Synthesis and Degradation of Rat Liver Lactate Dehydrogenase M₄

SYNTHESIS AND DEGRADATION OF Ratin Liver LACTATE DEHYDROGENASE ISOENZYMES*

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SUMMARY

For the measurement of lactate dehydrogenase (LDH) isoenzyme biosynthesis a new method is presented which is based on the quantitative hybridization of isoenzyme M₄ with isoenzyme H₄. After reversible dissociation the formation of active LDH M₄ was more rapid and occurred to a greater extent than the formation of LDH H₄. The formation of hybrid isoenzymes from the two subunits resulted in an intermediate total activity. In the reaction mixture, 1 mM NADH resulted in a quantitative recovery of all isoenzymes. Rat liver proteins were labeled after a single injection of [³H]leucine or in double labeling experiments after injections of [¹⁴C]leucine and [³H]leucine. LDH M₄ (LDH₅) of the liver homogenate was separated from the other isoenzymes by DEAE-Sephadex chromatography. The LDH M₄ fraction was subjected to a freeze-thaw cycle in neutral phosphate buffer containing 1 M NaCl and 1 mM NADH. A second DEAE-Sephadex chromatography was performed and the LDH M₄ fraction obtained was hybridized with nonradioactive LDH H₄ with the freeze-thaw technique. A third chromatography on DEAE-Sephadex was performed. The hybridized LDH M subunits attached to the column were eluted with a NaCl gradient. The radioactivity eluted was localized to the fractions containing LDH H₄M₀, LDH H₄M₂, and LDH H₄M₅. The radioactivity per unit of LDH M₄ activity in the liver homogenate was calculated. Using this technique, it was demonstrated that cycloheximide, but not actinomycin D, inhibited the incorporation of radioactive leucine into LDH M₄ isoenzymes, and the restoration of dehydrogenase activity during reassociation of the H and M monomers, and mixtures of them. The results demonstrate that the reassociation of M subunits is more rapid and more extensive than that of the H subunits and, furthermore, that the presence of 1 mM NADH increased the rate of reassociation of the H monomers to that of the M subunits. These results have been used to examine the turnover of rat liver LDH M₄, using the specific reassociation with H subunits for their isolation.

MATERIALS AND METHODS

Chemicals

NADH and NAD⁺ were purchased from Boehringer Mannheim, Sephadex G-25 and DEAE-Sephadex A-50 from Pharmacia, actinomycin D from Merck, Sharp & Dohme, and cycloheximide from Sigma.

Determination of Total Lactate Dehydrogenase Activity

The activity of lactate dehydrogenase was determined in 0.067 M phosphate buffer, pH 7.4, at 25°C in the presence of 1 mM pyruvate and 0.13 mM NADH. The reaction rate was measured by following the oxidation of NADH in a Gilford recording spectrophotometer. The enzyme activity is expressed as international units (i.u. = change in micromoles of substrate per min).

1 The abbreviations of lactate dehydrogenase (LDH) isoenzymes used as synonyms and the order of the migration rate of the isoenzymes toward the anode in electrophoresis: LDH₁ = LDH H₁ > LDH H₂ > LDH H₃ > LDH H₄ > LDH H₅. LDH H₆ = LDH H₅M > LDH H₆M > LDH H₇M > LDH H₄M > LDH H₃M > LDH H₂M > LDH H₁M = LDH M₄.

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Determination of Lactate Dehydrogenase Isoenzyme Pattern

The lactate dehydrogenase isoenzymes were separated by using cellulose acetate electrophoresis (Microzone). The same number of lactate dehydrogenase units, 0.25 µl of the enzyme solution containing 1500 i. u. per liter, was applied to the electrophoresis membrane by a spot application. The isoenzymes were visualized by staining with Nitro-BT (3). The percentage of distribution of the various isoenzymes was determined by scanning the electrophorograms with a Beckman model R-110 densitometer. The subunit M percentage of lactate dehydrogenase was calculated from the isoenzyme pattern by assuming that the subunit M forms 0, 25, 50, 75 and 100% of the lactate dehydrogenase isoenzymes 1, 2, 3, 4, and 5, respectively.

Separation of Rat LDH H4 and LDH M4

Rat heart and liver tissues were homogenized each 1:5 (w/v) in 0.05 M Tris-HCl, pH 7.5, with an Ultra-Turrax homogenizer and centrifuged at 15,000 × g for 30 min.

Ion exchange chromatography, resembling that used by Fritz et al. (4), was used for the separation of lactate dehydrogenase isoenzymes. A 2 ml of the sample were applied to a DEAE-Sephadex A-50 column, 1 × 20 cm, equilibrated with 0.05 M Tris-HCl, pH 7.5. The enzyme fractions were eluted from the column by a linear salt gradient up to 0.3 M NaCl, established by 200 ml of 0.05 M Tris-HCl and 200 ml of 0.05 M Tris-HCl-0.3 M NaCl. Fractions (3 ml) were collected from which lactate dehydrogenase activities and NaCl concentrations were determined. The isoenzyme LDH4 is not bound to the ion exchanger, but is eluted in the front with the initial buffer. LDH1 is eluted in the fractions containing 0.20 to 0.23 M NaCl.

For the rapid separation of rat LDH1 from LDH4, 2 ml of the sample in 0.05 M Tris, pH 7.5, prepared as above, were applied to a DEAE-Sephadex A-50 column, 1 × 10 cm, and were eluted with 30 ml of the same buffer. LDH1 was eluted in the first 6 ml. The rest of the eluate did not contain detectable amounts of lactate dehydrogenase. LDH4 was collected by adding 0.05 M Tris-HCl-0.3 M NaCl to the column. For the separation of LDH1 from LDH4, conditions were selected under which LDH4 flowed through the column with the buffer front, whereas LDH1 remained bound and could thereby be eluted. Only LDH1 remained bound to the ion exchanger when the column and sample were i 0.05 M Tris-HCl, pH 7.5, and 0.16 M NaCl. Izenzymes LDH4 were eluted in the first 10 ml. After all of the buffer-0.16 M NaCl solution had flowed through (30 ml), LDH1 was eluted with 0.05 M Tris-HCl-0.3 M NaCl.

Dissociation Procedure

The freeze-thaw dissociation method of Markert (6) was used. Since the dissociation was to take place in phosphate buffer, the Tris-HCl buffer of the enzyme sample was first changed to 0.1 M sodium phosphate, pH 7.0, using Sephadex G-25 columns equilibrated with this buffer. The samples were then divided into two 1-ml portions, one of which was quickly frozen by immersing the test tube into ethanol previously cooled to -70°, kept at this temperature for 30 min, and thereafter thawed in a water bath at 25°. The other sample was maintained at 25° and served as the control. The recovery of lactate dehydrogenase activity was followed by sampling the thawed specimen at various times as indicated in the text. The recovery of enzyme activity was expressed as a percentage of the control activity.

Labeling of Liver Protein in Vivo—Young adult male Sprague-Dawley rats weighing about 100 g were used in all incorporation experiments. In preliminary experiments, rats were given intraperitoneally 500 µCi of t-[4,5-3H]leucine (50 Ci/mmole, The Radiochemical Centre, Amersham). After 4 hours the animals were anesthetized with ether and bled, and livers were removed.

In another series of experiments involving double label analysis of protein turnover according to Arias et al. (6), the rats were administered 20 µCi of L-[U-14]Cleucine (331 µCi/mmole, The Radiochemical Centre, Amersham) in 0.9% sodium chloride on day 0, and 4 hours later an injection of 40 mg of cold l-leucine in 2 ml of 0.9% sodium chloride, after which the animals were given water containing 8 mg of cold l-leucine per liter and food ad libitum. The animals were given 100 µCi of t-[4,5-3H]leucine in 0.9% sodium chloride 4 hours before being killed on days 2, 4, 6, and 8.

Fig. 1. Principle for the separation of radioactive LDH1 (= LDH M4) subunits from other radioactive proteins by hybridization with nonradioactive LDH1 (= LDH H4).

About 2 g of liver were homogenized in 2 volumes of cold 0.2 M sodium acetate buffer, pH 4.2 (final pH 5.2), and centrifuged at 4° at 15,000 × g for 30 min. The supernatant was used for the analysis of both lactate dehydrogenase activity and isoenzyme pattern. Protein concentrations were assayed according to Lowry et al. (7).

Purification of Radioactive Rat Liver LDH M4 with Hybridization—The following procedure was adopted for the hybridization of labeled LDH M4 with unlabeled LDH H4. The steps are numbered and correspond to the ion exchange chromatographies in Fig. 1.

(I) The buffer of the liver homogenate was changed to 0.05 M Tris-HCl, pH 7.5, using a Sephadex G-25 column. The first DEAE-Sephadex A-50 chromatography was performed and the LDH M4 fraction was isolated as described above.

(II) The buffer of the lactate dehydrogenase fraction was changed to 0.1 M sodium phosphate, pH 7.0, with gel filtration through Sephadex G-25. A 1/4 volume of 5 mM NADH-5 M NaCl was added and the sample was frozen to -70°. After thawing, the buffer was exchanged to 0.05 M Tris-HCl, pH 7.5, with a Sephadex G-25 column. The second DEAE-cellulose chromatography was performed and the LDH M4 fraction was again collected. This step was found to be necessary in order to avoid nonspecific distribution of radioactivity in the next step chromatography.

(III) The buffer composition of the sample was again changed to 0.1 M sodium phosphate, pH 7.0. The enzyme activity was measured and an equal amount of units of nonradioactive LDH H4 was added. Thereafter a 1/4 volume of 5 mM NADH-5 M NaCl was added, and the samples were frozen at -70°. After thawing, the samples were run through Sephadex G-25 and equilibrated with 0.05 M Tris-HCl, pH 7.5. The sample was run through a third DEAE-Sephadex A-50 column and the LDH M4 fraction again collected. The DEAE-Sephadex column was then eluted with 50 ml of the Tris buffer and the hybridized fractions were collected, either by a linear salt gradient of up to 0.3 M NaCl or by 0.05 M Tris-HCl, pH 7.5, containing 0.3 M NaCl.

From the hybridized sample, lactate dehydrogenase isoenzyme patterns were determined by cellulose acetate electrophoresis. Ninety-five per cent of the total M subunits were found in the hybrid species. This is a higher percentage than would be expected (87.5%) after combination of equal amounts of both sub-
units. This distribution is due to the higher specific activity of LDH M4 when compared to LDH H4 (8) and to the fact that equal amounts of activity were combined. In calculations of the enzyme activity or radioactivity in the original liver sample, a correction for this skewness was applied.

Procedure for Measurement of Radioactivity of Lactate Dehydrogenase—On the fractions obtained from the DEAE-Sephadex chromatography, lactate dehydrogenase assays were performed, after diluting 200 μg of carrier albumin was added. Cold 20% trichloroacetic acid (2 ml) was added to each fraction, and the precipitates were collected on Whatman GF/A glass fiber filters, washed twice with 5 ml of 5% trichloroacetic acid-0.5 mM L-leucine, and the filters were transferred to polyethylene counting vials. The protein precipitate was wetted with 0.1 ml of water and the proteins were dissolved in 0.5 ml of NCS solubilizer (Amer sham-Corning Corp.).

Radioactivity of Rat Liver Total Proteins—Rat liver (200 mg) was homogenized 1:20 (w/v) in 0.14 M NaCl with Potter-Elvehjem homogenizer. The homogenate (2 ml) was mixed with 2 ml of ice-cold 10% trichloroacetic acid, incubated for 30 min in an ice bath, and centrifuged for 5 min at 4000 × g. To the precipitated pellet 5 ml of 5% trichloroacetic acid were added, thereafter the solution was maintained for 30 min at 95° in a water bath, cooled, and centrifuged for 5 min at 4000 × g. The precipitate was then washed successively with 2% (w/v) sodium acetate in 90% (v/v) ethanol, ethanol-ether (3:1), and ether, centrifuging after each wash. The precipitate was dried at 60° and dissolved in 1 ml of 0.3 M KOH overnight at 37°. From the alkali digest, a sample was taken for the determination of proteins. For measurement of radioactivity, 100 μl of the digest were mixed with 10 ml of Instagel (Packard).

Radioactivity was expressed as cpm per mg of protein or as cpm of 14C per cpm of 3H in the double labeling experiments. Radioactivity of Rat Liver RNA—Inoculation of 100 μCi of [5-3H]orotic acid (17 Ci per mmol, The Radiochemical Centre, Amersham) was made intraperitoneally 4 hours before killing the animals. Liver tissue was homogenized in 0.2 M sodium acetate buffer, pH 4.2. After centrifugation at the 15,000 × g, the buffer composition of the supernatant of the homogenate was changed to 0.05 M sodium phosphate, pH 6.0. After concentration by ultrafiltration, the sample was chromatographed on a Sephadex G-25 column with a linear gradient prepared with 0.05 M Tris-HCl, pH 7.5, and 0.05 M Tris-HCl-0.3 M NaCl. The LDH M4 fraction obtained was concentrated in the Amicon ultrafiltration apparatus. After changing the buffer as above, the sample was chromatographed on a DEAE-Sephadex A-50 column, 1 × 20 cm, with a linear salt gradient constructed with 0.05 M Tris-HCl, pH 7.5, and 0.05 M Tris-HCl-0.3 M NaCl. The LDH M4 fraction obtained was concentrated in the Amicon ultrafiltration apparatus. After changing the buffer as above, the sample was chromatographed on a Sephadex G-200 column. At each step, lactate dehydrogenase activity and protein concentration were measured. After the final purification step, the protein migrated as one band in polyacrylamide electrophoresis without (11) and with sodium dodecyl sulfate (12).

RESULTS

Dissociation-Association of Lactate Dehydrogenase

Time Dependence of Recovery Enzyme Activity—Rat LDH H4, LDH M4, and a mixture of the two (1:1) were diluted first with phosphate buffer to contain 5000 i.u. per liter and were then-diluted 4:1 with 5 M NaCl in phosphate buffer. At various times after the freeze-thaw, samples were taken for the measurement of lactate dehydrogenase activity and recoveries were calculated. Fig. 2 demonstrates that the activity of M4 is rapidly restored, whereas the activity of LDH H4 is restored slowly and reaches only 30% after 2 hours of incubation. Freeze-thaw of the mixture of the two homologous isoenzymes, LDH H4 and LDH M4 led to the formation of hybrid isoenzymes. The recovery of activity of the hybridized enzymes is between those of the homologous isoenzymes (Fig. 2).

Dependence of Recovery of Activity on Enzyme Concentration—The freeze-thaw dissociation was performed as in Fig. 2. The LDH M4 and LDH H4 concentrations varied from 400 to 4000 i.u. per liter. Reactivation time was 120 min. Fig. 3 demonstrates that the recovery of enzyme activity depends on enzyme concentration, as negligible amounts of LDH H4 are recovered with less than 1000 i.u. per liter present and the recovered LDH M4 is only a fraction of that found with a higher enzyme concentration.

From Figs. 2 and 3 it can be concluded that the association of lactate dehydrogenase into the active enzyme is dependent upon time and also enzyme concentration. The recovery of LDH M4 was always greater than that of LDH H4 and the formation of
hybrid lactate dehydrogenase molecules restored intermediate enzyme activities.

Effect of Nicotinamide Adenine Dinucleotide on Recovery of Lactate Dehydrogenase Activity after Freeze-Thaw Hybridization—In the absence of NADH in the reaction mixture, the recovery of LDH M₄ was 84% in 120 min (Fig. 4). When NADH was included in the incubation mixture, slightly more rapid activation could be obtained 10 min after the thawing, but the recovery of enzyme activity was unchanged after 2 hours.

The mixture of subunits M and H resulted in a recovery of 46% in the absence of NADH. However, when the reduced coenzyme was added to the mixture, an activating effect could be noted, with the recovery of 79% in the presence of 1 μM NADH (Fig. 5).

As shown in Fig. 6, 16% of LDH H₄ activity was recovered in the absence of the reduced coenzyme. The addition of various amounts of NADH to the reactivation mixture increased the recovery of the activity, so that in the presence of 1 mM NADH 81% of the original activity was restored.

When the freeze-thaw dissociation was performed in the presence of NAD⁺ (0.01 to 1.0 mM), no effect on the reactivation was seen (Fig. 7).

Validation of Hybridization Method for Lactate Dehydrogenase Turnover Studies—The principle of the use of hybridization in lactate dehydrogenase purification is given in Fig. 1. The hybridization method could be considered useful for the measurement of lactate dehydrogenase turnover only if it could be shown that radioactivity from the protein fraction containing the labeled LDH M₄ was hybridized only with the unlabeled LDH H₄. This means that, upon hybridization, radioactivity should be found only in the fractions containing lactate dehydrogenase. This condition could not be met by simple hybridization of the labeled M₄ fraction with unlabeled H₄ as shown in Fig. 8, in which the radioactivity did not fit the peaks of lactate dehydrogenase. If the freeze-thaw hybridization caused a degradation of proteins and thus altered their charge resulting in a new behavior in the chromatography, an additional freeze-thaw cycle and chromatography before the hybridization would eliminate the disturbing radioactivity. When this "sham" hybridization was performed, whereby LDH M₄ was dissociated and run once more through the DEAE-Sephadex column before its hybridization with LDH H₄, the expected distribution of radioactivity on the lactate dehydrogenase peaks was obtained in the third chromatography (Fig. 9).

In the following experiments of incorporation of leucine into LDH M₄, the background was first eluted with 50 ml of the
buffer and the hybridized M subunits attached to the column were eluted with high salt concentration as presented under "Materials and Methods."

**Effect of Protein Synthesis Inhibitors**

When the animals were treated with actinomycin D prior to the administration of the radioactive amino acid, no change in the incorporation as expressed as cpm per unit of lactate dehydrogenase or as cpm per mg of protein was noted. Actinomycin D inhibited the incorporation of orotic acid into RNA, indicating that the inhibitor reached the cells. However, when the rats were pretreated with cycloheximide, the incorporation of leucine to lactate dehydrogenase and proteins was almost totally inhibited (Table I).

**Degradation of Rat Liver LDH M₄**

The half-life of rat liver LDH M₄ was studied by injecting \( L-\text{[14C]} \)-leucine on day 0 and \( L-\text{[3H]} \)-leucine 4 hours before killing on days 2, 4, 6, and 8. All the liver samples were kept at 70°C and processed for radioactivity determinations at the same time. When the logarithm of radioactivity per unit of lactate dehydrogenase activity is plotted against the day after the start of the experiment (Fig. 10), a straight line is obtained, from which a half-life of 4.21 days is calculated. With the double labeling technique, the half-life for rat liver LDH M₄ is 4.04 days (Fig. 10).

For total protein, the half-life calculated from the specific radioactivity is 3.45 days, whereas that with the double labeling method is 3.74 days (Fig. 11).

**Synthesis Rate of Rat Liver M₄**

In the steady state, the synthesis and degradation of enzymes are equal. The degradation of an enzyme molecule is a first order process, depending on the concentration of the enzyme present, whereas, on the other hand, the synthesis of an enzyme molecule is a zero order process (1).
TABLE I
Effect of actinomycin D and cycloheximide on the synthesis of rat liver LDH M₄, total proteins, and RNA

Results of four experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LDH ¹</th>
<th>Protein ²</th>
<th>RNA ³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/unit</td>
<td>mg</td>
<td>cpm/mg</td>
</tr>
<tr>
<td>Control</td>
<td>4.92 ± 0.97</td>
<td>3343 ± 416</td>
<td>57,875 ± 5836</td>
</tr>
<tr>
<td>Actinomycin D, 2.5 mg per kg</td>
<td>5.48 ± 0.57</td>
<td>3585 ± 232</td>
<td>16,250 ± 4672</td>
</tr>
<tr>
<td>Cycloheximide, 8 mg per kg</td>
<td>0.12 ± 0.17</td>
<td>296 ± 40</td>
<td>37,250 ± 8462</td>
</tr>
</tbody>
</table>

- Actinomycin D, 2.5 mg per kg, was given 90 min before the isotope.
- Cycloheximide, 8 mg per kg, was given 15 min before the isotope.
- The synthesis measured as the specific radioactivity of rat liver LDH M₄ or proteins 4 hours after the injection of L-[3H]-leucine.
- The synthesis measured as the specific radioactivity of rat liver RNA 4 hours after the injection of [3H]orotate.
- The values, compared to the control group, n.s. = not significant (Student's t test).

TABLE II
Half-lives and degradation coefficients of LDH M₄ and total proteins in rat liver

<table>
<thead>
<tr>
<th></th>
<th>t₁/₂</th>
<th>kₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH M₄</td>
<td>4.01</td>
<td>0.173 day⁻¹</td>
</tr>
<tr>
<td>LDH M₄</td>
<td>4.21</td>
<td>0.165 day⁻¹</td>
</tr>
<tr>
<td>Total proteins</td>
<td>3.45</td>
<td>0.201 day⁻¹</td>
</tr>
<tr>
<td>Total proteins</td>
<td>3.74</td>
<td>0.185 day⁻¹</td>
</tr>
</tbody>
</table>

- Calculated from cpm of ¹⁴C per unit of lactate dehydrogenase or mg of protein.
- Calculated from cpm of ¹³C per cpm of ³H.

TABLE III
Lactate dehydrogenase activity in rat liver during the study of the degradation of the enzyme

Results of three experiments.

<table>
<thead>
<tr>
<th>Days</th>
<th>Lactate dehydrogenase activity</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>i.u./g dry wt.</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>570 ± 70</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>541 ± 61</td>
<td>n.s.</td>
</tr>
<tr>
<td>4</td>
<td>471 ± 39</td>
<td>n.s.</td>
</tr>
<tr>
<td>6</td>
<td>500 ± 62</td>
<td>n.s.</td>
</tr>
<tr>
<td>Mean</td>
<td>516 ± 59</td>
<td></td>
</tr>
</tbody>
</table>

- Difference not significant (p > 0.1), when compared to the 0-day value with Student's t test.

Taking the appropriate values from Tables II and III, we obtain

\[ kₐ = 0.173 \text{ day}^{-1} \times 516 \text{ units.g}^{-1} \]  \hspace{1cm} (4)
\[ kₐ = 89.3 \text{ units.g}^{-1} \cdot \text{day}^{-1} \]  \hspace{1cm} (5)

In order to convert units of enzyme activity into moles of enzyme, lactate dehydrogenase was extensively purified as described under “Materials and Methods.” The results of this purification are given in Table IV and indicate a purification at least as good as has been described elsewhere (8). When the specific activity of purified rat LDH M₄ is taken from Table IV, the equation can be rewritten as

\[ kₐ = 0.328 \text{ mg.g}^{-1} \cdot \text{day}^{-1} \]  \hspace{1cm} (6)

and, assuming the molecular weight of lactate dehydrogenase...
as 140,000 (8),

\[ k_2 = 2.34 \times 10^{-3} \text{ mol} \cdot \text{g}^{-1} \cdot \text{day}^{-1} \]  

(7)
multiplying with the Avogradro number

\[ k_1 = 1.41 \times 10^{14} \text{ molecules} \cdot \text{g}^{-1} \cdot \text{day}^{-1} \]  

(8)
and the number of cells per g in rat liver (13)

\[ k_4 = 7.27 \times 10^4 \text{ molecules} \cdot \text{cell}^{-1} \cdot \text{day}^{-1} \]  

(9)
and, finally,

\[ k_5 = 84 \text{ molecules} \cdot \text{cell}^{-1} \cdot \text{s}^{-1} \]  

(10)

**DISCUSSION**

In acid dissociation experiments with several subunit enzymes Cook and Koshland (2) found that foreign proteins did not interfere with the subunit association. Furthermore, no cross hybrids between the subunits of different enzymes were detected (2), which suggests that the intersubunit binding sites are highly specific.

In the present study, differences in the rates of formation of active isoenzymes from the two different monomers were found. The formation of active H4 isoenzymes was considerably slower than the formation of M4 isoenzymes, and the formation of hybrid isoenzymes resulted in activities approximately between those obtained in the homologous reaction mixtures. As shown in the present study, the differences in the recovery of enzyme activity from the monomers largely disappear in the presence of 1 mM NADH. Previously, the changes in activity after initiation of the reassociation has been shown to be accelerated by the presence of NADH (14–16). Chibon et al. (17) have suggested that there is an equilibrium between urea-denatured and folded polypeptide chains, and that the presence of coenzyme during the reassociation affects the rate of equilibration by stabilizing the folded state.

The presence of NAD+ in the incubation medium during the renaturation did not increase the rate or the degree of the renaturation. This may be explained by the tighter binding of the reduced form of the pyridine nucleotide to lactate dehydrogenase (18–20). Firm binding to the polypeptide moiety may be critical for the initiation, the direction, and the rate of folding to a thermodynamically stable form, which is the enzymatically active “native” form of the enzyme.

Since this study was designed to find reproducible conditions under which hybridization and restoration of lactate dehydrogenase activity could be obtained in vitro with high efficiency, the mechanism by which NADH effects the reactivation and tetramer formation from one of the subunit types (H) but not from the other, was not studied more closely. Whether the NADH effect has any significance in vivo cannot easily be decided on the basis of the present study. Similar requirements of nicotinamide adenine dinucleotide for folding of several dehydrogenases has been described by Deal (21).

The fact that many protein molecules, among them numerous enzymes, are oligomeric structures, has been used to advantage in the present study on the synthesis and degradation rates of lactate dehydrogenase M4. This approach resembles the immunological method in utilizing the specific interaction of two different protein peptide molecules, the M and H subunits, for isolation of one of them. The advantage of the present method lies mainly in the fact that extensive purification of the molecule to be studied is not necessary, since the interactions between subunits are highly specific (2), as also seen in Fig. 9.

The hybridization method described in this paper requires two freeze-thaw cycles in phosphate buffer, in the presence of 1 M NaCl and 1 mM NADH. The first freeze-thaw cycle is performed with the LDH M4 fraction obtained in the DEAE-Sephadex chromatography, and is followed by a second DEAE-Sephadex chromatography. This first freeze-thaw cycle was an essential procedure permitting the subsequent hybridization with LDH H4 to separate radioactive M subunits from other radioactive proteins. If this first freeze-thaw cycle was omitted, radioactivity was found over the entire chromatogram as shown in Fig. 8. Undoubtedly, this radioactivity was due to freeze-thaw induced breakdown of non-lactate dehydrogenase proteins, which were eluted with the hybridized M subunits. The intermediate freeze-thaw cycle thus serves as a simple means of removing undesirable background material. The second freeze-thaw cycle is the proper hybridization, where the radioactive LDH M4 is hybridized with the nonradioactive LDH H4. The nonradioactive radioactivity, still remaining in the LDH H4 fraction, is eluted with the LDH M4 in the solvent front in the third DEAE-Sephadex chromatography. The purity of the hybrid fractions (HM4, H2M4, H4M) was demonstrated by the coincident localization of the radioactivity and the enzyme activity.

It seems obvious that this technique could be useful also in the isolation of subunit peptides of other proteins, provided that the subunits can be isolated from one another and from the complete oligomer. In cases where an oligomeric protein consists of similar subunits it might be possible to attach covalent ligands to one population of subunits in such a way that they can be separated from native subunit peptides. Providing these groups do not alter binding and enzyme properties of these molecules, their isolation by the hybridization technique should be possible. For example, partial succinylation of hemerythrin alters its electrophoretic mobility (22). Mixtures of this modified protein and unmodified protein generate species of intermediate electrophoretic mobility.

Using the procedure described in this paper, it could be shown that the synthesis of rat liver LDH M4 was not inhibited by prior injection of actinomycin D. This is consistent with the concept of the relative stability of animal mRNA, reported to be 3 to 80 hours for the majority of mRNA species in rat liver (23). However, the incorporation was totally inhibited by cycloheximide, indicating that the radioactive fractions obtained require the translation step of protein synthesis and thus are synthesized de novo.

In studying rates of protein synthesis and degradation in vivo, the radioactive precursor is usually administered as a pulse of isotopically labeled amino acid after which the decrease in the specific activity of the enzyme can be studied and thus the half-life obtained. In pulse-labeling methods the apparent half-life
obtained may be too long due to the reutilization of amino acid (24–26). This may partly be overcome by the use of L-[guanido-14C]arginine, as the reutilization of the radioactive carbon is smaller in this compound (27).

A radioactive precursor can also be used in the measurement of saturation of the enzyme to a constant specific activity (e.g. [14C]carbonate (28), L-[guanido-14C]arginine (29), or [35S]cysteine (30)), where the reutilization problem is stated to be smaller (1).

The half-life of 4.1 days obtained for rat liver LDH M4 in this study is considerably shorter than the 16-day value obtained by the isotope saturation method followed by immunological purification (31). The long half-life is difficult to interpret, since the study is considerably shorter than the 16-day value obtained by Pool (26). This may partly be overcome by the use of L-[guanido-14C]arginine (33).

Using radioactive carbonate, Fritz et al. (34) found that different isoforms of LDH have different turnover rates and that the turnover rate of the same isoform varies among tissues. The question thus arises, whether the different subunits have different turnover rates.

Some light on these questions can be shed by studying the turnover of the subunits employing the same principle as described in the present study, i.e. hybridizing labeled LDH H4 with non-radioactive LDH M4.

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Synthesis and Degradation of Rat Liver Lactate Dehydrogenase M₄: HYBRIDIZATION IN THE PURIFICATION OF LACTATE DEHYDROGENASE ISOENZYMES
Seppo Lindy


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