The Amino Acid Sequence of the Tryptic Peptides Isolated from Dogfish M₄ Lactate Dehydrogenase*

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Lactate dehydrogenase, one of the major enzymes in the glycolytic pathway, catalyzes the reversible oxidation of l-lactic acid to pyruvate in vertebrate tissues. The enzyme is tetrameric and requires nicotinamide adenine dinucleotide (NAD) as a cofactor. The subunits are identical and have a molecular weight of 36,000 (1). Each subunit binds 1 molecule of substrate and 1 molecule of coenzyme; no cooperative effects of subunit interaction have been demonstrated (2, 3).

At least two unique major isoenzyme forms are known to exist in vertebrates (4, 5) as well as some minor isoenzymes (6). In terms of molecular weight, the isoenzyme forms are all very similar; however, significant differences exist in their amino acid compositions as well as in their kinetic and electrophoretic properties (7, 8). One of the major isoenzyme forms, the M type or type V, is found generally in anaerobic tissues such as skeletal muscle, whereas the other major isoenzyme, H type or type I, is more prominent in aerobic tissue such as heart (9). In general the M-type isoenzymes isolated from related species are more similar to one another than are the M-type and H-type isoenzymes found in any single species. Despite distinct differences, the isoenzyme forms have clearly evolved from common origins. A tryptic peptide containing an "essential" cysteine residue in lactate dehydrogenase has been isolated from both M-type and H-type isoenzymes and the sequence of this dodecapeptide is closely conserved in all cases (10, 11).

In addition, in most species the fully active tetrameric enzyme containing both M-type and H-type subunits is observed in vivo (4, 5) and can be formed in vitro under artificial folding conditions (12), again indicating a high degree of structural similarity between the isoenzyme forms. The kinetic and electrophoretic properties of these mixed hybrids correlate directly with the percentage of each subunit type present and emphasize the independent nature of each subunit during catalysis (7, 8).

The enzyme for which the primary structure has been characterized here is the M₄ isoform of lactate dehydrogenase from dogfish muscle (Squalus acanthius). The enzyme crystallizes readily, and the crystal structure of both the apoenzyme (13) and the ternary complex of the enzyme and NAD-pyruvate (14) have been solved at resolutions of 2.0 and 3.0 Å, respectively. Since each subunit contains 9 arginine residues and 30 lysine residues, 40 unique tryptic peptides can be anticipated following treatment of the enzyme with trypsin. In fact, 37 of these peptides have been isolated and characterized. Only three tryptic peptides were not isolated; the sequences of the tryptic peptides reported here account for the remaining portion of the molecule. The sequences of the three peptides which have not been isolated have been deduced from other proteolytic digests and will be presented elsewhere. A preliminary report of this sequence has been published (15).

MATERIALS AND METHODS

Reagents—Twice recrystallized trypsin was purchased from Worthington. Chymotrypsin and subtilisin were purchased from Sigma and thermolysin from Calbiochem. Iodo-¹³C lactic acid was obtained from New England Nuclear.

Lactate Dehydrogenase Purification—Lactate dehydrogenase was purified as described previously (15). More recently the enzyme has been purified by affinity chromatography using adenose monophosphate which has been covalently bound to Sepharose (16). The enzyme was stored as a crystalline suspension in ammonium sulfate at 4°. The purified enzyme showed a single protein band when subjected to electrophoresis in 6.5% polyacrylamide gels (17); this band correlated with the band of enzymatic activity which was stained according to the method of Fine and Costello (18). The specific activity of the purified protein was 800 i.u./mg, where 1 i.u. equals 1 µmol of NADH oxidized/min.

Alkylation—Prior to proteolytic digestion the enzyme was treated with iodo-¹³C lactic acid (2.1 x 10⁵ cpm/µmol) in order to carboxymethylate the cysteine residues (19). Two hundred milligrams of enzyme were dialyzed against 0.01 M Tris-HCl, pH 8.3, 0.5 mM EDTA for 15 hours. The solution was brought to a concentration of 8 M urea by

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adding solid enzyme grade urea. To ensure that all of the cysteine residues were fully reduced, a 2-fold excess of dithiothreitol (80 pmol) over total number of cysteine residues was added. The concentration of the enzyme was approximately 5 mg/ml. After incubation at room temperature for 2 hours, a solution of iodo-[\(^{14}\)C]acetate acid (400 pmol) was added dropwise. The solution was stirred continuously, and the pH was maintained at 8.0 by adding 0.1 M NaOH when necessary. The reaction was allowed to continue for 4 hours at room temperature and was then terminated by the addition of 800 pmol of 2-mercaptoethanol. The solution was dialyzed for 24 hours at 4°C against three 2-liter volumes of 0.05 M NaHCO\(_3\) (pH 8.3). A total of 8.2 × 10\(^6\) cpm (39.2 pmol) were incorporated into the protein.

**Proteolytic Digestion**—Prior to use the trypsin was treated with 4-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) to remove any residual chymotryptic activity (20). Trypsin digestion was carried out at a concentration of S-[\(^{14}\)C]carboxymethylated lactate dehydrogenase of 4 mg/ml. After adding the TPCK-treated trypsin (1/100 w/v), the solution was incubated for 4 hours at 37°C. The reaction was terminated by adding a 2-fold excess of soybean trypsin inhibitor over any residual chymotryptic activity (20). Trypsin digestion was carried out on a Beckman model 119 automatic amino acid analyzer.

**Cyanogen Bromide Treatment**—Two hundred milligrams of S-[\(^{14}\)C]carboxymethylated lactate dehydrogenase were treated with cyanogen bromide according to the method of Gross and Witkop (21). The lyophilized S-[\(^{14}\)C]carboxymethylated lactate dehydrogenase was redisolved in 30 ml of 0.1 M HCl. After adding 480 mg of solid cyanogen bromide, the solution was left stirring at room temperature for 24 hours. The solution then was lyophilized and redissolved in 50 mM NH\(_3\)OH. The cyanogen bromide fragments were then chromatographed on a column of Sephadex G-50 eluting with 50 mM NH\(_3\)OH.

**Paper Electrophoresis and Chromatography**—Peptides were purified by paper electrophoresis in the apparatus described by Ryler et al. (22). Four different buffer systems (pH 1.9, 6.5, 3.5, and 8.9) previously described by Ambler et al. (23) were used. In some cases descending chromatography was also utilized with the following solvent: butanol/acetic acid/water/pyridine (15/3/12/10). The mobilities of peptides at pH 1.9 and pH 6.5 relative to serine and aspartate, respectively, were recorded to determine their net charge by the method of Oloff (24). The reported mobilities at pH 6.5 are followed by A or B indicating, respectively, an acidic peptide migrating towards the anode or a basic peptide migrating in the direction of the cathode.

**Sequencing**—In all cases sequences were determined by using the subtractive dansyl procedure. The dansyl derivatives were identified by thin layer chromatography on polyamide sheets (9 x 8 cm) (25, 26). In the figure a solid line beneath an amino acid residue indicates that it has been positively identified by the sequential dansyl procedure. A dashed line indicates that the dansyl derivative of that residue was not identified sequentially in that peptide. Amino acid analyses were carried out on a Beckman model 119 automatic amino acid analyzer. Unless otherwise stated hydrolyses were carried out in 6 N HCl at 105°C. Tryptophan content was determined by the method of Liu and Yang (27).

**RESULTS**

Following the reaction of lactate dehydrogenase with iodo-[\(^{14}\)C]acetate, 7.0 pmol of \(^{14}\)C were incorporated/µmol of \(M_s = 36,000\) subunit on the basis of nondialyzable radioactivity associated with the protein. This was confirmed by amino acid analysis of the carboxymethylated lactate dehydrogenase which showed 6.8 µmol of carboxymethylcysteine per \(M_s = 36,000\) subunit. The fractionation of the peptides in the tryptic digest on a column of Sephadex G-75 (3.0 x 100 cm) is shown in Fig. 1. Pooled Fractions B and C of this eluate were further fractionated on a column of Sephadex G-50, whereas pooled

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**TABLE I**

<table>
<thead>
<tr>
<th>Amino acid composition in moles per subunit ((M_s = 36,000)) of carboxymethylated dogfish (M_s) lactate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine-Cm</td>
</tr>
<tr>
<td>Aspartic Acid</td>
</tr>
<tr>
<td>Threonine</td>
</tr>
<tr>
<td>Serine</td>
</tr>
<tr>
<td>Glutamic Acid</td>
</tr>
<tr>
<td>Proline</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Valine</td>
</tr>
<tr>
<td>Methionine</td>
</tr>
<tr>
<td>Isoleucine</td>
</tr>
<tr>
<td>Leucine</td>
</tr>
<tr>
<td>Tyrosine</td>
</tr>
<tr>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Histidine</td>
</tr>
<tr>
<td>Arginine</td>
</tr>
<tr>
<td>Tryptophan</td>
</tr>
</tbody>
</table>

Fractions D, E, and F were fractionated on a Sephadex G-25 column.

On the basis of the amino acid composition (Table I), 40 unique trypsic peptides were anticipated. In addition to three of the peptides whose sequences were published previously (1), 25 of the tryptic peptides (Table II) were sequenced in their entirety directly by the subtractive dansyl procedure. Details of the characterization and isolation procedures for these peptides are included in the appendix. Three peptide bonds

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Some of the data are presented as a miniprint supplement immediately following this paper. Material published in miniprint form can be easily read with the aid of a large field reading glass of a type readily available at most opticians. For the convenience of those who prefer to obtain supplementary material in the form of full size photocopies, these same data are available as JBC Document No. 75M-291. Orders for supplementary material should specify the title, authors, and reference to this paper and the JBC
containing lysine were never cleaved by trypsin; three other similar peptide bonds were only partially split by trypsin. In addition, three of the tryptic peptides were never isolated. The sequences of these peptides have been deduced from peptides which were isolated after fragmentation processes other than proteolytic digestion with trypsin and will be presented in the second paper of this series. Free lysine was also isolated from the tryptic digest. Three of the remaining tryptic peptides contained 16 or more residues and required additional cleavage procedures in order to characterize completely the sequence. In addition, two peptides (T6 and T34) had an amino acid composition which included 4 glutamic acid residues; these peptides required additional procedures to identify which residues were acids and which were amides. The characterization of the sequence of these latter six peptides will be discussed in detail.

**Tryptic Peptide T2**—The sequence of the COOH-terminal region of this peptide has been corrected from that previously published by Allison et al. (1). The Edman degradation of the entire peptide clearly indicated a terminal sequence of Glx-Glx-Pro-Arg rather than Glx-Pro-Glx-Arg. The tetrapeptide Glx-Glx-Pro-Arg was isolated after treating T3 with subtilisin and had the following amino acid composition: 1.0 Pro, 1.9 Glu, and 1.0 Arg. This peptide was neutral when subjected to electrophoresis at pH 6.5, indicating that the peptide contained one glutamic acid and 1 glutamine residue. After the NH$_2$-terminal amino acid residue was removed by Edman degradation, the remaining tripeptide was still neutral at pH 6.5, indicating that a glutamine residue had been removed. After a second Edman degradation, the residual dipeptide had a mobility of 0.62 B. Therefore, the sequence was deduced to be: Gln-Glu-Pro-Arg. This sequence is fully corroborated by the x-ray crystallography studies and, consequently, the sequence of the entire tryptic peptide T3 is as follows: Leu-Ile-Gly-His-Leu-Ala-Thr-Ser-Glu-Glu-Pro-Arg.

**Tryptic Peptide T5**—Tryptic peptide T5 contains 20 amino acids; the sequence is shown in Table III. The dansyl procedure successfully identified the first 18 residues of the peptide with the exception of the 6th residue, cysteine, and the 11th residue, which gave no detectable dansyl derivative. The 7th residue, Asx, gave a very faint spot; however, the composition indicates that this residue must be an asparagine or an aspartic acid. In addition, after treatment of lactate dehydrogenase with cyanogen bromide an octapeptide was isolated (CNBr 2) which accounted for 8 residues of peptide T5. CNBr 2 was completely sequenced by the dansyl procedure, as indicated in Table III. The mobility of this peptide at pH 6.5 confirmed that residue 13 was an aspartic acid. This sequence and the charge of the aspartic acid residue was confirmed by the chymotryptic peptide C6. The amino acid composition of the complete tryptic peptide as well as a second cyanogen bromide peptide beginning with NH$_2$-terminal lysine and a chymotryptic peptide C6 with the exception of the 6th residue, cysteine, and the 11th residue, which gave no detectable dansyl derivative. The 7th residue, Asx, gave a very faint spot; however, the composition indicates that this residue must be an asparagine or an aspartic acid. In addition, after treatment of lactate dehydrogenase with cyanogen bromide an octapeptide was isolated (CNBr 2) which accounted for 8 residues of peptide T5. CNBr 2 was completely sequenced by the dansyl procedure, as indicated in Table III. The mobility of this peptide at pH 6.5 confirmed that residue 13 was an aspartic acid. This sequence and the charge of the aspartic acid residue was confirmed by the chymotryptic peptide C6. The amino acid composition of the complete tryptic peptide as well as a second cyanogen bromide peptide beginning with NH$_2$-terminal lysine and a chymotryptic peptide C6 with the exception of the 6th residue, cysteine, and the 11th residue, which gave no detectable dansyl derivative. The 7th residue, Asx, gave a very faint spot; however, the composition indicates that this residue must be an asparagine or an aspartic acid.

### Table II

**Tryptic peptides of dogfish M$_1$ lactate dehydrogenase**

<table>
<thead>
<tr>
<th>Tryptic Peptide</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1,2,3</td>
<td>Acetyl-Thr-Ala-Leu-Lys-Asp-Leu-Ile-Gly-His-Leu-Ala-Thr-Ser-Glu-Glu-Pro-Arg (1)</td>
</tr>
<tr>
<td>T4</td>
<td>Ser-Tyr-Asn-Lys$^a$</td>
</tr>
<tr>
<td>T5</td>
<td>Ile-Thr-Val-Val-Gly(Cys,Asx)Ala-Val-Gly-Met-Ala-Asp-Ala-Val-Ser-Val-Leu-Met-Lys</td>
</tr>
<tr>
<td>T6,7</td>
<td>Asp-Leu-Ala-Asp-Glu-Val-Ala-Leu-Leu-Val-Ala-Leu-Glu-Leu-Asp-Glu-Lys$^a$</td>
</tr>
<tr>
<td>T9</td>
<td>Ile-Val-Ser-Gly-Lys$^a$</td>
</tr>
<tr>
<td>T10</td>
<td>Asp-tyr-Ser-Val-Ser-Ala-Gly-ser-Lys$^a$</td>
</tr>
<tr>
<td>T11</td>
<td>Leu-Val-Ala-Ile-Thr-Ala-Ser-Ala-Arg</td>
</tr>
<tr>
<td>T12</td>
<td>Glu-Gln-Glu-Gly-Glu-Ser-Arg</td>
</tr>
<tr>
<td>T13</td>
<td>Leu-Asn-Leu-Val-Gln-Ala-Arg</td>
</tr>
<tr>
<td>T14</td>
<td>Asn-Val-Ala-Asn-His-Asn-Arg</td>
</tr>
<tr>
<td>T15</td>
<td>Phe-Ile-Val-Pro-Asp-Ile-Val-Lys$^a$</td>
</tr>
<tr>
<td>T16</td>
<td>Glu-Leu-His-Pro-Glu-Leu-Glu-Thr-Asn-Lys-Asp-Lys-Gln-Asp-Trp-Lys$^a$</td>
</tr>
<tr>
<td>T17</td>
<td>Leu-Ser-Gly-Leu-Pro-Met-His-Arg</td>
</tr>
<tr>
<td>T18</td>
<td>Ile-Ile-Gly-Ser-Gly-Cys-Asn-Leu-Asn-Ser-Ala-Arg</td>
</tr>
<tr>
<td>T19</td>
<td>Phe-Arg</td>
</tr>
<tr>
<td>T20</td>
<td>Tyr-Leu-Met-Gly-Glu-Ala</td>
</tr>
<tr>
<td>T21</td>
<td>Leu-Glu-Gly-His-Ser-Cys-Ser-Gly-Val-Pro-Val-Val-Glu-Val-Ile-Lys-Leu-Lys$^a$</td>
</tr>
<tr>
<td>T22</td>
<td>Asn-Leu-Glu-Glu-Glu</td>
</tr>
<tr>
<td>T23</td>
<td>Val-Ser-Pro-Val-Val-Ser-Pro-Val-Pro-Val</td>
</tr>
<tr>
<td>T29</td>
<td>Asn-Leu-Asn-Arg</td>
</tr>
<tr>
<td>T30</td>
<td>Asp-Phe-Tyr-Gly-Ile-Lys$^a$</td>
</tr>
<tr>
<td>T31</td>
<td>Asp-Pro-Val-Leu-Asn-Leu-Asp-Ura-Glu-Ile-Ser-Arg-Ile-Ile-Val</td>
</tr>
<tr>
<td>T33</td>
<td>Met-Lys</td>
</tr>
<tr>
<td>T34</td>
<td>Leu-Lys-Pro-Pro-Asn-Glu-Glu-Glu-Gln-Leu-Gln-Gln</td>
</tr>
<tr>
<td>T35</td>
<td>Ser-Ala-Thr-Thr-Thl-Pro-Thr-Pro-Ile-Glu-Val-Ile-Lys</td>
</tr>
<tr>
<td>T36</td>
<td>Asp-Leu-Lys-Phe</td>
</tr>
</tbody>
</table>

$^a$ Indicates those peptides which have been sequenced in their entirety by the dansyl procedure.
**Table III**

**Amino acid sequence of Tryptic Peptide T5**

This peptide was found in fraction B after elution from Sephadex G-75. Following elution from Sephadex G-50, where it eluted just after the void volume, it was purified by paper electrophoresis at pH 8.9 and 3.5.

<table>
<thead>
<tr>
<th>Peptide No.</th>
<th>Mobility (6.5)</th>
<th>Amino acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>T5</td>
<td>-</td>
<td>1.0 Lys 0.5 Cys(Cm) 2.1 Asp 0.8 Thr 0.9 Ser 2.0 Gly 3.0 Ala 4.1 Val 1.2 Met 2.0 Ile 1.5 Leu</td>
</tr>
<tr>
<td>CNBr2</td>
<td>acidic</td>
<td>0.8 Asp 0.9 Ser 2.0 Ala 0.8 Val 1.4 Leu 1.1 Leu 1.0 Hse</td>
</tr>
<tr>
<td>C5</td>
<td>acidic</td>
<td>0.8 Asp 1.0 Ser 2.0 Ala 0.8 Val 1.4 Ile 1.2 Leu</td>
</tr>
</tbody>
</table>

* Explanation of Table III which will apply for all subsequent tables. A solid line (---) beneath an amino acid residue indicates that the residue has been positively identified by the sequential dansyl procedure. A dashed line (---) indicates that the dansylamino acid derivative of that residue was not unambiguously identified sequentially in that peptide.

* This tryptic peptide is apparently very insoluble at pH 6.5 and remains at the origin, where it was originally spotted. It was therefore not possible to determine a mobility for this peptide at that pH.

* These amino acid compositions were based on 24- and 48-hour hydrolysates in order to determine accurate values for valine and isoleucine.

from the COOH-terminal end. This residue was originally identified as valine; however, in both the cyanogen bromide peptide and in the chymotryptic peptide, the analysis and sequence indicated some isoleucine occurring at this position. The dansyl-Edman degradation at the preceding step gave a clear and strong dansylphenylalanine spot. After the next Edman degradation both isoleucine and valine derivatives were observed as well as some of the dansyl derivatives of the dipeptide valine-leucine. The following step gave only leucine.

**Tryptic Peptide T8**—The first 10 residues of tryptic peptide T8 (Table IV) were also sequenced by the dansyl procedure. In addition, four fragments were isolated following treatment of this peptide with thermolysin. The compositions, sequences, and electrophoretic mobilities at pH 6.5 of these thermolysin peptides (Th8a, 8b, 8c, and 8d) are shown in Table IV. Th8a and 8b were overlapped by the chymotryptic peptide C8a. The mobility of the tryptic peptide at pH 6.5 (0.18) is consistent with a peptide of molecular weight 1,900 having a net positive charge of 1. Since the molecule contains 2 histidine residues as well as a lysine, the mobility indicates that one amide and two acidic groups must be present. Th8a is an acidic peptide as indicated; therefore, the 5th residue must be aspartic acid. On the other hand, peptide Th8b is basic, which indicates that the 7th residue is in fact a glutamine. By elimination the 2nd fragment must be glutamic acid. This conclusion is confirmed by a cyanogen bromide fragment which ends in Gly-Glu-Hsr- and is consistent with an acidic charge of a glutamic acid residue.

**Tryptic Peptide T12**—Peptide T12 had the following amino acid composition: 3.8 Glu, 1.1 Gly, 1.0 Ser, 1.0 Arg. The sequence according to the dansyl procedure was as follows: Gix-Glx-Glx-Gly-Glx-Ser-Arg. The mobility of the peptide when subjected to electrophoresis at pH 6.0 was 0.28 A, which indicates that the peptide contained 2 glutamic acid residues and 2 glutamine residues. After a single Edman degradation step, the mobility of the remaining peptide was 0.28 A indicating that the 1st residue was glutamine. A second Edman degradation was done, and the mobility of the remaining peptide at pH 6.5 was 0.33 A. Since this is consistent with a peptide of molecular weight 8/6 having a net charge of -1, the 2nd residue was assigned as a glutamine. After a third Edman degradation, the residual peptide was neutral at pH 6.5. The NH₂-terminal residue was glycine which was confirmed by the peptide staining a strong yellow with ninhydrin. The final sequence was consequently deduced to be Gin-Gln-Glu-Gly-Glu-Ser-Arg.

**Tryptic Peptide T17**—This tryptic peptide (Table V) was always obtained as a complete peptide; no cleavage was ever observed after either of the internal lysine residues. The peptide was sequenced entirely by the dansyl procedure with the exception of the penultimate tryptophan, which gave no dansyl derivative. The fact that the total peptide was neutral at pH 6.5 indicated that three acidic and two amide groups were present. Although not enough of subtilisin peptide Sa was obtained after electrophoresis at pH 6.5.

**Table IV**

**Amino acid sequence of tryptic peptide T8 and of peptide fragments isolated from T8.**

After elution of the tryptic peptides from Sephadex G-75, peptide T8 was found in fraction E. When fractionated on Sephadex G-25, it eluted immediately after the void volume. Final purification was obtained after electrophoresis at pH 6.8.

<table>
<thead>
<tr>
<th>Peptide Mobility (6.5)</th>
<th>Amino acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>T8</td>
<td>0.18B 1.1 Lys 1.7 His 1.5 Asp 1.0 Thr 0.9 Ser 2.2 Glu 1.7 Gly 1.0 Ala 1.2 Met 2.8 Leu 0.8 Phe</td>
</tr>
<tr>
<td>T8a</td>
<td>0.55 0.9 Met 1.0 Asp</td>
</tr>
<tr>
<td>T8b</td>
<td>0.45B 1.0 Ser 1.0 Gly 1.1 Leu 1.0 His</td>
</tr>
<tr>
<td>T8d</td>
<td>0.50B 1.0 Thr 0.8 Leu 0.9 His</td>
</tr>
<tr>
<td>T8e</td>
<td>0.72B 1.0 Ala 1.0 Lys</td>
</tr>
<tr>
<td>C8a</td>
<td>Neutral 1.0 His 1.0 Asp 1.0 Ser 1.0 Gly 1.1 Gly 1.9 Leu 0.9 Phe</td>
</tr>
</tbody>
</table>

**Tryptic Peptide T17**—This tryptic peptide (Table V) was always obtained as a complete peptide; no cleavage was ever observed after either of the internal lysine residues. The peptide was sequenced entirely by the dansyl procedure with the exception of the penultimate tryptophan, which gave no dansyl derivative. The fact that the total peptide was neutral at pH 6.5 indicated that three acidic and two amide groups were present. Although not enough of subtilisin peptide Sa was available to do an amino acid composition, the peptide was sequenced and its mobility at pH 6.5 was established. This evidence indicated that two acidic groups were present in this peptide. Thermolysin peptide Tha was neutral and confirmed the acidic charge on the second glutamic acid. Another neutral thermolysin peptide Leu-Lys-Glu (Table VI) confirmed the charge of the 1st glutamic acid residue. The mobility of peptide Sc indicates a net basic charge of 1. After two Edman degradation steps the residual peptide was still basic, thereby identifying the 2nd residue as aspartic acid. After two more
Edman degradation steps the remaining tripeptide was neutral. Therefore, the 4th residue must be glutamine and the 5th aspartic acid.

**Tryptic Peptide T22** Peptide T22 (Table VII) contained 31 residues and as such was the largest tryptic peptide in the molecule. Since it also contains the essential histidine residue (His 195), it contains at least part of the active site region of the molecule. The essential nature of the histidine residue was identified by Woenckhaus et al. in pig heart and pig muscle lactate dehydrogenase (28), and the peptide which they isolated and sequenced from the pig lactate dehydrogenase isoenzymes is clearly homologous to a portion of this tryptic peptide from dogfish lactate dehydrogenase. The subtilisin peptide S22 and the thermolysin peptide Th22e were both neutral when subjected to electrophoresis at pH 6.5, therefore identifying the 2nd penultimate residue as asparagine. The thermolysin and chymotryptic peptides indicated in Table VII enabled us to determine the sequence of nearly all of this peptide with the exception of the 9th and 10th residues. The amino acid composition of T22 has established that these 2 residues are valine and leucine; however, the order in which these 2 residues occur has not yet been established.

**Tryptic Peptide T32**—The final large tryptic peptide (T32) contained 22 amino acid residues. Only the first 7 of these were sequenced directly. The remainder of the sequence was deduced from several internal chymotryptic, subtilisin, and thermolysin peptides (Table VII) which completely account for the entire tryptic peptide. The first 2 of these peptides (S34a and S34b) were overlapped by chymotryptic peptide S32a and thermolysin peptide S32a. Thermolysin peptide Th32h overlapped S32b and S32c. Peptide S32d ended in COOH-terminal lysine and was clearly the final peptide in the sequence. The mobility of S32a indicated that the peptide contained one aspartic acid and one asparagine. After removal of the 1st residue by the Edman procedure the remaining peptide was neutral, verifying the assignments indicated. Peptides S32c and C32b were both neutral. After the NH2-terminal residue was removed the peptide was still neutral. The 1st residue was therefore assigned as asparagine and the 2nd as aspartic acid. This tryptic peptide was quite hydrophobic and was difficult to isolate in large amounts.

**Tryptic Peptide 34**—This peptide (Table IX) contains a Lys-Pro sequence which was not susceptible to a cleavage with trypsin. Although this peptide was easily sequenced in its entirety, the amino acid composition indicated that it contained a total of 5 glutamic acids and 1 aspartic acid. Since the tryptic peptide was neutral at pH 6.5, four of these groups must exist as amides and only two as acids. Subtilisin peptide S5a had a mobility at pH 6.5 of 0.76 A; this indicates a net negative charge of 2 for a peptide having a molecular weight of 776. Carboxypeptidase identified the COOH-terminal residue of this peptide as glutamic acid. Peptide Sb contained 4 glutamates and 1 leucine and had a COOH-terminal leucine. This peptide had a mobility at pH 6.5 of 0.8 A, indicating that it,

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**Table V**

_Amino acid sequence of tryptic peptide T17_

This peptide was found in Fraction D following elution from Sephadex G-75. It was subsequently fractionated on a column of Sephadex G-25, where it eluted in the void volume. Final purification was achieved by electrophoresis at pH 6.5 and 1.9.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Mobility (6.5)</th>
<th>Amino acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>T17</td>
<td>Neutral</td>
<td>2.7 Lys 0.9 His 3.2 Asp 1.0 Thr 3.2 Glu 0.8 Pro 1.0 Gly 1.0 Leu Trp+</td>
</tr>
<tr>
<td>S-a</td>
<td>0.35A</td>
<td>2.9 Lys 1.9 Asp 1.0 Glu Trp+</td>
</tr>
<tr>
<td>S-c</td>
<td>0.39B</td>
<td>1.0 Glu 1.0 Pro 1.0 Leu 1.0 His</td>
</tr>
<tr>
<td>Th-a</td>
<td>0.55B</td>
<td>3.1 Asp 0.8 Thr 1.1 Glu 0.9 Gly 0.8 Leu 4.2 Lys</td>
</tr>
<tr>
<td>Th-b</td>
<td>0.30B</td>
<td>0.7 Lys 0.9 His 2.2 Asp 4.3 Ser 1.1 Glu 1.0 Pro 4.9 Gly 1.1 Ala 4.5 Val 0.7 Met 0.9 Ile 1.9 Leu 0.6 Cys (Cm) 0.6 Trp+</td>
</tr>
</tbody>
</table>

* Amino acid composition based on 24- and 48-hour hydrolysates.
**TABLE VIII**

Amino acid sequence of tryptic peptide T32 and of peptide fragments derived from T32

This peptide appeared in Fraction D after elution from Sephadex G-75 and eluted in the void volume when it was fractioned on a column of Sephadex G-25. It was subsequently purified by paper electrophoresis at pH 8.9 and 3.5 and by paper chromatography.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Mobility (6.5)</th>
<th>Amino acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>T32</td>
<td>0.9 Lys 0.9 His 5.1 Asp 2.0 Ser 1.0 Pro 1.1 Gly 2.3 Val 2.8 Leu 1.0 Phe*</td>
<td></td>
</tr>
<tr>
<td>T(P32)</td>
<td>1.0 Lys 0.9 His 2.6 Asp 1.1 Ser 1.0 Gly 1.5 Val 1.5 Ile 0.9 Leu Cys(Cm)+</td>
<td></td>
</tr>
<tr>
<td>S32a</td>
<td>0.76A 1.9 Asp 1.0 Val 1.1 Leu 1.0 Phe</td>
<td></td>
</tr>
<tr>
<td>S32b</td>
<td>0.38A 0.8 Cys(Cm) 0.9 Ser 1.0 Pro 1.0 Val 1.8 Leu</td>
<td></td>
</tr>
<tr>
<td>S32c</td>
<td>Neutral 0.9 His 1.7 Asp 1.1 Ser 1.4 Gly 1.1 Ile</td>
<td></td>
</tr>
<tr>
<td>S32d</td>
<td>0.49B 1.0 Asp 1.1 Val 0.8 Ile 1.1 Lys*</td>
<td></td>
</tr>
<tr>
<td>C32a</td>
<td>0.21A 1.0 Ser 1.0 Pro 0.6 Val 2.7 Leu</td>
<td></td>
</tr>
<tr>
<td>C32b</td>
<td>Neutral 0.8 His 2.2 Asp 1.0 Ser 0.9 Gly 0.9 Ile</td>
<td></td>
</tr>
<tr>
<td>Th32a</td>
<td>0.42A 1.0 Ser 1.1 Pro 1.8 Leu 0.4 Cys(Cm)</td>
<td></td>
</tr>
<tr>
<td>Th32b</td>
<td>Neutral 2.1 Asp 1.0 Gly 0.8 Val 0.9 Leu 0.8 His</td>
<td></td>
</tr>
<tr>
<td>Th32d</td>
<td>0.50B 1.0 Ile 1.0 Val 1.0 Lys*</td>
<td></td>
</tr>
</tbody>
</table>

* This peptide was not very soluble at pH 6.5 and remained at the origin following electrophoresis at that pH.

Amino acid composition based on 24- and 48-hour hydrolysates.

**TABLE IX**

Amino acid sequence of tryptic peptide T34

Following elution from Sephadex G-75 this peptide was found in Fraction E. After fractionation on Sephadex G-25 it eluted immediately after the void volume. Final purification resulted after electrophoresis at pH 6.5 and 1.9.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Mobility 6.5</th>
<th>CPA*</th>
<th>Amino acid composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>T32</td>
<td>Neutral 1.9 Lys 1.1 Asp 5.1 Glu 0.9 Pro 1.8 Leu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sa</td>
<td>0.76A Glu 4.9 Glu 1.0 Leu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sh</td>
<td>0.80A Leu 4.1 Glu 1.0 Leu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sc</td>
<td>0.72B 1.0 Glu 1.0 Lys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sd</td>
<td>Neutral 1.0 Glu 1.0 Leu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Th</td>
<td>0.34A 0.9 Lys 1.0 Asp 4.2 Glu 1.0 Pro 0.7 Leu</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* CPA indicates amino acid residues which were identified by treatment with carboxypeptidase A; residues so identified are indicated by the following designation: (−).

**DISCUSSION**

Of the total composite of tryptic peptides, there are several which stand out immediately as containing residues which are part of the active site. The first of these is peptide T19 which contains 2 such residues, a cysteine and (Ile-Ile-Gly-Ser-Gly-Cys*-Asn-Leu-Asp-Ser-Ala-Arg*) an arginine. Although this cysteine is relatively unreactive, it has been demonstrated that enzymatic activity is inhibited when this cysteine residue is modified by various chemical procedures. The sequence of this cysteine has been closely conserved in a number of lactate dehydrogenases isolated from a variety of species, including both M-type and H-type isoenzymes (10, 11). Although this cysteine has been identified in the crystal structure, it does not appear to play a direct role in the enzymatic mechanism, but rather occupies a position near the active site (29). Berghäuser and Falderbaum (30) have shown by chemical modification with phenylglyoxal that an arginine residue is involved in substrate binding. Using butanedione, Yang and Schwert (31) have confirmed that at least 2 arginine residues per subunit are essential for enzymatic activity. Although neither group has identified chemically the exact arginine residues which are essential, the crystal structure indicates that the terminal arginine residue in peptide T19 is involved in substrate binding.

The guanidinium group of this arginine clearly interacts electrostatically with the carboxyl group of pyruvate. On the basis of the crystallographic data it is apparent that this must be one of the essential arginine residues.
The other peptide which is of special interest is peptide T22. The 16th residue of this peptide is histidine. The essential nature of this particular histidine was first demonstrated by Woenckhaus et al. (28). After reacting pig M₄ lactate dehydrogenase with bromoacetyl pyridine (¹⁴C), they observed that the enzymatic activity was inhibited and that the inhibition could be accounted for by the reaction of a single histidine residue per subunit. They subsequently isolated a peptide containing this ¹⁴C-labeled histidine and demonstrated that it had the following sequence: Ile-Leu-Gly-Glu-His*-Gly-Asp-Ser-Val-Pro-Ser-Val-Trp. Although comparable experiments have not yet been done with dogfish M₄ lactate dehydrogenase, it is clear that the Woenckhaus peptide is homologous to the middle portion of tryptic peptide T22. Resolution of the crystal structure has shown that this histidine residue is most certainly involved in a proton transfer to, or from, the substrate since the imidazole ring of this histidine is oriented towards the carbonyl group of pyruvate. Peptides T19 and T22 therefore are both clearly involved at the active site region of the molecule.

This paper is the first in a series of two. In the second paper the complete amino acid sequence will be presented and the significance of some of the other regions of the molecule will be discussed more fully.

Acknowledgments—The technical assistance of S. S. Oxley and P. J. Lane is gratefully acknowledged. The continual interest and helpful advice of Dr. Michael Rossmann of Purdue University has greatly aided the progress of this work and is warmly acknowledged.

REFERENCES
In addition to the seven tryptic peptides which are discussed in detail in the text, the twenty-one tryptic peptides in Table I were isolated as described from dogfish M4 lactate dehydrogenase. The entire tryptic digest was initially fractionated on a column of Sephadex G-75. The indicated fractions were pooled and concentrated. Fractions B and C were further fractionated on Sephadex G-10 although not much additional purification was achieved. However, substantial purification was obtained after elution of fractions D, E, and F from Sephadex G-25 and these elution profiles are indicated in Figure 1. The amino acid composition of each peptide is given as well as the purification procedure used to isolate each peptide. The RF's of each peptide following electrophoresis at pH 6.5 and/or pH 2.1 are also given, and in all cases these mobilities are consistent with the calculated molecular weight for each peptide. Each of these peptides was sequenced in its entirety by the dansyl-Edman procedure. On the basis of the peptide mobilities at pH 6.5, it was possible to unambiguously assign acidic and/or amide residues in each peptide without fragmenting the peptide further.

In two instances a partial resistance to cleavage with trypsin was observed. Tryptic Peptide T6,7 was isolated as indicated, however, T6 and T7 were also isolated in significant amounts. The mobility of peptide T6 at pH 6.5 was 0.8 which is also consistent with all of the glutamates and aspartates being in the acid form. The carboxy-terminal peptide, T36, was isolated in large yields; however, Asp-Leu-Lys and free phenylalanine were also found among the tryptic peptides.
The amino acid sequence of the tryptic peptides isolated from dogfish M4 lactate dehydrogenase.
S S Taylor, W S Allison and N O Kaplan