Protein Science (1997), 6:2568–2577. Cambridge University Press. Printed in the USA.

Structural characterization of human hemoglobin crosslinked by bis(3,5-dibromosalicyl) fumarate using mass spectrometric techniques

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(RECEIVED April 22, 1997; ACCEPTED August 11, 1997)

Abstract

Diaspirin crosslinked hemoglobin (DCLHb) was analyzed by mass spectrometric-based techniques to identify the protein modifications effected by the crosslinking reaction with bis(3,5-dibromosalicyl) fumarate. DCLHb consists of two principal components. These components were isolated by size-exclusion chromatography and identified by measurement of their molecular weight using electrospray mass spectrometry and subsequent peptide mass mapping and mass spectrometric sequence analysis of their individual digests. Three major RP-HPLC fractions were observed from the major hemoglobin in DCLHb. Their MWs matched the MW of heme, intact hemoglobin β-chain, and two hemoglobin α-chains crosslinked by a fumarate moiety, respectively. The minor HPLC peaks of DCLHb were also separated, and characterized by mass spectrometric methods. These minor components revealed additional details of the structural nature of covalent modification of DCLHb.

Keywords: blood substitute; hemoglobin; mass spectrometry; peptide mass mapping; protein crosslinking; structural characterization

The search for a blood substitute has been pursued for decades (Amberson, 1937; Moffat, 1991). Historically, this search has focused on the development of fluids that could be used in place of blood, primarily to reduce concerns about its availability and safety (Moffat, 1991; O'Donnell et al., 1993). However, in recent years, the objectives of this search have changed, and the current goal is the development of a therapeutic fluid that will restore both oxygen delivery and perfusion following either global or focal oxygen deficit (ischemia). This more comprehensive objective has the potential to encompass clinical applications that range from traditional uses as a resuscitation fluid following acute, severe blood loss, where blood transfusion is a standard of care, to future applications as a treatment for the ischemia associated with myocardial infarction or stroke, where blood loss is not indicated.

Hemoglobin, the iron-containing protein that transports oxygen from the lungs to the tissues, is a natural candidate for this purpose. Human hemoglobin is a tetrameric protein of about 64.5 kDa, consisting of two α-chains and two β-chains that are tightly associated with each other but not covalently linked (Perutz et al., 1960). A heme group is embedded in each of the protein subunits. Because it constitutes about 95% of the mass of the red blood cell, large quantities of this natural protein are readily available. In addition, hemoglobin has been well studied and characterized extensively (Bunn & Forget, 1986).

In the red blood cell, hemoglobin reversibly binds and releases oxygen via a conformational change from a high-affinity to low-affinity state that is facilitated by the polyanionic effector, 2,3-diphosphoglycerate (Perutz, 1979). However, purified hemoglobin, also known as stroma-free hemoglobin, lacks this capability in the absence of an effector. In addition, SFHb has been found to be nephrotoxic (Bunn et al., 1969). SFHb dissociates into α,β-dimers that are filtered in the glomerulus of the kidney; high concentrations of dimers overwhelm the filtration capacity of this organ and are toxic. The combination of toxicity and high oxygen-binding

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Abbreviations: DCLHb, diaspirin crosslinked hemoglobin; SFHb, stroma-free hemoglobin; RP-HPLC, reverse-phase HPLC; SEC, size-exclusion chromatography; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; MS, mass spectrometry; PSD, post source decay; DBBF, bis(3,5-dibromosalicyl) fumarate; STP, pentasodium tripolyphosphate; TFA, trifluoroacetic acid.

affinity renders SFHb not clinically useful (Vandergriff et al., 1989; Roth et al., 1993).

The shortcomings of SFHb may be corrected by chemical modification of hemoglobin. For example, modifications of purified human hemoglobin by bis(3,5-dibromosalicyl) fumarate, in the presence of pentasodium tripolyphosphate, has been shown to yield an intramolecularly crosslinked hemoglobin analogue called diaspirin crosslinked hemoglobin (Snyder et al., 1987; Jones, 1989; Roth et al., 1993).

Size-exclusion analysis of DCLHb shows that it consists of two principal crosslinked hemoglobins, both of which are capable of reversible oxygen binding and release. The major component in DCLHb comprises about 96% or more of the product and has an apparent molecular weight of about 65 kDa; this crosslinked hemoglobin does not break into dimers spontaneously. The other minor hemoglobin in DCLHb comprises about 2–3% of the product and has an apparent molecular weight of about 130 kDa; this crosslinked hemoglobin, like its 65-kDa counterpart, does not spontaneously break into smaller hemoglobin subunits.

DCLHb has an oxygen-binding capability comparable to hemoglobin in fresh, whole blood. In addition to its oxygen-carrying properties, DCLHb functions as an active oncotic and pharmacological agent. Preclinical and clinical studies indicate that DCLHb is capable of restoring blood pressure and circulation following acute hypotension after insults such as hypovolemic shock. Moreover, preclinical studies suggest that DCLHb may be useful to restore and maintain perfusion of key organs following myocardial infarction, stroke, or septicemia (Cole et al., 1993; Farmer et al., 1995).

Comprehensive characterization of DCLHb is one aspect of the therapeutic development of this oxygen-carrying plasma-volume expander. Structural analysis of DCLHb and other chemically modified hemoglobins has been performed previously using HPLC in combination with enzymatic digestions (Jones, 1989). However, this approach lacked the detailed level of identification that appeared to be necessary for rigorous characterization of the hemoglobins that are present in DCLHb.

X-ray crystallography also has been employed to characterize the structure and crosslinking site of DCLHb (Chatterjee et al., 1986; Fernandez, 1995). Chatterjee et al. have shown that the epsilon amino groups of the lysine residues in position 99 of the α subunits (Lys-99) are covalently crosslinked to each other by a fumaryl bis(amide) bridge. Further crystal structure studies have verified the crosslinking site at higher resolution (Fernandez, 1995). Nonetheless, besides the major effort required in crystallization, data collection, and structural interpretation, this technique only focuses on the major crosslinked hemoglobin that is present in DCLHb. Information concerning the identity of minor components is missing.

In the last few years, mass spectrometry has become the method of choice for the characterization of proteins and protein modifications (Burlingame et al., 1996). This is, in large measure, due to the discovery of two new ionization techniques, namely electrospray ionization (Fenn et al., 1989) and matrix-assisted laser desorption ionization (Karas & Hillenkamp, 1988), which provide both the high sensitivity and extended mass range required for a detailed structural characterization of covalently modified proteins. These techniques already have been used extensively in the characterization of hemoglobin variants responsible for a large number of hemoglobin-based genetic diseases (Green et al., 1990; for review, see Shackleton & Witkowska, 1996). In this paper, we report the structural characterization of both major and minor components of DCLHb using advanced methods of mass spectrometry.

Results and discussion

Separation of the principal DCLHb components

Separation of DCLHb by SEC (Fig. 1) revealed that it is comprised of two principal components: a major fraction (96%) having a molecular weight of approximately 65 kDa, and a minor fraction (2–3%) having an apparent MW of about 130 kDa. Because the size-exclusion HPLC conditions that were used did not disrupt the tetrameric association of this crosslinked protein, it would be reasonable to assume that the major fraction is an intramolecularly crosslinked hemoglobin molecule, and that the 130-kDa peak is a covalent dimer of the crosslinked hemoglobin. However, these assumptions required structural verification.

Analysis of these two components using SDS-PAGE mini-gels provided more detailed information about their composition. The 65-kDa component resolved into two bands having MW of approximately 15 and 30 kDa. The 130-kDa component separated into four bands having MW of approximately 15, 30, 45, and 60 kDa (data not shown). Given that the molecular weight of the human hemoglobin α-chain is 15,126 Da and the β-chain is 15,868 Da, the band at 15 kDa most likely corresponds to either an α-chain or a β-chain, or a mixture of the two polypeptides, because the resolution of SDS-PAGE under these conditions was not sufficient to separate these two components. The 30-kDa band could be assumed as a covalent product of α-α, β-β, or α-β crosslinking, simply based upon the molecular weight information. The 45-kDa band probably corresponded to a covalently linked trimer, whereas the 60-kDa band probably represented a tetramer of the α-chain and/or β-chain subunits. However, all these assumptions required structural clarification.

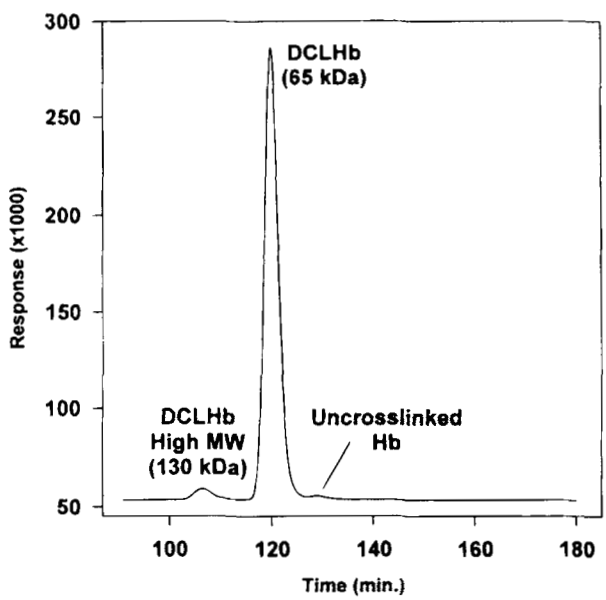


Fig. 1. Size-exclusion chromatogram of DCLHb. SEC analysis was performed using a Superose 12 column and a mobile phase containing 0.75 M $MgCl_2$ delivered at a flow rate of 0.1 mL/min.

Molecular weight determination of the principal DCLHb components

Samples of the 65-kDa and 130-kDa components of DCLHb, along with the starting material SFHb, were separated by RP-HPLC, and the individual fractions were collected for mass spectrometric analysis. ESI-MS was used to obtain molecular weight information.

As expected, the RP-HPLC chromatogram for SFHb contained three peaks (data not shown). The first peak had an *m/z* value of 616.6, which corresponds to the MH^+ value of heme. The second peak had a molecular weight of 15,867 ± 1, which corresponds to the mass of a single β-chain subunit of hemoglobin. The third peak had a molecular weight of 15,126 ± 1, which corresponds to the molecular weight of a single α-chain subunit.

RP-HPLC separation of the 65-kDa component of DCLHb gave three peaks (Fig. 2). The retention times of the first two of these peaks were the same as those of heme and the β-chain of SFHb, respectively. In addition, they showed the same molecular weight information from ESI-MS as the corresponding SFHb peaks; subsequent peptide mapping and sequencing of Peak B (Fig. 2) after digestion with Lys-C confirmed its identity as a hemoglobin β-chain. The third peak (Fig. 2, Peak C) eluted significantly later. Its MW was determined to be 30,333 ± 1 (Fig. 3). This value corresponds to the sum of the expected molecular weights of two α-subunits plus an additional moiety of mass 81 ± 1 Da. Subsequent peptide mass mapping and mass spectrometric peptide sequencing established that Peak C (Fig. 2) contained peptides derived exclusively from the hemoglobin α-subunit.

The 130-kDa component of DCLHb was isolated by gel permeation chromatography. Analysis of this component by RP-HPLC showed the presence of five major peaks (Fig. 4). The earliest eluting two components (Fig. 4, Peaks A and B) were identified as heme and the single β-chain, respectively. Electrospray mass analysis of Peak C (Fig. 4) revealed a molecular weight of 46,287 ± 7 (Fig. 5). This mass value corresponds to the sum of two α-chains plus one β-chain plus 167 ± 7 Da. Subsequent peptide mapping confirmed the presence of both α- and β-chain peptides. Peak D had the same retention time as Peak C in Figure 2, and their molecular weights also matched. The fifth peak (Fig. 4, Peak E) showed a molecular weight of 60,758 ± 14 (Fig. 6), which is equal to four α-chains plus 252 ± 14 Da. This fraction was shown by digestion to contain peptides from the α-chain exclusively.

These results may be rationalized by consideration of the reaction of DBBF with nucleophilic side chains among hemoglobin subunits (see Scheme I). Nucleophilic displacement of the 3,5-dibromosalicylate leaving group of DBBF by an amino function yields a fumaramide. If each of the two dibromosalicylate leaving groups of DBBF is displaced by an amino group on separate protein subunits, these subunits will become crosslinked by a bis(fumaramide) bridge. Hence, the molecular weight of the resulting crosslinked species will be equal to the sum of the molecular weights of the individual subunits plus 80 Da, the incremental mass corresponding to incorporation of the fumarate residue

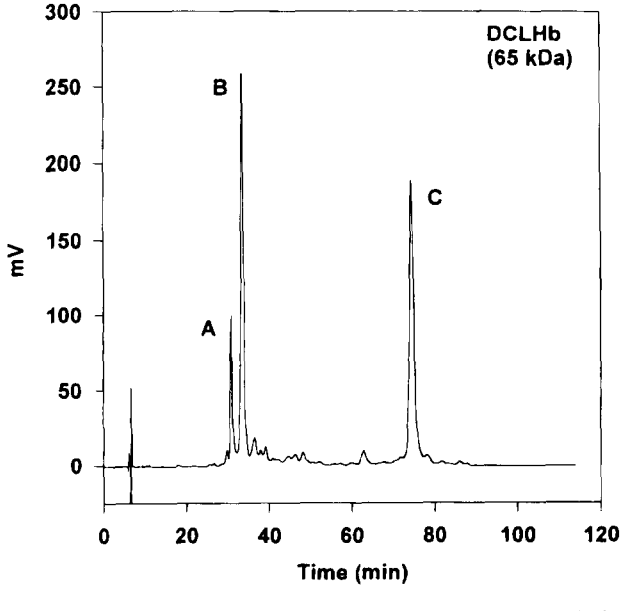
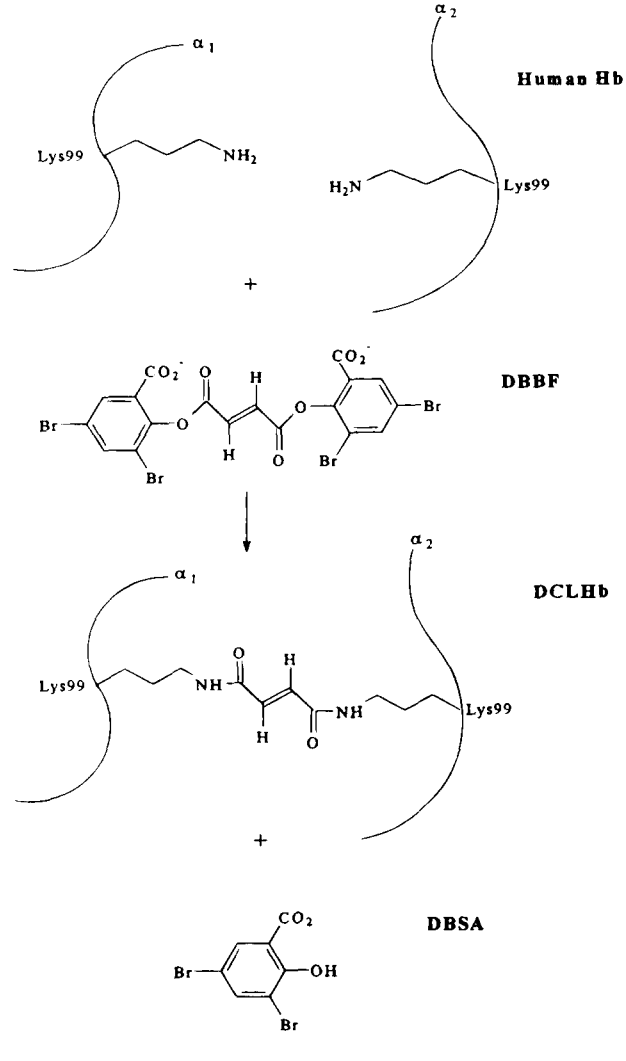


Fig. 2. RP-HPLC chromatogram of DCLHb, 65-kDa component. A slow gradient of 40–60% B (acetonitrile + 0.08% TFA) in 150 min was applied to obtain a good separation. Under these conditions, uncrosslinked protein subunits are resolved, but covalent bonds are not disrupted.



Scheme I.

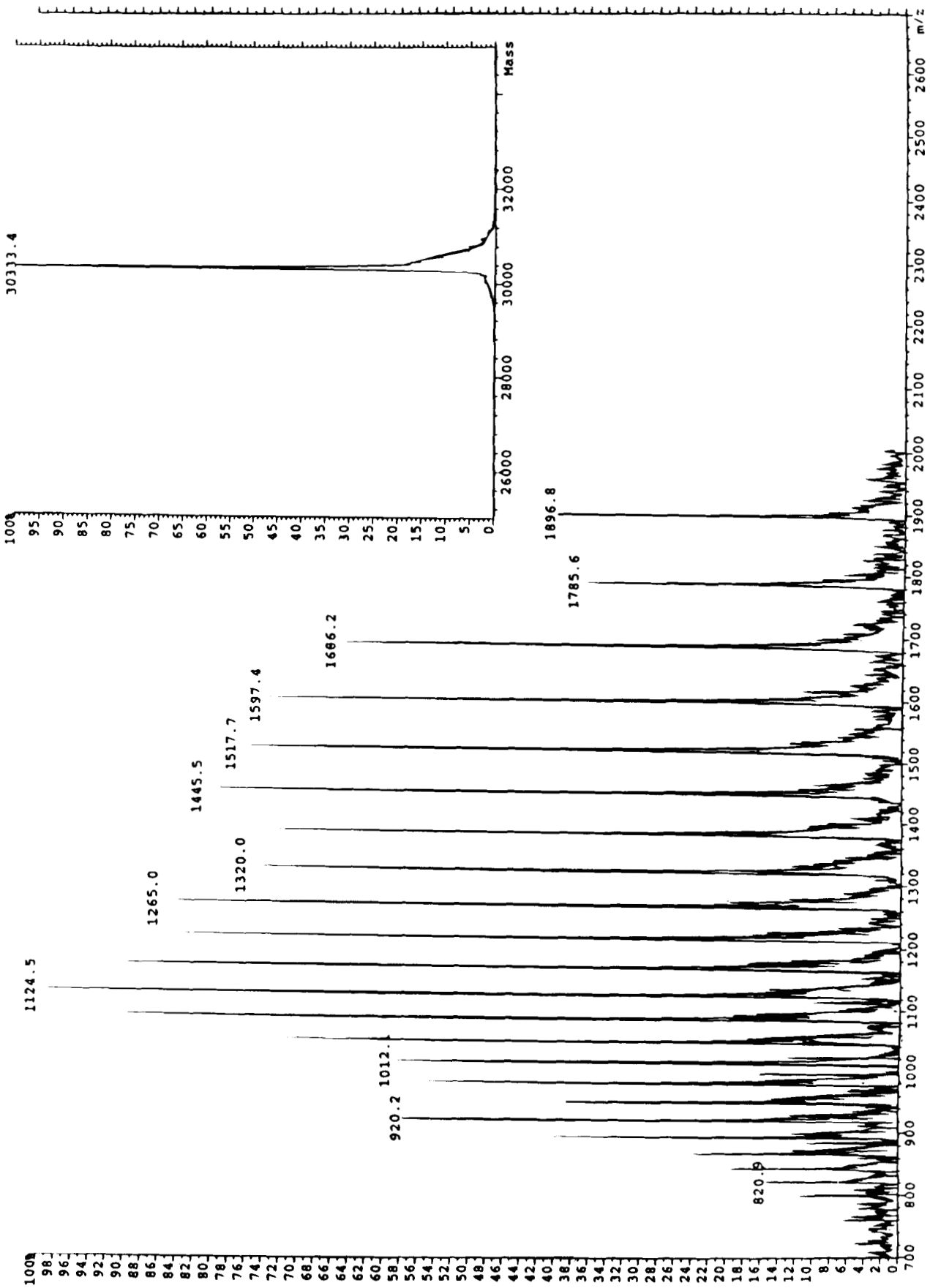


Fig. 3. Mass spectrum of Peak C in Figure 2. The MW, 30,333, corresponds to the mass of two α-chains plus a fumarate.

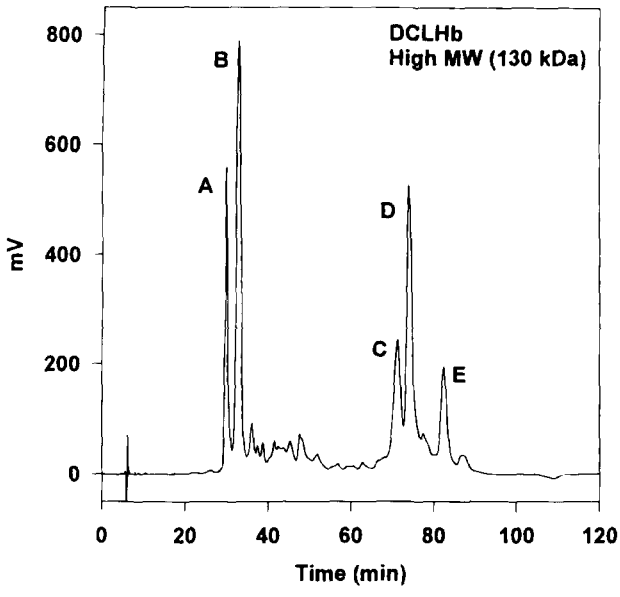


Fig. 4. RP-HPLC chromatogram of DCLHb, 130-kDa component. This component was purified previously by SEC. A slow gradient of 40–60% B (acetonitrile + 0.08% TFA) in 150 min was applied to obtain a good separation. Under these conditions, uncrosslinked protein subunits are resolved, but covalent bonds are not disrupted.

OC—CH=CH—CO minus two protons, one from each lysine ϵ-amino group that are substituted by the fumarate.

However, if one of the dibromosalicylate leaving groups of DBBF is hydrolyzed by water, that carboxyl moiety of DBBF will be converted to a carboxylic acid residue that will not undergo nucleophilic displacement. Under these conditions, the other dibromosalicylate leaving group of DBBF may be displaced by a protein amino group, yielding a modified protein species increased in molecular weight by 98 Da, the mass corresponding to the fumarate residue OC—CH=CH—CO—OH. Of course, if the second carboxyl moiety of DBBF is hydrolyzed as well, no covalent bond to the protein results.

Therefore, a component having MW 30,333 (Fig. 2, Peak C; Fig. 4, Peak D) equals the mass of two α-chains and one fumarate molecule exactly. Taken together, the ESI-MS data indicate that the 65-kDa component of DCLHb observed in SEC (Fig. 1) is a hemoglobin tetramer ($\alpha_2\beta_2$) in which the α-subunits are covalently crosslinked by fumarate.

A similar analysis of the major components of the gel permeation purified 130-kDa fraction (Fig. 1) of DCLHb indicates that Peak C (Fig. 4, MW 46,287 Da) is a species that consists of one fumarate crosslink between two α-chains and a second fumarate crosslink between one of the α-chains and a β-chain (expected MW 46280). Further, the measured molecular weight of Peak E (Fig. 4) of this 130-kDa fraction was established as 60,758

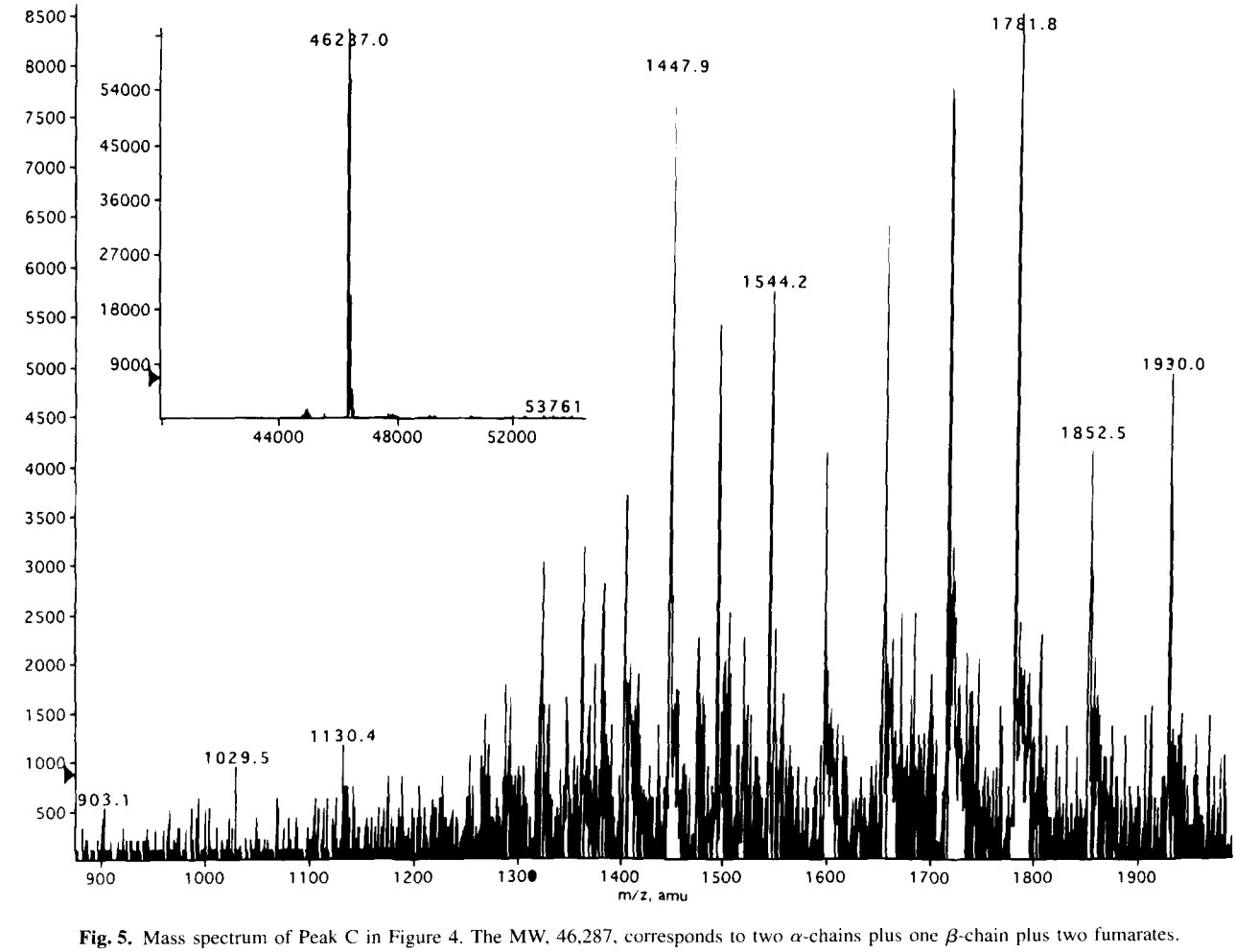


Fig. 5. Mass spectrum of Peak C in Figure 4. The MW, 46,287, corresponds to two α-chains plus one β-chain plus two fumarates.

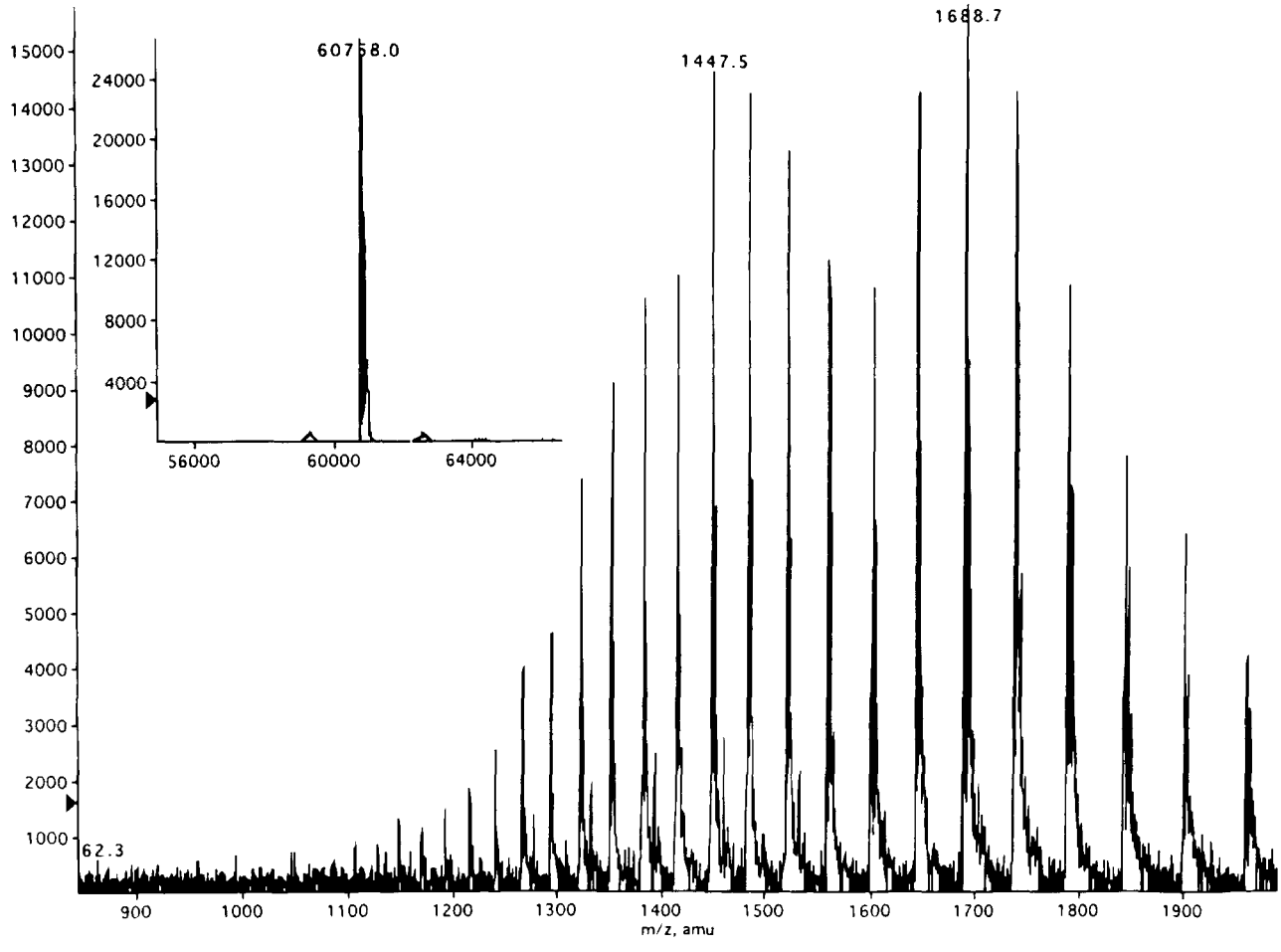


Fig. 6. Mass spectrum of Peak E in Figure 4. The MW, 60,758, corresponds to four α-chains plus three fumarates.

Da. This mass value corresponds to four α-chains plus three fumarate crosslinker moieties (expected MW 60,746). This species clearly contains one intermolecular fumarate crosslink between two crosslinked α-chains in addition to the two intramolecular crosslinks between the α-chains. Taken together, the ESI-MS data indicate that the 130-kDa component of DCLHb comprises dimers of intramolecularly crosslinked tetramers ($\alpha_2\beta_2$) in which the α-subunits are covalently crosslinked by fumarate and intermolecular fumarate crosslinks covalently join an α-subunit to either a β-subunit or an α-subunit on a second intramolecularly crosslinked hemoglobin.

Identification of the crosslinking sites

Interpretation of the molecular weight information obtained by ESI-MS established that the major component observed in SEC (Fig. 1) of DCLHb (i.e., the 65-kDa component) is a hemoglobin ($\alpha_2\beta_2$) tetramer having its two α-subunits crosslinked by a single fumarate moiety. Further investigations were undertaken to identify the crosslinking site(s) between these two α-chains as described below.

To locate the site of crosslinking in the covalent α-α component, a sample corresponding to Peak C (Fig. 2) was reduced and carboxymethylated and then digested using the highly specific protease Lys-C from *Achrombacter lyticus* (Medzihradszky et al., 1992). The resulting digest was separated into peptide fractions by RP-HPLC (Fig. 7). The molecular weights of the peptides were determined employing on-line LC/ESI-MS, fraction splitting most of the effluent for collecting. These fractions were analyzed by MALDI-MS (Matsui et al., 1997) as well.

The molecular weights obtained from the ESI-MS and MALDI-MS experiments were then compared with the values expected for the peptide components obtained by a Lys-C enzymatic digestion of the α-chain of human hemoglobin. This comparison revealed that all of the peptides in the α-α subunit originated from the α-chain, establishing that this modified protein is composed solely of α-subunits. The majority of these peptides were further characterized by MALDI-PSD/MS (Matsui et al., 1997) to establish unambiguously their amino acid sequence assignment. The peptide map incorporated the entire amino acid sequence of the α-chain except one major region (91–127) and four small peptides. However, the presence of these four small peptides was established from a different mass map obtained from a complementary enzymatic digestion using chymotrypsin (data not shown). Only the particular peptide expected from cleavage at the lysine residue in position 99 (K99) of the α-chain was not detected. However, one additional peptide was observed at relatively high molecular weight, m/z 8,157, that did not match any expected linear peptide sequence

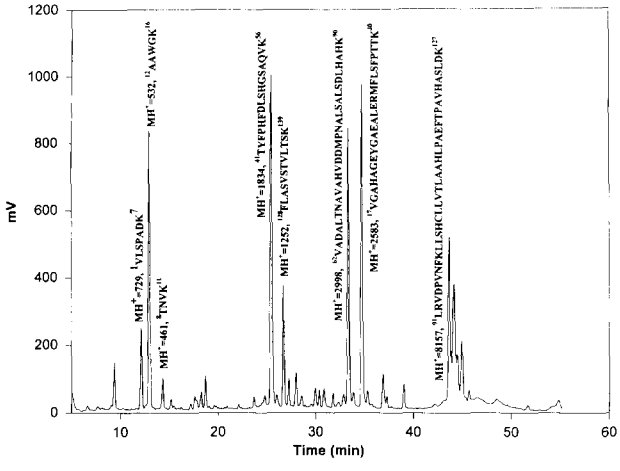


Fig. 7. RP-HPLC chromatogram of the crosslinked α-α subunit (Fig. 6, Peak C) of DCLHb cleaved by Lys-C protease. Molecular weights of the peptides and the amino acid sequence of each peptide, obtained by MALDI and MALDI-PSD, are labeled above the peaks.

from the α-chain of hemoglobin. This mass value corresponds exactly to the mass of two Leu 91–Lys 127 peptides crosslinked by a fumarate moiety (calc. m/z 8,157). This finding suggested that this component contained the crosslink sought.

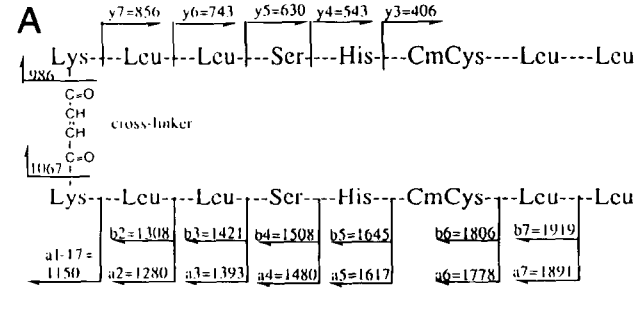
The m/z 8,157 peptide fraction was subdigested using pepsin, and the digestion mixture was analyzed by MALDI-MS to obtain molecular weight information. A number of peptides were detected, and their sequences were identified by MALDI/PSD experiments. These results established that these sequences were all present within the region of Leu 91–Lys 127, except for one peptic peptide having a mass of 2,050 m.u. The molecular weight did not match any linear peptides from the Leu 91–Lys 127 region of the α-chain. MALDI/PSD analysis of this peptide demonstrated unequivocally that it is comprised of two identical peptide chains (sequence Lys-Leu-Leu-Ser-His-CmCys-Leu-Leu) linked by a fumarate bridge at the side chains of the lysine residues (Fig. 8). These lysine residues correspond to position 99 of the hemoglobin α-chain. Therefore, these data establish that the major crosslinking has occurred between the side chains of lysine 99 of both hemoglobin α-subunits.

As discussed above, the 130-kDa gel permeation fraction is comprised of the intermolecular crosslinking of two α-α chain intramolecular crosslinked hemoglobin molecules. Peaks C and E of Figure 4 indicate that the intermolecular crosslinking is either between an α,α-crosslinked chain and an α,β-chain, or between two α,α-crosslinked chains. In an effort to locate potential site(s) of this intermolecular crosslinking, the fractions containing these intermolecular crosslinked species ($\alpha\alpha\beta$ and $\alpha\alpha\alpha\alpha$) were digested by Lys-C protease and analyzed subsequently by LC-ESI-MS and MALDI-MS. Results showed that all the expected Lys-C peptides were present, including the m/z 8,157 K99-K99 peptide, which represents the major intramolecular crosslinked species (see above). However, no additional crosslinked peptides were observed in addition to the m/z 8,157 peptide. These experiments were repeated several times, and similar results were obtained. Based on these studies, it appears that this minor intermolecular crosslinking is not site-specific. Hence, apparently different crosslinking species are formed with common isobaric molecular weights. As a result, proteolytic digestion of these species generated peptides that covered the whole sequence. The basis of nonspecific crosslinking is that, unlike the intramolecular crosslinking, the hemoglobin molecules are not sterically restricted to favor or forbid one specific form of intermolecular crosslinking, therefore small amounts of a variety of intermolecular crosslinking species may be formed.

Characterization of minor components of DCLHb

The RP-HPLC chromatogram of DCLHb contains several peaks of low relative abundance in addition to the two major components of DCLHb that have been characterized (see above) (Fig. 2). These peaks are likely to correspond to α- and β-subunits of hemoglobin that have been modified by reaction of DBBF at sites other than Lys 99α. Such minor modifications are expected for several reasons. Foremost among these is the fact that DBBF is a crosslinking agent that has high affinity for the central water cavity of hemoglobin and the binding site within it that contains the Lys 99α amino acids; however, this affinity is merely sufficient to provide high selectivity in reactions of DBBF with hemoglobin, but is not sufficient to ensure complete reaction specificity. In addition, hemoglobin contains many other lysine residues having surface-exposed amino groups available for potential reaction with DBBF. Reaction of DBBF with these residues will result in fumarate modification of the surface of the protein. In the synthesis of DCLHb, a small molar excess of DBBF is added to the hemoglobin to ensure extensive crosslinking; thus, sufficient DBBF is available to both crosslink and surface-modify a low percentage of the hemoglobin.

When DCLHb was separated after deliberately overloading the RP-HPLC column by injecting a large amount of sample, the major peaks were saturated, and the intensity of several low-abundance peaks was increased sufficiently to permit their isolation (Fig. 9). Each of these components was analyzed by ESI-MS to establish their molecular weights and then was digested by Lys-C. The digests then were analyzed by ESI-MS and MALDI-MS to ascertain information on possible sites of covalent modification. For a few peptides, MALDI/PSD experiments were performed to confirm their sequence identity. From these results (see Table 1), some of the other, minor hemoglobin subunit modifications were defined. These may occur during the crosslinking reaction, subsequent processing steps, or storage of DCLHb. In these data, an increase of 98 units in the mass of a polypeptide corresponds to amide bond formation between the protein and one carboxyl group of the fumarate. This modification was observed for both the α-chain (Peak 6) and β-chains (Peaks 3, 4). Peptide mass mapping and



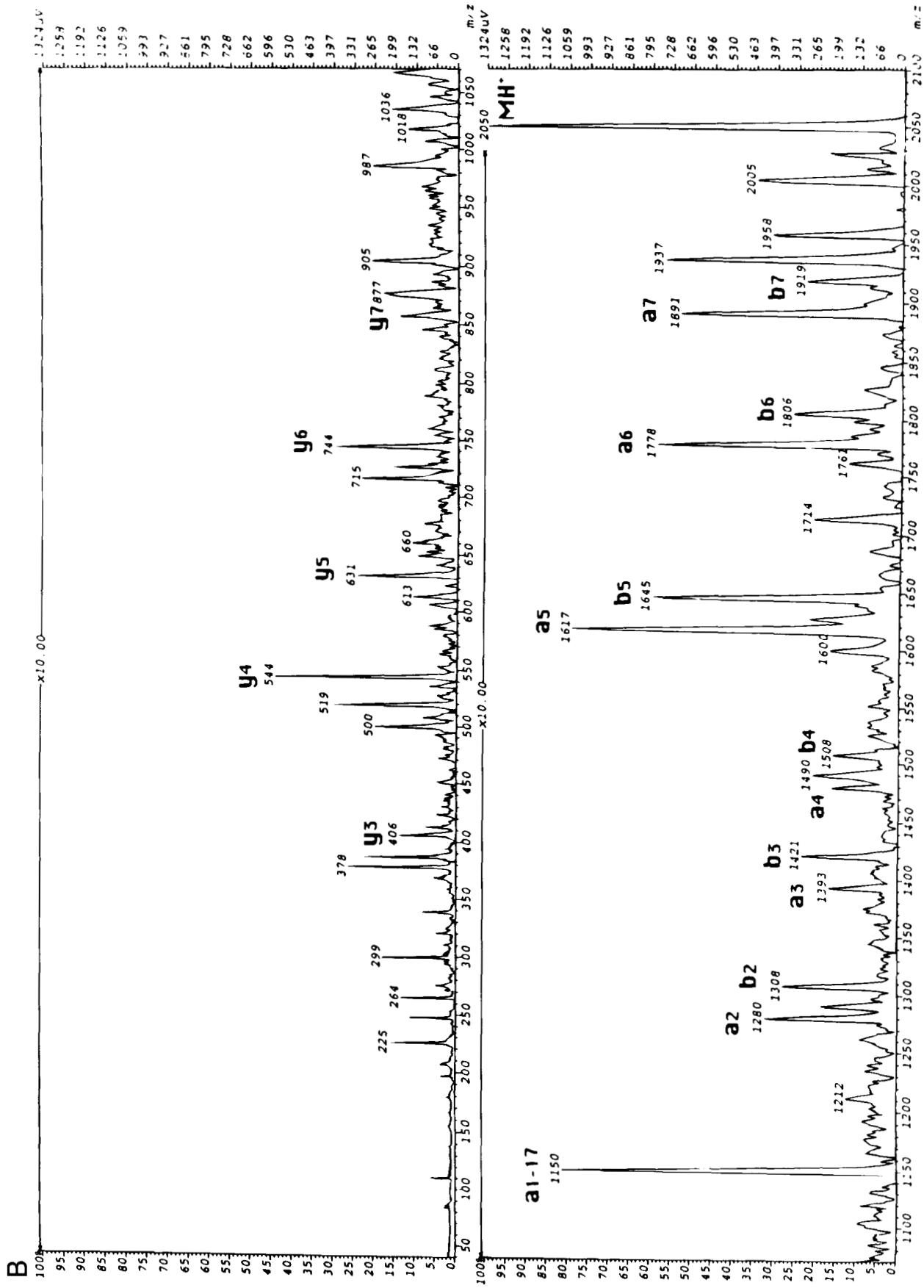


Fig. 8. MALDI-PSD spectrum of the 2,050 m.u. peptide, obtained by peptic digestion of the 8,156-Da Lys-C product of the DCLHb crosslinked α-α component. Peak assignment is shown on the upper panel.

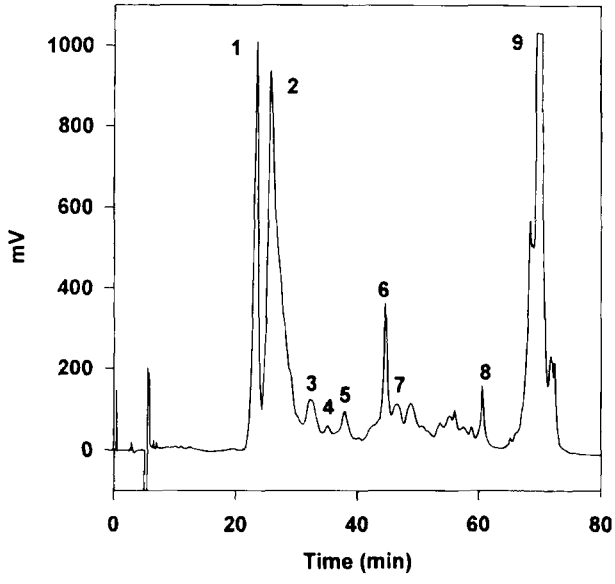


Fig. 9. RP-HPLC chromatogram of DCLHb. The column was overloaded in order to observe minor protein subunits of DCLHb; thus, the major peaks were either saturated or distorted.

MALDI/PSD experiments indicated that Lys 132 or Lys 144 of the β-chain was modified. Attachment of a heme moiety to a β-chain was also observed (Peak 4). In addition, subunit crosslinking through a disulfide bridge is indicated in Peak 5 from the molecular weight obtained (31,730 Da). The observation of a Lys-C peptide at MH^+ 5,656 indicates that Cys 112 of the two β-chains occurs partially in the disulfide bridge linkage. In a separate experiment, a minor component of MW 30,430 was observed, which corresponds to the mass of the α-α crosslinked species plus one fumarate. A molecular weight species of 32,014 Da was also observed from this separate experiment, and this represents a species with two β-chains crosslinked by the fumarate and modified by fumarate at two sites. All these components, however, represent a very minor portion of DCLHb, as indicated above.

Conclusion

This study provides a comprehensive description of the crosslinking reaction between purified human hemoglobin and DBBF. The dominant reaction is fumaryl bis(amide) crosslinking between the epsilon amino functions of two α-chains at Lys-99, as shown previously by X-ray analysis. Therefore, the major component of DCLHb is a tetrameric protein with only one intramolecular covalent crosslink. The high molecular weight component of DCLHb is the result of intermolecular crosslinking between two intramolecularly crosslinked hemoglobin molecules through a (crosslinked) α-subunit of one hemoglobin and a (crosslinked) α- or β-subunit of a second hemoglobin tetramer.

The power and versatility of mass spectrometric-based techniques is well demonstrated in this work. Accurate molecular weight information on the proteins provided by ESI-MS allowed assignment of the molecular identities of the major and minor components of DCLHb, together with a comprehensive characterization of the protein modifications effected by the crosslinking process. In addition, the sensitivity of these techniques enabled a detailed investigation of the structures of both the major and minor products. Peptide sequencing by mass spectrometry provides direct information establishing the modified amino acid residues precisely.

Table 1. *Molecular weight information and structural assignment of the DCLHb subunits identified by RP-HPLC in Figure 9*

Peak no.	Molecular weight (Da)	Sequences of modified peptides	MH^+ of modified peptides (m.u.)	Modification sites
1	617 (heme)	NA[a]	NA	NA
2	15,868 (β-chain)	NA	NA	None
3	15,966 (β-chain + fumarate)	Val 133–His 146	1,548.7	Lys 144
		Glu 121–Lys 144	2,606	Lys 132
4	15,966 (β-chain + fumarate)	Val 133–His 146	1,548.7	Lys 144
		Glu 121–Lys 144	2,606	Lys 132
	16,500 (β-chain + heme)	Val 133–His 146	2,066	Lys 144
5	15,966 (β-chain + fumarate)	Val 133–His 146	1,548.7	Lys 144
		Glu 121–Lys 144	2,606	Lys 132
	31,730 (2β-chains crosslinked by disulfo bridge)	Leu 96–Lys 120	5,656	Cys 112
6	15,947 (β-chain + fumarate)	NI	NI	NI
	15,226 (α-chain + fumarate)			
7	15,126 (α-chain)	NA	NA	None
8	15,966 (β-chain + fumarate)	Val 133–His 146	1,548.7	Lys 144
		Gly 83–Lys 120	4,326.7	Lys 95
9	30,333 (2 α-chains + fumarate)	Leu 100–Leu 116	2,050	Lys 99

[a]NA = not applicable; NI = not identified.

Materials and methods

DCLHb (lot number 95I15AD11, 96B23AD11, 96A26AD11), DCLHb high molecular weight fraction (lot number PBS-1-96-023), and SFHb (lot number PBS-1-95-044) were prepared by Baxter. Endopeptidase Lys-C was purchased from Wako Bioproducts, Japan. Bovine chymotrypsin and porcine pepsin were purchased from Sigma (St. Louis, Missouri). HPLC grade water and acetonitrile were from Fisher Scientific.

Protein isolation purification and digestion

DCLHb was separated by SEC using a Sephadex 200 preparative column (Pharmacia). The SEC fractions were analyzed by SDS-PAGE to obtain their approximate molecular weights. Analytical RP-HPLC was performed using a C-18 column (Vydac 4.6 × 250 mm). Proteins were eluted by running a gradient using water containing 0.1% TFA as solvent A and acetonitrile containing 0.08% TFA as solvent B. Gradient conditions were varied as necessary to achieve separations of the desired peak fractions. The HPLC fractions were either reduced and alkylated by iodoacetic acid, and then enzymatically cleaved by Lys-C protease, or were digested

directly by Lys-C. Prior to digestion, the protein fractions were dissolved in 8 M urea, which was then diluted with ammonium bicarbonate solution to 1.6 M urea and 80 mM ammonium bicarbonate as the digestion buffer. A substrate:protease ratio of about 100:1 was used, and the reaction was continued 12 h at 37 °C. Chymotryptic digestion was performed under the same conditions as the Lys-C digestion. In some cases, subdigestion was performed using pepsin. For peptic digestion, samples were dissolved in 70% formic acid and then diluted by adding 20 volumes of 1 mM HCl. A two-hour reaction was performed at 37 °C with a substrate to enzyme ratio of 100:1. For direct HPLC separation of the peptides, the enzymatic digests were injected onto a C-18 HPLC column (Vydac 4.6 × 250 mm) at a gradient of 1% solvent B per min (solvents A and B are the same as described above).

Mass spectrometry

Molecular weight information of the proteins was obtained either by direct LC/ESI-MS analysis of the samples, or batch mode ESI-MS analysis on an Autospec 5000 mass spectrometer equipped with an electrospray ion source, or a PE Sciex API 300 electrospray mass spectrometer. When LC/ESI-MS experiments were performed, a microbore C-18 column (Vydac 1 × 150 mm) was used to separate the samples at gradients suitable for proteins. Molecular weight information on the enzymatic peptides was obtained by LC/ESI-MS using a C-18 microbore column at the gradient described for peptide separation. The HPLC fractions were also analyzed by MALDI-MS on a VG TofSpec SE MALDI mass spectrometer, equipped with a nitrogen laser and operated in either reflectron or linear mode. Peptides were co-crystallized in a matrix consisting of 100 mM α-cyano-4-hydroxycinnamic acid. For sequence information, the peptides from the digestions were analyzed by MALDI/PSD (Matsui et al., 1997) on the VG-TofSpec SE MALDI mass spectrometer.

Acknowledgments

Financial support was furnished by NIH NCRR BRTP grant RR 01614 and Baxter Healthcare Corp.

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