A Peptide Containing the Essential Sulfhydryl Group of Beef Heart Lactic Dehydrogenase*

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ABSTRACT: The sulfhydryl group reagent *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM) has been used to label specifically the essential sulfhydryl groups of beef heart lactic dehydrogenase. A single DDPM-labeled pentadecapeptide was isolated from the trypsin-hydrolyzed, DDPM-treated enzyme and a partial amino acid sequence of the peptide determined. The results provide further evidence for the tetrameric nature of the enzyme molecule and suggest that the several active sites of the enzyme are identical in structure.

The successful use of serine-specific reagents for comparative studies of the primary structure of the active sites of serine-dependent mutases, esterases, and proteases (Koshland, 1960) suggested the possibility of utilizing sulfhydryl-specific reagents to compare the active sites of sulfhydryl-dependent enzymes. Previous reports from this laboratory have described the use of the yellow-colored maleimide derivative, DDPM,[1] for the specific labeling of the essential sulfhydryl groups of rabbit muscle GDH and the isolation of a labeled peptide from the pepsin-hydrolyzed DDPM-alkylated enzyme (Segal and Gold, 1963; Gold and Segal, 1964a). In the present study, DDPM was used to label specifically the essential sulfhydryl groups of a second sulfhydryl enzyme, beef heart LDH. The DDPM-labeled peptide from the trypsin-hydrolyzed, DDPM-labeled enzyme was isolated and part of the amino acid sequence was determined.

The LDH's of several vertebrate organisms have been shown to exist in five isozymic forms, two of which consist of all-heart and all-muscle chains and the other three of a mixture of chains (Markert and Møller, 1959; Cahn *et al.*, 1962; Markert, 1963; Pesce *et al.*, 1964). The beef heart enzyme is composed of four subunits (Appella and Markert, 1961) and contains a total of about 16 titratable sulfhydryl groups (Di Sabato *et al.*, 1963), 4 of which appear to be essential for enzyme activity and are concerned with the binding of the pyridine nucleotide coenzyme to the enzyme molecule (Di Sabato and Kaplan, 1963; Winer, 1963).

The present investigation provides further information regarding the chemical composition of the beef heart LDH molecule and demonstrates that each of the essential sulfhydryl groups is a component of the same peptide sequence from the several monomers.

Among the sulfhydryl-dependent dehydrogenases thus far investigated, GDH (Harris *et al.*, 1963; Perham and Harris, 1963; Gold and Segal, 1964a), alcohol dehydrogenase (Li and Vallee, 1964), and LDH, as reported here, no similarity of amino acid sequences at the active sites has become evident. Similar studies with sulfhydryl-dependent proteinases, ficin (Wong and Liener, 1964), papain (Light *et al.*, 1964), and a streptococcal proteinase (Liu *et al.*, 1965), have revealed a similarity between the two plant enzymes, but not the bacterial enzyme.

A preliminary report of some of the results in this paper has been presented (Gold and Segal, 1964b), as has one by Fondy and Everse (1964) regarding a similar, but apparently smaller peptide from frog muscle LDH.

Experimental Procedure and Results

Materials. Crystalline beef heart LDH, trypsin, DFP-treated carboxypeptidase A, and pyruvic acid, as the sodium salt, were obtained from Sigma Chemical Co., St. Louis, Mo. Carboxypeptidase B was obtained from Worthington Biochemical Corp., Freehold, N.J. DDPM was generously donated by Professor H. Tuppy. All other reagents and solvents used were the purest quality and were obtained from either Eastman Organic Chemicals or Fisher Scientific Co.

LDH and carboxypeptidases A and B were used without further purification. Trypsin was treated with dilute HCl to inactivate any contaminating chymotrypsin (Redfield and Anfinsen, 1956). The equipment and procedures used for the paper ionophoretic and talc and paper chromatographic purification steps have been previously described (Gold and Segal, 1964a).

Assay of Enzyme Activity. LDH activity was determined, as previously described (Di Sabato and Kaplan, 1963), by measuring the rate of reduction of pyruvate with DPNH at 340 mμ with a Cary recording

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[1] Abbreviations are: DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; LDH, lactic dehydrogenase; GDH, glyceraldehyde 3-phosphate dehydrogenase; PTC, phenyl isothiocyanate; PTH, phenylthiohydantoin.

spectrophotometer. The 3-ml cuvet contained 2.9 ml of 0.1 M sodium phosphate buffer, *p*H 6.9, 0.03 ml of 0.02 M sodium pyruvate, adjusted to *p*H 7.0, and 0.03 ml of 0.014 M DPNH. The reaction was initiated by adding to the cuvet 0.04 ml of an appropriate dilution of the enzyme. The enzyme activity was linear with time and the change in optical density was determined for the first 1–2 minutes of the reaction period.

Inactivation of the Enzyme with DDPM. The titration of LDH activity with DDPM was carried out by reacting 1–4 equivalents of the alkylating reagent with the enzyme. Simple mixing of the enzyme with DDPM resulted in no loss of enzyme activity, in confirmation of the previously observed refractoriness of beef heart LDH to inactivation with maleimide-alkylating reagents (Nielands, 1954). The dissociation of the enzyme into subunits upon freezing in salt solution (Markert, 1963) suggested the possibility that this treatment might expose the essential sulfhydryl groups to the alkylating reagent. This proved to be the case.

The suspension of the crystalline enzyme was centrifuged at 10,000 rpm for 20 minutes, and the pellet was dissolved in cold 0.01 M sodium phosphate buffer, *p*H 6.9, containing 0.25 M NaCl, to a concentration of about 10 mg of enzyme/ml.[2] To each reaction tube, which contained 0.015 μmole of enzyme, a small quantity of Methyl Cellosolve containing 1–4 equivalents of DDPM was added. Control tubes were prepared, one containing the enzyme alone and the other containing the enzyme plus Methyl Cellosolve. In all cases the final concentration of Methyl Cellosolve was less than 1% by volume. A test of coenzyme protection against DDPM inactivation was performed by adding 0.2 μmole of DPNH to a reaction tube containing the enzyme plus 4 equivalents of DDPM. The tubes were alternately frozen in a dry ice–Methyl Cellosolve bath and thawed in a room-temperature water bath several times. A comparison of the enzyme activity of the control and DDPM-treated enzyme mixtures showed that three to five freeze-thaw cycles were usually sufficient to produce maximum inactivation of the enzyme.

Figure 1 shows a titration of the enzyme activity with DDPM. It may be seen that there was a loss of about one-fourth of the LDH activity per equivalent of DDPM added. (In later experiments with 0.5 M NaCl instead of 0.25 M, 85–90% inactivation was routinely obtained with 4 equivalents of DDPM and fewer freeze-thaw cycles were required to produce maximum inactivation.) It was also noted that in the presence of DPNH the enzyme is completely protected against DDPM inactivation. Since Chilson *et al.* (1965) have shown that DPNH does not prevent dissociation of the enzyme into subunits under similar conditions, it may be concluded that it is the alkylation step itself which is blocked by the coenzyme. The activity of the control enzyme sample (Methyl Cellosolve but no DDPM) showed a loss of only 5–10% of enzyme activity. These results are in good accord with the previously reported *p*-mercuribenzoate titration of the beef heart enzyme (Di Sabato and Kaplan, 1963). It thus appears that DDPM reacts specifically with the essential sulfhydryl groups of LDH under these conditions and that these groups are protected by the presence of coenzyme.

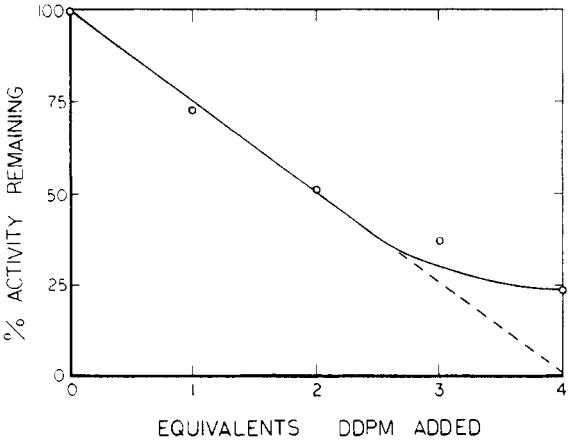

FIGURE 1: Titration of beef heart LDH activity with DDPM. Enzyme, 0.015 μmole, was present in 0.214 ml of 0.01 M phosphate buffer, *p*H 6.9, containing 0.25 M NaCl. Methyl Cellosolve, 0.001 ml, was added containing the appropriate amount of DDPM. Each reaction tube was frozen and thawed and assayed for enzyme activity after each freeze-thaw cycle. After three to five freeze-thaw cycles, maximum inactivation for the amount of DDPM added was obtained and no further change was noted; 0.2 μmole of DPNH completely protected the enzyme against inactivation with 4 equivalents of DDPM.

Preparation of DDPM-Peptide. For isolation of the DDPM-peptide for amino acid characterization, batch preparations employing 500 mg of the crystalline LDH were used. The enzyme was centrifuged, and the pellet was dissolved in cold 0.01 M phosphate buffer, *p*H 6.9, containing 0.5 M NaCl, to an enzyme concentration of approximately 10^{-4} M. An aliquot of a 50-mM solution of DDPM in Methyl Cellosolve was added to the enzyme at a molar ratio of 4.4 moles of DDPM/mole of LDH. The final concentration of Methyl Cellosolve was about 1% by volume. Uniform freezing was achieved by placing the mixture in a round-bottom flask and rapidly rotating the flask in a dry-ice bath. The frozen material was rapidly thawed by rotating the flask in a room-temperature water bath. A control aliquot of the enzyme (no DDPM) was similarly treated. Three freeze-thaw cycles were usually sufficient to label the enzyme fully with DDPM as demonstrated by a loss of about 85–90% of the enzyme activity. The absorbancy of the DDPM-treated enzyme solution at 440 mμ (the absorbtion maximum of DDPM-cysteine [Gold and Segal, 1964a]) was determined and was in agreement with the value expected.

The DDPM-labeled enzyme was precipitated by

[2] Based on a 280-mμ extinction coefficient of 2×10^3 M^{-1} cm^{-1} and a molecular weight of 135,000 (Di Sabato and Kaplan, 1963).

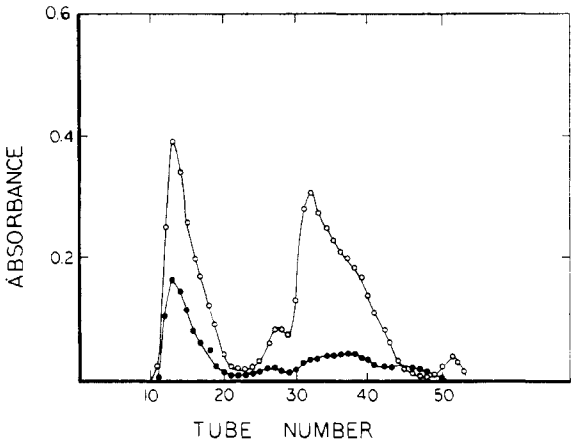

FIGURE 2: Sephadex G-25 chromatography of the trypsin-hydrolyzed DDPM-enzyme. Fractions of 5 ml each were eluted with 0.001 N ammonium acetate buffer, *p*H 8.0. Open circles, 440 mμ absorbance; filled circles, 280 mμ absorbance. The 280-mμ absorbancy values were divided by 100 for plotting.

adding solid ammonium sulfate to 90% saturation and centrifuging the mixture at 10,000 rpm for 20 minutes. The bright-yellow pellet was dissolved in 20–25 ml of cold deionized water and dialyzed in the cold against several changes of deionized water for 24 hours, during which time the clear yellow solution became turbid. The temperature of the yellow solution was brought to 37° and the *p*H was adjusted to 8.0 with 0.01 N NH_4OH. Trypsin, equivalent to 2% of the calculated weight of the LDH, was added to the solution and proteolytic digestion was allowed to proceed at 37° with periodic adjustment of the *p*H to 8.0. Trypsin proteolysis was discontinued when no further change in *p*H occurred (6–8 hours). The digestion resulted in a clear yellow solution.

Removal of trypsin and undigested DDPM-enzyme was carried out by chromatographing the digestion mixture on a Sephadex G-25 (fine beads) column, 2.5 × 80 cm in size. The trypsin digest was reduced to a volume of about 10 ml by evaporation in a rotary flash evaporator, the *p*H was adjusted to 8.0, and the material was placed on the Sephadex column which had been previously equilibrated with 0.001 N ammonium acetate buffer, *p*H 8.0. Fractions of 5 ml each were eluted from the column with the ammonium acetate buffer and the absorbancies at 280 and 440 mμ were determined on each fraction. The results of the Sephadex chromatography are shown in Figure 2. The first peak that emerged from the column representing trypsin and undigested DDPM-enzyme was subjected to a second trypsin digestion with one-half the amount of trypsin originally used. Prior to the second digestion, the volume of the pooled fractions was reduced by evaporation to 25 ml and the material was heated at 90° for 2 minutes. The heating resulted in the formation of some precipitate which was dispersed by rapid mixing. The *p*H of the solution was readjusted to 8.0 with dilute NH_4OH and the proteolytic digestion was allowed to proceed at 37° with maintenance of the *p*H at 8.0. When no further change in *p*H occurred, the digestion mixture was reduced to a volume of approximately 10 ml, applied to the Sephadex column, and eluted from the column as described. Suitable 440 mμ-absorbing fractions from the slower-moving peak were pooled and added to pooled fractions from the slower-moving major peak obtained from the first Sephadex chromatography. The combined pooled fractions represented 70 and 40% of the 440 and 280 mμ-absorbing material, respectively, subjected to the first Sephadex chromatography.

The pooled Sephadex fractions were evaporated under reduced pressure to a volume of about 10 ml, applied to a talc column, 2 × 12 cm, and washed with 4–5 column volumes of deionized water. The yellow material remained adsorbed to the upper two-thirds of the column throughout the water washings and was eluted from the column as a single band with a 50% ethanol–2% acetic acid solution. Approximately 50% of the 280 mμ-absorbing material and 10% of the 440 mμ-absorbing material were discarded in the water washings from the talc column. The *p*H of the ethanol–acetic acid fraction was adjusted to 6.5 with triethylamine and the material was evaporated to dryness, *in vacuo*, over P_2O_5. Evaporation of the ethanol–acetic acid fraction without prior neutralization resulted in the conversion of a large fraction of the yellow material into an insoluble form, possibly owing to esterification or rearrangement.

The dried yellow material was placed in solution with a small amount of water and subjected to *p*H 2 paper ionophoresis for 2 hours at a 5-kv potential on a 120-cm sheet (Gold and Segal, 1964a). In addition to the yellow band which moved out from the origin, a minor band of yellow material remained at the origin, as is characteristic with DDPM-peptides (Witter and Tuppy, 1960). The major band of yellow material was cut out, eluted from the paper with a 10% acetic acid solution, and dried *in vacuo*. Upon developing the rest of the paper strip with a 0.25% solution of ninhydrin in acetone, it was seen that the yellow material was well separated from several colorless, ninhydrin-positive bands, but was contaminated with a light background of ninhydrin-positive material. The dried yellow material was subjected to a second *p*H 2 paper ionophoresis for 3 hours. The yellow material again moved as a single band and was now separated from the background ninhydrin-positive material. When part of the yellow material was left on the paper and subjected to the ninhydrin development procedure, it became dark brown. The DDPM-peptide was further purified by two successive descending paper chromatography steps with butanol–acetic acid–water (4:1:5) and butanol-pyridine-water (4:1:5) as the developing solvents. In both cases the major portion of the yellow material migrated as a single, well-defined band separated from several minor, yellow-colored bands as well as several colorless, ninhydrin-positive bands. The purified DDPM-peptide, in about a 50% yield of the 440 mμ-absorbing material

recovered from the Sephadex chromatography steps, was tested for purity by *p*H 4.7 paper ionophoresis (Gold and Segal, 1964a). The yellow band remained at the origin and no other ninhydrin-positive or yellow material appeared.

Amino Acid Characterization of the DDPM-Peptide. The quantitative amino acid analyses of the peptides isolated from four separate enzyme preparations are presented in Table I.[3] Hydrolysis of the peptide was carried out in sealed evacuated glass tubes with constant-boiling HCl–1% thioglycolic acid solution at 105° for 24 hours. Examination of the amino acid ratios relative to 2-amino-2-carboxyethylmercaptosuccinic acid, the hydrolysis product of DDPM-cysteine, indicated that the DDPM-peptide isolated from DDPM-labeled LDH was a pentadecapeptide. Tryptophan analysis of sample 3, according to the method of Spies and Chambers (1949), showed the presence of only 0.18 μmole of the amino acid / μmole of the peptide. The neutral behavior of the peptide at *p*H 4.7 indicates that 2 of the 3 aspartic acid residues exist in the peptide as the amides.

The sequence of the three amino acids at the amino-terminal end of the peptide was determined by three successive steps of the quantitative subtractive Edman reaction as described by Konigsberg and Hill (1962). The chromatographic purification step between each cycle of the reaction was omitted, and after reaction of the peptide with PTC, cyclization, and extraction of the PTH amino acid derivative, as previously described (Gold and Segal, 1964a), the peptide was subjected to a quantitative amino acid analysis. The results are shown in Table II, where the ratio of amino acids remaining, relative to 2-amino-2-carboxyethylmercaptosuccinic acid, is tabulated after each cycle. A repetition of the Edman degradation gave substantially identical results. The results indicate that the sequence of the amino acids at the amino-terminal end of the peptide is Val-Ile-Asn-. The assignment of asparagine rather than aspartic acid is based on the fact that the DDPM-peptide was still neutral upon *p*H 4.7 paper ionophoresis after the third cycle of the reaction.

The partial sequence of amino acids at the carboxy terminus was determined from a quantitative analysis of the rate of release of amino acids from the peptide as a result of the simultaneous action of carboxypeptidases A and B. Carboxypeptidase B was utilized for the removal of arginine (Folk and Gladner, 1958) to permit further hydrolysis of the peptide by carboxypeptidase A. That carboxypeptidase B did hydrolyze arginine from the peptide was noted from the observation that the peptide behaved as an acidic compound upon *p*H 4.7 paper ionophoresis after incubation with the enzyme according to the procedure of Winstead and Wold (1964). The simultaneous incubation of the peptide with the two enzymes was carried out with 0.2–0.3 μmole of

TABLE I: Amino Acid Composition of DDPM-Peptide.[a]

Amino Acid[b]	Ratio Relative to 2-Amino-2-carboxyethylmercaptosuccinic Acid (ACSA)[c] I	II	III	IV	Average[e]
ACSA[c]	1[d]	1[d]	1[d]	1[d]	1
Arginine	1.36	1.40	1.33	1.13	1
Aspartic acid	2.90	3.09	2.83	2.83	3
Serine	2.10	1.78	2.28	1.76	2
Glycine	2.70	3.03	2.85	3.21	3
Alanine	1.85	1.58	1.49	1.37	1
Valine	1.36	1.09	1.27	1.56	1
Isoleucine	1.10	1.46	1.10	1.32	1
Leucine	1.99	1.93	1.78	2.20	2

[a] Peptide (0.2 μmole), as calculated from the 440-mμ absorbancy, was hydrolyzed and an equal portion applied to each column of the analyzer. [b] No more than trace amounts of other amino acids were found except glutamic acid, which was present in amounts approaching 1 residue in hydrolysates I and IV. Since glutamic acid was not present in significant amounts in hydrolysates II and III, it is considered to be a contaminant. [c] The hydrolysis product of DDPM-cysteine. [d] The actual analyses of ACSA were 0.112, 0.112, 0.107, and 0.113 μmole, respectively. [e] To nearest integer.

TABLE II: Amino Acid Composition of DDPM-Peptide after Three Cycles of the Edman Reaction.[a]

Amino Acid	Ratio Relative to ACSA[b] Cycle 1st	2nd	3rd
ACSA[b]	1[c]	1[c]	1[c]
Aspartic acid	2.76	3.23	*1.68*[d]
Serine	2.07	2.05	2.59
Glycine	2.79	3.34	3.40
Alanine	1.42	1.74	2.00
Valine	*0.36*[d]	0.55	Nil
Isoleucine	1.35	*0.61*[d]	0.62
Leucine	1.52	1.94	2.31

[a] Peptide (0.3 μmole), as determined from the 440-mμ absorbancy, was taken as the starting material. One-third was hydrolyzed after each cycle. [b] See Table I, footnote *c*. [c] The actual analyses for ACSA were 0.072, 0.062, and 0.042 μmole, respectively. [d] The amino acid removed in each cycle is italicized.

[3] The authors are indebted to Mr. Mas Yamada of the Botany Department, Washington University, St. Louis, Mo., for these analyses.

the peptide. A carboxypeptidase A solution was prepared by adding a suspension of the enzyme to cold H_2O, dissolving the enzyme with 0.1 N NH_4OH, and quickly adjusting the *p*H of the solution to 8.0 with dilute acetic acid. An aliquot of the enzyme solution was added to 0.2 μmole of the peptide such that a molar ratio of substrate to enzyme of 50:1 resulted (based on a molecular weight of 34,400 for the carboxypeptidase). To this solution, 20 μg of carboxypeptidase B was added, and the mixture was allowed to incubate at 37° for 0.5-hour and 12-hour periods. At the end of each reaction period, aliquots were withdrawn and the hydrolysis was stopped by heating the mixture at 100° for 1 minute. The heated samples were cooled and centrifuged, and the resultant clear supernatant fractions were removed, dried *in vacuo*, and subjected to a quantitative amino acid analysis. The results of the analyses are shown in Table III. There was a rapid release of alanine followed by a slower release of leucine and asparagine. The combined amino-terminal and carboxy-terminal analyses indicate the sequence: Val-Ile-Asn-(Asp, Gly_3, Leu, Ser_2, DDPM-Cys)-Asn-Leu-Ala-Arg.

TABLE III: Quantitative Analysis of Neutral and Acidic Amino Acids Released from DDPM-Peptide by Carboxypeptidases A and B.[a]

Amino Acid[b]	Time of Incubation	
	0.5 hr	12 hr
Alanine	0.108	0.145
Leucine	0.070	0.142
Asparagine	Trace	0.070

[a] Peptide (0.1 μmole), as determined by the 440-mμ absorbancy, was incubated for each time period. Results are expressed as μmoles of amino acids released. [b] Only small amounts of other amino acids were found.

Discussion

The ability to describe the mechanism of an enzyme-catalyzed reaction in terms applicable to other organic reactions requires a knowledge of the participating atoms and their orientation. Identification of the amino acid sequences in the peptide chains containing groups which take part in the reaction represents progress in this direction. Studies of this type with enzymes containing serine in the active site have permitted certain conclusions as to how these enzymes might function (Koshland, 1960; Hummel and Kalnitsky, 1964). Analogous investigations with sulfhydryl-dependent enzymes have appeared in the cases of the dehydrogenases, GDH (Harris *et al.*, 1963; Perham and Harris, 1963; Gold and Segal, 1964a), liver alcohol dehydrogenase (Li and Vallee, 1964), and the present report, and the proteinases, ficin (Wong and Liener, 1964), papain (Light *et al.*, 1964), and a streptococcal proteinase (Liu *et al.*, 1965). Unlike many of the serine-specific enzymes thus far studied, the amino acid composition and sequences of the peptides from sulfhydryl-dependent enzymes has shown no similarity in the primary structure of the active sites except for the two plant proteinases.

In the present investigation the specificity of labeling of the essential sulfhydryl groups of beef heart LDH was shown by the stoichiometric relation between equivalent of reagent and inhibition of LDH activity. As in all such experiments, an uncertainty exists as to whether the sulfhydryl group in question is functionally or merely structurally essential, although the protection by DPNH lends support to the former interpretation. The purification of the labeled peptide was facilitated by the adsorbability of DDPM on talc and the ready visual localization of the labeled peptides in the paper ionophoretic and chromatographic isolation procedures. The recovery of 70% or more of the theoretical yield in the peptide mixture obtained after trypsin digestion and the observation of, at most, minor yellow contaminants in the several subsequent purification steps indicates that substantially all of the essentia. sulfhydryl groups are constituents of the same peptide. This result is consistent with earlier evidence to the effect that the enzyme isolated from beef heart is largely a tetramer composed of identical chains (Appella and Markert, 1961; Markert, 1963; Di Sabato and Kaplan, 1964).

Inspection of the amino acid composition of the DDPM-peptide shows a relatively high proportion of nonpolar residues in the vicinity of the essential sulfhydryl group in contrast to the case with rabbit muscle GDH (Harris *et al.*, 1963; Perham and Harris, 1963; Gold and Segal, 1964a), and suggests that this portion of the protein chain is submerged in a hydrophobic phase in the interior of the molecule. Such an orientation would be consistent with the marked lack of reactivity of the essential sulfhydryl groups of the native enzyme with alkylating reagents. A similarly observed lack of reactivity of rabbit muscle LDH with iodoacetamide (Dube *et al.*, 1963) suggests that the essential sulfhydryl groups of this enzyme may also be contained in largely nonpolar peptides.

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Increased Specific Activity and Formation of an Inhibitor from the LDH_5 Isozyme of Lactate Dehydrogenase*

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ABSTRACT: The specific activity of the LDH_5 isozyme of crystalline rabbit muscle lactate dehydrogenase can be increased 200% by means of sucrose density gradient centrifugation or Sephadex G-200 molecular sieving of the enzyme in the presence of low concentrations of β-mercaptoethanol. Also separated by the procedure is a denser protein fraction that is inhibitory to LDH_5. In contrast to the original LDH_5, the LDH_5 that is activated by β-mercaptoethanol treatment contains no detectable reduced nicotinamide–adenine dinucleotide X (NADH-X), while the inhibitor fraction contains approximately three times as much presumed NADH-X per mole of protein. The addition of excess NADH to the sucrose density gradient prevents the separation of LDH_5 into the activated and inhibitory fractions. The LDH_1 isozyme is not affected by the β-mercaptoethanol treatment and is not inhibited by the LDH_5 inhibitor fraction.

We have found that in the presence of low concentrations of β-mercaptoethanol there are substantial and significant changes in the properties of the molecule of LDH_5 isozyme of lactate dehydrogenase. In the studies described here it is shown that LDH_5 in the presence of β-mercaptoethanol can be separated by sucrose density gradient sedimentation and Sephadex G-200 chromatography into essentially two protein fractions, one with a high specific activity and the other with no enzymatic activity and an inhibitory action to LDH_5.

Methods

Rabbit muscle lactate dehydrogenase, substantially free of pyruvate kinase, was obtained as a three-times-crystallized preparation from Sigma Chemical Co. The enzyme was dialyzed against 0.001 M Tris-HCl buffer, *p*H 7.5, and separated into the five isozyme fractions by DEAE-cellulose chromatography as previously described (Gelderman *et al.*, 1965). LDH_5 is defined as the cathodal isozyme by electrophoresis or that isozyme not adsorbed to DEAE-cellulose. A value of 1.45/mg

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