DHA, dihydroxyacetophenone; 2,3-DPG, 2,3-diphosphoglycerate; ESI, electron spray ionization; FA, formic acid; deoxyHb, deoxyhaemoglobin; Hb*, ferric Hb; HbA0, HbA2; HbCN, cyanomet Hb; HbO2, oxyHb; IHP, inositol hexaphosphate; IMT, 2-iminothiolane; MALDI, matrix-assisted laser-desorption ionization; PEG, poly(ethylene glycol); MAL-PEG, maleimido-PEG; MS/MS, tandem MS; NEM, N-ethylmaleimide; p50, oxygen affinity (the oxygen partial pressure producing 50% saturation); RSA, relative solvent accessibility.
increases the p groups introduced by the reaction with IMT, as shown in Scheme 1. The reaction with NEM prevented the ring closure of the thiol maleimido derivative that reacts rapidly with thiols. Furthermore, the anaerobic reaction with NEM, a low-molecular-mass complex. The added thiol groups were then blocked in step b carried out reaction step a of Scheme 1 on the deoxyHb–IHP binding prevents the CO2 and cyanate reactions with the tertiary structure of the Cys β93 residues to thiol-specific reagents. Thus we 93 residues not treated with NEM and provided indirectly a means of checking the efficacy of the deoxyHb–IHP complex in stabilizing the T tertiary and quaternary conformations. At the end of the cycle a stable product is obtained, which can be used for the analysis of the physicochemical properties and the identification of the sites of the chemical modification. Alternatively, the product can be used for another cycle of reactions with IMT and NEM. The reactions with IMT and MAL-PEG were carried out by the same procedure with slight changes due to the PEG high molecular mass and the slow MAL-PEG reaction with thiols.

Reagents

IMT and IHP (dodecasodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); NEM was from Fluka Chemie (Buchs, Switzerland); HPLC-grade acetonitrile, bi-distilled water, FA (formic acid) and TFA (trifluoroacetic acid) were from JT Baker (Milan, Italy); Tos-Phe-CH2Cl (tosylphenylalanilylchloromethane; ‘TPCK’)-trypsin was from Promega (Madison, WI, U.S.A.); pepsin was from Sigma–Aldrich (St. Louis, MO, U.S.A.); MAL-PEG (molecular mass 5.6 kDa) was from Nektar Molecule Engineering (Nektar Therapeutics, San Carlos, CA, U.S.A.) and gases were from SAPIO (Monza, Italy).

Reactions under anaerobic conditions

Reactions with IMT and NEM

Purified HbA0 (Hb A0) [Hb+ (ferric Hb) < 1%] was used [18]. Chemical modifications of the deoxyHb–IHP complex (0.50 mM, tetramer; IHP/Hb molar ratio, 1.1:1) in 50 mM potassium phosphate, 100 mM KCl and 0.5 mM EDTA (pH 7.0) were carried out in a reactor thermostatically maintained at 20 °C under a nitrogen flow using various IMT concentrations and a NEM/Hb molar ratio of 6:1. Anaerobic gel filtration to stop the reactions was carried out using Sephadex G-25 columns equilibrated with deoxygenated reaction buffer (50 mM potassium phosphate, 100 mM KCl and 0.5 mM EDTA, pH 7.0.) and eluted anaerobically at 20–25 °C. Since the reaction between the deoxyHb–IHP complex and IMT and NEM continued during column loading and elution before achieving a complete separation, the overall contact times, calibrated using a mixture of Hb and p-nitrophenol, were: ≤ 8 min for NEM and ≤ 15 min for IMT.

Reactions with IMT and MAL-PEG

IMT and MAL-PEG, added together to the deoxyHb–IHP complex, were made to react for 23–25 min in the reactor before removal of the IMT excess by gel filtration. The MAL-PEG reaction continued during chromatography and anaerobic collection of the eluate to which more deoxygenated IHP solution was added (1 mol/Hb tetramer). The reaction was terminated after 45 min (against 2–3 min required by NEM) by the addition of a deoxygenated cysteine solution. PEGylated cysteine was removed by ultrafiltration using an Amicon membrane with 30 kDa cut-off.

Determination of the number of NEM- and MAL-PEG-modified moieties

A sample of deoxyHb–IHP solution was incubated separately under the same conditions in the absence of the maleimido compounds. Thiol titration in this sample after IMT removal by gel filtration yielded the number of thiol groups added by thiolation plus those of the Cysβ93 residues. Titration of the thiol groups in the sample treated with NEM or MAL-PEG provided the number of
thiols left on the protein. The difference between the two titrations yielded the number of groups modified by conjugation with NEM or Mal-PEG. Different topological distributions of PEGylated residues were obtained by treating the thiolated protein in a first cycle with NEM and in a second cycle with MAL-PEG. Conjugation with NEM and MAL-PEG was determined at each cycle.

Thiol titration

HbO₂ samples were treated with 4-PDS (4,4′-dithiopyridine) for 12 min at 25 °C until the absorbance change at 324 nm was less than 0.005 min⁻¹. OxyHbₐₒ yielded 2.3 ± 0.1 thiol groups/tetramer [20]. To check the accessibility to the reaction with NEM of the Cys⁴⁹⁹ residues in the deoxyHb–IHP complex, a constant amount of deoxygenated NEM solution was added to samples of 0.5 mM deoxyHb–IHP solution in phosphate buffers of varying pH (50 mM potassium phosphate, 100 mM KCl and 0.5 mM EDTA, pH 7.0–8.0). At the end of the incubation period a deoxygenated solution of 2-mercaptoethanol (20 mol/mol of NEM) was added to quench the NEM reaction with the protein. The solution was transferred to a column for aerobic gel filtration to remove the reagents in excess and the thiol titre of the Hb eluate was determined.

IHP removal

The pH of the Hb solution was increased from 7 to 8.3 by the addition of 2 M Tris and the solution was gel filtered on a Sephadex G-25 column equilibrated with 50 mM potassium phosphate, 100 mM KCl and 0.5 mM EDTA (pH 8.3). The eluate was then gel filtered again in the standard buffer (50 mM potassium phosphate, 100 mM KCl and 0.5 mM EDTA, pH 7.0).

Oxygen affinity and autoxidation rate measurements

Oxygen affinity (p50; the oxygen partial pressure producing 50 % saturation) at 20°C was measured with a precision of ±1–2 % of the value from the spectral determination of the composition in deoxyHb, HbO₂ and HbCN (cyanomet Hb) of the Hb solution (2 mM haem concentration) in a buffer containing 0.2 mM cyanide (Figure 1). The Hill coefficient, h, was calculated with a ±5 % precision using oxygen saturation data in the 35–65 % range. Enzymes for radical scavenging were not added. The Hb⁺ increase, determined as HbCN, was less than 2 % of the total after equilibration. The Bohr effect (measurements of pH versus p50 versus pH) and the effect of 2,3-DPG addition at pH 7 were obtained similarly. The rate of Hb⁺ formation by autoxidation was obtained from the spectrum of Hb samples incubated at 20°C in the standard phosphate buffer (pH 7) under aerobic conditions in the presence of cyanide, as described for the measurement of the oxygen saturation.

Trypsin and pepsin digestions

Proteolytic digestions were carried out with: (i) trypsin, using a 1:20 (w/w) trypsin/Hb ratio, at 37°C for 12 h in 100 mM ammonium carbonate (pH 8.5); (ii) pepsin, 1:25 (w/w) pepsin/Hb ratio, for 2 and 6 h at 30°C and pH 2; and (iii) trypsin followed by pepsin after trypsin removal by ultrafiltration using 10 kDa molecular-mass cut-off Centronics (Millipore, Bedford, MA, U.S.A.). Digestions were stopped by freezing at –80°C.

HPLC for MS experiments

Samples (20 µl) were analysed using an HPLC apparatus and a Hypersil C18 column (150 mm × 0.55 mm, 5 µm particle size) equilibrated with solvent A (water + 0.025 % FA). After 2 min of isocratic elution, the proportion of solvent B (acetonitrile + 0.025 % FA) was raised from 0 to 35 % in 27 min, to 60 % in 10 min, to 95 % in 3 min. This condition was maintained for 2 min and in 1 min the proportion of solvent B was lowered from 95 to 0 %.

MS

Voyager DE-PRO mass spectrometer (Applied Biosystems) was used to obtain the MALDI (matrix-assisted laser-desorption ionization) spectra of whole protein or products of protein enzymatic digestion. The matrixes were α-CHC (α-cyano-4-hydroxycinnamate) for the analyses of digests and DHA (dihydroxyacetophenone) for undigested Hb. The matrix solvents for dissolving α-CHC and DHA were 50:50 water/acetonitrile plus 0.1 % FA and 70:30 water/acetonitrile plus 0.1 % FA. The digested or whole protein sample (1 µl) was dried on the MALDI target before the addition of matrix solution (0.5 µl) and dried again. The ESI (electron spray ionization) mass spectra and chromatograms were obtained using an Bruker HCT™ (high-capacity trap) Ion Trap mass spectrometer (Bruker Daltonics, Billerica, MA, U.S.A.). The entrance capillary temperature was 250°C. The capillary voltage

Figure 1  Spectra of 0.5 mM Hb solutions in a 100 mM KCl and 50 mM phosphate buffer, containing 0.2 mM cyanide, measured by using a 2-mm-optical-pathlength cell sealed to a 250 ml tonometer bottle

(A) (—–), Spectrum of the deoxygenated solution introduced into the tonometer purged with nitrogen; (– – – –), best fitting composite spectrum using Matlab 6.5, <1 % HbO₂, <1 % HbCN, > 99 % deoxyHb. (B) (—–), same solution as (A) equilibrated for 15 min after the introduction of 7 ml of air; (— – – –), best fitting composite spectrum, 70.6 % HbO₂, 1.1 % HbCN, 28.3 % deoxyHb. Equilibrium pO₂ (oxygen partial pressure) was calculated knowing the oxygen saturation, the amount of Hb and oxygen (mol) introduced into the tonometer.
was 5 kV. MS/MS (tandem MS) spectra of selected peptide ions were obtained by Collision Induced Dissociation.

MS data analysis and RSA (relative solvent accessibility) data

MALDI data were converted into ASCII format and elaborated using the Profound PMF (Peptide Mass Fingerprint) approach (http://prowl.rockefeller.edu/). Modified and unmodified peptide masses were identified with a mass accuracy of 20 p.p.m. and from approx. 3.4 to 2.4 after three cycles using the high (60:1) IMT/deoxyHb–IHP molar ratio.

RESULTS

Assessment of the conditions for deoxyHb–IHP thiolation

Since thiolation requires a non-protonated amino group (Scheme 1), the reaction proceeds faster with a higher pH. However, the stability of the deoxyHb–IHP complex, which constrains the protein T tertiary and quaternary conformations, decreases with increasing pH. The kinetics at 20°C of the NEM reaction with the Cys93 residues of the deoxyHb–IHP complex were studied in the pH range 7.0–8.5 (Figure 2). At pH 7.0, using 6 mol NEM/tetramer, less than 2% of the Cys93 residues were NEM-modified after 15 min reaction. A greater proportion of the Cys93 residues reacted at pH > 7.0. Thus thiolation was carried out at pH 7.0 and the timing of the various phases of the procedure was standardized, as described in the Experimental section. In particular, the overall NEM reaction time was < 8 min.

Thiol groups added to deoxyHb–IHP with each IMT reaction cycle

The net number of thiols added at each reaction cycle using different IMT/deoxyHb–IHP molar ratios and averaged from five or more independent experiments is listed in Table 2. A progressive decrease was observed, from approx. 1.7 to 1.3 after five cycles using the low (20:1) IMT/deoxyHb–IHP molar ratio.
Table 2  Properties of the products of the IMT and NEM reactions with the deoxyHb–IHP complex at 20°C and pH 7.0 using IMT/deoxyHb–IHP molar ratios of 20:1 and 60:1

The net number of thiol groups added to the haemoglobin tetramer per reaction cycle was obtained by subtracting the thiol titre after the NEM addition (+NEM) from the titre determined without the NEM addition (−NEM). The p50 values (in torr; 1 torr = 133.3 Pa) in the absence of 2,3-DPG were measured at 20°C in 100 mM KCl, 0.5 mM EDTA, 0.2 mM KCN and 50 mM potassium phosphate (pH 7.0). In parentheses are the values of the Hill coefficient h. The increase in p50 value (Δp50) upon the addition of a 20% stoichiometric excess of 2,3-DPG was determined under the same pH conditions. Bohr protons were determined from p50 measurements in phosphate buffers of varying pH. Also shown are the properties of the product of HbO2 modification by one cycle of IMT and NEM reactions at pH 7.0, 4°C and 6 h reaction time. The 2,3-DPG effect of this product was determined with a 100% excess of Pi. –, Not measured; I/I, IMT/deoxyHb–IHP ratio; RCN, reaction cycle no.

<table>
<thead>
<tr>
<th>I/I</th>
<th>10:1</th>
<th>20:1</th>
<th>DeoxyHb–IHP</th>
<th>DeoxyHb–IHP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Thiol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−NEM</td>
<td>2.30±0.10</td>
<td>4.71</td>
<td>4.04±0.13</td>
<td>3.78±0.22</td>
</tr>
<tr>
<td>+NEM</td>
<td>0.10±0.05</td>
<td>0.04</td>
<td>2.29±0.06</td>
<td>2.26±0.05</td>
</tr>
<tr>
<td>Added thiol/cycle</td>
<td>1.74±0.14</td>
<td>1.52±0.23</td>
<td>1.65±0.13</td>
<td>1.20±0.17</td>
</tr>
<tr>
<td>Total added thiol</td>
<td>1.74</td>
<td>3.26</td>
<td>4.91</td>
<td>6.11</td>
</tr>
<tr>
<td>p50 (−2,3-DPG)</td>
<td>8.54±0.09 (2.5±0.1)</td>
<td>1.8 (1.8)</td>
<td>8.44 (2.5)</td>
<td>7.24 (2.4)</td>
</tr>
<tr>
<td>Δp50 (+2,3-DPG)</td>
<td>0.90</td>
<td>&lt;-0.10</td>
<td>0.80</td>
<td>0.80</td>
</tr>
<tr>
<td>Bohr H+ /tetramer</td>
<td>2.6</td>
<td>2.2</td>
<td>2.1</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Figure 3  Mass identification of the native chains and chains modified by the addition of single and multiple 227.31 Da mass units [IMT + NEM + H+] in MALDI spectra of non-digested Hb

(A) Product of four cycles of reactions with a 20:1 IMT/deoxyHb–IHP ratio. (1) α-chain; (2) α-chain plus one mass unit; (3) α-chain plus two mass units; (4) α-chain plus three mass units; (5) β-chain; (6) β-chain plus one mass unit; (7) β-chain plus two mass units. (B) Product of two cycles of reactions with a 60:1 IMT/deoxyHb–IHP ratio. (1) α-chain; (2) α-chain plus one mass unit; (3) α-chain plus two mass units; (4) α-chain plus three mass units; (5) α-chain plus four mass units; (6) β-chain; (7) β-chain plus one mass unit; (8) β-chain plus two mass units; (9) β-chain plus three mass units.

An 11-residue peptide corresponding to sequence 1–11 of the α-chain modified by a single 227.31 Da addition, [IMT + NEM + H+], was found in the products of all reaction cycles at low and high IMT/deoxyHb–IHP ratio. The MALDI technique could not distinguish whether the modified residue was Valα1 or Lysα7. The identification of Lysα7 as the
modified residue was made by the ESI technique. Figure 4 shows the MS/MS spectrum obtained by fragmenting the [V1LSPADK³TNVK¹ + IMT + NEM + 2H]⁺ ion, the sequence of which was assigned with a high statistical confidence (P value = 3.05 × 10⁻⁷). Besides this +2 ion at m/z 699.8 containing the 227.31 Da adduct, two abundant fragments were obtained at m/z 815 with sequence V1LSPADK³ and at m/z 937 with sequence K²TNVK¹ both modified at Lys¹. The identification of Lys¹ as the modified residue was supported by the presence of fragmentation ions modified at Val¹. Furthermore, such an identification was consistent with the absence in the MALDI spectrum of the digested chains of a peptide with the sequence corresponding to the first seven residues of the α-chain modified at Val⁰.

Mass spectra of the pepsin digests

ESI analyses of the pepsin digests agreed with the MALDI analyses of the tryptic digests on the identification of five modified lysine residues of the α-chain (Lys⁷, Lys¹¹, Lys¹⁶, Lys⁵⁶ and Lys¹³⁹) and five of the β-chain (Lys⁴, Lys¹⁷, Lys⁶⁰, Lys⁶¹ and Lys⁶⁶) in each of the products of four reaction cycles at low IMT/deoxyHb–IHP ratio and in the products of two reaction cycles at high ratio. Using the high IMT/deoxyHb–IHP ratio, additional modified lysine residues were found in the products of three cycles (Lys⁶⁰ and Lys⁹⁰, α-chain; Lys⁶⁵, β-chain) and four cycles (Lys⁶⁰, α-chain; Lys⁹⁵ and Lys¹⁴¹, β-chain) (see Supplementary Table 2 of the Supplementary material at http://www.BiochemJ.org/bj/403/bj4030189add.htm).

NEM addition to the Cys⁹³ residues

A peptide with the NEM addition to Cys⁹³ (1–2% of the total) was observed in the MALDI spectrum of the tryptic-digested chains of some of the cyclic reaction products at 60:1 IMT/deoxyHb–IHP molar ratio, although such a NEM modification was not detected in the MALDI spectrum of the whole chains (Figure 3). Details on the approximate quantification of this peptide are presented in Supplementary Figure 1 of the Supplementary material at http://www.BiochemJ.org/bj/403/bj4030189add.htm.

Functional properties of the products of the IMT/NEM reactions

The p⁵⁰ values at pH 7.0 in the absence and presence of 2,3-DPG and the Bohr effect were determined for the products of each reaction cycle using the low (20:1) and the high (60:1) IMT/deoxyHb–IHP molar ratios. The data are listed in Table 2, where comparison is also made with the properties of a product obtained by treating oxyHbA₀ with IMT and NEM for 6 h at 4°C. Such a product was similar to NES-Hb (Hb made to react with NEM at both Cys⁹³ residues) with regard to oxygen affinity, Bohr effect and total absence of 2,3-DPG effect.

Table 3 Properties of the products of the IMT/MAL-PEG reactions

PEG-conjugated Hbs were prepared by treating the thiolated protein with MAL-PEG only or with NEM in a first cycle and MAL-PEG in a second cycle. Different numbers of PEG chains per tetramer, from 5 to 7, were obtained by increasing the IMT reaction time (>20 min). Size-exclusion chromatography indicated that the amount of residual unmodified Hb in all these products was below the detection limit (<5% of the total; results not shown). The functional properties of some of these products are listed in Table 3 together with the properties of the reference product prepared by treating under the same conditions the thiolated protein with NEM only. The MS analyses of the peptide mixture obtained by digesting the reference product with pepsin indicated that the only significantly modified residues were Lys⁷, Lys¹¹, Lys⁶⁰, Lys⁶¹ and Lys⁶⁶ of the α-chain and Lys⁴, Lys¹⁷, Lys⁵⁶, Lys⁶¹ and Lys⁶⁶ of the β-chain.

Stability to autoxidation

The rates of Hb+ formation by autoxidation were measured at 20°C in the standard phosphate buffer (pH 7.0) and in the

<table>
<thead>
<tr>
<th>Species</th>
<th>IMT thiol titre</th>
<th>Cys⁹³ thiol titre</th>
<th>[p⁵⁰] ᵃ₋₇</th>
<th>2,3-DPG</th>
<th>+2,3-DPG</th>
<th>h</th>
<th>Bohr H⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>DeoxyHb–NEM</td>
<td>7.3 (NEM)</td>
<td>2.3</td>
<td>5.6</td>
<td>–</td>
<td>1.9 ± 0.1</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>DeoxyHb–PEG₅</td>
<td>4.7 (MAL-PEG)</td>
<td>2.5</td>
<td>5.8</td>
<td>–</td>
<td>1.6 ± 0.1</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>DeoxyHb–PEG₆</td>
<td>5.8 (MAL-PEG)</td>
<td>2.6</td>
<td>4.6</td>
<td>5.2</td>
<td>1.3 ± 0.1</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>DeoxyHb–PEG₇</td>
<td>7.5 (MAL-PEG)</td>
<td>2.3</td>
<td>4.5</td>
<td>–</td>
<td>1.4 ± 0.1</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>DeoxyHb–PEG₈</td>
<td>1.7 (NEM), 5.8 (MAL-PEG)</td>
<td>2.5</td>
<td>3.8</td>
<td>–</td>
<td>1.3 ± 0.1</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>DeoxyHb–PEG₉</td>
<td>2.7 (NEM), 6.6 (MAL-PEG)</td>
<td>2.3</td>
<td>5.2</td>
<td>5.9</td>
<td>1.5 ± 0.1</td>
<td>1.4</td>
<td></td>
</tr>
</tbody>
</table>

Hill coefficient (h) data are average values from measurements in the pH range 6.8–7.6. The thiol titre of the IMT-modified products (IMT thiol titre column) equals the number of the haemoglobin-conjugated NEM/MAL-PEG molecules (the conjugated species is indicated in parentheses). The Cys⁹³ thiol titre was determined after the NEM/MAL-PEG reactions. Thiol titre and Bohr effect refer to tetrameric haemoglobin. –, Not measured.
absence of radical scavenging enzymes, using purified native Hb, Hb thiolated under conditions in which about six thiol groups were added per tetramer and then made to react with NEM or MAL-PEG. The rates are compared in Figure 5 with the rate of autoxidation of Hb modified under the oxy conditions, for which the functional data are listed in Table 2.

DISCUSSION

Identification of the thiolation sites

The identification of the sites of Hb thiolation, which are also the sites of potential PEG attachment, was made possible by the strategy adopted to (i) stabilize the thiolation sites by the NEM reaction; (ii) carry out a progressive thiolation through cycles of reactions under two different kinetic conditions, and (iii) complement the MS analyses of the trypptic digests with those of the pepsin digests. We predicted that the small size, 227.31 Da, of the [IMT + NEM + H⁺] adduct would not interfere with trypsin proteolysis at sites flanking a modified one provided that the cleavage site was not next to the modified site. This prediction was confirmed, e.g., by the finding of a peptide with sequence 60–65 of the β-chain modified at Lys⁶¹ clearly indicating trypsin recognition of both cleavage sites at Lys⁶⁰ and Lys⁶⁵.

The relative intensities of the mass peaks of the species identified in the spectra of the modified undigested proteins in Figure 3 support the correlation between species distributions and number of modified sites. Such a correlation was consistent with indications of the analyses of the trypptic digests. After 2–4 reaction cycles using the low IMT/deoxyHb–IHP ratio, yielding up to six thiol groups/tetramer, a peptide modified at Lysα₁₁ with the sequence 8–16 of the α-chain and a peptide modified at Lysβ₁₆ with the sequence 12–31 of the same chain were found (Supplementary Table 1). The same modified peptides were found in the products of up to four reaction cycles at 20:1 IMT/deoxyHb–IHP molar ratio and two cycles at 60:1 molar ratio due to the presence of enough unmodified cleavage sites at Lys¹¹. When the modification of Lys¹¹ was also brought about by additional cycles at 60:1 IMT/deoxyHb–IHP molar ratio, the next potential site for cleavage was Lys¹⁶, yielding a peptide with mass of 3667 Da, beyond the detection range of the MALDI technique. This interpretation is supported by the persistence, even after three cycles at high IMT/deoxyHb–IHP molar ratio, of the peptide modified at Lys¹⁵ of the β-chain, due to the trypsin recognition of some still unmodified Lys⁹⁸ on one side and of the non-modifiable Arg¹³⁰ on the other side of the peptide (Table 1). These difficulties were overcome by digesting the protein with less site-specific pepsin (Supplementary Table 2). The consistency and reproducibility of these findings, which were duplicated in independent experiments, together with the evidence that the type of modified peptides did not depend on the kinetics of the modification reaction, validate the identification of the isolated peptides.

Residue accessibility

The observed absence of Val¹¹ modification, despite the high RSA value of this residue (Table 1), was predicted because of the protective effect of the interaction of deoxyHb with IHP. Similarly, the observed absence of Valα₁ modification was the likely effect of the protection by the network of salt bridges under anaerobic and high chloride concentration conditions [24]. The use of cycles of reactions under different kinetic conditions allowed the identification and reactivity differentiation of the modified lysine residues. The MS analyses of the trypptic and pepsin digests agreed in the identification of five lysine residues of the α-chain and five of the β-chain as the most accessible out of the 11 potentially modificable lysine residues. Within this class, the analyses of the trypptic digests (Supplementary Table 1) suggest slight differences in reactivity: Lys⁵ > Lys¹¹, Lys¹⁶ > Lys¹₉ > Lys₂⁶ in the α-chain and Lys⁵ > Lys¹⁷, Lys⁹⁰, Lys⁶¹, Lys⁶⁶ in the β-chain. The analyses of the pepsin digests indicated three modificable, but distinctly less accessible sites, on each chain with a reactivity order: Lys⁹⁰, Lys⁹⁰ > Lys⁶⁰ in the α-chain and Lys⁶⁰ > Lys³⁰ > Lys⁴⁴ in the β-chain (Supplementary Table 2).

Residues with poor RSA located far from the N- and C-termini, such as Lys⁶⁶ and Lys¹³⁷ in the α-chain and Lys¹³⁷ in the β-chain (Table 1), were not found modified. Conversely, residues with high solvent accessibility values, such as Lys⁴⁰ in the α-chain and Lys¹₂₀ in the β-chain, were not modified by thiolane, a positive ion, presumably due to both steric and charge effects. Lys²³ in the β-chain, a residue with moderate solvent accessibility, was not modified because of its participation in the IHP-binding pocket. Finally, the different residue accessibility indicated by the MS analyses was consistent with the finding that the number of thiols added per tetramer decreased progressively with the thiolation extent (Table 2).

Functional properties of the IMT/NEM-modified Hbs

The oxygen affinity, in the absence of 2,3-DPG, increased progressively with successive thiolation cycles and was paralleled...
by a progressive decrease in co-operativity. The increase in p50 due to the addition of a 20% stoichiometric excess of 2,3-DPG to the chemically modified Hbs was similar to that measured with native Hb and independent of the extent of thiolation. Instead, the decrease in Bohr effect depended on the extent of thiolation. The Bohr effect was reduced to 85–80% of the value of native Hb after the first reaction cycle, but was still 70–60% of that value after the modification of 8–9 residues/tetramer, irrespective of the IMT/deoxyHb–IHP molar ratio used for the modification (Table 2).

Functional properties of the IMT/MAL-PEG-modified Hbs

The data listed in Table 3 show that all Hb derivatives maintained a good to high proportion of the oxygen affinity and effects of the modulators of native Hb. However, co-operativity was significantly decreased by the attachment of five PEG chains/tetramer, compared with that of the reference protein modified at a greater number of sites using NEM. The reaction with NEM of part of the sites more accessible to thiolation before conjugation with MAL-PEG, in the attempt to obtain different topological distributions of the conjugation sites, did not yield products with improved co-operativity. This suggests that the co-operativity deterioration was not solely due to the chemical modification of the residues but to some specific effect(s) on the stability of the tertiary and/or quaternary structures of conjugation with long, flexible and hydrated PEG chains.

Despite the partial loss of co-operativity the products obtained by Hb PEGylation under anaerobic conditions in the presence of IHP showed remarkable stability to autoxidation, as compared with the product prepared under aerobic conditions (Figure 5). This was the expected result of the protection of groups crucial for the stability of the protein, such as the Cys995 residues. Such a property, together with the residual co-operativity and significant response to allosteric modulation, could be valuable for comparative in vivo studies of such products using other Hb-based oxygen carriers currently undergoing clinical trials.

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