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[1]Abbreviations:

PEG, poly(ethylene glicol); MAL-PEG, maleimido-PEG; IMT, 2-iminothiolane; 2,3-DPG, 2,3-diphosphoglycerate; IHP, inositolhexaphosphate; p-MB, p-hydroxymercuribenzoate; NEM, N-ethylmaleimide; HbA_0, haemoglobin A_0; Hb, deoxy haemoglobin; HbO_2, oxy haemoglobin; Hb^+, ferric haemoglobin; HbCN, cyanomet haemoglobin; NES-haemoglobin, haemoglobin reacted with NEM at both Cysβ93's; MS, mass spectrometry; MALDI, matrix assisted laser desorption ionization; ESI, electron spray ionization; FA, formic acid; TFA, trifluoroacetic acid; CHCA, alpha-cyano-4-hydroxy-cinnamic acid; DHA, dihydroxyacetophenone; 4-PDS, 4,4'-dithiopyridine; PMF, peptide mass fingerprint; RSA, relative solvent accessibility.

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ABSTRACT

The 2-iminothiolane reaction with protein amino groups adds a spacer arm ending with a thiol group, which can be further reacted with molecules carrying a maleimido ring. This approach is currently used for the preparation of a candidate 'blood substitute' in which human haemoglobin is conjugated with long chains of polyethylene glycol. To identify the thiolation sites by mass spectrometry, we have carried out the reaction using deoxy haemoglobin bound to inositol hexaphosphate to protect some of the residues crucial for function and N-ethyl maleimide to block and stabilize the thiol groups prior to enzymatic digestion by trypsin and pepsin. Under the conditions for the attachment of 5-8 polyethylene glycol chains per tetramer, the thiolated residues were K_7, K_{11}, K_{16}, K_{56}, K_{139}, and, with lower accessibility, K_{90}, K_{99}, K_{60} of the α chain and K_8, K_{17}, K_{59}, K_{61}, K_{66}, and, with lower accessibility, K_{65}, K_{95}, K_{144} of the β chain. The α-amino groups of α and β chains were not modified and the reaction of the Cysβ93's with N-ethylmaleimide was minor or absent. After the modification with thiolane and N-ethylmaleimide of up to 5-8 lysines per tetramer, the products retained a large proportion of the properties of native haemoglobin, such as low oxygen affinity, cooperativity, effect of the modulators and stability to auto-oxidation. Under identical anaerobic conditions, the conjugation of the thiolated haemoglobin tetramer with 5-6 chains of the maleimido derivative of 6kDa polyethylene glycol yielded products with diminished cooperativity, Hill's n=1.3-1.5, still retaining a significant proportion of the effects of the modulators of oxygen affinity and stability to auto-oxidation. Cooperativity was apparently independent of the topological distribution of the pegylated sites as obtained by reacting partly the thiolated protein with N-ethyl maleimide prior to pegylation.

KEY WORDS

Haemoglobin-based oxygen carriers

Blood substitute

Protein thiolation

Protein pegylation

Protein mass spectroscopy

N-ethyl maleimide-modified haemoglobin

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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 poly(ethylene glycol)[1], 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 weight 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 the recognition by antibodies and proteolytic enzymes. Several chemical strategies are known for coupling activated PEG to protein amino, carboxyl or thiol groups [2,3]. In the two-step sequence of reactions of Scheme 1(*a,b*), the amino groups are reacted with IMT, which adds a spacer arm ending with a thiol group [4]. The thiols are then reacted with MAL-PEG. The procedure has been applied for the stabilization of enzymes of pharmaceutical interest and haemoglobin to be used as an acellular, injectable oxygen carrier (so-called 'blood substitute' or 'oxygen therapeutic') [5-7].

Concerning the use of haemoglobin 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 CO_2 and chloride, and the α-amino groups of the β chains, involved in the interactions with CO_2 and 2,3-DPG [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 haemoglobin quaternary structures with the result of changing oxygen affinity and cooperativity. The chemical modification or mutation of the Lysβ82's participating in 2,3-DPG binding alters haemoglobin function [9,10]. Mutations of other lysines are known, which change oxygen affinity or protein stability. However, residue thiolation may not be equivalent to a mutation. Furthermore, haemoglobin has thiol groups on

both the α and β chains. It is known that sulfhydryl reagents, such as p-MB and NEM, reacting readily with the Cysβ93's of HbO_2, 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 haemoglobin is transfused, the integrity of most of the residues involved in the interactions with the modulators is crucial for maintaining the native haemoglobin oxygen affinity and stability with regard to the auto-oxidation 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 auto-regulation of oxygen transport to tissues [13,14], the efficacy of acellular haemoglobin 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 haemoglobin 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(*a,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 haemoglobin pegylation, an experimental approach has been adopted which, 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 haemoglobins described in this paper should help in the understanding and evaluation of the *in vivo* properties of similar products currently undergoing clinical trials.

EXPERIMENTAL

Cyclic modification procedure

Inositol hexaphosphate binds human Hb with high affinity in the same site where the physiological organic phosphate, 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 Lysβ82's, increases the pK 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 Hb-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 Cysβ93's to sulfhydryl reagents. Thus, we carried out reaction step *a* of Scheme 1 on the Hb-IHP complex. The added thiol groups were then blocked in step *b* by the anaerobic reaction with NEM, a small molecular weight maleimido derivative which 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*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 Cysβ93's not reacted with NEM and provided indirectly a means of checking the efficacy of the Hb-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 physico-chemical 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 weight and the slow MAL-PEG reaction with thiols.

Reagents

IMT and IHP (dodecasodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); NEM from Fluka Chemie Gmbh (Buchs, CH); HPLC grade CH_3CN, bi-distilled water, FA and TFA from JT Baker (Milan, IT); TPCK-trypsin from Promega (Madison, WI, USA); pepsin from Sigma-Aldrich Corporation (St.

Louis, MS); MAL-PEG (m.W. 5.6 kDa) from Nektar Molecule Engineering (USA); gases from SAPIO (Monza, IT).

Reactions under anaerobic conditions

Reactions with IMT and NEM. Purified HbA_0 (Hb^+<1%) was used [18]. Chemical modifications of the Hb-IHP complex (0.50 mM, tetramer; IHP/haemoglobin molar ratio, 1.1/1) in 50 mM K-phosphate, 100 mM KCl, 0.5 mM EDTA, pH 7.0, were carried out in a reactor thermostatted at 20°C under a nitrogen flow using various IMT concentrations and a NEM/haemoglobin molar ratio of 6/1. Anaerobic gel filtration to stop the reactions was carried out using Sephadex G25 columns equilibrated with deoxygenated reaction buffer and eluted anaerobically at 20-25°C. Since the reaction between the Hb-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 haemoglobin 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 Hb-IHP complex, were reacted 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 mole/haemoglobin 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 Hb-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 SH groups added by thiolation plus those of the Cysβ93's. 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 reacting 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

HbO_2 samples were reacted with 4-PDS for 12 min at 25°C until the absorbance change at 324 nm was less than 0.005/min. Oxy HbA_0 yielded 2.3 ± 0.1 SH group/tetramer [20]. To check the accessibility to the reaction with NEM of the Cysβ93's in the Hb-IHP complex, a constant amount of deoxygenated NEM solution was added to samples of 0.5 mM Hb-IHP solution in phosphate buffers of varying pH. At the end of the incubation period a deoxygenated solution of β-mercaptoethanol (20 moles/mole 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 haemoglobin eluate was determined.

IHP removal

The pH of the haemoglobin solution was increased from 7 to 8.3 by the addition of 2 M TRIS and the solution was gel filtered on a Sephadex G25 column equilibrated with 50 mM K-phosphate, 100 mM KCl, 0.5 mM EDTA, pH 8.3. The eluate was then gel filtered again in the standard buffer.

Oxygen affinity and auto-oxidation rate measurements

Oxygen affinity (P_{50}) at 20°C was measured with a precision of ±1-2% of the value from the spectral determination of the composition in Hb, HbO_2 and HbCN of the haemoglobin solution (2 mM, heme concentration) in buffer containing 0.2 mM cyanide (Fig. 1). The Hill coefficient, *n*, 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} vs pH) and the effect of 2,3-DPG addition at pH 7 were obtained similarly. The rate of Hb^+ formation by auto-oxidation

was obtained from the spectrum of haemoglobin 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: a) trypsin, using a 1/20 (w/w) trypsin/haemoglobin ratio, at 37° for 12 h in 100 mM ammonium carbonate, pH 8.5; b) pepsin, 1/25 (w/w) pepsin/haemoglobin ratio, for 2 and 6 h at 30°C, pH 2; and c) trypsin followed by pepsin after trypsin removal by ultrafiltration using 10 kDa mw cut-off centricons (Millipore Corporation, Bedford, MA). Digestions were stopped by freezing at -80°C.

HPLC for mass spectrometry experiments.

Samples (20 µl) were analysed using an HPLC apparatus and a Hypersil column C18 (150x0.55 mm, 5 µm particle size) equilibrated with solvent A (H_2O + 0.025% FA). After 2 min of isocratic elution the proportion of solvent B (CH_3CN + 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 minutes and in 1 minute the proportion of solvent B was lowered from 95% to 0%.

Mass spectrometry

Voyager DE-PRO mass spectrometer (Applied Biosystems, USA) was used to obtain the MALDI spectra of whole protein or products of protein enzymatic digestion. The matrixes were CHCA for the analyses of digests and DHA for undigested haemoglobin. The matrix solvents for dissolving CHCA and DHA were 50:50 H_2O/CH_3CN plus 0.1% FA and 70:30 H_2O/CH_3CN 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 mass spectra and chromatograms were obtained using an HCT Bruker Ion Trap mass spectrometer (Bruker Daltonics, Germany). The entrance capillary temperature was 250°C. The capillary voltage was 5 kV. MS/MS spectra of selected peptide ions were obtained by Collision Induced Dissociation.

MS data analysis and RSA data

MALDI data were converted in ASCII format and elaborated using the Profound Peptide Mass Fingerprint approach (http://prowl.rockefeller.edu/). Modified and unmodified peptide masses were identified with a mass accuracy of 20 ppm and the proteins with a pValue < 10^{-7}. Δmass units were obtained from ppm using FindMod on-line software (http://www.expasy.ch). ESI spectra and LC-ESI-MS/MS mass chromatograms were converted to mzXML and mgf formats respectively before elaboration. RSA values listed in Table 1 were calculated for the structures of deoxy haemoglobin either organic phosphate-free or bound to IHP using polyview server (http://poliview.cchmc.org) [21-23].

RESULTS

Assessment of the conditions for Hb-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 Hb-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 Cysβ93's of the Hb-IHP complex was studied in the pH range 7.0-8.5 (Fig. 2). At pH 7.0, using 6 moles NEM/tetramer, less than 2% of the Cysβ93's were NEM-modified after 15 min reaction. A greater proportion of the Cysβ93's 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 Cysβ93's were not modified by MAL-PEG for up to 45 min under anaerobic conditions.

Thiol groups added to Hb-IHP with each IMT reaction cycle

The net number of thiols added at each reaction cycle using different IMT/Hb-IHP molar ratios and averaged from five or more independent experiments are listed in Table 2. A progressive decrease was observed, from about 1.7 to 1.3 after five cycles

using the low (20/1) IMT/Hb-IHP molar ratio and from about 3.4 to 2.4 after three cycles using the high (60/1) IMT/Hb-IHP molar ratio.

Mass spectra of haemoglobin chains

MALDI spectra of whole haemoglobin modified by four cycles of IMT/NEM reactions using the low (20/1) IMT/Hb-IHP molar ratio are shown in Fig. 3A, and by two reaction cycles using the high (60/1) ratio in Fig. 3B. Both products had a similar number (about 6) of thiol groups added per haemoglobin 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 haemoglobin (data 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 cysteines or NEM-reacted cysteines 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 analyze 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 haemoglobin 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 Δmass between the observed and matching peptide were ≤20 ppm. Some of the identifications were confirmed by ESI analyses of the peptides (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 high (60/1)

IMT/Hb-IHP ratios, but, due to differences in residue accessibility, peptides isolated after one reaction cycle using the high IMT/Hb-IHP ratio required more reaction cycles to reach the same significance when using the low ratio (Table 1, Supplemental material).

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/Hb-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 $[V_1LSPADK_7TNVK_{11}+IMT+NEM+2H]^{++}$ ion, the sequence of which was assigned with a high statistical confidence (pValue=3,05x10^{-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 K_7. The identification of K_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 Fig. 4, none of which contained a modified residue, and by the absence of fragmentation ions modified at V_1. Furthermore, such identification was consistent with the absence in the MALDI spectrum of the digested chains of a peptide with the sequence corresponding to the first 7 residues of the α chain modified at Valα1.

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 5 modified lysine residues of the α chain (K_7, K_{11}, K_{16}, K_{56}, K_{139}) and 5 of the β chain (K_8, K_{17}, K_{59}, K_{61}, K_{66}) in each of the products of four reaction cycles at low IMT/Hb-IHP ratio and in the products of two reaction cycles at high ratio. Using the high IMT/Hb-IHP ratio additional modified lysines were found in the products of three cycles (K_{90} and K_{99} , α chain; K_{65} , β chain) and four cycles (K_{60}, α chain; K_{95} and K_{144}, β chain) (Table 2, Supplemental material).

NEM addition to the Cysβ93's

A peptide with the NEM addition to Cysβ93 (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/Hb-IHP molar ratio, although such a NEM modification was not detected in the MALDI spectrum of the whole chains (Fig. 3). Details on the approximate quantification of this peptide are presented in Fig 1 of the Supplemental material.

Functional properties of the products of the IMT/NEM reactions

The P_{50} 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/Hb-IHP molar ratios. The data are listed in Table 2, where comparison is also made with the properties of a product obtained by reacting oxy HbA_0 with IMT and NEM for 6 h at 4°C. Such a product was similar to NES-haemoglobin 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 haemoglobins were prepared by reacting 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, 5 to 7, were obtained by increasing the IMT reaction time (>20 min). Size exclusion chromatography indicated that the amount of residual unmodified haemoglobin in all these products was below the detection limit (<5% of the total, data 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 reacting 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 K_7, K_{11}, K_{16}, K_{56}, K_{139} of the α chain and K_8, K_{17}, K_{59}, K_{61}, K_{66} of the β chain.

Stability to auto-oxidation

The rates of Hb^+ formation by auto-oxidation were measured at 20°C in the standard phosphate buffer, pH 7.0, and in the absence of radical scavenging enzymes, using purified native haemoglobin, haemoglobin thiolated under conditions in which about 6 thiol groups were added per tetramer and then reacted with NEM or MAL-PEG. The rates are compared in Fig. 5 with the rate of auto-oxidation of haemoglobin 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 haemoglobin thiolation, which are also the sites of potential PEG attachment, was made possible by the strategy adopted to (a) stabilize the thiolation sites by the NEM reaction; (b) carry out a progressive thiolation through cycles of reactions under two different kinetic conditions, and (c) 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 K_{61} clearly indicating trypsin recognition of both cleavage sites at Lysβ59 and Lysβ65.

The relative intensities of the mass peaks of the species identified in the spectra of the modified undigested proteins in Fig. 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/Hb-IHP ratio, yielding up to 6 SH groups/tetramer, a peptide modified at Lysα11 with the sequence 8-16 of the α chain and a peptide modified at Lysα16 with the sequence 12-31 of the same chain were found (Table 1, Supplemental material). The same modified peptides were found in the products of one-two reaction cycles using the high IMT/Hb-IHP ratio, also yielding up to six SH groups/tetramer. Similarly, the peptide map of the β chain after four reaction cycles at low IMT/Hb-IHP ratio showed the masses of three peptides modified at Lysβ17, Lysβ61 and

Lysβ66 (Table 1, Supplemental material), which were also observed in the peptide map after two reaction cycles using the high ratio (Table 1, Supplemental material). Additional thiolation cycles (>2) using a high IMT/Hb-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 K_7 of the α chain with mass of 1398 Da was isolated in the products of up to four reaction cycles at 20/1 IMT/Hb-IHP molar ratio and two cycles at 60/1 molar ratio due to the presence of enough unmodified cleavage sites at K_{11}. When the modification of K_{11} was also brought about by additional cycles at 60/1 IMT/Hb-IHP molar ratio the next potential site for cleavage was K_{16}, 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/Hb-IHP molar ratio, of the peptide modified at K_{17} of the β chain, due to the trypsin recognition of some still unmodified Lysβ8 on one side and of the non-modifiable Argβ30 on the other side of the peptide (Table 1). These difficulties were overcome by digesting the protein with less site-specific pepsin (Table 2, Supplemental material). 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 Valβ1 modification, despite the high relative solvent accessibility value of this residue (Table 1), was predicted because of the protective effect of the interaction of deoxy haemoglobin 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 tryptic and peptic digests agreed in the identification of 5 lysines of the α chain and 5 of the β chain as the most accessible out of the 11 potentially modifiable lysines.

Within this class, the analyses of the tryptic digests (Table 1, Supplemental material) suggest slight differences in reactivity: $K_7 > K_{11}$, $K_{16} > K_{139} > K_{56}$ in the α chain and $K_8 > K_{17}$, K_{59}, K_{61}, K_{66} 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: K_{90}, $K_{99} > K_{60}$ in the α chain and $K_{65} > K_{95}$, K_{144} in the β chain (Table 2, Supplemental material).

Residues with poor relative solvent accessibility located far from the amino and carboxyl termini, such as K_{40} and K_{127} in the α chain and K_{132} in the β chain (Table 1), were not found modified. Conversely, residues with high solvent accessibility values, such as K_{61} in the α chain and K_{120} in the β chain, were not modified by thiolane, a positive ion, presumably due to both steric and charge effects. K_{82} 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 haemoglobins

The oxygen affinity, in the absence of 2,3-DPG, increased progressively with successive thiolation cycles and was paralleled by a progressive decrease in cooperativity. The increase in P_{50} due to the addition of a 20% stoichiometric excess of 2,3-DPG to the chemically modified haemoglobins was similar to that measured with native haemoglobin 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 haemoglobin 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/Hb-IHP molar ratio used for the modification (Table 2).

Functional properties of the IMT/MAL-PEG modified haemoglobins

The data listed in Table 3 show that all haemoglobin derivatives maintained a good to high proportion of the oxygen affinity and effects of the modulators of native haemoglobin. However, cooperativity was significantly decreased by the attachment

of 5 PEG chains/tetramer, compared to 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 cooperativity. This suggests that the cooperativity 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 cooperativity the products obtained by haemoglobin pegylation under anaerobic conditions in the presence of IHP showed remarkable stability to auto-oxidation, as compared with the product prepared under aerobic conditions (Fig. 5). This was the expected result of the protection of groups crucial for the stability of the protein, such as the Cysβ93's. Such a property, together with the residual cooperativity and significant response to allosteric modulation, could be valuable for comparative *in vivo* studies of such products using other haemoglobin-based oxygen carriers currently undergoing clinical trials.

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Table 1

Amino acid sequence of the α and β chains of deoxy haemoglobin, in blocks of 10 residues, indicating the modifiable sites (V_1 and K's) and the trypsin cleavage sites (K's and R's). The RSA values of the modifiable sites are shown in brackets. The RSA values range from 0 (fully buried) to 9 (fully exposed).

α chain

V_1[9]LSPADK$_7$[2]TNV K$_{11}$[4]AAWGK$_{16}$[5]VGAH AGEYGAEALE
R$_{31}$MFLSFPTTK$_{40}$[2] TYFPHFDLSH GSAQVK$_{56}$[4]GHGK$_{60}$[5]
K$_{61}$[7]VADALTNAV AHVDDMPNAL SALSDLHAHK$_{90}$[6] LR$_{92}$VDPVNFK$_{99}$[6]L
LSHCLLVTLA AHLPAEFTPA VHASLDK$_{127}$[1]FLA SVSTVLTSK$_{139}$[3]Y R$_{141}$

β chain

V_1[3]HLTPEEK$_8$[3]SA VTALWGK$_{17}$[6]VNV DEVGGEALGR$_{30}$ LLVVYPWTQR$_{40}$
FFESFGDLST PDAVMGNPK$_{59}$[5]V K$_{61}$[5]AHGK$_{65}$[4]K$_{66}$[7]VLGA
FSDGLAHLDN LK$_{82}$[4]GTFATLSE LHC$_{93}$[0]DK$_{95}$[6]LHVDP ENFR$_{104}$LLGNVL
VCVLAHHFGK$_{120}$[9] EFTPPVQAAY QK$_{132}$[3]VVAGVANA LAHK$_{144}$[3]YH

Table 2

Properties of the products of the IMT and NEM reactions with the Hb-IHP complex at 20°C and pH 7.0 using IMT/Hb-IHP molar ratios of 20/1 and 60/1. The net number of SH groups added to the haemoglobin tetramer per reaction cycle was obtained by subtracting the SH titre after the NEM addition (+NEM) from the titre determined without the NEM addition (-NEM). The P_{50} values (torr) in the absence of 2,3-DPG were measured at 20°C in 100 mM KCl, 0.5 mM EDTA, 0.2 mM KCN, 50 mM K-phosphate, pH 7.0. In parentheses are the values of the Hill coefficient *n*. The increase in P_{50} value (ΔP_{50}) upon the addition of a 20% stoichiometric excess of 2,3-DPG was determined under the same pH conditions. Bohr protons were determined from P_{50} measurements in phosphate buffers of varying pH. Also shown are the properties of the product of HbO_2 modification by 1 cycle of IMT and NEM reactions at pH 7.0, 4°C and 6 hr reaction time. The 2,3-DPG effect of this product was determined with a 100% excess of organic phosphate.

IMT/Hb-IHP: 20/1		**Reaction cycle no**					
		Hb-IHP					**HbO_2**
SH titration	**HbAo**	**1**	**2**	**3**	**4**	**5**	**1**
SH (- NEM)	2.30 ± 0.10	4.04±0.13	3.78±0.22	3.77±0.13	3.44±0.15	3.46	4.71
SH (+ NEM)	0.10 ± 0.05	2.29±0.06	2.26±0.05	2.12±0.02	2.24±0.08	2.16	0.04
Added SH / cycle		1.74±0.14	1.52±0.23	1.65±0.13	1.20±0.17	1.3	
Total added SH		1.74	3.26	4.91	6.11	7.41	
P_{50} (-2,3-DPG)	8.54±0.09 (2.5±0.1)	8.44 (2.5)	7.24 (2.4)	6.97 (2.2)	6.44 (1.9)	6.14 (1.7)	1.8 (1.8)
ΔP_{50} (+2,3-DPG)	0.90	0.80	0.80	0.80	0.80	/	<0.10
Bohr H^+ / tetramer	2.6	2.2	2.1	2.0	1.9	/	1.8

IMT/Hb-IHP: 60/1		**Reaction cycle no**		
		Hb-IHP		
SH titration	**HbAo**	**1**	**2**	**3**
SH (- NEM)	2.30 ± 0.10	5.73±0.08	4.98±0.01	4.64±0.01
SH (+ NEM)	0.10 ± 0.05	2.3 ± 0.06	2.26±0.06	2.23±0.07
Added SH / cycle		3.43±0.10	2.72±0.06	2.41±0.07
Total added SH		3.43	6.15	8.56
P_{50} (-2,3-DPG)	8.54 (2.5)	7.00 (2.1)	5.83 (2.1)	5.07 (1.9)
ΔP_{50} (+2,3-DPG)	0.90	1.0	0.95	0.80
Bohr H^+ / tetramer	2.6	2.1	1.8	1.8

Table 3

Properties of the products of the Hb-IHP reactions with IMT and NEM (Hb-NEM) and/or varying chains of MAL-PEG, at 20°C and pH 7.0. Hill's *n* data are average values from measurements in the pH range 6.8-7.6. The SH titre of the IMT modified products (IMT SH titre column) equals the number of the haemoglobin-conjugated NEM/MALPEG molecules (the conjugated species is indicated in parentheses). The Cysβ93 SH titre was determined after the NEM/MAL-PEG reactions. SH titre and Bohr effect refer to tetrameric haemoglobin.

Species	IMT SH titre	Cysβ93 SH titre	$[P_{50}]_{pH=7}$ -2,3-DPG	$[P_{50}]_{pH=7}$ +2,3-DPG	*n*	Bohr H^+
Hb-NEM	7.3(NEM)	2.3	5.6	/	1.9±0.1	1.9
Hb-PEG_1	4.7(MALPEG)	2.5	5.8	/	1.6±0.1	1.9
Hb-PEG_2	5.8(MALPEG)	2.6	4.6	5.2	1.3±0.1	1.4
Hb-PEG_3	7.5(MALPEG)	2.3	4.5	/	1.4±0.1	
Hb-PEG_4	1.7(NEM) 5.8(MALPEG)	2.5	3.8	/	1.3±0.1	1.4
Hb-PEG_5	2.7(NEM) 6.6(MALPEG)	2.3	5.2	5.9	1.5±0.1	

FIGURE LEGENDS

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 an SH group. Step *b*, the SH group reacts with the maleimido ring of NEM, or MAL-PEG, to yield a protonated adduct. Step *c*, ring closure of the spacer arm.

Fig. 1 Spectra of 0.5 mM haemoglobin solutions in a 100 mM KCl, 50 mM phosphate buffer, 0.2 mM cyanide, measured using a 2 mm o.p, 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% HbO_2, <1% HbCN, >99% Hb. B) (——), same solution as A equilibrated for 15 min after the introduction of 7 ml of air; (------), best fitting composite spectrum, 70.6% HbO_2, 1.1% HbCN, 28.3 % Hb. Equilibrium P_{O2} was calculated knowing the oxygen saturation, the haemoglobin and oxygen moles introduced into the tonometer.

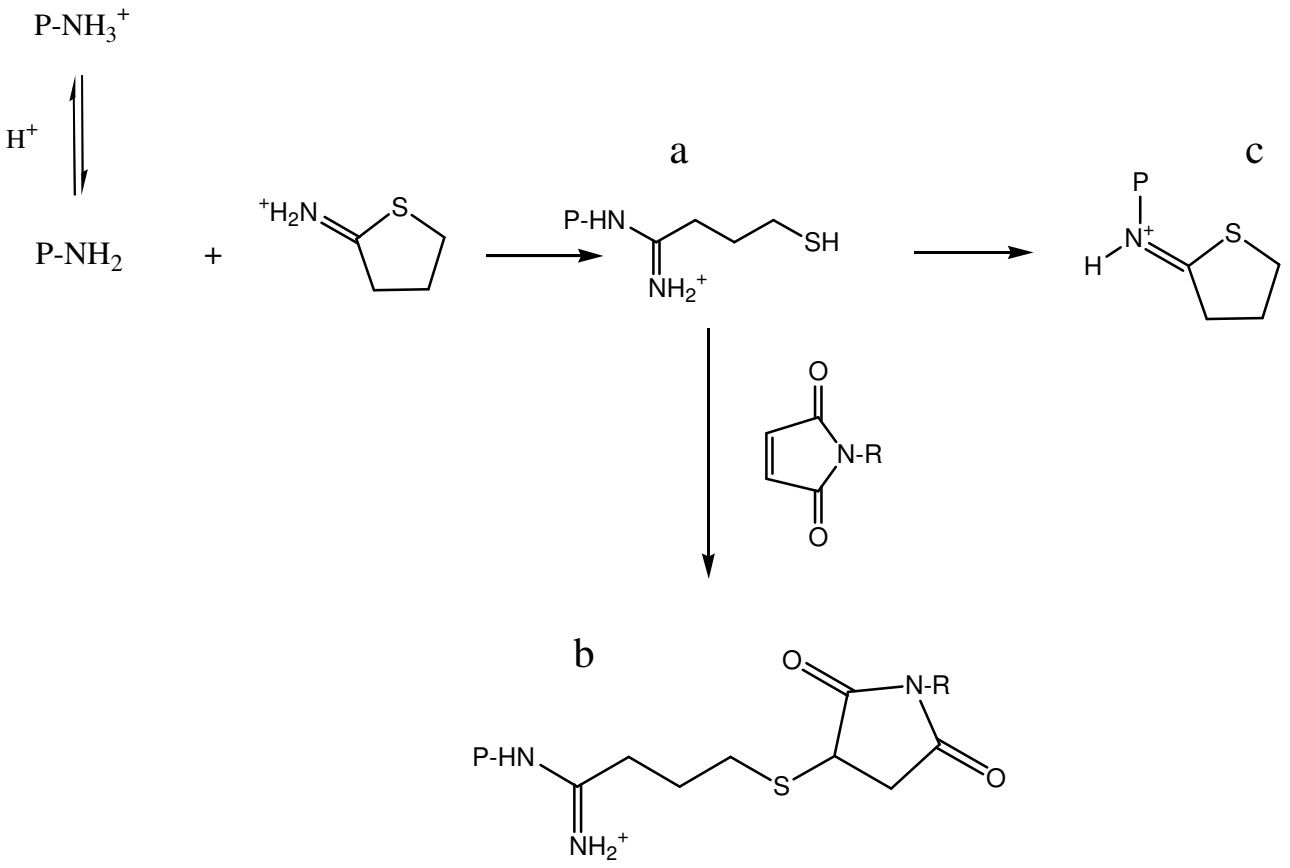
Fig. 2 Kinetics of the reactions of the Hb-IHP complex with NEM (6 moles/mole Hb-IHP) at 20°C in phosphate buffers of varying pH. The NEM in excess was extinguished by the addition of deoxygenated mercaptoethanol (20 moles/mole NEM) before aerobic gel filtration on a Sephadex G25 column. The SH titre value of native tetrameric haemoglobin was 2.3 ± 0.1.

Fig. 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 haemoglobin. A), product of 4 cycles of reactions with a 20/1 IMT/Hb-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 2 cycles of reactions with a 60/1 IMT/Hb-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.

Fig. 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 Lysα7 (labelled with an asterisk). Other less abundant fragmentation ions also indicated modification of Lysα7.

Fig. 5 Rate of Hb^+ formation from oxy haemoglobin, at 20°C and pH 7.0. Ferric haemoglobin 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 (Fig. 1). (○), HbA_0; (□), HbA_0 modified in two cycles of anaerobic reactions using a 60/1 IMT/Hb-IHP molar ratio; (Δ), HbA_0 modified by aerobic reaction with IMT and NEM for 6 h at 5°C and pH 7.4; (●), pegylated haemoglobin derivative Hb-PEG_3 (Table 3).



Scheme 1

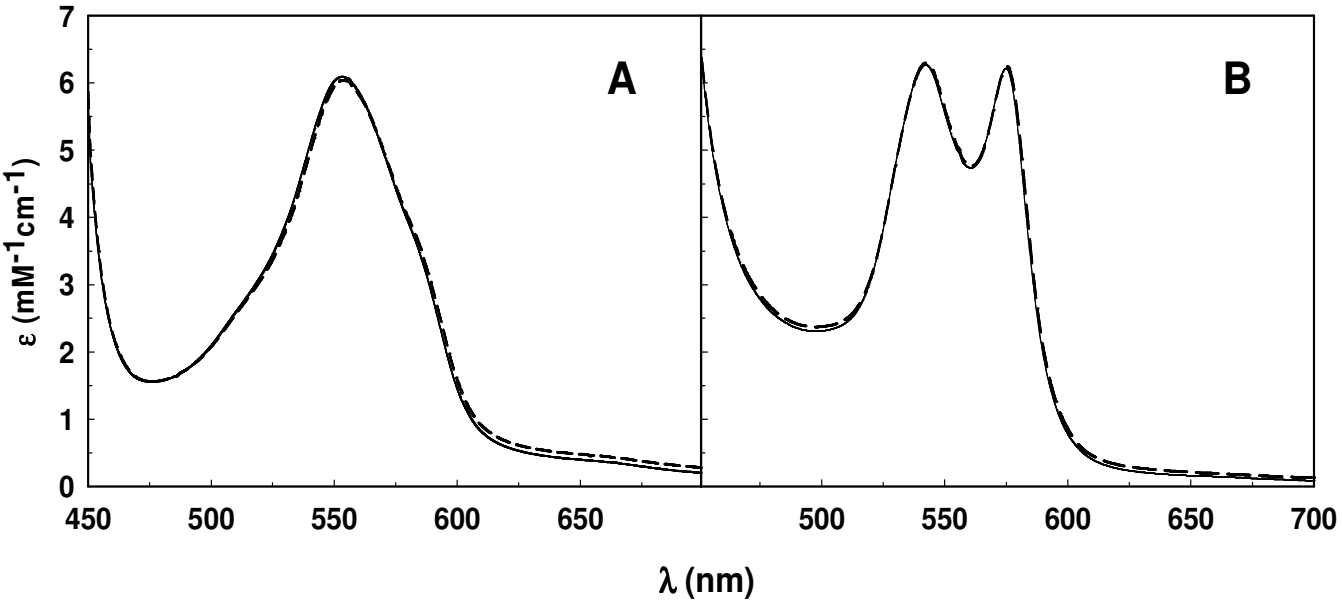


Figure 1

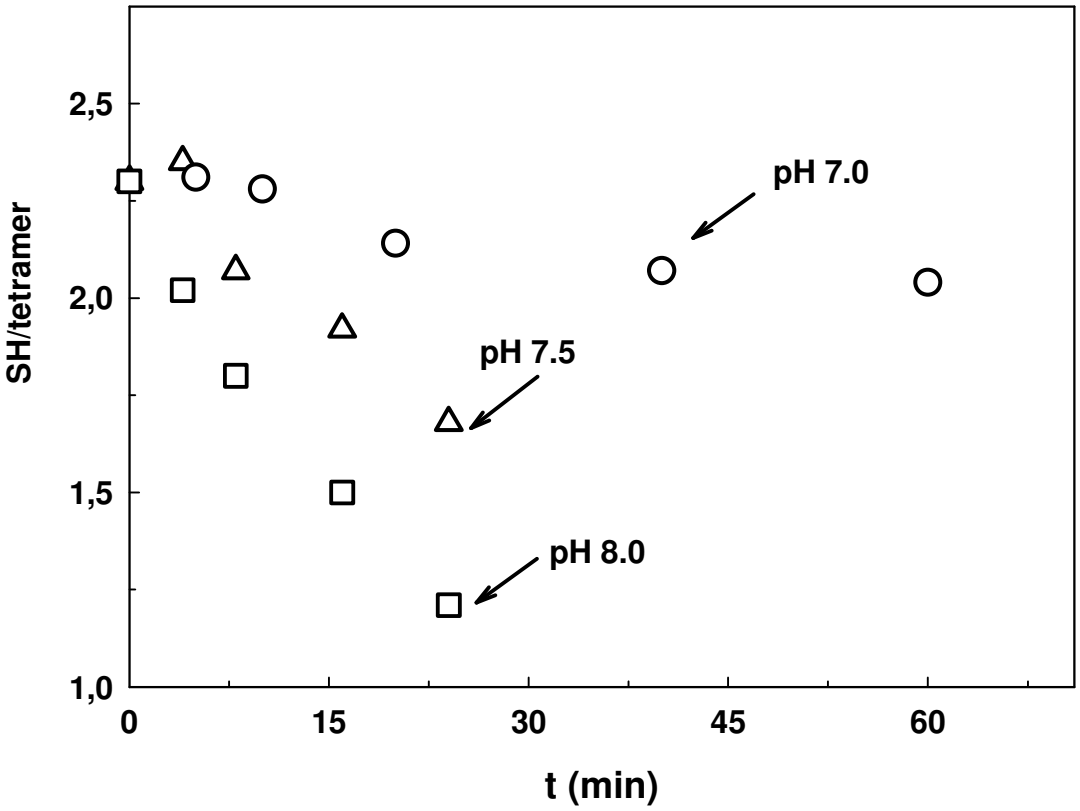


Figure 2

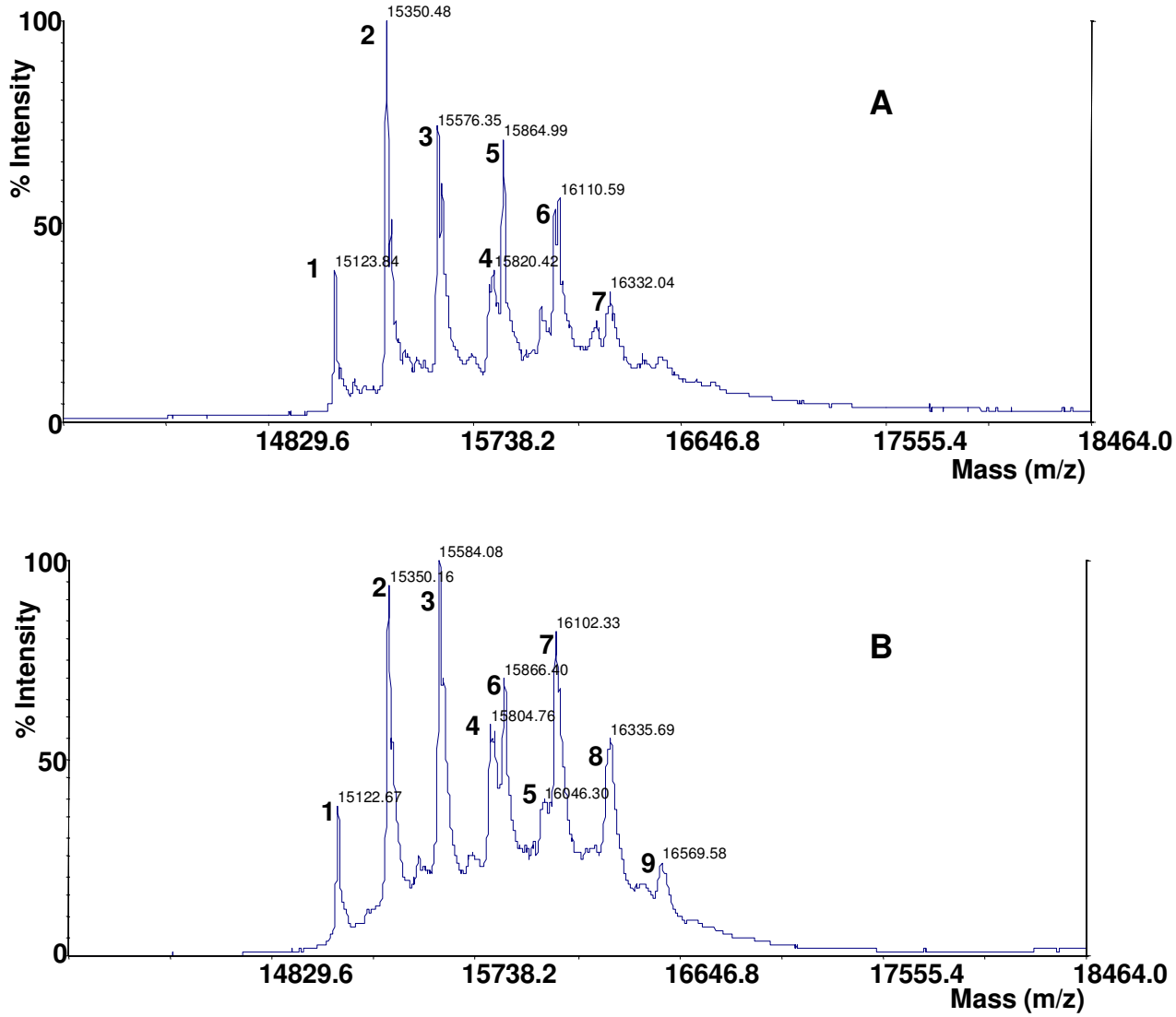


Figure 3

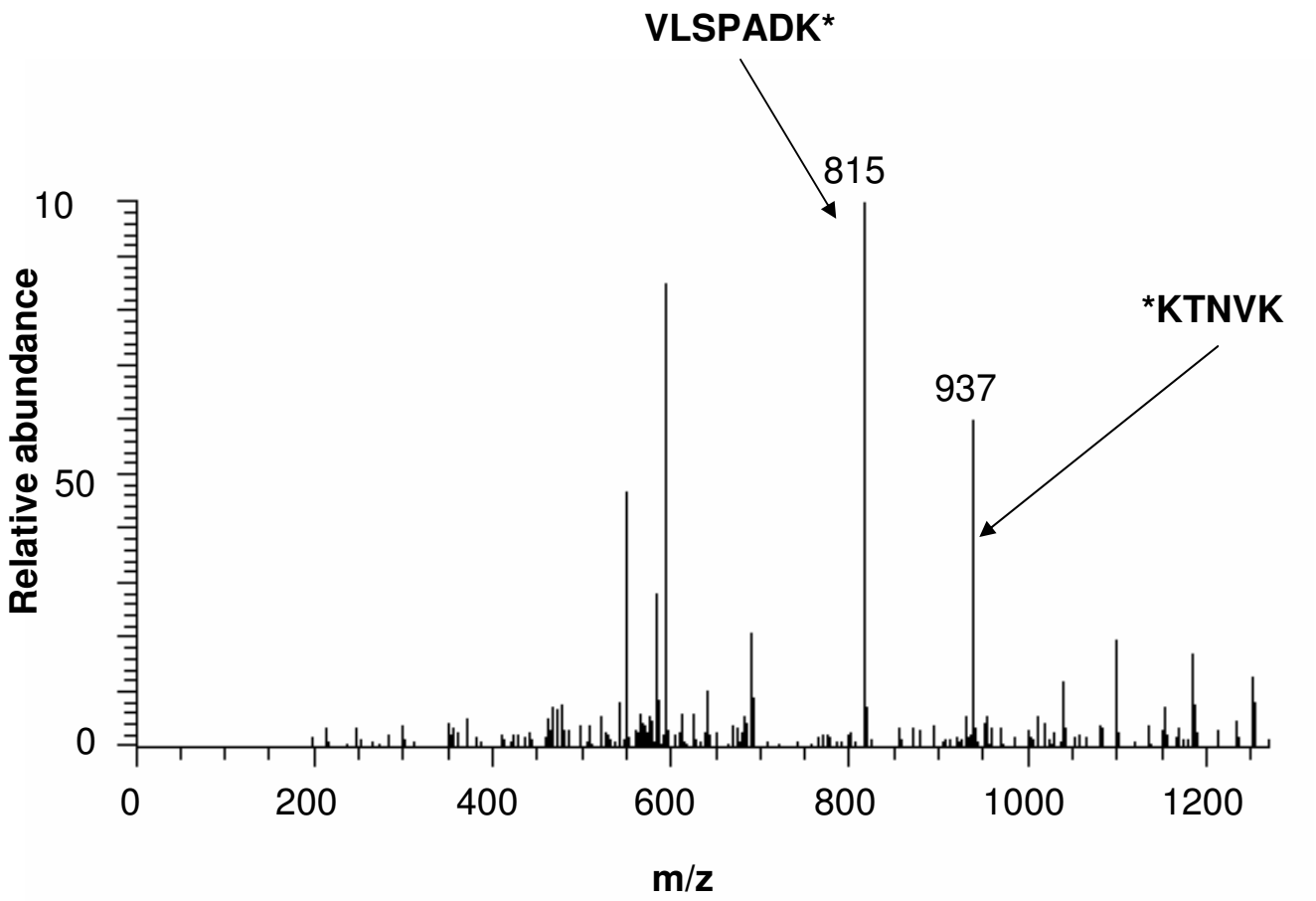


Figure 4

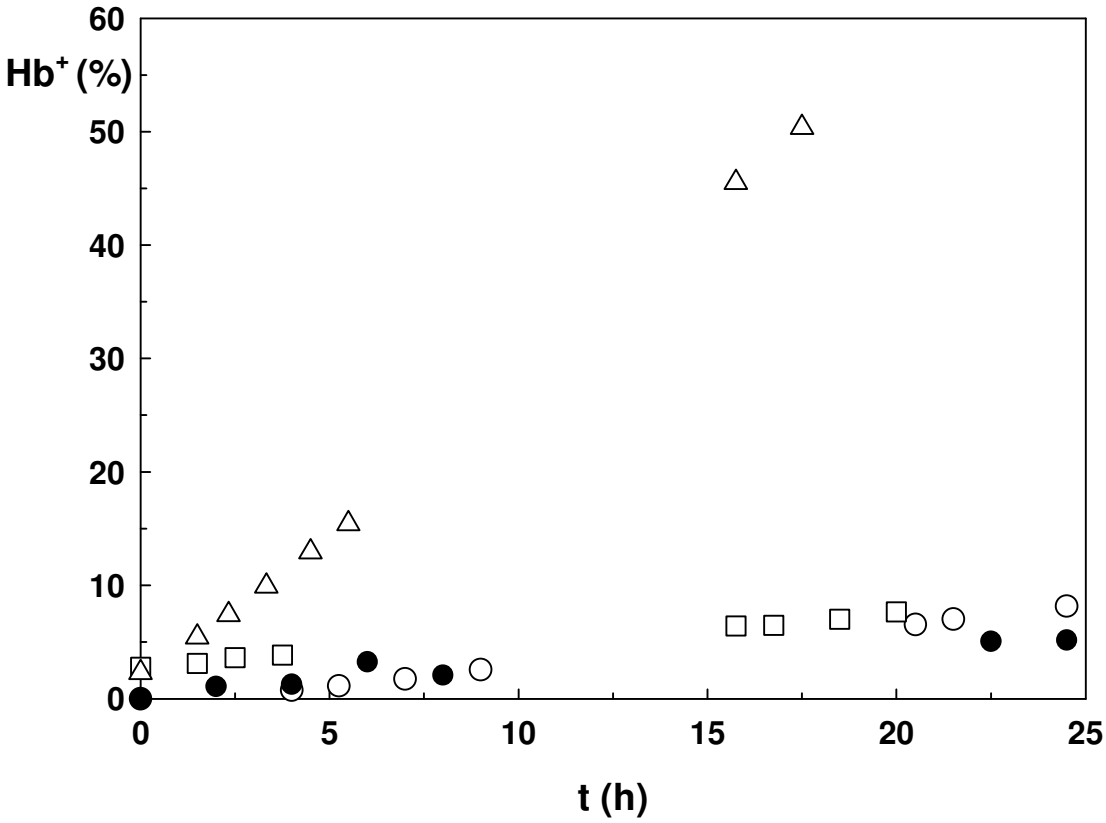


Figure 5