

# Mass Spectrometric Peptide Mapping Analysis and Structural Characterization of Dihydrodiol Dehydrogenase Isoenzymes

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The direct molecular weight determination and structural analysis of polypeptides and peptide mixtures have become amenable by the recent development of fast atom bombardment (FABMS) and  $^{252}\text{Cf}$ -plasma desorption (PDMS) mass spectrometry. FABMS and PDMS peptide mapping, i.e., the direct analysis of peptide mixtures resulting from proteolytic digestion, have been developed as powerful methods for the structural characterization of epoxide-metabolizing isoenzymes. The major advantage of this approach is provided by the selectivity of the endoproteolytic cleavage, combined with the specific and accurate molecular weight determination of complex digest mixtures containing peptides up to several thousand daltons in size. Furthermore, the mass spectrometric peptide mapping analysis can be combined with a range of protein-chemical modification reactions and with sequential degradation such as by carboxypeptidases. Both FABMS and PDMS peptide mapping have already been successfully applied to the structural differentiation of glutathione transferase and epoxide hydrolase isoenzymes in cases where reference sequence data for at least one isoenzyme form was available. In the application described here, for a series of dihydrodiol dehydrogenase (DDH) isoenzymes with hitherto undetermined primary structures, a direct correlation between the structural differentiation from peptide mapping data and differences in their substrate specificities could be demonstrated. The mass spectrometric peptide mapping analysis of isoenzymes proved to be an efficient basis for the elucidation of the structure of one major DDH isoenzyme form; partial sequence data for this protein are reported.

## Introduction

The occurrence and biochemical significance of a multitude of isoenzyme forms of important hepatic epoxide-metabolizing enzymes has been demonstrated in a number of studies in the last years (1,2). Thus, besides the already well-known variety of glutathione transferases (GST) (2), several isoenzyme forms of cytosolic and microsomal epoxide hydrolase (EH), and, more recently, dihydrodiol dehydrogenase (DDH) have been isolated from rat and rabbit liver (3-5). The development of efficient separation techniques for purifying isoenzymes to apparent homogeneity requires highly specific analytical methods for the structural characterization of these often closely related forms. A structural differentiation has become of particular interest for isoen-

zymes with distinct differences in substrate specificity, as recently demonstrated for a number of DDH isoenzyme forms (5,6).

The successful development of fast-atom bombardment (FABMS) and, more recently,  $^{252}\text{Cf}$ -plasma desorption mass spectrometry (PDMS) in the last years has enabled the direct, general analysis of abundant molecular ions of biological polypeptides of up to small proteins in size (7-9). While direct structural (sequence) information from fragment ions in FABMS and PDMS is normally limited and/or scarce, the capability of both methods for providing unequivocal, precise molecular weight determinations has recently led to new applications for the analysis of polypeptide mixtures produced by specific proteolytic digestion (peptide mapping). FABMS and PDMS peptide mapping have been initially employed as a complementary method for the verification (and in some cases correction) of DNA-derived protein sequences (10), but these methods have recently emerged as powerful, general tools in several applications to protein structural chemistry (7,8,11).

Both FABMS and PDMS peptide mapping of specific enzymatic and chemical digest mixtures have been suc-

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cessfully applied to the characterization of glutathione transferase and epoxide hydrolase isoenzyme forms with closely related primary structures (12,13). In these studies, structural differentiation of substantial parts of conservative and variable sequences was possible by comparison with one single reference structure available from DNA sequence data. However, the utility of mass spectrometric peptide mapping has also been demonstrated in studies of dihydrodiol dehydrogenase isoenzymes isolated from rabbit liver (5) with hitherto unknown primary structures. Peptide mapping analyses described here served to differentiate common and unique peptide fragments of these isoenzymes and were used as a basis for the complete elucidation of the structure of one major DDH isoenzyme form. Partial sequence data for this protein were determined by a combination of conventional sequence analysis and mass spectrometry.

## Materials and Methods

### Isolation and Purification of DDH Isoenzymes

Rabbit liver cytosolic dihydrodiol dehydrogenases were isolated by a five-step procedure with final FPLC on phenylsuperose and were separated into eight isoenzyme forms (5). For proteolytic digestion and mass spectrometry, all isoenzymes were dialyzed against 0.01 mM potassium phosphate (pH 7.0) and then extensively against H<sub>2</sub>O and 0.02% trifluoroacetic acid (TFA) and were finally concentrated in solution on a Millipore VS molecular size exclusion membrane or lyophilized directly.

### Proteolytic Digestion

Lyophilized porcine trypsin and *S. aureus* V8 protease were obtained from Sigma (St. Louis, MO); carboxypeptidases (Cpases) A, B, P, and Y were from Boehringer (Mannheim, FRG) and were dialyzed against water and/or digestion buffer. The activity of all enzymes was tested with model peptides. Trypsin digestion was performed in 50 mM NH<sub>4</sub>HCO<sub>3</sub> (pH 8.2) for 16 hr, 37°C with enzyme:substrate ratios of 1:20 to 1:50. V8 protease digestion was carried out in NH<sub>4</sub>HCO<sub>3</sub> buffer at 25°C for 18 hr with an enzyme:substrate ratio of 1:10. Conditions for carboxypeptidase digestion and preparation of aliquots for mass spectrometry were as previously described (14). For mass spectrometric analysis or HPLC separation of tryptic peptides digest mixtures were either lyophilized directly or concentrated with a speed-vac microcentrifuge.

### Isolation of DDH Peptide Fragments

HPLC separation of tryptic peptides from the major DDH isoenzyme (CM-2) was performed with a Waters LC-244 chromatograph on a thermostated 25 × 0.4 cm reverse-phase (RP)-tetraalkylsilyl column at 35°C, us-

ing a linear gradient program of 0.04% trifluoroacetic acid:acetonitrile (1:10) starting eluant, up to 80% acetonitrile at approximately 1%/min. A Waters 490E multiwavelength detector and Gilson-CPR programmed fraction collector were used for detection and isolation of peptides; approximately 0.1 to 0.8 nmole aliquots of peptides were used for PDMS and sequence determinations. N-Terminal sequence and amino acid analyses were performed with an automated Edman gas phase sequenator and RP-octadecylsilyl HPLC of phenylthiohydantoin (PTH) amino acids (15).

### Mass Spectrometry

FABMS peptide mapping analyses were performed with a modified Finnigan MAT 312/AMD-5000 double focusing spectrometer equipped with a laminated high-field magnet, a 15 kV cesium primary ion source, and a thermostatted 2-mm<sup>2</sup> stainless-steel target. Details of instrumental and experimental conditions were as previously reported (12,16). Samples were prepared with 0.5 to 1 µg/µL aliquots of peptide fragment mixtures in 1% TFA mixed with 1 µL of glycerol, or 3-nitrobenzylalcohol as matrix on the FAB target. PD spectra were obtained with a BIO-ION-20K time of flight spectrometer (Uppsala, Sweden) equipped with a 10 µCi <sup>252</sup>Cf-primary ion source (9,17). The sample target consisted of an Al/Mylar foil onto which an approximately 3-mm<sup>2</sup> nitrocellulose (NC) backing surface was prepared by electrospraying (17). Sample solutions of 0.1 to 1 µg/µL in 1% TFA were applied to the NC target with a microliter syringe. Carboxypeptidase digestion of several peptide fragments isolated by HPLC was performed by addition of enzyme directly to the NC target (12,18).

## Results and Discussion

Both FABMS and PDMS peptide mapping have been applied to the structural characterization of several hepatic glutathione transferases and epoxide hydrolases, for which a reference structure of one isoenzyme form was available from either DNA or protein sequence data. For example, in a comparative study of the tryptic peptide digests of three closely related GST 4-4 isoenzyme forms, a direct characterization of homologous and variable partial sequences and structural differentiation for approximately 70% of the entire protein were obtained relative to the previously reported sequence of the GST-4 subunit [(18), H. Thomas, F. Oesch, M. Przybylski et al., manuscript in preparation]. In contrast to the GST isoenzymes, FABMS and PDMS peptide mapping analyses have been applied to a series of dihydrodiol dehydrogenases isolated from rabbit liver for which no primary structural data were known. The catalytic properties of eight chromatographically distinct DDH isoenzymes have been evaluated in detail and could be assigned to isoenzymes with carbonyl reductase activity (forms CF-1; CF-2) pronounced aldehyde reductase activity (forms CF-5; CF-6), while a major DDH form

(CM-2) (Fig. 1) showed markedly different properties resembling a quinone reductase (6,20).

Typical FABMS analyses (positive ions) of the peptide fragment mixtures obtained by trypsin digestion of three DDH isoenzymes are compared in Figure 1. At the strongly acidified matrix conditions used (12,13), abundant protonated molecular ions ( $MH^+$ ) of several common peptide fragments can be identified (e.g.,  $m/z$  686, 841, 994). However, other  $MH^+$  ions of peptides in the spectrum of isoenzyme CF-5 were not present in the spectra of forms CM-1 and CM-2 (e.g.,  $m/z$  548, 876), and major peptide ions from CM-2 had no match in CF-5, indicating the structural relation as well as distinct differences between the isoenzymes. The molecular weight determinations and assignments of tryptic peptides were ascertained (a) by FABMS analysis of digest mixtures after additional Cpase B digestion, resulting in molecular ion shifts of 128 and 156 amu due to removal of C-terminal Lys and Arg residues (12), and (b) by

PDMS peptide mapping, which revealed additional peptide fragments from incomplete digestion. Only a few identical peptides indicative of highly conserved regions were common to all isoenzymes. It is notable that a structural correlation derived from the tryptic peptide mapping data (comprising approximately 30% of the primary structures) showed good agreement with the substrate specificity differences of the isoenzymes and their classification into distinct subgroups (20).

The mass spectrometric peptide mapping data for the dihydrodiol dehydrogenases of hitherto unknown structure were then employed as a basis for the complete structure elucidation of the major isoenzyme, CM-2. Initial structural studies on the CM-2 protein clearly showed the advantage of this approach in that precise molecular weights of isolated proteolytic peptides could be immediately assigned and partial sequences established; homogeneities of peptide fragments and possible artifacts were determined directly; and sequence de-

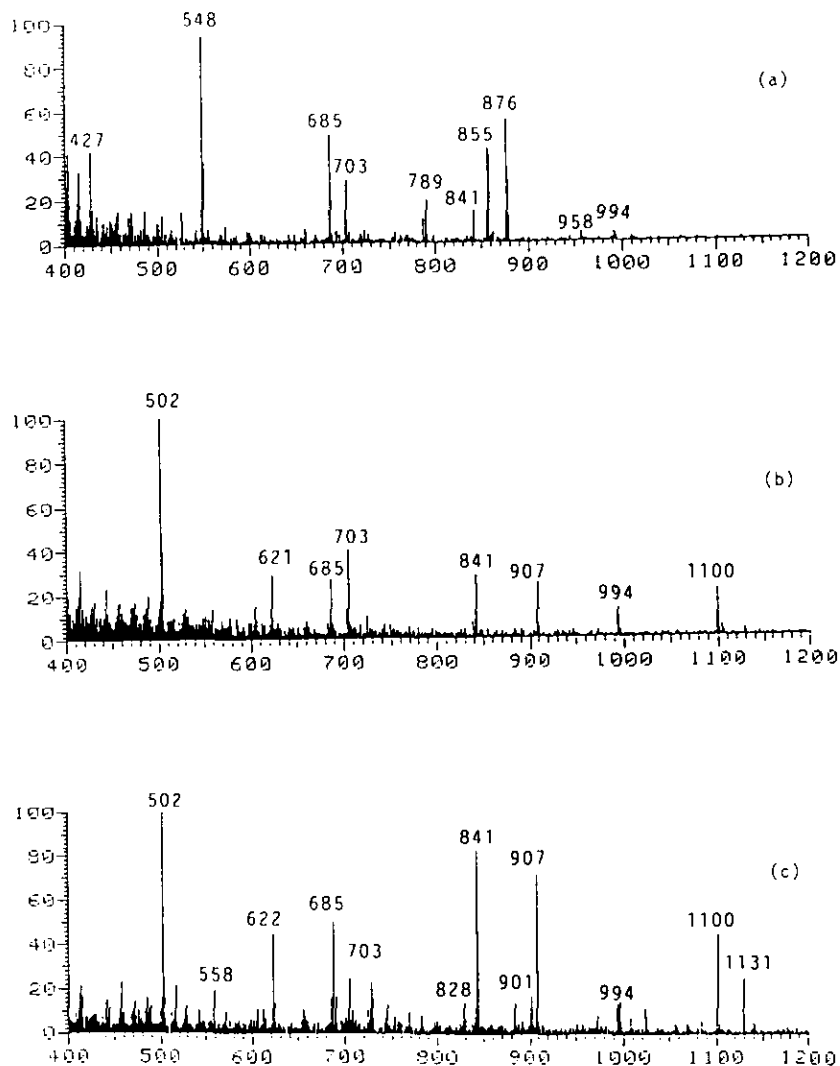


FIGURE 1. FAB mass spectrometric peptide mapping analyses (positive ions) of the tryptic peptide fragment mixtures from rabbit liver DDH isoenzymes CF-5 (a), CM-1 (b), and CM-2 (c). Aliquots of digest mixtures corresponding to approx. 0.5 nmole of protein were dissolved in the matrix, 1% TFA: glycerol, 1:1; conditions for enzymatic digestion were as described in "Materials and Methods."

terminations reveal directly partial structures for all isoenzymes. Furthermore, initial attempts to obtain a partial N-terminal sequence by automated Edman analysis failed due to a blocked N-terminus of CM-2 (as well as of all other DDH isoenzymes). The separation by reversed-phase HPLC of a peptide fragment mixture obtained by complete trypsin digestion (Fig. 2) and molecular weight determinations of aliquot fractions by PDMS yielded a good agreement with the peptide mapping data and the total amino acid determination. A molecular weight for the entire protein of approximately 38 kDa was derived. This is slightly higher than the subunit molecular weight of 35 kDa estimated from gel electrophoresis data (20). Preliminary attempts to obtain a direct mass spectrometric molecular weight de-

termination have failed so far, which might be due to residual salt and/or detergent contamination of the protein.

The structures of a series of tryptic peptides isolated by HPLC were determined by a combination of FABMS and PDMS with partial Cpsase digestion, N-terminal Edman sequencing, and amino acid analysis, using samples of 1 to 3  $\mu\text{g}$  of peptide fractions. Partial sequence data for approximately 30% of the entire protein could be derived from the identification of tryptic peptides. Although the molecular ions of several HPLC fractions showed the presence of incompletely separated peptide mixtures (Fig. 2), unequivocal sequences were obtained for unique peptides in the CM-2 protein as well as for peptides common to all isoenzymes, as is summarized

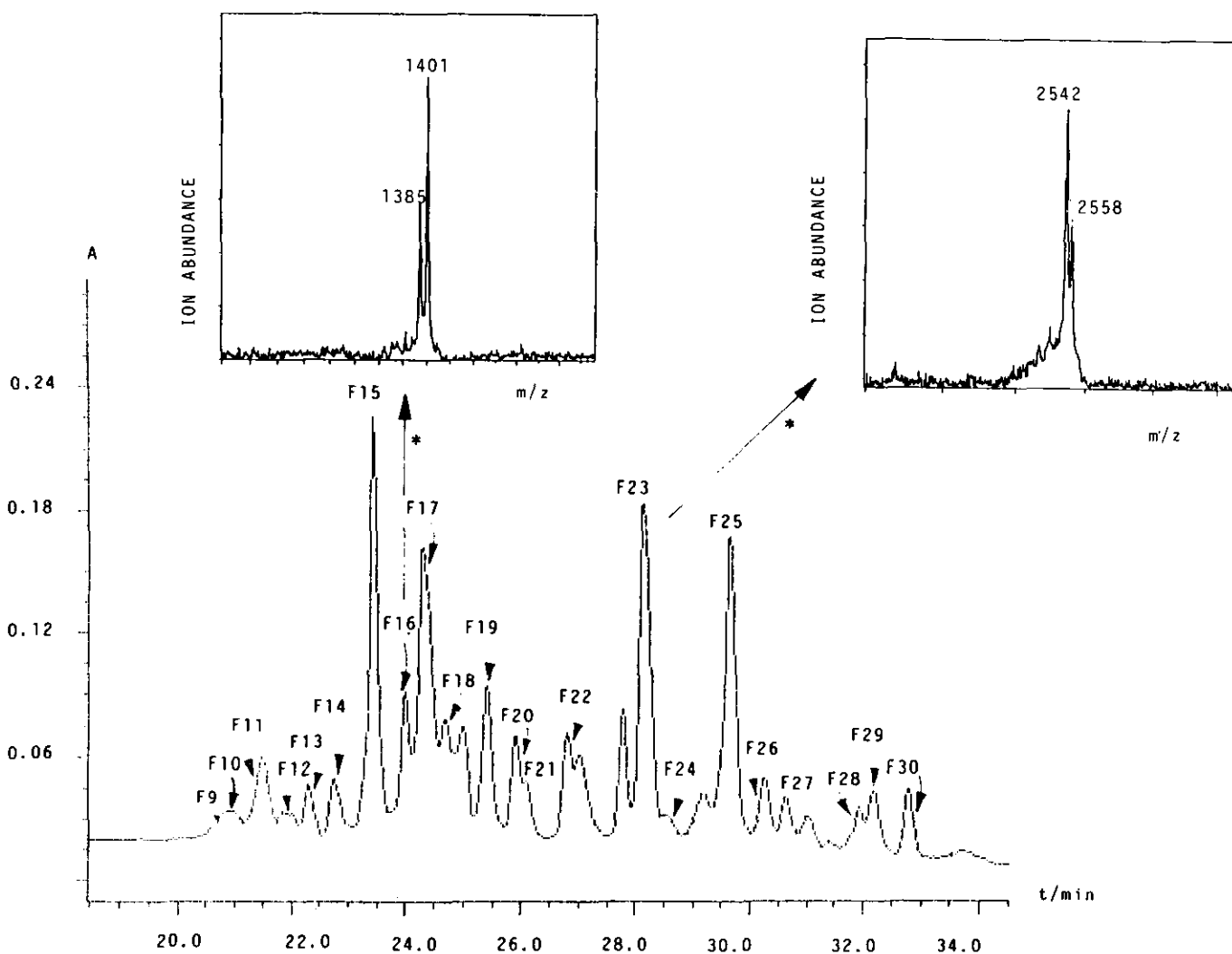


FIGURE 2. Separation of tryptic peptide fragments obtained from DDH isoenzyme CM-2 by RP-HPLC on a tetraalkylsilyl (C4) column. A linear gradient of 0.04% TFA/acetonitrile was employed as described in "Materials and Methods." Numbers in the chromatogram denote peptide fractions isolated for mass spectrometric and sequence analysis; see Table 1 for structural data of fractions marked by an asterisk. Peptide fractions F16 (upper left panel) and F23 (upper right panel) contain partial oxidation products at methionine residues, as shown by PDMS and sequence data. Molecular weights of peptide fractions determined by FAB and PD mass spectra were: F9, 605; F10, 621; F11, 686; F12, 828, 883, 900, 1100; F13, 906, 1130; F14, 996; F15, 840; F16, 1385/1401; F17, 793, 973; F18, 1466; F19, 1820, 1433, 767; F20, 1141; F21, 2076; F23, 2543/2559; F24, 2936.

**Table 1. Molecular weights, amino acid composition, and partial sequences of DDH isoenzyme CM-2, determined from tryptic peptide fragments isolated by HPLC.**

HPLC		Molecular weight, MH <sup>+</sup> <sup>a</sup>	C-terminal residue <sup>b</sup>	Amino acid composition	Partial sequence (molecular weight)
Peptide fraction no.	Retention time/min				
6	5.5	708	Arg	L <sub>1</sub> (E/Q) <sub>2</sub> Y <sub>1</sub> R <sub>1</sub>	Y-Q-L-Q-R-OH (707)
10	21.2	621	Lys	(D/N) <sub>1</sub> I <sub>1</sub> V <sub>1</sub> F <sub>1</sub> K <sub>1</sub>	V-I-D-F-K-OH (620)
11	21.6	686	Lys	(E/Q) <sub>1</sub> A <sub>2</sub> V <sub>1</sub> L <sub>1</sub> G <sub>1</sub> K <sub>1</sub>	— <sup>c</sup>
16	24.0	1385/1401 <sup>d</sup>	Lys	—	Q-L-E-M-I-L-N-K <sup>f</sup> -P-G-L-K-OH (1384)
19	25.4	1821	Arg <sup>e</sup>	—	L-Q-L-D-Y-V-D-(S-R) <sup>e</sup>
21	26.4	2076	ND	—	I-I-S-F-P-A-A-K(P) <sup>f</sup>
23	28.2	2543/2559 <sup>d</sup>	Lys	—	M-A-L-N-D-G-H-F-I-P-A-L-G-G-Y-A- -W-K-OH

<sup>a</sup>Protonated molecular ions MH<sup>+</sup> from FABMS and/or PDMS data.

<sup>b</sup>From carboxypeptidase B digestion and PDMS data; ND, not determined.

<sup>c</sup>Not amenable to Edman degradation.

<sup>d</sup>Molecular ion due to partial oxidation at methionine residues.

<sup>e</sup>Tentative assignments.

<sup>f</sup>Lys-Pro sequence not amenable to trypsin digestion.

in part in Table 1. For example, the PDMS data and sequences of the peptide fractions F16 and F23 revealed their partial oxidation to the methionine-sulfoxides (Fig. 2). In several peptides such as in F16 (molecular weight 1384), -Lys-Pro-sequences not amenable to trypsin digestion were found. The partial methionine oxidation was also reflected in the PDMS peptide mapping analysis of BrCN fragments, in which multiple cleavage yielded additional, larger polypeptide ions than expected from the estimated number of Met residues.

Peptide mapping with different proteolytic digestion procedures was used for the analysis of specific, complementary polypeptides, e.g., by comparison of PD spectra from trypsin and *S. aureus* V8 protease digest mixtures (data not shown). The complementary information obtained from peptide ions in FAB and PD spectra is in agreement with the tendency in FABMS of peptide mixtures to discriminate molecular ion abundances according to hydrophilicity/hydrophobicity differences (21); by contrast, PD spectra showed a tendency to discriminate for peptide components with high positive charge. Further studies aimed at the complete structure elucidation of the CM-2 isoenzyme by isolation and structural identification of corresponding overlapping sequences are being carried out at present.

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