Characterisation of S-nitrosohaemoglobin by mass spectrometry

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Received 19 September 1996; revised version received 21 October 1996

Abstract Recent studies have demonstrated the biological importance of the interaction of S-nitrosothiols, which can be considered as nitric oxide (NO) protein donors, especially haemoglobin, at the level of Cys residues. It was recently proposed that S-nitrosohaemoglobin is formed within red blood cells and serves as a regulatory function. In human haemoglobin the α-subunit contains one Cys residue and the β-subunit contains two Cys residues, one of which (β-Cys^{93}) is highly reactive and conserved among species, although its function has remained unknown. Electrospray ionization mass spectrometry was used to monitor the results of exposure of haemolysates to S-nitrosocysteine under different conditions and thus addressed some aspects of NO–haemoglobin interaction. When an equimolar ratio of S-nitrosothiol was added to haemoglobin, only a single NO molecule was added. Peptide mapping by liquid chromatography–mass spectrometry located the nitrosyl group at the level of β-Cys^{93} demonstrating that this was the preferred site of formation of S-nitrosohaemoglobin. The present data also suggest that electrospray mass spectrometry can allow quantification and characterisation of S-nitrosoproteins in blood.

Key words: Haemoglobin; Nitric oxide; S-nitrosothiol; S-nitrosocysteine; S-nitrosohaemoglobin; Mass spectrometry

1. Introduction

The interaction of nitric oxide (NO) with proteins is known to play a critical role in several different physiological systems extending from blood pressure regulation to neurotransmission [1–3]. In addition, NO produced in infected and inflamed tissue could contribute to the process of carcinogenesis [4,5]. Although NO has been known since the late 1970s to be among the ligands that activate soluble guanilate cyclase and cause vascular smooth muscle relaxation, it has not been appreciated until more recently that NO is also an endogenous vasorelaxant. NO and endothelium-derived relaxant factor (EDRF) comparably relax vascular smooth muscle and are similarly quenched by haemoglobin and superoxide. Therefore it was proposed that EDRF is NO or a labile compound releasing NO, such as S-nitrosothiols (RSNO), the most abundant being S-nitrosocysteine (Cys–NO) and S-nitrosoglutathione. The target of NO on proteins are Cys and Tyr residues and bound metals such as heme-Fe^{2+} (for a recent review see [6]). Although much work has been performed in characterising the NO–Fe^{2+} interaction [7,8], there is considerably less data on the Cys–NO interactions. In fact, RSNOs are unstable in aqueous solution. It has been assumed that the lability of RSNOs is due to their propensity to undergo homolytic cleavage of the S–N bond with release of NO [9]. The quantitative contribution of labile NO donors such as RSNOs to EDRF is not yet well established, but it is clear that such compounds are formed in vivo and have biological activity.

Thiol formation is considered a likely outcome of Cys–NO interaction at acidic pH. At physiological pH, RSNO formation is thought to be less favourable, although RSNO on proteins has been found in vitro [10] and in vivo [11]. The β-chain of haemoglobin possesses a highly reactive thiol group at position 93 [12] which is conserved among mammalian species and, although its function remains unknown, it has been recently proposed that it can interact with endogenous RSNOs to form NO–Hb, suggesting that this may serve for regulation of blood pressure and facilitates efficient delivery of oxygen to tissues [13]. However, as yet no structural data have been produced to support the preferential formation of NO–Hb at β-Cys^{93}.

We used ES/MS to monitor the exposure of haemoglobin to Cys–NO under different conditions and to determine the cysteine residue involved in the formation of NO–Hb. We demonstrated that β-Cys^{93} is the preferred site of in vitro NO–Hb formation, whereas β-Cys^{112} and α-Cys^{104} form NO–Hb only in the presence of very high levels of RSNOs.

2. Materials and methods

2.1. Materials

Blood samples were collected from normal volunteers immediately prior to their use for in vitro incubation. Pepsin, sodium nitrite, L-cysteine, glycerol and thioglycerol were purchased from Sigma (St. Louis, MO). Sodium phosphate, sodium ethylenediaminetetracetate (EDTA), hydrochloric acid, HPLC-grade solvents and reagents were obtained from Carlo Erba (Milan, Italy). Phenylisothiocyanate (PITC) was purchased from Fluka (Buchs, Switzerland).

2.2. Preparation of S-nitrosothiol solutions

Solutions (50 mM) of Cys–NO were prepared by mixing equal volumes of 100 mM L-cysteine in 250 mM HCl, 0.1 mM Na_2EDTA with 100 mM $NaNO_2$ in water at room temperature. Previous studies have established that essentially no nitrite remains in the nitrosothiol solutions using this synthetic method [9].

2.3. In vitro incubation

Human erythrocytes from a normal volunteer were washed in isotonic saline as described previously [14] and then lysed by addition of an equal volume of water. This oxy-Hb (about 1 mM) was estimated by SDS-gel electrophoresis and by isoelectric focusing (IEF) to be about 95% pure and was prepared immediately prior to its use.

Incubations of Cys–NO with these fresh haemolysates (200 μl) were performed at 25°C for 30 min, using 1 : 10 and 1 : 100 haemoglobin/

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PII S0014-5793(96)01258-6

Cys–NO, respectively, in 1 ml total volume by addition of aerate phosphate buffer, pH 7.4, 0.5 mM EDTA and subsequently of the RSNO solution. The reactions were stopped by dilution of the samples with 400 μl of 0.1% TFA. A total of 200 μl of this solution was injected on the HPLC column.

2.4. HPLC separation of globin chains

Globin chains were purified by RP-HPLC using the procedure developed by Shelton et al. [15] with the following modifications: the samples (500 μg of globin dissolved in 100 μl of TFA 0.1% in water) were loaded onto a Vydac C4 large-pore column equilibrated in the following buffer: (A) 80:20 water/acetonitrile, 0.1% TFA; (B) 40:60 water/acetonitrile, 0.1% TFA. The column was equilibrated at 52% of buffer B. After a 2-min hold, buffer B was raised to 69% in 24 min. Before analysis globin samples were filtered through a 45 μm filter (Millipore). For analytical runs 100 μg samples were used. The column effluent was monitored at 280 nm. Protein fractions were manually collected and directly injected into the ES/MS source.

2.5. Electrospray mass spectrometry

ES/MS analyses of intact globin chains were performed with a PLATFORM mass spectrometer (Fisons, Manchester, UK). Samples from the HPLC separation (10 μl, 50 pmol) were injected into the ion source at a flow rate of 10 μl/min; the ES-mass spectra were scanned from 1600 to 600 u at a scan cycle of 10 s/scan. The source temperature was 65°C. Mass scale calibration was carried out using myoglobin as reference compound. Quantitative analysis of components was performed by integration of the multiple charged ions of the single species.

2.6. Peptic digestion

Peptic hydrolysis was carried out in 5% formic acid, at 37°C for 1.5 h in a haemoglobin/enzyme ratio of 50 : 1 (w/w). The reaction was stopped by freeze-drying.

2.7. LC/ES/MS peptide mapping

Liquid chromatography was performed using a 2.1 mm i.d.×250 mm, C18, 5 μm (Vydac) reverse-phase column with a flow rate of 0.2 ml/min on a Kontron modular system. The column effluent was split 1 : 25 with a Valco tee, to give a flow rate of about 8 ml/min into the electrospray nebuliser. The bulk of the flow was run through the detector for peak collection as measured by following the absorbance at 220 nm. Solvent A was 0.03% TFA (v/v) in water; solvent B was 0.02% TFA in acetonitrile. Separation of the peptides was effected with a gradient 5–45% B in 60 min. The ES-mass spectra were scanned from 1800 to 400 u at a scan cycle of 5 s/scan. The source temperature was 120°C. Different conditions of the orifice voltage were employed (20–80 V).

2.8. Assignment of modified cysteine residues

The HPLC fractions containing the nitrosylated peptides were lyophilised and redissolved in 0.4% ammonium bicarbonate, pH 9.0, for 2 h. The reaction was stopped by freeze-drying and the samples were analysed by FAB-MS/MS.

2.9. FAB mass spectrometry

FAB mass spectra were performed on a ZAB 2SE (Fisons, Manchester, UK) double-focusing, reversed-geometry mass spectrometer equipped with a caesium gun operating at 25 kV (2 mA). The sample was dissolved in 0.1 M HCl and loaded on to a glycerol/thioglycerol-coated probe tip.

High-energy collision induced-dissociation (CID) mass spectra were obtained on a ZAB-T (Fisons, Manchester, UK) four-sector (B1E1B2E2) mass spectrometer under control of an OPUS V3.1X data system and equipped with a 2048 channel-linear photo diode focal plane array detector. The sample was bombarded with a beam of Cs^+ ions having an energy of 30 keV. Analyses were performed at 8 kV accelerating potential. For collision-induced decomposition experiments, argon collision gas was used up to 50% attenuation of the parent ion beam. The collision cell was held on 50% of the accelerating potential. For each spectrum, 100–200 pmol of sample was dissolved in 1 μl of 5% acetic acid, and this solution was placed on the glycerol/thioglycerol (1 : 1) matrix on the probe tip. Signals recorded in the spectra were assigned to the corresponding peptides on the basis of the expected molecular weight by using a suitable computer program reference.

2.10. Edman degradation

Manual Edman degradation steps were carried out directly on the peptide mixtures using 5% phenylisothiocyanate as coupling agent, as described previously [16].

3. Results

Globins in the haemolysate, following incubation with Cys–NO in a 1 : 10 molar ratio, were separated by reverse phase HPLC (Fig. 1). When compared with a standard haemolysate

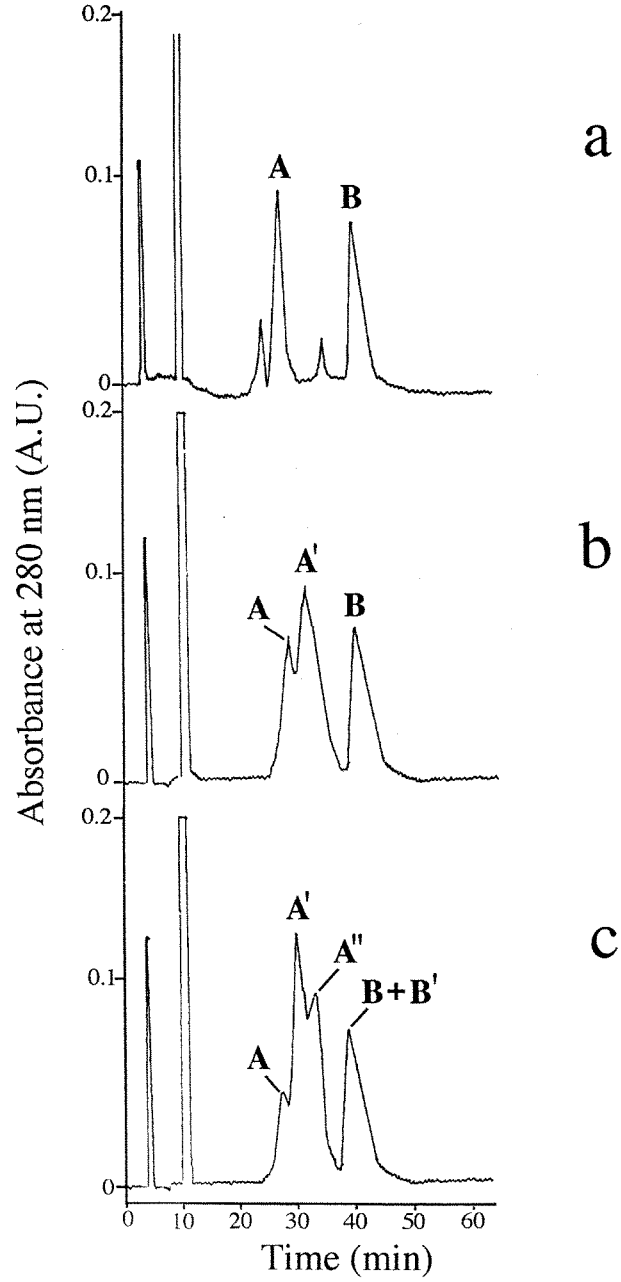

Fig. 1. HPLC separation of S-nitrosohaemoglobin. a: Standard haemolysate. b: Sample incubated with a Hb/Cys–NO ratio (1 : 10). c: Sample incubated with a Hb/Cys–NO ratio (1 : 100). A,A′,A″ = α-globin and nitrosylated derivatives; B,B′ = β-globin and nitrosylated derivative.

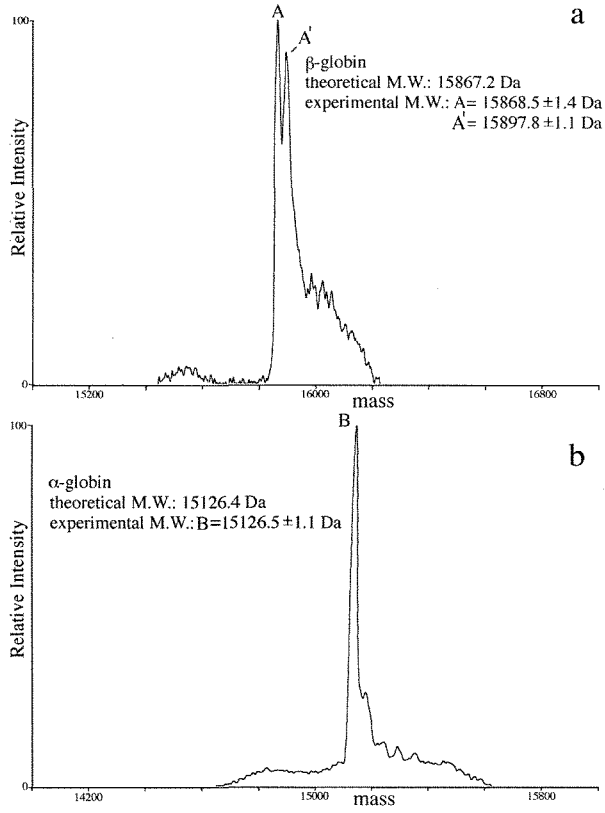

Fig. 2. ES/MS spectra in the transformed mass scale of globin chains from haemoglobin incubated with Hb/Cys–NO ratio (1 : 10). a: β-Globin chain. b: α-Globin chain.

(Fig. 1a), the HPLC profile showed a double shaped peak at the β position (Fig. 1b). Direct analysis by ES/MS of the modified samples collected during the HPLC run allowed accurate molecular weight determination as shown in Fig. 2. The mass measured for the α-chain (15126.5 ± 1.1) peak (Fig. 2b) perfectly matched with that of the native globin (15126.4). Of the two species present at the β position (Fig. 2b), the first was the native β-globin (15868.5 ± 1.4, expected mass 15867.2) whereas the second had a mass of 15897.8 ± 1.1 with a shift of +29 units compared with that measured for the β-globin, indicating the addition to the protein of a single NO moiety.

In contrast, Fig. 1c shows the HPLC profile and Fig. 3 the corresponding mass spectra obtained after incubation at a higher Hb/Cys–NO ratio (1 : 100). ES/MS analysis showed that both α- and β-globins had a mass shift with respect to the native chains. The α-chain was present either as the unmodified and the mono-nitrosylated species (measured mass: 15125.5 ± 1.1 and 15164.1 ± 2.2, respectively), while in the ES spectrum of the β-chain components were observed containing zero, one and two nitrosyl-groups (15867.2 ± 0.7, 15896.0 ± 0.5 and 15924.0 ± 1.6, respectively). These data indicated that at this level of nitrosylating agent, a non-selective modification occurred involving the two cysteines of the β-chain and the single cysteine of the α-chain. Therefore we focused our attention on the NO–Hb obtained by using the lower Cys–NO ratio, in order to determine the preferential site of the β-globin nitrosylation.

The peptic digest of the modified globins after incubation with 1 : 10 RSNO was separated by LC/ES/MS. The chromatogram obtained for the peak of the modified β-globin is shown in Fig. 4. HPLC peaks were assigned to peptides within the globin sequence on the basis of the measured molecular weight (Table 1). When necessary the assignments were confirmed by submitting the collected HPLC peaks to FAB/MS analysis prior to and after one or more manual Edman degradation steps, as previously described [16]. It can be observed that the entire β-globin sequence was actually checked out by this procedure (see Table 1).

The peak at 30.9 min (fraction 19) in the chromatogram of Fig. 4a contained a peptide with a molecular weight of 2052.6 ± 0.2 which was assigned to peptide 89–105 (molecular weight: 2052.3) containing β-Cys^{93}. This peak was also present in the spectrum of unmodified β-globin (Fig. 4b). A novel peak was instead found at a higher retention time (31.9 min, fraction 21) containing two mass signals 2052.3 ± 0.7 and 2081.3 ± 0.7 ($\Delta m = 29$ mass units) corresponding to the mass of unmodified and nitrosylated peptide 89–105, respectively.

Fraction 21 was collected and analysed by ES/MS at different cone voltage values. In Fig. 5 the ES mass spectra obtained at 20, 40 and 80 V are shown. At the lower voltage essentially the modified peptide was present (2081.6 ± 0.7). Increasing of the cone voltage led to a higher fragmentation and at 80 V only the peak at 2052.5 ± 0.3 was observed, whereas at the higher voltage only the native peptide was found. The above findings unambiguously demonstrated that the signal at the lower mass was due to a fragmented species arising from the nitrosylated peptide.

The identity of the modified peptide 89–105 was also confirmed by incubation at alkaline pH, after which the peptide

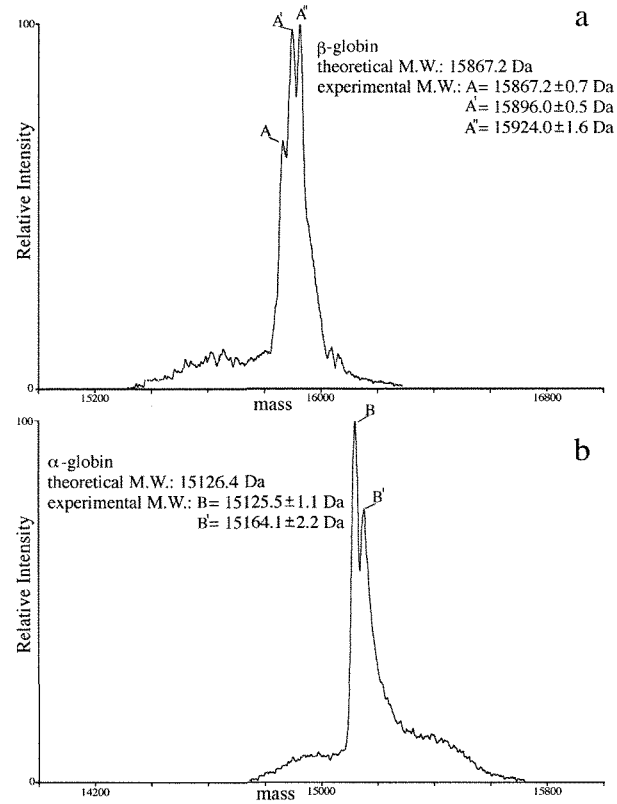

Fig. 3. ES/MS spectra in the transformed mass scale of globin chain from haemoglobin incubated with Hb/Cys–NO ratio (1 : 100). a: β-Globin chain. b: α-Globin chain.

Table 1
Analysis by LC/ES/MS of the products of digestion of beta-Hb-NO with pepsin

HPLC peak[a]	Theoretical mass	Peptide[c]	Measured mass[b]	Note
1	654.7	142–146	654.7 ± 0.2	
2	709.7	97–102	709.6 ± 0.2	
3	767.9	141–146	767.6 ± 0.2	
4	706.9	130–135	706.6 ± 0.2	
5	1306.4	92–102	1306.5 ± 0.9	
6	1335.4	(92–102)+NO	1336.3 ± 0.1	Cys^{93}-NO
	1758.0	22–37	1757.2 ± 0.9	
7	1119.3	130–140	1118.6 ± 0.3	
8	1819.1	32–45	1818.9 ± 0.7	
	2063.3	43–62	2063.6 ± 0.5	
9	2205.6	49–70	2205.3 ± 0.2	
10	1635.7	89–102	1635.1 ± 0.2	
11	n.d.	n.d.	=	
12	1664.7	(89–102)+NO	1664.2 ± 0.1	Cys^{93}-NO
	817.9	7–14	817.8 ± 0.2	
13	2543.9	122–145	2542.5 ± 0.1	
14	1342.5	33–42	1341.5 ± 0.5	
15	1180.3	136–146	1180.5 ± 0.1	
16	1494.7	1–14	1494.9 ± 0.5	
17	1869.1	130–146	1870.5 ± 0.9	
18	1229.4	86–96	1229.7 ± 0.6	
19	1472.6	15–28	1473.1 ± 0.7	
	2052.3	89–105	2052.6 ± 0.2	
	3525.0	33–63	3524.7 ± 0.5	
20	2205.6	49–70	2205.8 ± 0.7	
	1980.2	118–135	1980.9 ± 0.8	
21	2052.3	89–105	2052.3 ± 0.7	
	2081.3	(89–105)+NO	2081.6 ± 0.7	Cys^{93}-NO
22	3001.4	42–70	3001.5 ± 0.8	
	2738.1	115–140	2738.7 ± 0.7	
23	1634.8	71–85	1634.3 ± 0.2	
	816.0	107–114	815.8 ± 0.2	
24	929.2	106–114	928.1 ± 0.2	
25	3046.5	49–78	3046.0 ± 0.4	
26	1634.9	19–41	1634.6 ± 0.4	
27	3600.1	15–45	3601.1 ± 0.9	
	3388.9	49–81	3388.5 ± 0.8	
28	2205.6	49–70	2205.3 ± 0.1	
	3821.4	45–81 and 49–85	3821.5 ± 0.2	
29	3675.2	46–81 and 49–84	3675.1 ± 0.5	
30	1308.5	32–41	1308.8 ± 0.9	
31	4107.7	45–84	4016.9 ± 0.3	
32	3674.9	46–81 and 49–84	3674. ± 0.7	

[a]Numbers refer to the peaks of the chromatogram shown in Fig. 4.
[b]Average molecular masses in DA (mean ± S.D.) obtained by integrating the multiple peaks corresponding to each molecular species, differing only in the total number of charges, measured by LC/ES/MS.
[c]Numbers indicate the amino acid residues at the extremes of each peptide.

at 2081.6 shifted to 2052.3, due to hydrolysis of the S–NO bond. FAB–MS/MS analysis (not shown) fully confirmed the sequence of peptide 89–105.

The only modified peptides were those including β-Cys^{93} (peptides 92–102, 89–102 and 89–105). It should be noted that no trace of modified peptides containing β-Cys^{112} was detected, which indicated that nitrosylation selectively occurred at the level of β-Cys^{93}.

The globin chains modified following incubation with 1 : 100 Hb–NO were analysed with the same procedure (not shown). This analysis confirmed that at this higher ratio, all the cysteine residues in the α- and β-chains were partially nitrosylated.

4. Discussion

4.1. Analytical model for the determination of nitrosylated cysteines in haemoglobin

The need for methods to evaluate the action of exogenous as well as endogenous agents has stimulated studies on their reactivity with biomolecules. Ideally, methods are desirable which detect any modification in the intact protein easily and rapidly and identify the specific nature and sites of modification. Recent advances have made mass spectrometry the method of choice to address structural problems involving covalently modified proteins [17]. Furthermore, mass spectrometric methods are virtually independent from the type of modification to be identified and therefore ideally suited to the study of covalent modification of proteins. Molecular weight measurement by ES/MS was instrumental in the immediate evaluation of the degree of modification of the globin chains. This technique can be applied to the analysis of purified globin as well as to direct examination of haemolysates without the need for adduct purification. Modified species can be identified even in the case of NO, where the mass difference due to the reaction is only 29 mass units, as previously demonstrated in the case of nitrosylated peptides [18].

The present study based on the analysis of the adducts of

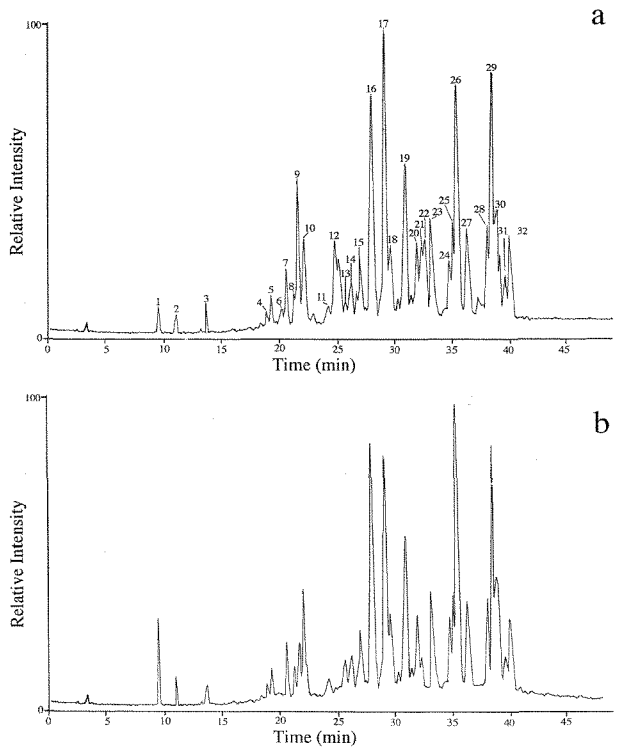

Fig. 4. LC/ES/MS of the peptic digest of the mononitrosyl β-globin (a), compared with that of native β-globin (b).

haemoglobin–NO was aimed at (i) verifying the overall reactivity of haemoglobin towards nitric oxide and at evaluating the level of modification, and (ii) identifying the actual modified cysteine(s) among those contained in the haemoglobin sequence. This study reports the identification of the unique reactive cysteine of haemoglobin following in vitro exposure of haemolysates at low Cys–NO/Hb ratio.

4.2. *Nitric oxide induces modification of a specific cysteine residue in haemoglobin*

A recent study [13] reported that NO–Hb can be formed in vitro and in vivo in the presence of RSNOs, and NO–Hb has been involved in blood pressure regulation. It was reported that no modified residue occurred different from β-Cys^{93}, but no structural data were available on the nature of the NO–Hb. These conflicting observations prompted us to undertake this study. To selectively nitrosylate the thiol group of cysteine residues a procedure involving RSNO intermediates was chosen [9]. This allowed us to monitor the reactivity of cysteine residues within the haemoglobin sequence. We varied the ratio of reagent to protein to determine some degree of selectivity. In fact, the use of NO at two different concentrations allowed determination of the level at which side chain reactivity was modulated by the NO/haemoglobin ratio. We actually demonstrated that under the conditions used by Jia et al. [13] only a single cysteine residue, β-Cys^{93}, is almost completely modified in vitro after 30 min. At longer times (2 h) the reaction is complete and no further modification is observed (not shown). It is worth mentioning that using a higher concentration of nitrosothiol (1 : 100), modification at β-Cys^{112} and α-Cys^{104} also occurred. However, this concentration appears to be far from showing real physiological significance [13]. Therefore one can reasonably conclude that only β-Cys^{93} is actually reversibly modified by RSNOs in red blood cells. A similar result for another highly reactive electrophilic agent such as methyl bromide was recently observed [19]. The different behaviour exhibited by β-Cys^{93} compared to the other cysteine residues is in agreement with the already reported in vivo reactivity of this residue in oxy-haemoglobin which readily undergoes oxidation to form mixed disulphides and other thioethers due to its position within the protein quaternary structure [20].

Once formed, Hb–NO adducts are readily degraded at neutral or alkaline pH [9]. For this reason nitrosylated peptides were not observed after tryptic hydrolysis (data not shown). A much greater stability of the nitrosylated globins was instead observed at low pH. Samples stored at pH < 3 showed minimal degradation over a period of several weeks. For this reason a procedure based on the use of pepsin as the proteolytic agent was chosen, which allowed us to preserve nitrosylated peptides during hydrolysis at low pH values.

The peptic mixture was directly analysed in LC/ES/MS. Enzymatic digestion of modified haemoglobin resulted in a mixture of native and nitrosylated peptides, the relative abundance depending on the extent of overall nitrosylation. The presence of overlapping peptides due to incomplete digestion was useful in obtaining a complete peptide map and in the accurate location of the modified cysteines. Nitrosylated peptides were readily identified from their molecular weight compared to the native ones and their identity was confirmed by

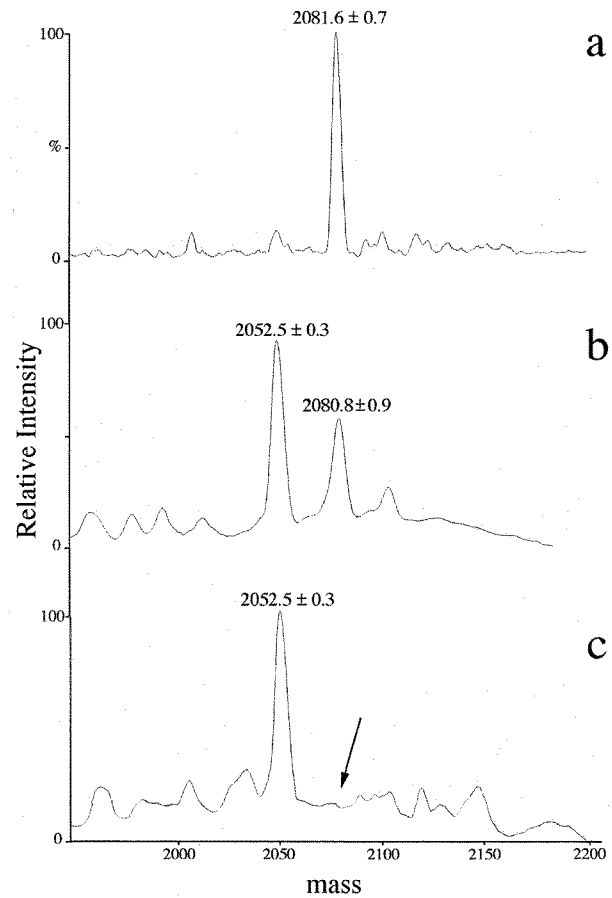

Fig. 5. ES/MS spectra of nitrosylated peptide (89–105) obtained at different values of cone voltage: a = 20 V, b = 40 V, c = 80 V.

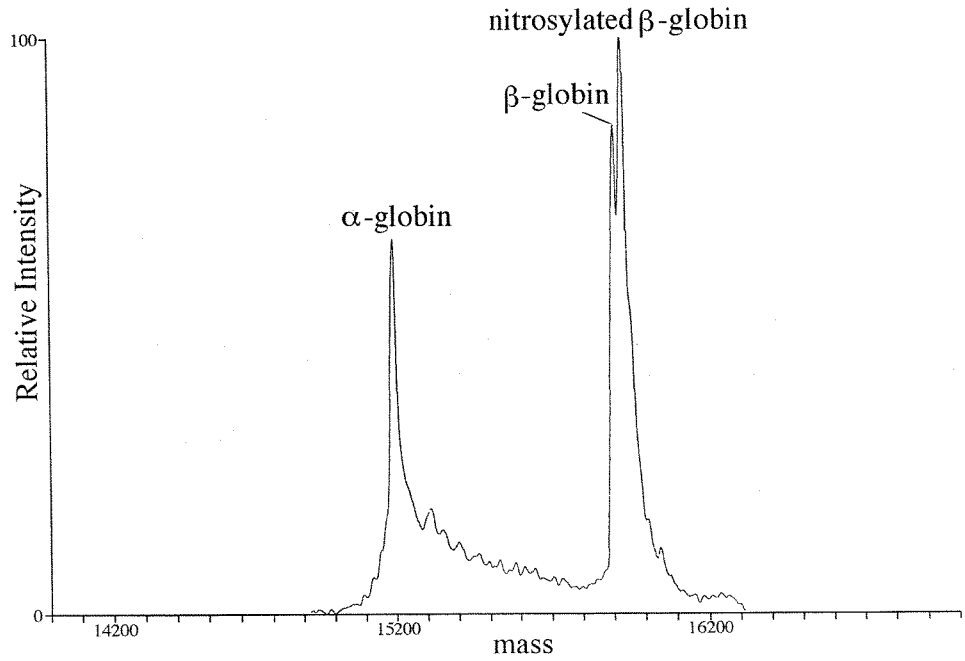

Fig. 6. ES/MS spectrum of haemolysate incubated with Hb/Cys–NO ratio (1 : 10). The nitrosyl derivative haemoglobin can be observed.

selective fragmentation at the S–NO bond by increasing the cone voltage.

In addition, the proposed approach can also be applied to monitor in vivo modification. In fact, in Fig. 6, the spectrum of a whole haemolysate treated with 1 : 10 Cys/NO is reported, which demonstrates the possibility to extend the in vivo the direct analysis the NO–adducts in blood. Considering the extremely variable and little-known in vivo reactivity of NO with proteins, the proposed methodology may represent a practical approach both for the analysis of the NO–protein adducts and for the assignment of NO-modified residues in polypeptide chains.

Acknowledgements: This work was supported by EU Grants ERB GE1CT92-0045 and ERB FMGE CT95-0061 and carried out under the auspices of the Istituto Nazionale di Chimica dei Sistemi Biologici of CNR. The authors are indebted to Dr. Umberto Breme for his skilful guidance in setting up the LC/ES/MS procedure.

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