Mass Spectrometric Mapping of Protein Epitope Structures of Myocardial Infarct Markers Myoglobin and Troponin T[†]

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ABSTRACT: Monoclonal antibodies are widely used analytical tools in biochemical research. The knowledge of their corresponding epitopes is of major interest. One possible approach for epitope characterization is the application of protein antigen proteolysis in combination with mass spectrometric peptide mapping analysis. Two complementary analytical strategies were applied: (a) limited proteolysis of antibody-bound antigen followed by removal of nonbound peptides and detachment of the antigenic peptides (epitope excision) and (b) enzymatic digest of the antigen followed by extraction of the antigenic peptides with the antibody and detachment of antigenic peptides after removal of nonbinding fragments (epitope extraction). In the few examples published so far, immobilized antibodies were used for these studies. In this study we present a method for characterization of the epitope sequences without prior immobilization of the monoclonal antibody. The separation of nonepitope peptides from antibody-bound peptides was carried out by ultrafiltration. The epitope and nonepitope fractions were analyzed by MALDI-MS without further purification, and the epitope sequences were identified. The method was developed using a model system consisting of the synthetic C-terminal cyanogen bromide fragment CB3 of myoglobin and the commercial monoclonal anti-myoglobin MG1. In further investigations the epitope sequence of a synthetic 32 amino acid peptide derived from heart muscle protein troponin T toward a monoclonal antibody MAb-M7, which was raised against the intact protein, was characterized. With this approach the epitope binding site of this antibody was determined, and selective shielding of potential cleavage sites in the immune complex could be observed. Furthermore, statements about the three-dimensional structure of the bound antigen were made.

Monoclonal antibodies are extremely selective and sensitive tools in biochemistry and molecular biology. Structures recognized by antibodies are called epitopes. Protein epitopes are generally divided in continuous amino acid sequences (linear epitopes) and epitopes resulting from the structural environment of several amino acids (conformational epitopes) (Harlow & Lane, 1988; Jin et al., 1992; Atassi & Smith, 1978). Linear epitopes are typically composed of 5−10 amino acids (Laver et al., 1990). Conformational epitopes originate from the folding of a protein that can spatially assemble a considerably larger number of amino acid residues, which are distant in the sequence. The exact knowledge of epitope structures is important for identifying cross-reactivities and for the study of structure−function relationships.

To date, only a few methods for determination of antibody binding sites have been developed. The classical methods use synthetic or recombinant peptides, which cover the sequence of interest, for binding assays (Geysen et al., 1984; Chen et al., 1993; Devlin et al., 1990). Furthermore, some examples of X-ray crystallographic determination of epitope structures and antibody binding sites are known (Amit et al., 1986; Colman et al., 1987; Sheriff et al., 1987). These methods are very sensitive but frequently time consuming, of limited specificity, and usually (with the exception of the X-ray crystallography) limited to linear epitopes. In previous work we have developed a different approach (Suckau et al., 1990) which combines proteolytic degradation of a protein or peptide whereas its epitope region is protected from proteolytic degradation by the bound antibody, with mass spectrometric peptide mapping analysis. Antibodies typically show a high resistance against proteolytic enzymes, thus protecting the epitope from proteolytic degradation (Moelling et al., 1980; Schwyzer et al., 1980). While this method of epitope identification was developed using plasma desorption mass spectrometry (PD-MS), similar studies have been subsequently reported using matrix-assisted laser desorption/ionization (MALDI-MS)[1] (Papac et al., 1994; Zhao & Chait, 1994; Krone et al., 1995; Parker et al., 1996), which provides higher sensitivity than PD-MS (Karas et al., 1991). One disadvantage of this previous method is the necessity

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[1] Abbreviations: MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; bc-TnT, bovine heart muscle troponin T; IgG, immunoglobulin G; MAb-M7, monoclonal anti-troponin T antibody M7; MAb-MG1, monoclonal anti-myoglobin antibody MG1; TPCK-trypsin, tosylphenylalanyl chloromethyl ketone treated trypsin; MWCO, molecular weight cutoff; PBS, phosphate-buffered saline; HCCA, 4-hydroxy-α-cyanocinnamic acid, TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; DFP, diisopropyl fluorophosphate; Fmoc, fluorenylmethyloxycarbonyl; DMSO, dimethyl sulfoxide; MAb, murine monoclonal antibody.

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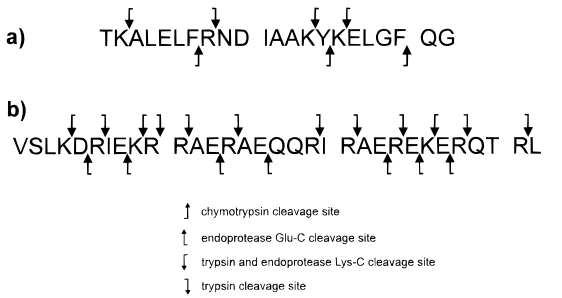

FIGURE 1: Sequences of the synthetic peptides myoglobin−CB3 (a) and bc-TnT(118−149) (b). Possible proteolytic cleavage sites are marked by arrows.

of using an immobilized antibody which is difficult to reproduce and generates a complex analytical system. In the present study we describe a method for rapid, unequivocal investigation of antibody binding sites which avoids an immobilized antibody as matrix for proteolytic degradation and uses "native" monoclonal antibodies as molecular shields.

The monoclonal antibodies investigated here have been designed for diagnostic applications of myocardial infarct (Katus et al., 1992; Wu et al., 1994; Block et al., 1983; Roxin et al., 1983). Therefore, the knowledge of epitope structures is of major interest to avoid cross-reactivities and hence possible misinterpretation of diagnostic data. The first antigen−antibody system studied consists of heart muscle myoglobin and a commercially available anti-myoglobin monoclonal antibody (MG1), which is used in myocardial infarct diagnostics. In previous investigations its epitope-containing sequence could be restricted to the C-terminal cyanogen bromide fragment CB3 (Fiedler et al., 1995) consisting of 22 amino acids. The amino acid sequence of CB3 is presented in Figure 1a. The use of a myoglobin peptide fragment has the advantage of better proteolytic cleavage since native myoglobin is not cleaved by trypsin or by α-chymotrypsin even at high enzyme:substrate ratios. As shown by Parker et al., it is well feasible to use the mass spectrometric epitope mapping strategy on intact proteins.

A further application is the epitope determination of bovine heart muscle troponin T (bc-TnT) with a anti-troponin T murine monoclonal antibody (MAb-M7) which was raised against the homologue human heart muscle protein (hc-TnT). It was found that this antibody recognizes not only hc-TnT but also bc-TnT but does not recognize the human skeletal TnT and is therefore suitable for immunological differentiation of heart and skeletal protein. The epitope-containing sequence of heart muscle troponin T could be previously restricted to the partial sequence (118−149) of the bovine protein (Przybylski, 1995). This sequence differs from hc-TnT only by residues A^{139} and T^{147}, instead of two Asn residues in the human sequence (Leszyk et al., 1987; Mesnard et al., 1993). The sequence also contains a number of proteolytic cleavage sites (Figure 1b), making it a suitable system for studying the accuracy of epitope mapping by limited proteolysis. Furthermore, the information obtained by mass spectrometric epitope mapping of bc-TnT was combined with circular dichroism spectroscopy (CD) data, which enabled the development of a three-dimensional model of the antigen epitope region.

MATERIALS AND METHODS

All chemicals used were of analytical grade or otherwise of the highest commercially available purity. TPCK-treated trypsin was purchased from Sigma (St. Louis, MO). Endoproteinase Glu-C and endoproteinase Lys-C (sequencing grade) were from Boehringer Mannheim (Mannheim, FRG). α-Chymotrypsin was from Sigma (St. Louis, MO). Trifluoroacetic acid was from Merck (Darmstadt, FRG). Diisopropyl fluorophosphate was purchased from Aldrich (Milwaukee, WI). Fmoc- and side-chain-protected amino acid derivatives as well as PyBOP were purchased from NovaBiochem (Bad Soden, FRG). Purified IgG of anti-myoglobin antibody MAb-MG1 was purchased from ICN (Costa Mesa, CA). Purified IgG of anti-TnT-antibody MAb-M7 was a gift from Boehringer Mannheim (Penzberg, FRG).

Peptide Synthesis. Synthesis of the bc-TnT(118−149) and myoglobin−CB3 fragment peptides was carried out with an Abimed EPS 221 peptide synthesizer using a standard Fmoc strategy with a NovaSyn TGA resin and PyBOP activation (Hudson, 1988). The peptide was cleaved from the resin by treatment with 5 mL of TFA−H_2O−triethylsilane (90:5:5) for 2 h. After addition of 90 mL of *tert*-butyl methyl ether the crude peptides were precipitated overnight at −23 °C. The solution was centrifuged at 4000*g* for 30 min, the supernatant discarded and the peptide-containing pellet dried. The precipitated peptide was then purified by semipreparative RP-C_{18} HPLC on a Waters μ-Bondapak column (Eschborn, FRG) using a gradient of 0.1% TFA in water and 0.07% TFA in acetonitrile in the case of the bc-TnT(118−149) and 0.04% TFA in water and 0.03% TFA in acetonitrile for myoglobin−CB3. Sequences and homogeneity of the peptides were confirmed by mass spectrometry.

CD Spectroscopy. CD spectra were recorded on a JASCO J-500C CD spectropolarimeter using a 100 μM solution of bc-TnT(118−149) in 50 mM sodium phosphate (pH 7.3). A solvent spectrum was subtracted from the original spectra. The scan range was from 190 to 250 nm, and the scan speed was 1 nm/min.

Epitope Excision and Extraction. All enzymatic digests were performed using an enzyme to substrate ratio (E:S) of 1:50 in PBS buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 6.8) at 37 °C at an antigen concentration of 0.05 μg/μL. The E:S ratio was calculated on the basis of the antigen peptide.

For epitope excision 10 μg (5 μg/mL) of antigen and 200 μg (1 μg/μL) of IgG (which corresponds to a molar antigen antibody ratio of 2:1) were mixed and allowed to bind for 15 min. Then 0.2 μg (0.1 μg/μL) of proteolytic enzyme was added. Proteolysis was carried out for 60 min at 37 °C (except for trypsin where degradation was performed for 30 min). Reactions were stopped by addition of 5 μL of a 10% diisopropyl fluorophosphate solution in DMSO (due to the high toxicity of DFP this procedure should be carried out in a well-ventilated hood). Then 200 μL of PBS buffer was added to the mixture. The digest mixture was then filtered through Amicon Microcon 30000 microconcentrators within 20 min at 5000*g*. The retentate was washed with 350 μL of PBS buffer and again centrifuged for 20 min. The immune complex in the retentate was then dissociated by addition of 400 μL of 0.1% TFA. After incubation for 15 min a third

centrifugation step was performed. The retentate was washed with 350 μL of 0.1% TFA.

For epitope extraction 10 μg (5 μg/μL) of antigen and 0.2 μg (0.1 μg/μL) of proteolytic enzyme were mixed and stored at 37 °C for 60 min (except for trypsin, see above). Digests were stopped by addition of 5 μL of a solution of 10% diisopropyl fluorophosphate in absolute DMSO. Then 200 μg (1 μg/μL) of IgG (which corresponds to a molar antigen antibody ratio of 2:1) was added and allowed to form the immune complex for 15 min. The digest mixture was then filtered through Amicon Microcon 30000 microconcentrators within 20 min at 5000*g*. The retentate was washed with 350 μL of PBS buffer and again centrifuged for 20 min. The immune complex in the retentate was then dissociated by adding 400 μL of 0.1% TFA. After incubation for 15 min a third centrifugation step was performed. The retentate was washed once with 350 μL of 0.1% TFA.

Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS). For MALDI-MS analysis all samples were lyophilized and dissolved in 50 μL of 0.1% TFA in an ultrasonic bath for 15 min. MALDI-MS was carried out on a Bruker Biflex linear TOF mass spectrometer equipped with a SCOUT source and video system, a nitrogen UV laser (λ_{max} = 337 nm), and a dual channel plate detector. One microliter of a freshly prepared saturated solution of HCCA in acetonitrile−H_2O (40:60) with 0.1% TFA was placed on the target, and 1 μL of sample solution was added. The drop was then allowed to dry at room temperature. Spectra were recorded at an acceleration voltage of 25 kV using a deflection pulse of 1 μs duration for deflecting matrix ion (2 kV perpendicular to the ion flight path). Sixty to 300 single laser shots were added into an accumulated resultant spectrum. Calibration was carried out using the singly and doubly protonated ion signals of bovine insulin as internal standard.

RESULTS

Analytical Features of Epitope Extraction and Excision. For epitope identification, the antigen is degraded by a specific enzyme. The corresponding antibody was added to the proteolytic mixture and allowed to bind to the antigenic peptides. Nonbinding peptides were removed by ultrafiltration; the filtrate contained the nonepitope peptides and is therefore called the nonepitope fraction. The remaining immune complex in the retentate was dissociated by addition of diluted acid, and the mixture was again subjected to ultrafiltration. The filtrate contained the antigenic peptides and is therefore called the epitope fraction.

Epitope excision analysis was carried out in a similar manner. The immune complex was formed by incubation of antigen with antibody, and the complex was subjected to limited proteolysis followed by ultrafiltration. The filtrate contained the nonepitope peptides (nonepitope fraction). Epitope peptides were isolated by acid dissociation of the truncated immune complex and ultrafiltration; the retentate contained the epitope peptides (epitope fraction). Control experiments were carried out in an analogous manner but without addition of antibody. All fractions were analyzed by MALDI-MS without further purification.

Epitope Extraction and Excision of Myoglobin−CB3. In the present study, myoglobin from horse heart was chosen as a model system with the aim of developing a simplified

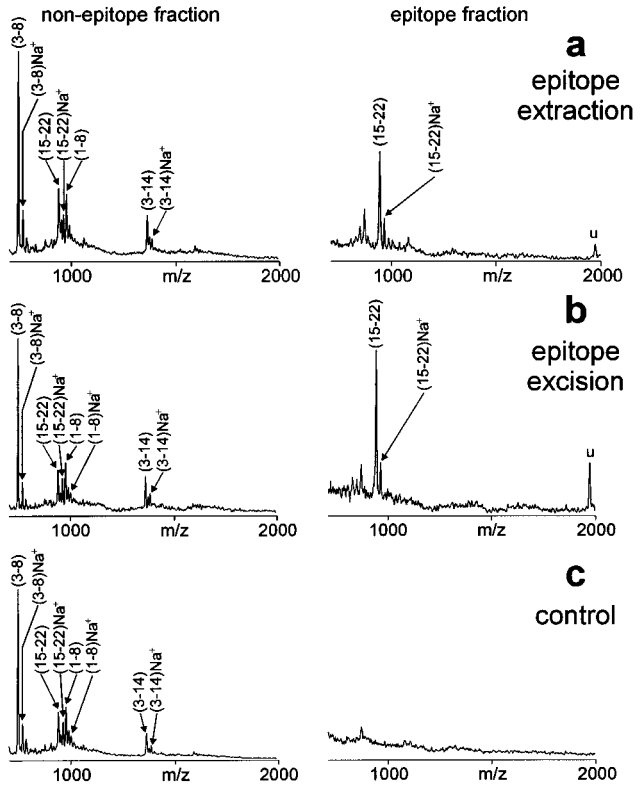

FIGURE 2: Epitope extraction/excision experiments and MALDI mass spectra of nonepitope and epitope fractions upon tryptic digestion of myoglobin−CB3: (a) digest followed by addition of antibody (epitope extraction), (b) digest in the presence of the antibody (epitope excision), and (c) control digest without antibody.

method for identification of epitope sequences. A commercially available anti-myoglobin antibody MAb-MG1 designed for myocardial infarct diagnostics was used. Cross-reactivity of this antibody with horse heart myoglobin has been previously observed (Fiedler et al., 1995). Also, previous investigations using proteolytic cleavage of myoglobin with cyanogen bromide and subsequent Western blot analysis of the resulting fragments showed the location of the epitope sequence in the C-terminal BrCN-cleavage fragment CB3, which consists of 22 amino acids. This peptide was synthesized by solid-phase peptide synthesis (SPPS) and employed as an antigen. Figure 1a shows the amino acid sequence of myoglobin−CB3 and its potential cleavage sites with different proteolytic enzymes. In this study, myoglobin-CB3 was cleaved by trypsin and α-chymotrypsin.

The epitope extraction experiment with tryptic digestion is shown in Figure 2a. The spectrum of the nonepitope fraction showed the protonated molecular ions $[M + H]^+$ of the partial peptides 1−8, 3−8, 3−14, and 15−22, as well as ions of their corresponding sodium adducts. These peptides cover the complete sequence of myoglobin−CB3. The spectrum of the epitope fraction showed the $[M + H]^+$ ion of peptide 15−22 as well as its $[M + Na]^+$ ion. This identification was confirmed by applying the epitope excision method, which yielded nearly identical spectra (Figure 2b). The mass spectra clearly showed the location of the epitope within the sequence 15−22.

Corresponding spectra of the supernatant, nonepitope fraction also showed protonated molecular ions for partial peptides 1−8, 3−8, 3−14, and 15−22. No significant differences of relative ion intensities to the epitope extraction

Table 1: Mass Spectrometric Identification of Myoglobin Epitopes Obtained from Epitope Extraction and Excision Experiments

	epitope extraction			epitope excision		
protease	fragments	MW_{exp}	MW_{calc}	fragments	MW_{exp}	MW_{calc}
trypsin	15−22	942	942	15−22	943	942
				15−22, Na^+	965	965
chymotrypsin	no signals observed			8−22	1710	1711

and excision were observed. This result was surprising since in the epitope extraction experiment a significant decrease of signal intensities for the epitope peptides should be expected at the antigen:antibody ratio employed. However, a substantial relative reduction of the epitope ion intensity was found by experiments employing an excess of antibody (data not shown), suggesting an avidity of the immune complex below the theoretical 2:1 stoichiometry.

Corresponding experiments were performed using α-chymotrypsin as protease. In the nonepitope fraction of the control experiment, peptide fragments 1−7, 8−15, 16−20, and 16−22 were identified. The spectra of the corresponding epitope fraction showed no ion signals due to a specific cleavage of myoglobin−CB3. The epitope fraction in the excision experiment contained peptide fragment 8−22, thus showing effective protection of cleavage sites Y^{15} and F^{20} by the antibody (Table 1). In the extraction experiment, the epitope fraction contained no peptide fragment. This result is well explained by the destruction of the epitope upon cleavage at Y^{15}, which therefore represents an essential residue of the epitope sequence. The molecular ion signals found after tryptic and α-chymotryptic digest are summarized in Table 1. Consistent with the above results, the epitope is assigned to the partial sequence 15−22.

Epitope Identification of a Bovine TnT Polypeptide. For detailed characterization of the MAb-M7 epitope, the bc-TnT polypeptide 118−149 was synthesized. Epitope-extraction and -excision experiments were carried out with trypsin, endoproteinase Lys-C, and endoproteinase Glu-C, while α-chymotrypsin was not employed because of the lack of specific cleavage sites in the partial TnT sequence 118−149 (Figure 1b).

Figure 3 shows the results of the epitope-extraction and -excision experiments using trypsin as proteolytic enzyme. In the epitope-extraction experiment peptide fragments 1−10, 1−12, 11−19, 20−28, 22−31, and 23−32 were detected in the nonepitope fraction, while only the peptide 1−10 was

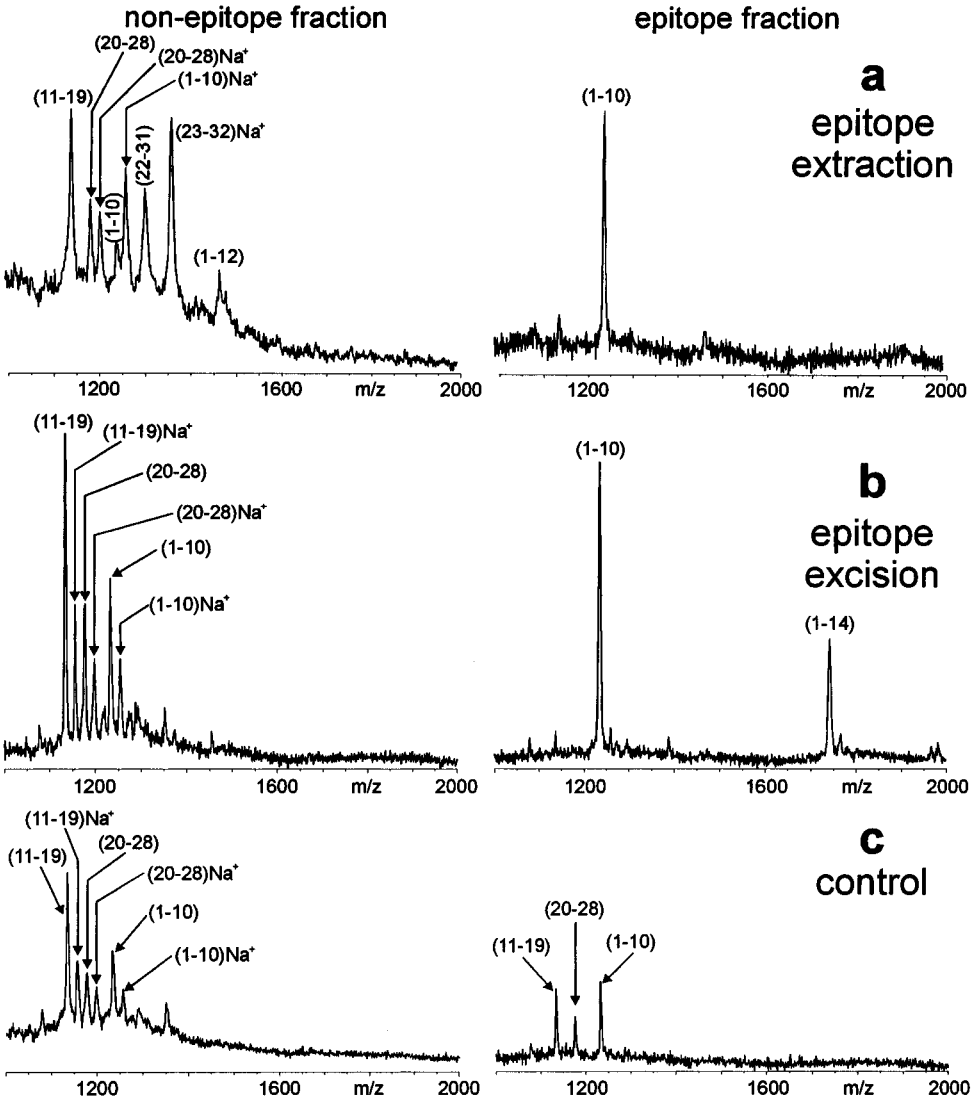

FIGURE 3: Epitope extraction/excision experiment with bc-TnT(118−149). Trypsin was used for proteolytic degradation of (a) antigen with antibody added after proteolytic digest (epitope extraction), (b) antigen with antibody added prior to digest (epitope excision), and (c) antigen without antibody (control).

Table 2: Epitope Sequences of bc-TnT(118−149) Found in Extraction and Excision Experiments

	epitope extraction			epitope excision		
protease	fragment	MW_{exp}	MW_{calc}	fragment	MW_{exp}	MW_{calc}
trypsin	1−10	1244	1244	1−10	1244	1244
				1−14	1756	1757
V8 protease	1−13	1602	1601	1−16	1959	1957
	1−16	1959	1957			
LysC protease	no signals observed			1−26	3252	3252
				unknown	3480	

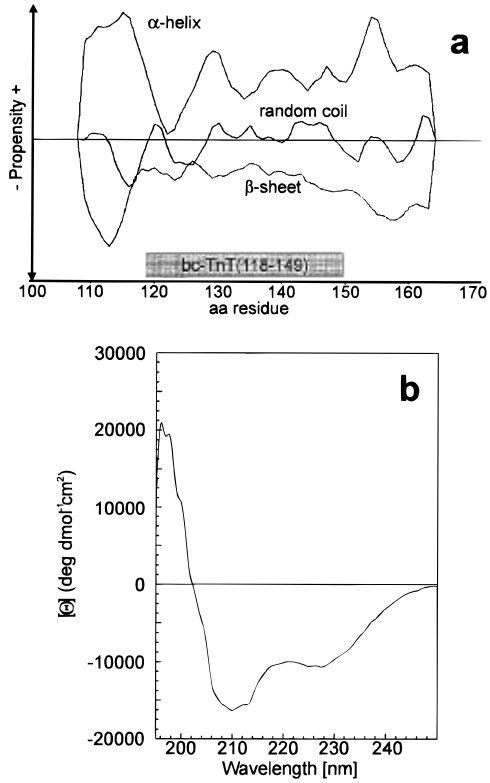

FIGURE 4: (a) Secondary structure prediction of bc-TnT according to Garnier (1990). The graph shows the region from amino acid 100 to 170. The synthesized sequence bc-TnT(118−149) is indicated by the gray bar. (b) CD spectra of bc-TnT(118−149). The spectrum was recorded at pH 7.3 in phosphate buffer.

identified in the epitope fraction (Figure 3a). In the epitope-excision experiment, very similar results were obtained for the nonepitope fraction; the epitope fraction contained exclusively fragments 1−10 and 1−14 upon dissociation from the antibody (Figure 3b). In the control experiment (Figure 3c) without addition of antibody $[M + H]^+$ ion signals of peptides 1−10, 11−19, and 20−28 as well as their sodium adducts were observed in the spectra of the supernatant fraction. In the spectra of the epitope fraction molecular ion signals due to three fragments, 1−10, 11−19, and 20−28, were also observed with low intensity. The occurrence of these peptides is explained by unspecific adsorption (Suckau et al., 1990).

Further studies with additional proteases provided complementary data to ascertain the epitope structure and its essential residues. Digestion with endoproteinase Glu-C provided peptide fragment 1−16 as the antigenic peptide by epitope extraction, as well as in the epitope-excision experiment. In addition, in the epitope-extraction experiment peptide 1−13 was found in the epitope fraction. The Lys-C digest revealed that residues K^4 and K^9 were not cleaved in

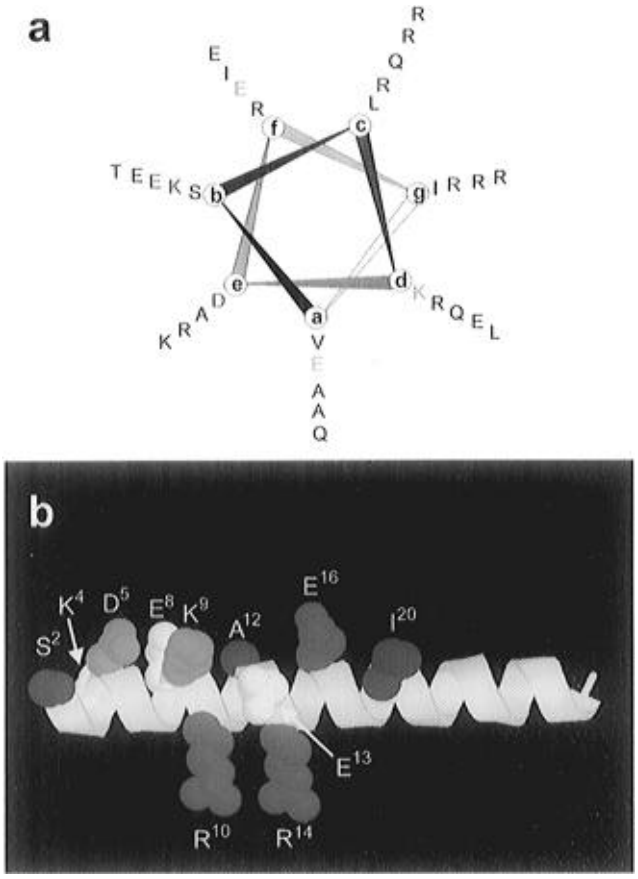

FIGURE 5: (a) Sequence of bc-TnT(118−149) in the helical wheel representation. Mutations from cardiac to skeletal TnT are marked in blue and green, respectively. Four mutation sites are located in the N-terminal part of the peptide which represents the epitope sequence. Cleavage sites protected from degradation in the immune complex are colored in yellow and green; those which are cleaved while the peptide is bound to the antibody are indicated in red. (b) Structure model based on CD measurements, secondary structure prediction, and the observed protected and nonprotected cleavage sites. The color scheme is identical to that in panel a.

the presence of the antibody but were completely cleaved within 15 min in the control experiment. Therefore, peptide 1−26 was found by the epitope excision in the epitope fraction. Epitope extraction with Lys-C yielded no molecular ion signals in the epitope fraction, most likely because peptides 1−4 and 5−9 are too small for binding to the antibody (Table 2). With these results the epitope sequence could be restricted to the first ten N-terminal amino acid residues of the bc-TnT(118−149) polypeptide. Furthermore, residues K^4, K^9, and E^{13} are effectively shielded in the immune complex against proteolytic cleavage. In contrast, amino acid residues R^{10} and R^{14} are accessible to proteolysis in the immune complex.

Secondary Structure Characterization of bc-TnT. Secondary structure prediction indicates a high α-helical content of the region 118−149 in the intact troponin T protein (Figure 4a) (Garnier, 1990). The helical content of the intact cardial TnT protein was estimated to be 57% (data not shown). In bovine heart muscle TnT a segment of high α-helicity (108−172), which binds to tropomyosin, could be identified (Pearlstone & Smillie, 1977). CD spectra show an α-helical amount of approximately 60% for the synthetic polypeptide, which correlates well with secondary structure predictions for the isolated peptide sequence 118−149 in bc-TnT (Figure 4b).

Table 3: Sequence Comparison of Bovine Heart Muscle (bc), Human Heart Muscle (hc), and Human Skeletal Muscle (hsk) Troponin T in the Region of Amino Acid Residues 118−149 (with Respect to bc-TnT)[a]

sequence position	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
helix position	a	b	c	d	e	f	g	a	b	c	d	e	f	g	a	b	c	d	e	f	g	a	b	c	d	e	f	g	a	b	c	d
bc-TnT	V[118]	S	L	K	D	R	I	E	K	R	R	A	E	R	A	E	Q	Q	R	I	R	A	E	R	E	K	E	R	Q	T	R	L[149]
hc-TnT	V[121]	S	L	K	D	R	I	E	K	R	R	A	E	R	A	E	Q	Q	R	I	R	**N**	E	R	E	K	E	R	Q	**N**	R	L[152]
hsk-TnT	V[86]	**A**	L	K	**E**	R	I	E	**R**	R	R	**S**	E	R	A	E	Q	Q	R	**F**	R	**T**	E	**K**	E	**R**	E	R	Q	**A**	**K**	L[117]

[a] Determined epitope sequence is underlined, and the mutation sites are typed in bold letters.

DISCUSSION

The mass spectrometric method for protein epitope mapping described in this study is characterized by the direct use of an antibody without the need for prior immobilization, which makes the analytical system as simple as possible and provides a significant reduction in analysis time compared to an immobilization procedure. Furthermore, additional purification steps are not required with the present procedure; nevertheless, HPLC purification can be performed for quantification and separation of epitope peptides.

In the previously published studies on epitope mapping with mass spectrometry (Suckau et al., 1990; Papac et al., 1994; Zhao & Chait, 1994; Krone et al., 1995), only identifications of sequences of epitopes binding to monoclonal antibodies were obtained. In addition to the epitope properties, this can be explained by the limited size of peptides previously used for mass spectrometric analysis with sequences of approximately 20 amino acids, in most cases. In this study a 22-residue myoglobin fragment and a considerably larger 32-residue polypeptide of bc-TnT were investigated. Since a defined conformation is only developing in peptides larger than approximately 20−25 amino acids, this would well explain the lack of secondary structure information in studies with smaller peptides. This is supported by the recent report of epitope mapping on an intact protein, HIV-1 p26, using immobilized antibodies, which suggested the presence of a conformational epitope (Parker et al., 1996).

On the basis of the circular dichroism measurements and secondary structure prediction an structure model with an α-helical conformation was generated for the bc-TnT epitope peptide in this study (Hyperchem Release 3 for Windows using an AMBER force field; Weiner et al., 1984). This structure model is consistent with the data obtained by epitope excision experiments, showing that amino acid residues K^4, D^5, E^8, K^9, and E^{13} all are inaccessible to proteolytic enzymes in the immune complex, suggesting their effective shielding by the antibody. These residues are all located on one side of the α-helix as shown by the helical wheel representation, where all residues are on the left-hand side (Figure 5a, positions e, b, and f). In contrast, amino acid residues R^{10} and R^{14}, which are recognized and cleaved by the serine proteases, are located at the opposite side of the helix, indicating that this side of the α-helical sequence is not shielded by the antibody (helix positions a, c, d, and g). Furthermore, the cleavage found by endoprotease Glu-C at amino acid residue E^{16} in the immune complex indicates that this position is outside the antigen−antibody interaction region. This model of the antigen structure and epitope site (Figure 5b) is further supported by comparison of the sequences of bovine cardiac (bc-TnT) with the human cardiac (hc-TnT) and the human skeletal (hsk-TnT) proteins (Table 3). Residues S^2, D^5, K^9, and A^{12} of the cardiac protein are mutated in the skeletal protein (bold letters in Table 3); these substitutions lead to the complete loss of binding affinity to the MAb-M7 antibody. They are all located on the same side of the helix, which is protected from proteolytic degradation. Hence, these substitutions well explain the immunological discrimination of the skeletal protein. Residues E^5 and R^9 can be considered as substitutions merely comprising the spatial microenvironment, while the interchange substitutions of residues A^2 and S^{12} comprise functional groups. $S^2 \rightarrow A^2$ substitution may lead to the loss of a hydrogen bond in the antigen−antibody complex of the bc-TnT peptide, which is providing further suggestion to the binding to the antibody in an α-helical conformation.

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