Identification of the sites of deoxyhaemoglobin PEGylation

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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 Lys⁷, Lys¹¹, Lys¹⁶, Lys⁵⁶ and 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 Cys⁸⁹³ 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, co-operativity, 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 co-operativity, 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)ation].

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.

INTRODUCTION

The short blood circulation lifetime limits the efficacy of transfusion of pharmacologically active peptides and proteins. The attachment to these substances of long chains of PEG [poly(ethylene glycol)], PEGylation, in short, is a promising approach to circumvent this problem, provided that the chemical modification does not impair basic functional properties of the molecule. PEGylation increases the molecular mass and hydrodynamic volume, which prevent loss through renal filtration and possibly slow down extravasation. Furthermore, while PEG itself is reported to have low antigenic activity [1], PEGylation can shield the protein from recognition by antibodies and proteolytic enzymes. Several chemical strategies are known for coupling activated PEG to protein amino, carboxy or thiol groups [2,3]. In the two-step sequence of reactions of Scheme 1 (steps a and b), the amino groups are made to react with IMT (2-iminothiolane), which adds a spacer arm ending with a thiol group [4]. The thiols are then made to react with MAL-PEG (maleimido-PEG). The procedure has been applied for the stabilization of enzymes of pharmaceutical interest and Hb to be used as an acellular, injectable oxygen carrier (so-called ‘blood substitute’ or ‘oxygen therapeutic’) [5–7].

Concerning the use of Hb derivatives as candidate blood substitutes, two important issues need to be addressed. The first concerns the effects of the chemical modifications on protein function and stability. In this respect, thiolation with IMT according to

Scheme 1 can modify the α -amino groups of the α -chains, involved in the Bohr effect and in the interactions with CO₂ and chloride, and the α -amino groups of the β -chains, involved in the interactions with CO₂ and 2,3-DPG (2,3-diphosphoglycerate) [8]. Also available for reaction are 11 lysines on each α - and β -chain. Thiolation and PEGylation of some of these α - and ε -amino residues can also influence the stability of the Hb quaternary structures with the result of changing oxygen affinity and co-operativity. The chemical modification or mutation of the Lys⁸⁸² residues participating in 2,3-DPG binding alters Hb function [9,10]. Mutations of other lysine residues are known, which change oxygen affinity or protein stability. However, residue thiolation may not be equivalent to a mutation. Furthermore, Hb has thiol groups on both the α - and β -chains. It is known that thiol-specific reagents, such as *p*-hydroxymercuribenzoate and NEM (*N*-ethylmaleimide), reacting readily with the Cys⁸⁹³ residues of HbO₂ (oxyHb), alter the oxygen affinity, Bohr effect and tetramer stability [8,11,12]. These groups are also a target for the MAL-PEG reaction. Although the interactions of the oxygen affinity modulators with the protein are diminished or abolished when acellular Hb is transfused, the integrity of most of the residues involved in the interactions with the modulators is crucial for maintaining the native Hb oxygen affinity and stability with regard to the autoxidation reaction. Thus, even if a high oxygen affinity, as result of the chemical modifications, was not detrimental to oxygen delivery to tissues, as predicted by a theory on the autoregulation of oxygen

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, Hb A₀; 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; *p*50, oxygen affinity (the oxygen partial pressure producing 50% saturation); RSA, relative solvent accessibility.

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Scheme 1 Reactions involved in the amino group modification

Step a: the unprotonated amino group of protein P reacts with protonated IMT to yield a spacer arm ending with a thiol group. Step b: the thiol group reacts with the maleimido ring of NEM, or MAL-PEG, to yield a protonated adduct. Step c: ring closure of the spacer arm.

transport to tissues [13,14], the efficacy of acellular Hb as an oxygen carrier depends on the redox properties of the protein, which can be impaired by the modifications.

The other important issue that relates to the transfusion of Hb derivatives is the chemical characterization of the products, which is needed to complement the functional characterization [15]. Since several classes of potentially reactive groups can be modified according to Scheme 1 (steps a and b), some chemical heterogeneity of the derivatives is unavoidable. However, a varying degree of accessibility within each class of reactive groups is predictable, which could be exploited by suitable strategies. Such products should be identified and more specifically characterized. In the present study of the sites of Hb PEGylation, an experimental approach has been adopted that, while addressing the issue of maintaining the maximal functional integrity of the protein during the chemical modifications, also facilitates the identification of some of the modified residues by MS. The studies of the PEGylated Hbs described in the present paper should help in the understanding and evaluation of the *in vivo* properties of similar products currently undergoing clinical trials.

EXPERIMENTAL

Cyclic modification procedure

IHP (inositol hexaphosphate) binds human deoxyHb (deoxyhaemoglobin) with high affinity in the same site where the physiological P_i , 2,3-DPG, binds [16]. The interaction between the phosphate negative charges and the positive charges of the residues forming the binding site, in particular the α -amino groups of the β -chains and ϵ -amino groups of the Lys⁸² residues, increases the pK_a of the amino groups with the effect of slowing down their reaction with IMT (Scheme 1, step a). In fact, IHP binding prevents the CO_2 and cyanate reactions with the α -amino groups of the β -chains [17,18]. The deoxyHb–IHP interaction stabilizes the T quaternary structure and, by constraining the tertiary structure of the β -chains, hinders the exposure of the thiol groups of the Cys⁹³ residues to thiol-specific reagents. Thus we carried out reaction step a of Scheme 1 on the deoxyHb–IHP complex. The added thiol groups were then blocked in step b by the anaerobic reaction with NEM, a low-molecular-mass maleimido derivative that reacts rapidly with thiols. Furthermore, the reaction with NEM prevented the ring closure of the thiol groups introduced by the reaction with IMT, as shown in Scheme 1

(step c) [19]. To terminate the IMT and NEM reactions, both reagents were removed by anaerobic gel filtration. Titration of the thiols at the end of the procedure yielded the thiols of the Cys⁹³ 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 (tosylphenylalanylchloromethane; '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 Cys⁹³ residues. Titration of the thiol groups in the sample treated with NEM or MAL-PEG provided the number of

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 *p*O₂ (oxygen partial pressure) was calculated knowing the oxygen saturation, the amount of Hb and oxygen (mol) introduced into the tonometer.

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. OxyHbA₀ yielded 2.3 ± 0.1 thiol groups/tetramer [20]. To check the accessibility to the reaction with NEM of the Cys⁹³ 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 (*p*50; 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 *p*50 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 Centricons (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 HCTTM (high-capacity trap) Ion Trap mass spectrometer (Bruker Daltonics, Billerica, MA, U.S.A.). The entrance capillary temperature was 250 °C. The capillary voltage

Table 1 Amino acid sequence of the α - and β -chains of deoxyHb, in blocks of ten residues, indicating the modifiable sites (Val¹ and lysines) and the trypsin cleavage sites (lysines and arginines)

The RSA values of the modifiable sites are shown in square brackets. The RSA values range from 0 (fully buried) to 9 (fully exposed).

α -Chain

V¹[9]LSPADK⁷[2]TNV K¹¹[4]AAWVK¹⁶[5]VGAH AGEYGAEAL
R³¹MFLSFPTTK⁴⁰[2]TYFPFDLSH GSAQVK⁵⁶[4]GHGK⁶⁰[5]
K⁶¹[7]VADALTNV AHVDDMPNAL SALSDLHAHK⁹⁰[6]LR⁹²VDPVNFK⁹⁹[6]L
LSHCLLVTLA AHLPAEFTPA VHASLDK¹²⁷[1]FLA SVSTVLTSK¹³⁹[3]Y R¹⁴¹

β -Chain

V¹[3]HLTPEEK⁸[3]SA VTALWGK¹⁷[6]VNV DEVGGEALGR³⁰LLWVYPWTQR⁴⁰
FFESFGDLST PDAVMGNPK⁵⁹[5]V K⁶¹[5]AHGK⁶⁵[4]K⁶⁶[7]VLGA
FSDGLAHLDN LK⁸²[4]GTFATLSE LHC⁹³[0]DK⁹⁵[6]LHVDP ENFR¹⁰⁴LLGNVL
VCVLAHFGK¹²⁰[9]EFTPPVQAAY QK¹³²[3]VVAGVANA LAHK¹⁴⁴[3]YH

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 the proteins with a *P* value < 10⁻⁷. The Δ mass units were obtained from p.p.m. using FindMod online software (<http://www.expasy.ch>). ESI spectra and LC (liquid chromatography)–ESI–MS/MS mass chromatograms were converted into mzXML and mgf formats respectively before elaboration. RSA values listed in Table 1 were calculated for the structures of deoxyHb either P_i-free or bound to IHP using Polyview server (<http://poliview.cchmc.org>) [21–23].

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. The Cys⁹³ residues were not modified by MAL-PEG for up to 45 min under anaerobic conditions.

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

Figure 2 Kinetics of the reactions of the deoxyHb–IHP complex with NEM (6 mol/mol of deoxyHb–IHP) at 20 °C in phosphate buffers of varying pH

The NEM in excess was extinguished by the addition of deoxygenated mercaptoethanol (20 mol/mol of NEM) before aerobic gel filtration on a Sephadex G-25 column. The thiol titre value of native tetrameric Hb was 2.3 ± 0.1 .

and from approx. 3.4 to 2.4 after three cycles using the high (60:1) IMT/deoxyHb–IHP molar ratio.

Mass spectra of Hb chains

MALDI spectra of whole Hb modified by four cycles of IMT/NEM reactions using the low (20:1) IMT/deoxyHb–IHP molar ratio are shown in Figure 3(A), and by two reaction cycles using the high (60:1) ratio in Figure 3(B). Both products had a similar number (~6) of thiol groups added per Hb tetramer and the mass spectra were also similar. The lower intensities of the signals from the β -chains with respect to the signals of the α -chains were also observed in the control spectra of native Hb (results not shown). In each spectrum, the intensities of the peaks were approximately indicative of the relative proportions of the species identified by the mass. Small amounts of unmodified α - and β -chains were present together with chains modified by the single and multiple addition of a 227.31 Da unit, which corresponds to an amino residue modified by IMT and NEM plus a proton, as depicted in Scheme 1. The peaks indicating the addition of either NEM alone to the protein cysteine residues or NEM-treated cysteine residues plus single or multiple 227.31 Da units added to amino groups could not be distinguished from the background noise.

Mass spectra of the tryptic digests

MALDI MS was used to analyse the tryptic digests of the products of the IMT and NEM reactions. Similar results were obtained using either the whole protein or the isolated α - and β -chains. Only masses in the 800–3000 Da range were isolated and selected by the automatic scanning procedure, using as a reference the mass spectrum of native Hb digested under the same conditions. The observed peptide masses were matched by sequences containing only one modified residue. Confidence in peptide identification was assumed if values of Δ mass between the observed and matching peptide were ≤ 20 p.p.m. Some of the identifications were confirmed by ESI analyses of the peptides (results not shown). A non-automatic search did not reveal a significant presence of peptides with double amino group modifications. The same peptides were identified using both the low (20:1) and the

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 ($\Delta p50$) 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 P_i. –, Not measured; I/I, IMT/deoxyHb–IHP ratio; RCN, reaction cycle no.

		I/I . . .	10:1	20:1					60:1		
		HbA ₀	HbO ₂	DeoxyHb–IHP					DeoxyHb–IHP		
Thiol titration		RCN . . .	1	1	2	3	4	5	1	2	3
Thiol											
– NEM	2.30±0.10		4.71	4.04±0.13	3.78±0.22	3.77±0.13	3.44±0.15	3.46	5.73±0.08	4.98±0.01	4.64±0.01
+ NEM	0.10±0.05		0.04	2.29±0.06	2.26±0.05	2.12±0.02	2.24±0.08	2.16	2.3±0.06	2.26±0.06	2.23±0.07
Added thiol/cycle				1.74±0.14	1.52±0.23	1.65±0.13	1.20±0.17	1.3	3.43±0.10	2.72±0.06	2.41±0.07
Total added thiol				1.74	3.26	4.91	6.11	7.41	3.43	6.15	8.56
p50 (–2,3-DPG)	8.54±0.09 (2.5±0.1)		1.8 (1.8)	8.44 (2.5)	7.24 (2.4)	6.97 (2.2)	6.44 (1.9)	6.14 (1.7)	7.00 (2.1)	5.83 (2.1)	5.07 (1.9)
Δ p50 (+2,3-DPG)	0.90		<0.10	0.80	0.80	0.80	0.80	—	1.0	0.95	0.80
Bohr H ⁺ /tetramer	2.6		1.8	2.2	2.1	2.0	1.9	—	2.1	1.8	1.8

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.

high (60:1) IMT/deoxyHb–IHP ratios, but, due to differences in residue accessibility, peptides isolated after one reaction cycle using the high IMT/deoxyHb–IHP ratio required more reaction cycles to reach the same significance when using the low ratio (see Supplementary Table 1 of the Supplementary material at <http://www.BiochemJ.org/bj/403/bj4030189add.htm>).

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 Val α 1 or Lys α 7. The identification of Lys α 7 as the

Figure 4 ESI fragmentation spectrum of the $[V^1LSPADK^7TNVK^{11} + IMT + NEM + 2H]^{++}$ ion (m/z 699) from the α -chain

The two most abundant ions at m/z 815 and 937 were both modified at Lys α 7 (labelled with an asterisk). Other less abundant fragmentation ions also indicated modification of Lys α 7.

modified residue was made by the ESI technique. Figure 4 shows the MS/MS spectrum obtained by fragmenting the $[V^1LSPADK^7TNVK^{11} + IMT + NEM + 2H]^{++}$ ion, the sequence of which was assigned with a high statistical confidence (P value = 3.05×10^{-9}). 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 $V^1LSPADK^7$ and at m/z 937 with sequence K^7TNVK^{11} both modified at Lys α 7. The identification of Lys α 7 as the modified residue was supported by the m/z values of other less abundant fragmentation ions derived from the N- and C-termini, not labelled in Figure 4, none of which contained a modified residue, and by the absence of fragmentation ions modified at Val α 1. 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 Val α 1.

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 (Lys α 7, Lys α 11, Lys α 16, Lys α 56 and Lys α 139) and five of the β -chain (Lys β 8, Lys β 17, Lys β 59, Lys β 61 and Lys β 66) 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 (Lys α 90 and Lys α 99, α -chain; Lys β 65, β -chain) and four cycles (Lys α 60, α -chain; Lys β 95 and Lys β 144, β -chain) (see Supplementary Table 2 of the Supplementary material at <http://www.BiochemJ.org/bj/403/bj4030189add.htm>).

NEM addition to the Cys β 93 residues

A peptide with the NEM addition to Cys β 93 (1–2% of the total) was observed in the MALDI spectrum of the trypsin-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 oxyHbA $_0$ 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 Cys β 93 residues) with regard to oxygen affinity, Bohr effect and total absence of 2,3-DPG effect.

Functional 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 Lys α 7, Lys α 11, Lys α 16, Lys α 56 and Lys α 139 of the α -chain and Lys β 8, Lys β 17, Lys β 59, Lys β 61 and Lys β 66 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 3 Properties of the products of the deoxyHb-IHP reactions with IMT and NEM (deoxyHb-NEM) and/or varying chains of MAL-PEG, at 20 °C and pH 7.0

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 Cys β 93 thiol titre was determined after the NEM/MAL-PEG reactions. Thiol titre and Bohr effect refer to tetrameric haemoglobin. –, Not measured.

Species	IMT thiol titre	Cys β 93 thiol titre	$[p50]_{pH=7}$		h	Bohr H $^{+}$
			– 2,3-DPG	+ 2,3-DPG		
DeoxyHb-NEM	7.3 (NEM)	2.3	5.6	–	1.9 ± 0.1	1.9
DeoxyHb-PEG $_1$	4.7 (MAL-PEG)	2.5	5.8	–	1.6 ± 0.1	1.9
DeoxyHb-PEG $_2$	5.8 (MAL-PEG)	2.6	4.6	5.2	1.3 ± 0.1	1.4
DeoxyHb-PEG $_3$	7.5 (MAL-PEG)	2.3	4.5	–	1.4 ± 0.1	–
DeoxyHb-PEG $_4$	1.7 (NEM), 5.8 (MAL-PEG)	2.5	3.8	–	1.3 ± 0.1	1.4
DeoxyHb-PEG $_5$	2.7 (NEM), 6.6 (MAL-PEG)	2.3	5.2	5.9	1.5 ± 0.1	–

Figure 5 Rate of Hb⁺ formation from HbO₂, at 20 °C and pH 7.0

Hb⁺ was measured as HbCN by the addition of cyanide (0.2 mM) to the buffer, as described for the spectroscopic determination of the oxygen saturation (Figure 1). (○) HbA₀; (□) HbA₀ modified in two cycles of anaerobic reactions using a 60:1 IMT/deoxyHb–IHP molar ratio; (Δ) HbA₀ modified by aerobic reaction with IMT and NEM for 6 h at 5 °C and pH 7.4; (●) pegylated Hb derivative deoxyHb–PEG₃ (Table 3).

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 tryptic 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 Lys⁶⁵⁹ and Lys⁶⁶⁵.

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 tryptic digests. After 2–4 reaction cycles using the low IMT/deoxyHb–IHP ratio, yielding up to six thiol groups/tetramer, a peptide modified at Lys^{α11} with the sequence 8–16 of the α-chain and a peptide modified at Lys^{α16} with the sequence 12–31 of the same chain were found (Supplementary Table 1). The same modified peptides were found in the products of 1–2 reaction cycles using the high IMT/deoxyHb–IHP ratio, also yielding up to six thiol groups/tetramer. Similarly, the peptide map of the β-chain after four reaction cycles at low IMT/deoxyHb–IHP ratio showed

the masses of three peptides modified at Lys^{β17}, Lys^{β61} and Lys^{β66} (Supplementary Table 1), which were also observed in the peptide map after two reaction cycles using the high ratio (Supplementary Table 1). Additional thiolation cycles (> 2) using a high IMT/deoxyHb–IHP ratio led to an apparent loss of signals in the tryptic digests due to modification also of the sites flanking the modified one. To illustrate this point, a peptide modified at Lys⁷ of the α-chain with mass of 1398 Da was isolated 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 Lys¹⁷ of the β-chain, due to the trypsin recognition of some still unmodified Lys^{β8} on one side and of the non-modifiable Arg^{β30} 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 Val^{β1} 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 Val^{α1} 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 tryptic and peptic 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 modifiable lysine residues. Within this class, the analyses of the tryptic digests (Supplementary Table 1) suggest slight differences in reactivity: Lys⁷ > Lys¹¹, Lys¹⁶ > Lys¹³⁹ > Lys⁵⁶ in the α-chain and Lys⁸ > Lys¹⁷, Lys⁵⁹, Lys⁶¹, Lys⁶⁶ in the β-chain. The analyses of the peptic digests indicated three modifiable, but distinctly less accessible sites, on each chain with a reactivity order: Lys⁹⁰, Lys⁹⁹ > Lys⁶⁰ in the α-chain and Lys⁶⁵ > Lys⁹⁵, Lys⁴⁴ in the β-chain (Supplementary Table 2).

Residues with poor RSA located far from the N- and C-termini, such as Lys⁴⁰ and Lys¹²⁷ in the α-chain and Lys¹³² in the β-chain (Table 1), were not found modified. Conversely, residues with high solvent accessibility values, such as Lys⁶¹ 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. Lys⁸² 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 Cys⁸⁹³ 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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