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Research Article

Antibody Epitope and Affinity Determination of the Myocardial Infarction Marker Myoglobin by SPR-Biosensor Mass Spectrometry

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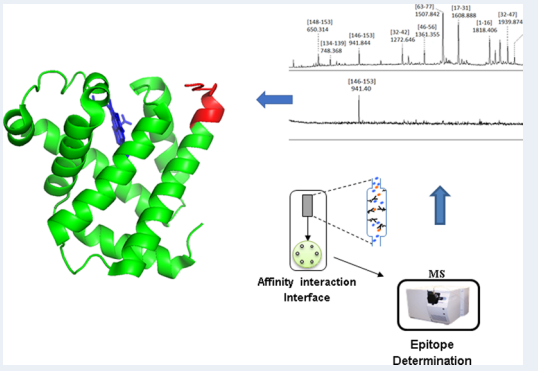
ABSTRACT: Myoglobin (MG) is a biomarker for heart muscle injury, making it a potential target protein for early detection of myocardial infarction. Elevated myoglobin levels alone have low specificity for acute myocardial infarction (AMI) but in combination with cardiac troponin T have been considered highly efficient diagnostic biomarkers. Myoglobin is a monomeric heme protein with a molecular weight of 17 kDa that is found in skeletal and cardiac tissue as an intracellular storage unit of oxygen. MG consists of eight α-helices connected by loops and a heme group responsible for oxygen-binding. Monoclonal antibodies are widely used analytical tools in biomedical research and have been employed for immunoanalytical detection of MG. However, the epitope(s) recognized by MG antibodies have been hitherto unknown. Precise molecular identification of the epitope(s) recognized by antibodies is of key importance for the development of MG as a diagnostic biomarker. The epitope of a monoclonal MG antibody was identified by proteolytic epitope extraction mass spectrometry in combination with surface plasmon resonance (SPR) biosensor analysis. The MG antibody was immobilized both on an affinity microcolumn and a gold SPR chip. The SPR kinetic analysis provided an affinity-binding constant K_D of 270 nM for MG. Binding of a tryptic peptide mixture followed by elution of the epitope from the SPR-MS affinity interface by mild acidification provided a single-epitope peptide located at the C-terminus [146−153] [YKELGFQG] of MG. The specificity and affinity of the epitope were ascertained by synthesis and affinity-mass spectrometric characterization of the epitope peptide.



INTRODUCTION

The oxygen-binding protein myoglobin (MG) is one of the major proteins in skeletal and cardiac muscle. Myoglobin is a cytoplasmic heme protein consisting of a single polypeptide chain of 153 amino acids with a molecular weight of 17.8 kDa. Expressed solely in cardiac myocytes and oxidative skeletal muscle fibers (Figure 1),[1] myoglobin was named according to its functional and structural similarity to hemoglobin. However, unlike hemoglobin, MG reversibly binds oxygen and facilitates oxygen transport from red blood cells to mitochondria during periods of increased metabolic activity, with a single O_2-binding site and a hyperbolic saturation curve. Normal human myoglobin levels in blood range from 0 to 70 ng/mL. Myoglobin was the first protein for which a three-dimensional structure was determined.[2] Its structure has been extensively studied, with 75% of the main chain being folded in an α-helical conformation (Figure 1).

Myoglobin has recently gained increased interest due to its potential role as a biomarker for acute myocardial infarction (AMI), in comparison to previously characterized biomarkers.[3−5] Following cardiac muscle injury, both cardiac and skeletal serum levels of MG are dramatically increased and are also elevated in certain disorders caused by the release of myoglobin from damaged or necrotic muscle cells.[4,5] Due to its small molecular size, myoglobin is able to translocate into the vascular system without processing in the lymphatic system. Acute myocardial infarction (AMI) affects millions of people each year, and rapid, precise, and timely diagnosis is considered of key importance for therapeutic intervention, particularly within the first 6 h after AMI onset.[6] A principal tool for diagnosis of AMI is the development and application of monoclonal MG antibodies that can be employed as specific reagents in immunoassays for determination of MG in serum or plasma of AMI patients. Immunoassays have been employed for diagnosis and quantification of myocardial necrosis and infarction;[7−10] however, the binding epitope(s) of MG antibodies and their affinities have not been previously characterized. For the identification and affinity determination of biomolecular interaction epitopes, efficient methods have

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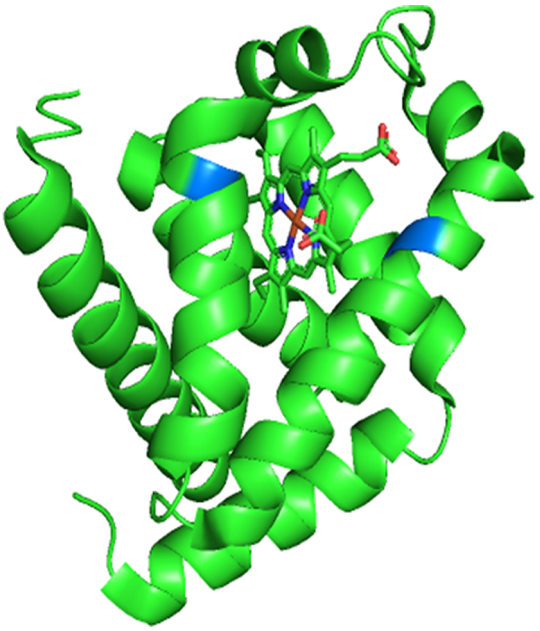

Figure 1. Structure of native human heme-myoglobin, ribbon presentation. Myoglobin consists of a backbone and heme-binding domain.[2] The backbone is composed of eight α-helices (blue) that wrap around a central pocket containing the heme group (red), capable of binding various ligands including oxygen, carbon monoxide, and nitric oxide.

been developed and employed in our laboratory using a combination of proteolytic excision and extraction electrospray (ESI) and MALDI mass spectrometry (MS) as well as surface plasmon resonance (SPR) biosensor analysis within an SPR-MS interface[11−17] (PROTEX-SPR-MS; Supporting Figure S1).

In this study, a monoclonal anti-MG antibody was immobilized on a self-assembled monolayer (SAM)-SPR affinity chip as well as on a Sepharose affinity microcolumn, and both were incubated with a proteolytic digestion mixture of MG. After nonbinding peptides were washed off, the fraction eluted by mild acidification was analyzed by mass spectrometry, and the presence of a single-epitope peptide was revealed. SPR determinations of the MG-antibody complex with intact myoglobin and the epitope peptide ascertained high, specific affinity. These results show that the anti-MG antibody-myoglobin epitope determination is an efficient diagnostic analysis for AMI.

EXPERIMENTAL SECTION

Materials and Samples. Myoglobin, native heme protein from equine heart (M1882), purity ≥ 90% (by SDS-PAGE), essentially salt-free, lyophilized powder was obtained from Sigma-Aldrich (Hamburg, Germany). Equine MG apoprotein, sequencing grade, was obtained from Sigma (München, Germany). The mouse monoclonal anti-MG antibody against apo-myoglobin was obtained from Santa Cruz Biotechnology (Heidelberg, Germany), with cross-reactivity for equine, human, and rat MG.[18] The vial contains 200 μg of IgG1 in 1.0 mL of PBS with <0.1% sodium azide. Other reagents and chemicals were of highest available purity from Sigma-Aldrich (München, Germany) and from Merck (Darmstadt, Germany).

Mass Spectrometric Characterization of Horse Heart Myoglobin. ESI-MS analysis of MG was performed on a Waters TripleQuad Ultima mass spectrometer by direct infusion in the positive ion mode. The ion source parameters employed were as follows: capillary voltage −4500 V, end plate offset −500 V, capillary exit voltage 278.5 V, nebulizer gas flow 12 psi, dry gas flow 9 L min^{-1}, and drying temperature 250 °C. Spectra were recorded in the full scan mode with a scan range of m/z 200−3000. The influence of different solvents on the ionization of MG was tested by preparation of 10 mM solutions of MG with the following solvents: 50% (v/v) acetonitrile, 50% (v/v) H_2O, and 0.1% (v/v) trifluoroacetic acid (TFA) and ammonium acetate buffer (10 mM ammonium acetate, pH 7.0) (Figure S2). Samples were introduced to the ESI source either by direct infusion with a syringe, operated with a pump system at a constant flow rate of 100 μL min^{-1}, or by infusion through the SPR-MS interface. Mass spectra were evaluated using the GPMAW 7.0 software,[19] which provides determination of molecular weights of proteins and peptides with known sequences, average or monoisotopic masses of multiply charged proteins or peptides, as well as masses of fragments.

Proteolytic Digestion. Prior to proteolytic digestion, the heme group of MG was removed in order to obtain a higher digestion yield and better sequence coverage. Removal of the heme group was performed by acidification with 0.1% TFA and passage through an Amicon Filter Ultra-15 (10 K). High pressure proteolytic digestion was performed with a Barocycler 2320EXT instrument (Pressure Biosciences; South Easton, USA) at a temperature of 37 °C and 25 kpsi pressure, using 120 cycles at 50 s/cycle pressure and 10 s off pressure, and a total digestion time of 120 min. A solution of 100 μg (1 mg/mL) of MG in 100 mM ammonium hydrogen carbonate was introduced in a PCT microtube and diluted with the same buffer to a 50 μg/μL final concentration. Keeping a protease-to-substrate ratio of 1:50, a solution of 1 μL of trypsin (0.5 mg/mL) from Sigma GmbH, München and Serva AG, Heidelberg, Germany was added to the protein. As a comparison, the same ratio of sample to enzyme was used for standard digestion (18 h, 37 °C). Tryptic digestion mixtures were characterized by MALDI-TOF-MS, using 2,5-dihydroxybenzoic acid (DHB) as a matrix.

Determination of digestion yields was performed by quantitative mass spectrometric determination of two specific peptide fragments within the digestion mixture. The abundance ratio was determined for the peptide fragment (146−153) (YKELGFQG) (m/z 941) to a synthetic reference peptide (FKELGFQG, m/z 925) spiked to aliquots of the digestion mixture at a concentration of 50 μL/min at 10, 20, 30, 60, and 90 min within the high pressure digestion. This evaluation in comparison to digestion at standard conditions showed that the high pressure proteolytic digestion proved to be most effective. The digestion yield was estimated to be >90% at 120 min, and no intact protein was detectable at this time by gel electrophoresis.

Antibody Immobilization. The antimyoglobin antibody was immobilized on a self-assembled monolayer (SAM) of the activated surface of an SPR chip, using amine coupling as previously described.[16,20] SAM chips were washed three times with alternating water and 70% aqueous ethanol and then immersed for 15 s in piranha solution (H_2SO_4/H_2O_2, 2:1). This step was repeated three times, and the final washing was performed with deionized water and 70% ethanol. The cleaned chip was incubated for 12 h at 20 °C with shaking in a solution of 16-mercaptohexadodecanoic acid in 99% chloroform (1

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mg/mL) and dried under N_2 for 24 h. All reactions were carried out in the microfluidic cell of the SPR biosensor with reagents injected via the autosampler.

The surface of the chip was first washed with 20 mL of PBST buffer (50 mM Na_2HPO_4, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.5). Subsequently, activation of the carboxyl groups of the SAM was obtained by injection of 250 μL of a freshly prepared aqueous solution of 0.2 M EDC and 0.1 M NHS over the SAM chip at a constant flow rate of 25 μL min^{-1}. Coupling of the antibody was carried out with PBST buffer at pH 5.2. A solution of 25 μg of the monoclonal antibody in 10 mM sodium acetate buffer was injected over the chip surface at a flow rate of 25 μL min^{-1}. Unreacted NHS-esters were blocked by injection of 250 μL of ethanolamine solution (1 M, pH 8.5).

SPR-Biosensor Analysis. Affinity determinations of myoglobin and the anti-MG antibody were performed by SPR with the SPR-MS interface, using a dual-channel SR7500DC biosensor (Ametek- Reichert, Buffalo, USA). Determination of affinity-binding constants was performed by real-time analysis with activated gold chips as described above. Binding experiments were carried out at 25 °C with PBST buffer (50 mM Na_2HPO_4, 150 mM NaCl, 0.05% (v/v) Tween, pH 7.5). A 57 mM MG solution was freshly prepared and injected via the autosampler over the chip surface at a constant flow rate of 25 μL min^{-1}. Association and dissociation kinetics were evaluated using Trace Drawer software (Ridgeview Instruments AB, Vange, Sweden).[21] K_D values were calculated from association and dissociation rate constants (k_a and k_d) determined from duplicate affinity measurements at each concentration.

SPR-MS Interface for Epitope Analysis (Figure S1). For validation of the analytical accuracy of the SPR-MS interface, a comparison of (i), direct analysis of MG by direct ESI-MS, and (ii), analysis through the SPR-MS interface, was carried out. The instrumental combination of SPR with ESI-MS comprises valves that enable affinity isolation, mass spectrometric identification, and quantification of affinity-bound ligands from a protein-ligand complex immobilized on the gold chip. The SPR-MS interface provides sample concentration and in situ desalting for direct MS analysis of the ligand eluate.[14,20] Both ESI-ion trap and triple quad-MS systems can be coupled to the SPR-MS interface. Applications of the SPR-MS combination were previously described in studies of a mixed-disulfide antibody epitope of the rheumatoid target protein, HLA-B27, and the epitope identification of chaperone complexes of lysosomal enzymes.[14,17,22]

Epitope Identification by Proteolytic Epitope Extraction-MS Using an Affinity Column. The anti-MG antibody was immobilized on CNBr-activated Sepharose 4B (GE Healthcare Europe, Freiburg, Germany), by suspending the Sepharose (15.1 mg) in 0.3 mL of 1 mM HCl and incubating for 15 min at 20 °C with stirring. Subsequently, the suspension was extensively washed to remove the HCl using 10 mL of coupling solution (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3), which was then discarded. The Sepharose was then resuspended in 500 μL of coupling solution and incubated for 3 h with 10 μg of antibody at 25 °C with vigorous shaking. The matrix was then washed extensively with 10 mL of washing buffer (0.2 M NaOAc, 0.5 M NaCl, pH 4) and 10 mL of blocking solution (0.1 M ethanolamine, 0.5 M NaCl, pH 8.0) for 3 h to remove unreacted cyanogenic. The column matrix was equilibrated by washing with 10 mL of Milli-Q water and used directly for the next step. After preparation, the affinity column was tested with intact MG and the tryptic digestion mixture.

A solution of 20 μg of the tryptic digestion mixture was incubated in the affinity column for 2 h. Subsequently, the first drop (30−40 μL) was collected, and the matrix was washed with 10 mL of deionized water, of which the last elution drop (30−40 μL) was collected. The eluates were analyzed by MALDI-MS, which showed unbound peptides in the first elution drop, and no peptide signal in the final elution. Subsequently, elution from the antibody was performed with 300 μL of 0.1% TFA (500 μL final volume) followed by washing with 10 mL of water and a 1 mL aliquot collected for MS analysis. After concentration of the elution and last washing fraction to 20 μL, the elution fraction was analyzed by MALDI-MS. The final washing was also analyzed for control of the background prior to reuse of the affinity column.

For longer storage, the column was kept in deionized water at 0 °C. Several microcolumns were prepared in the same format.

Synthesis of Epitope Peptides. The epitope peptide (YKELGFQG) and modified epitope peptide sequences were synthesized by solid-phase peptide synthesis (SPPS) on an ABI 433A peptide synthesizer, using a preloaded PS-PBH-Arg-(PMC)-Fmoc resin (Rapp Polymere, Tuebingen, Germany). The following chemicals used in SPPS were obtained from Applied Biosystems (Darmstadt, Germany): *N*,*N*-dimethylformamide (DMF), *N*-methyl-2-pyrrolidone (NMP), dichloromethane (DCM), 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium-hexafluorophosphate (HTBU), methyl *tert*-butyl ether (MTBE), diisopropylethylamine (DIPEA), and TFA. Fmoc-amino acid derivatives were purchased from Nova-Biochem (Darmstadt, Germany), piperidine was purchased from Acros Organics (Fisher Scientific), and tri-isopropylsilane (TIS) was purchased from Alfa Caesar (Landau, Germany). Deprotection of the peptides from the resin was carried out by the addition of 5 mL of 95% TFA and 2.5% TIS to the resin for 3 h at 20 °C. Peptides were then precipitated by addition of the reaction mixture to 20 mL of MTBE, centrifugation, and subsequent decanting. The final purification was performed by HPLC on a semipreparative C18 column, and the homogeneity of peptides was confirmed by MALDI-MS.

RESULTS AND DISCUSSION

Affinity Characterization of Myoglobin by SPR Analysis. The binding affinities for the interaction of MG with the monoclonal antibody against equine apo-myoglobin were determined by SPR analysis. Immobilization of the antibody on the SPR chip surface and injection of a dilution series of the MG heme protein and apoprotein was carried out via the autosampler of the SPRMS interface, individually, as described in the Experimental Section. Binding constants were determined by subtraction of unspecific interactions using the reference channel of the dual-channel SPR detector. The binding association curve for the MG hemeprotein complex is shown in Figure 2. Kinetic evaluation using the Trace Drawer program[21] provided a dissociation constant K_D of 270 ± 14 nM. A comparable binding constant was determined for the MG-apoprotein (350 ± 13.5 nM), thus confirming the high affinity of the protein to the antibody, independent of the native helical structure.

Structural Characterization of Myoglobin by Mass Spectrometric Peptide Mapping. Prior to proteolytic

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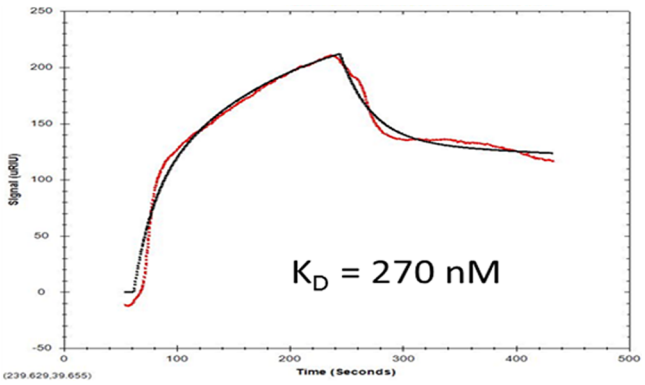

Figure 2. SPR analysis of the affinity interaction between intact native myoglobin and the mouse monoclonal anti-MG antibody (A6, Santa Cruz, Heidelberg, Germany). Following antibody immobilization, the K_D value obtained by direct SPR was comparable to that obtained by analysis in the SPRMS interface (K_D = 270 nM).

digestion, native equine heme-myoglobin was subjected to removal of the heme group under mild acidic conditions as described in the Experimental Section. ESI-MS analysis of the holoprotein (pH 7) and the apoprotein (pH 3) provided a characteristic most abundant $[M + 8H]^{8+}$ ion for the intact heme protein and the multiply charged ion distribution together with the dissociated heme ion for the apoprotein (Figure 3). Likewise, MALDI-MS provided an accurate characterization of the denatured apoprotein (Figure S2) as a basis for the determination of proteolytic fragments. Using the SPR-MS affinity interface, the apo-MG showed a sharp TIC elution signal at approximately 5 min and the multiply protonated molecular ions by ESI-MS, thus confirming the specific affinity-binding to the antibody microcolumn (Figure S3).

Apo-MG was digested with trypsin both at standard conditions at ambient pressure and at high pressure using a high pressure digestion instrument (Barocycler-2320EXT, Pressure Biosciences, Boston, USA). Digestion of MG with trypsin was performed at 37 °C for 18 h and at 30 kpsi for 120 min. Both proteolytic conditions provided complete sequence coverage for MG, albeit with significant differences of digestion yields. High pressure digestion provided a digestion yield >90% as estimated by monitoring the abundance ratio of the C-terminal peptide [146−153] (YKELGFQG) to a synthetic reference peptide (FKELGFQG). In contrast, digestion yields of 70−80% were estimated for ambient pressure digestion, and several miscleaved peptide fragments were observed. Corresponding MALDI-MS peptide mapping analyses at ambient pressure and at high pressure digestion are compared in Figure 4, and the identified tryptic peptides summarized in Table 1. At the high pressure digestion, all tryptic peptide fragments were identified within the MG sequence; in addition, several peptides were found resulting from cleavage at repeat lysine residues (Table 1 and Figure S4).

In contrast, in the tryptic digest mixture at ambient pressure, additional larger tryptic peptide fragments were found that resulted from miscleavages, such as peptides [78−96], [119−147], and [97−133]. In summary, the mass spectrometric peptide mapping provided the characterization of tryptic fragments over the complete sequence of myoglobin and thus were found suitable for unequivocal identification of antibody epitopes by proteolytic epitope extraction-MS.

Identification of the Antibody Epitope of Myoglobin. Identification of the epitope of MG to the monoclonal anti-MG antibody was obtained by proteolytic epitope extraction-MS using the SPR-MS interface in two modes: (i) with the Sepharose-immobilized antibody bound in an affinity microcolumn and (ii) binding of the MG tryptic mixture to the SPR chip containing the immobilized antibody and subsequent elution (Experimental Section). The epitope identification from the affinity microcolumn is shown in Figure 5. Following incubation of a 20 μg aliquot of the tryptic digestion mixture, unbound peptides were washed away, the corresponding supernatant peptides were verified by mass spectrometry, and the final washing fraction did not show any residual peptides (Figure 5a,b). Subsequent elution with 0.1% TFA (pH 3) provided a single peptide (Figure 5c), which was identified as the C-terminal epitope peptide sequence [146−153] (YKELGFQG). This peptide [146−153] was also found in the supernatant fraction (Figure 5a), which is readily explained

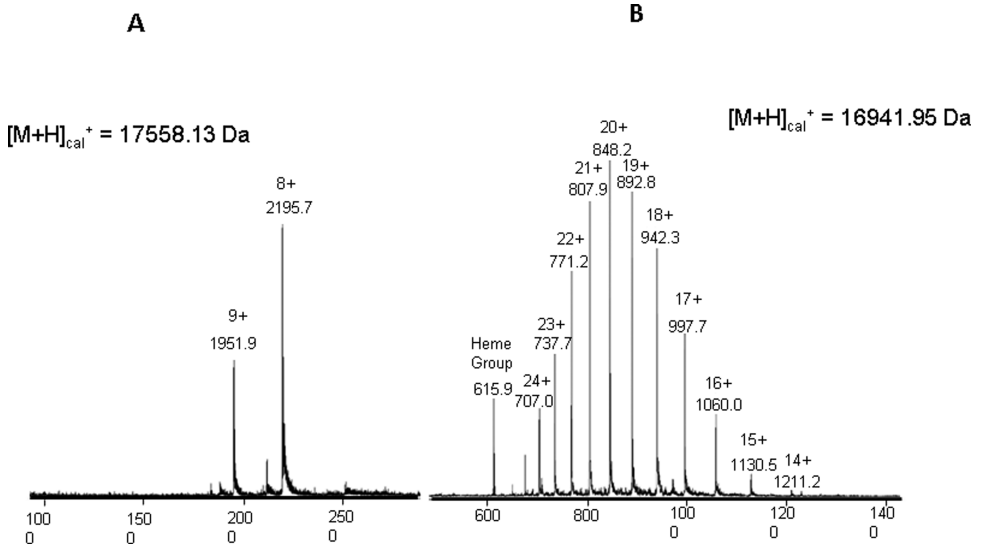

Figure 3. ESI-MS of (A) equine heme-myoglobin under native conditions (pH 7) (molecular mass, 17 557 Da); (B) myoglobin, apoprotein at acidic conditions (pH 2) (molecular mass, 16 941 Da).

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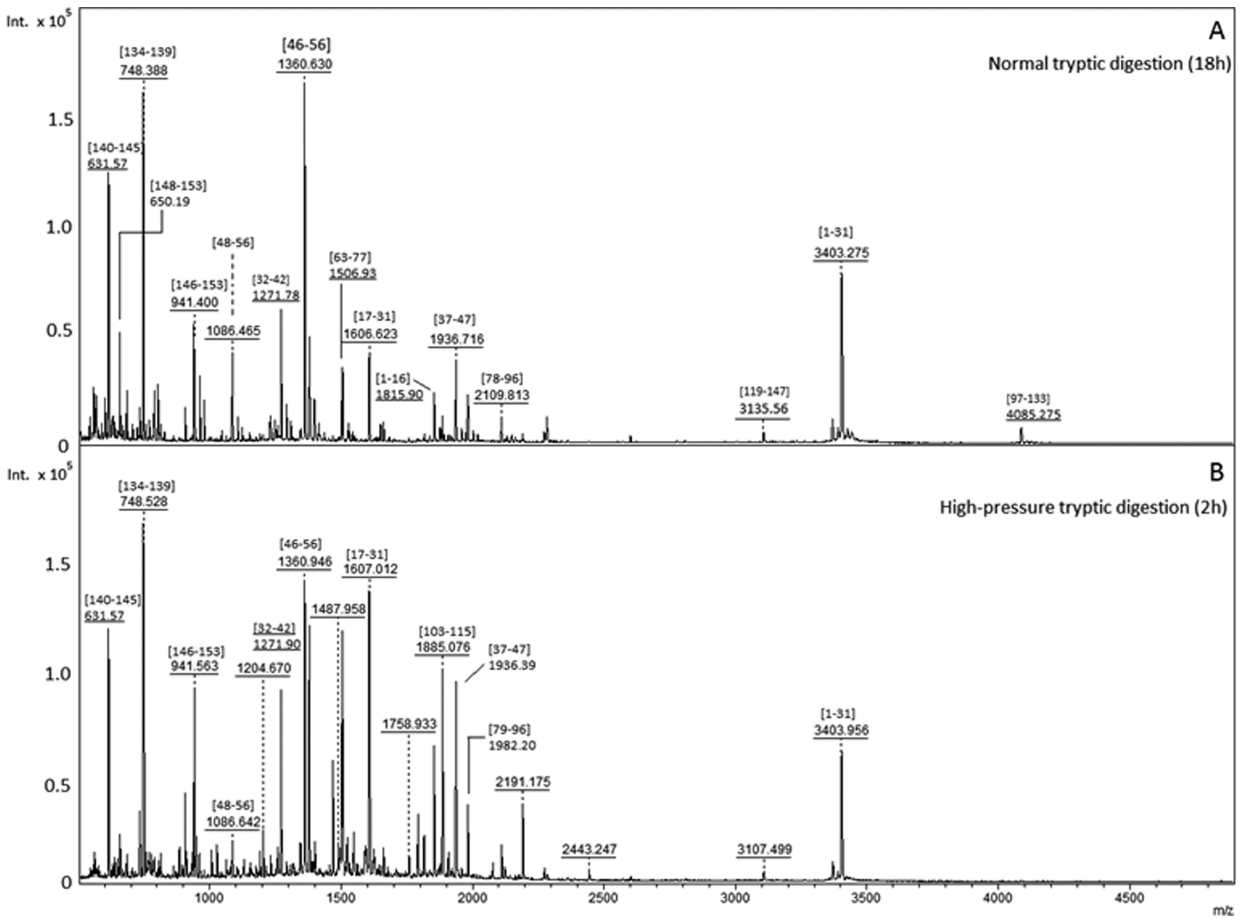

Figure 4. Proteolytic peptide mapping of MG (A) upon digestion at atmospheric pressure (37 °C, 12 h); (B) high pressure digestion using the Barocycler 2320EXT (37 °C, 90 min, 25 kpsi). Both digestions provided a sequence coverage > 92%.

Table 1. Tryptic Peptide Fragments of MG Identified by MALDI-MS Peptide Mapping upon High Pressure Digestion[a]

no.	*m/z*	AA sequence	sequence no.
1	1815.9024	GLSDGEWQQVLNVWGK.v	[1−16]
2	3403.7393	GLSDGEWQQVLNVWGKVEADIAGHGQEVLIR.l[4]	[11−31]
3	1606.8547	k.VEADIAGHGQEVLIR.l	[17−31]
4	1271.6630	r.LFTGHPETLEK.f	[32−42]
5	1661.8533	r.LFTGHPETLEKFDK.f[5]	[32−45]
6	1937.0167	r.LFTGHPETLEKFDKFK.h[6]	[32−47]
7	684.3715	k.FDKFK.h[1]	[43−47]
8	1086.5612	k.HLKTEAEMK.a	[48−56]
9	1857.9739	k.HLKTEAEMKASEDLKK.h[7]	[48−63]
10	3217.7977	k.HLKTEAEMKASEDLKKHGTVVLTALGGILK.k	[48−77]
11	790.4305	k.ASEDLKK.h	[57−63]
12	1378.8417	k.HGTVVLTALGGILK.k	[64−77]
13	1506.9366	k.HGTVVLTALGGILKK.k	[64−78]
14	2110.1516	k.KKGHHEAELKPLAQSHATK.h	[78−96]
15	1853.9617	k.GHHEAELKPLAQSHATK.h	[80−96]
16	735.4876	k.HKIPIK.y[2]	[97−102]
17	1885.0218	k.YLEFISDAIIHVLHSK.h	[103−118]
18	1502.6693	k.HPGDFGADAQGAMTK.a	[119−133]
19	748.4352	k.ALELFR.n	[134−139]
20	1360.7583	k.ALELFRNDIAAK.y	[134−145]
21	941.4727	k.YKELGFQG.[3]	[146−153]

[a]See Experimental Section.

by the nonstoichiometric ratio of the MG digest-to-antibody containing a molar excess of the MG digestion mixture. The background from the final washing following the elution was also analyzed and did not show remaining peptides (Figure

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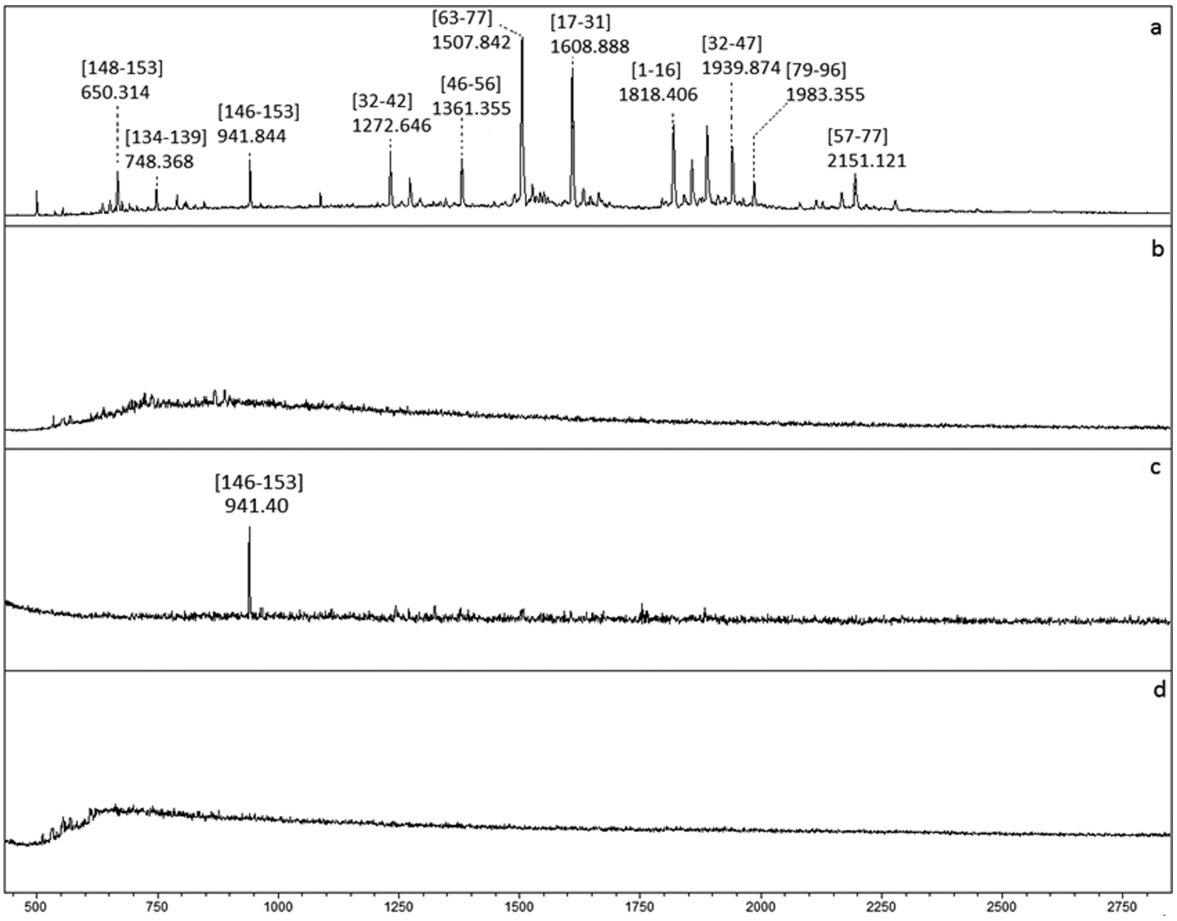

Figure 5. Identification of the apo-MG epitope by proteolytic extraction and MALDI- MS from the SPR-MS interface containing the immobilized anti-MG antibody. (a) Supernatant peptides from the antibody affinity column; (b) background spectrum before elution; (c) identification of epitope peptide, [146−153] (m/z 941.4), upon elution with 0.1% TFA; (d) background spectrum of last washing fraction after elution.

5d). In contrast to trypsin digestion, an epitope extraction-MS experiment using α-chymotrypsin as a protease did not provide a detectable epitope, due to the cleavage of MG at Y146 and F151 that cleaves the C-terminal epitope peptide sequence (data not shown).

Epitope analysis by SPR-MS upon binding and elution of the tryptic digestion mixture from the SPR chip containing the immobilized antibody provided the identical epitope peptide, [146−153] (Figure S5). The specificity of the epitope was ascertained by synthesis and affinity-MS characterization of the C-terminal peptide. In an affinity-MS experiment of a mixture of the C-terminal peptide (146−153) and the chymotryptic peptide (147−150), only the epitope peptide was retained, while the peptide (147−150) was found in the flowthrough.

CONCLUSIONS

In this study, the molecular epitope structure and affinity of equine heme-myoglobin and apo-myoglobin to a monoclonal antibody were determined using proteolytic epitope extraction mass spectrometry in combination with surface plasmon resonance (SPR) biosensor analysis. The sequence of the identified C-terminal epitope peptide is highlighted in Figure 6 in the structure of myoglobin. The epitope is shown to comprise the C-terminal sequence at the end of the final eighth α-helical loop of MG. The identified epitope, YKELGFQG, comprises the uncleaved residue lysine-146, suggesting the importance of the N-terminal sequence of the epitope for

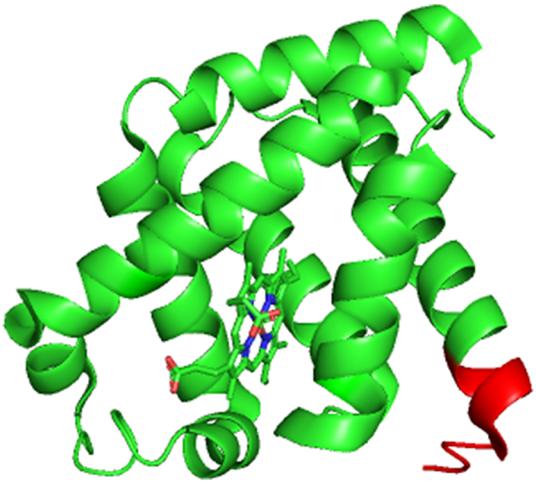

Figure 6. Structure of myoglobin with the C-terminal epitope peptide [146−153] highlighted in red.

affinity-binding. The detailed affinity determination of the C-terminal epitope by SPR is carried out at present.

The C-terminal epitope sequence is also in agreement with the binding affinity of the antibody to the denatured MG apoprotein. Moreover, the epitope is consistent with the cross-reactivity of the antibody for equine myoglobin with human MG and several other myoglobin proteins, described in

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previous publications.[6,18] These results confirm the efficiency of the molecular epitope characterization by proteolytic affinity-MS and the use of MG as a suitable model system in the development of the SPR-MS combination.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jasms.0c00234.

Figure S1: Schematic representation of the SPR-MS interface. (1) Autosampler/sample injector. (2) Interface containing microaffinity column or microchip with immobilized antibody. (3) SPR and SPR chip. SPR analysis and affinity-binding of immobilized antibody are performed at the interface and operation of the multiport valves. (4,5) Multiport valves for sample transfer and desalting/buffer change are located before affinity column and after elution before transfer to ESI-MS or SPR chip. (6) ESI-MS insertion port. Figure S2: MALDI-MS of (A) apo-myoglobin, (B) ESI-MS of apo-MG (pH 2; for details, see Experimental Section). Average molecular mass determined by MALDI-MS: 16 952.6 Da; monoisotopic mass determined by ESI-MS: 16 942.5 Da. Figure S3: Total ion chromatogram (A) and ESI-MS (B) of apomyoglobin upon affinity separation and elution in the SPR-MS interface. The TIC showed a sharp elution signal around 5.3 min. The multiply protonated molecular ions provide a molecular mass of 16.942 Da. Figure S4: Amino acid sequences of (A) horse heart myoglobin (PDB 1WLA), (B) human heart myoglobin (PDB 3RGK). Lysine and arginine residues are highlighted in red. Single mutation sites in human myoglobin are highlighted in blue. Figure S5: Identification of the apo-MG epitope [146−153] (m/z 941.4) by proteolytic extraction-ESI-MS upon elution from the SPR chip containing the immobilized anti-MG antibody (PDF)

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Notes

The authors declare no competing financial interest.

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