Existence of Three Forms of H_2M_2 Isoenzyme of Lactate Dehydrogenase

Tomoji KOCHA,*[,a] Teruo FUKUDA,[a] and Tsuneo OKUYAMA[b]

Department of Hygienic Chemistry, Showa College of Pharmaceutical Sciences,[a] 3-3165, Higashi-tamagawagakuen, Machida-shi, Tokyo 194, Japan and Biochemistry Laboratory, Department of Chemistry, Faculty of Science, Tokyo Metropolitan University,[b] 2-1-1, Fukasawa, Setagaya-ku, Tokyo 158, Japan. *Received May 15, 1990*

Lactate dehydrogenase (LDH) was purified from rat and bovine tissues by affinity chromatography on immobilized colchicine and used for the separation of isoenzymes by high-performance anion-exchange liquid chromatography (HPLC). This analysis showed the splitting of rat H_2M_2 into three peaks and of bovine H_2M_2 into two peaks. The heat stability, inactivation rate of urea and electrophoretic mobility of isoenzymes were examined and these analyses indicated differences in physicochemical properties for the respective peaks of rat and bovine H_2M_2. In hybridization experiments, the splitting of H_2M_2 into three peaks was achieved only with the combination of rat H_4 and rat M_4, while the other combinations of bovine H_4 and bovine M_4, of rat H_4 and bovine M_4 and of bovine H_4 and rat M_4 resulted in two H_2M_2 peaks.

These results demonstrate that H_2M_2 of LDH in normal rat and bovine tissues is always split into two or three peaks by HPLC and that these H_2M_2 peaks have different physicochemical propertites, suggesting the existence of three possible geometrical isomers of H_2M_2.

Keywords LDH; HPLC; three geometrical isomers; H_2M_2 isoenzyme; hybridization

Introduction

It is well established in mammals and birds that lactate dehydrogenase (EC 1.1.1.27) is a tetramer composed of H and M subunits and contains five isoenzyme forms (H_4, H_3M, H_2M_2, HM_3, M_4) from possible combinations of the two subunits. Recently, the separation of lactate dehydrogenase (LDH) isoenzymes in human serum has been studied by the enzymatic post-column reactor system on high-performance anion-exchange liquid chromatography (HPLC).[1-4] This technique has the advantages of mere rapid analysis (within 30 min) and higher resolution than electrophoretic analysis.

There have been several reports of abnormal electrophoretic patterns of serum LDH having extra isoenzyme bands,[5-11] and the existence of extra bands for a single LDH isoenzyme has been ascribed to the presence of allelic variation[5,6] and the noncovalent association of LDH isoenzyme with other serum proteins.[9-11] In addition, Nagamine and Okochi reported that H_2M_2 in normal sera of humans and other animals is always separated into two sub-bands on polyacrylamide gel electrophoresis.[12] Schlabach *et al.* showed that H_2M_2 in normal sera and tissues of humans is separated into three peaks on HPLC.[3] Although the multiplicity of H_2M_2 observed in normal serum has been discussed in a few reports,[12-14] the interpretations do not always agree.

To avoid the occurrence of multiple isoenzyme peaks associated with other proteins, we used purified LDH for the separation of isoenzymes by HPLC and studied the existence and nature of multiple H_2M_2 forms in LDH.

Materials and Methods

Animals Bovine brains were obtained from a slaughterhouse. Bovine heart and skeletal muscle were obtained from a meat store. Male Wistar strain rats (about 180 g in body weight) were purchased from Nippon Bio-Supply. Center.

Materials Lithium DL-lactate, pyruvic acid, nitroblue tetrazolium, nicotinamide adenine dinucleotide (NAD) and NAD reduced form (NADH) were purchased from Sigma. The urea used was ultra-pure grade from Schwarz/Mann. Other chemicals were reagent grade from Wako.

Purification of LDH LDH was purified from various tissues of bovine (brain, heart and skeletal muscle) and rat (brain, heart, lung, liver, kidney, skeletal muscle and testis) by affinity chromatography on an immobilized colchicine column.[15] The isolated LDH was pure on the analysis of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein concentration was based on $E_{280} = 13.8\,cm^{-1}$.[16]

HPLC HPLC was performed using a Shimadzu Model LC-6A liquid chromatograph with detection at 280 nm (or 230 nm) by a Shimadzu Model SPD-6A detector. Data were processed using a Shimadzu Model C-R4A chromatopac. A TSK gel DEAE-5PW column (75 × 7.5 mm i.d., Tosoh) was used for the separation of LDH isoenzymes at room temperature. The buffers for gradient elution were 20 mM Tris–HCl buffer, pH 8.0 (buffer A) and 20 mM Tris–HCl buffer containing 0.5 M NaCl, pH 8.0 (buffer B). The gradient was begun automatically 5 min after injection and 35% buffer B was achieved in 40 min. The column was washed with buffer B for 5 min and then with buffer A for at least 10 min before each injection. The flow rate was 1.0 ml/min.

Heat Stability The mixture contained potassium phosphate buffer (pH 7.0, 0.1 M), bovine serum albumin (0.5 mg/ml) and LDH (sufficient to give an initial activity of 0.2 to 0.3 μmol/min/ml). After incubation for 30 min at various temperatures, samples (0.1 ml) were added to 2.9 ml of the assay solution in cuvettes to give final concentrations of 0.5 mM for pyruvate, 0.1 mM for NADH and 0.1 M for potassium phosphate buffer (pH 7.0). The linear decrease in absorbance at 340 nm for the first 1 min was used to calculate activity.

Urea Inactivation The mixture contained potassium phosphate buffer (pH 7.0, 0.1 M), bovine serum albumin (0.5 mg/ml), LDH (sufficient to give an initial activity of 0.02 to 0.03 μmol/min/ml) and urea at various concentrations. After incubation at 25 °C for various intervals, a part (0.9 ml) of the sample was added to 0.1 ml of assay solution in cuvettes to give final concentrations of 0.5 mM for pyruvate, 0.1 mM for NADH and 0.1 M for potassium phosphate buffer (pH 7.0). Thus, the final concentration of urea in each of the assays was 90% of that during the inactivation period. The linear decrease in the absorbance at 340 nm for the first 1 min was used to calculate activity. The inactivation rate of LDH isoenzymes on urea concentration was calculated according to the method of Chan and Shanks.[17]

Hybridization Hybridization of H_4 and M_4 was achieved by the freeze-thaw method.[18] A mixture of H_4 and M_4 (equal amounts, total protein 0.6 mg/ml) in 25 mM Tris–HCl buffer (pH 8.0) containing 10 mM NaSCN was quickly frozen at −50 °C and thawed at room temperature. One freeze-thaw cycle was carried out and the hybridization mixture was analyzed by HPLC.

Electrophoresis Polyacrylamide gel electrophoresis (PAGE) was performed on a 6% slab gel (separating gel: 14 cm, wide × 10 cm, long × 0.1 cm, thick) according to the method of Ogita and Markert.[19] Electrophoresis was carried out with a constant current of 15 mA for 4 h. 0.1% (SDS)–PAGE was performed on a 10% slab gel according to the method of Laemmli.[20]

LDH Staining Isoenzymes were visualized by incubaing the gels at 37 °C in the dark for 30 min in a staining solution (100 ml) described by

Vonwyl and Fischberg.[21] The solution was prepared by mixing 0.5 M potassium phosphate buffer (pH 7.4, 60 ml), 0.1 M NaCl–5 mM $MgCl_2$ (6 ml, 1 M lithium DL-lactate (pH 7.4, 6 ml) and purified water (28 ml) containing nitroblue tetrazolium (NBT, 14 mg), phenazine methosulfate (PMS, 2 mg) and NAD (60 mg). NBT, PMS and NAD were dissolved in purified water immediately before incubation.

Results

Separation of LDH Isoenzymes by HPLC Purified LDHs, which were obtained from three types of bovine tissue (brain, heart and skeletal muscle) and seven types of rat tissue (brain, heart, lung, liver, kidney, skeletal muscle and testis) by affinity chromatography on immobilized colchicine column,[15] were used for the separation of isoenzymes by HPLC. Figure 1A shows the separation of isoenzymes from bovine heart LDH by HPLC. All LDHs obtained from bovine tissues (brain, heart and skeletal muscle) were separated into six peaks with the same retention times of 2.4, 16.5, 23.3, 27.2, 30.6 and 35.0 min, respectively, and these were identified as M_4, HM_3, H_2M_2, H_3M and H_4 from their relative amounts of H and M subunits on SDS-PAGE (Fig. 2A). This HPLC analysis showed splitting of H_2M_2 into two peaks and these peaks were designated H_2M_2-a (23.3 min) and H_2M_2-b (27.2 min) based on their retention times. Figure 1B shows the separation of isoenzymes from rat heart LDH by HPLC. All of the LDHs obtained from rat tissues (brain, heart, kidney, skeletal muscle and testis) were separated into seven peaks with the same retention times of 2.2, 17.1, 26.2, 28.2, 30.0, 36.0 and 41.6 min. Figure 2B indicates that the isoenzymes were eluted in the order of M_4, HM_3, H_2M_2 (3 peaks), H_3M and H_4. This HPLC analysis showed splitting of H_2M_2 into three peaks, which were designated H_2M_2-1 (26.2 min), H_2M_2-2 (28.2 min) and H_2M_2-3 (30.0 min) on the basis of their retention times. Lung H_2M_2 was also separated into three peaks. Because the amount of H subunit in liver LDH was very low compared to the M subunit, and SDS-PAGE of liver LDH showed only a M subunit band,[15] a small amount of liver LDH did not permit the detection of H_2M_2, H_3M and H_4 on HPLC.

Rabbit H_2M_2 was split into two peaks by HPLC (data not shown). These observations indicate that H_2M_2 in LDHs of bovine, rat and rabbit tissues is always split into two or three peaks by HPLC.

Physicochemical Properties of LDH Isoenzymes Multiplicity of H_2M_2 in normal mammalian sera and tissues

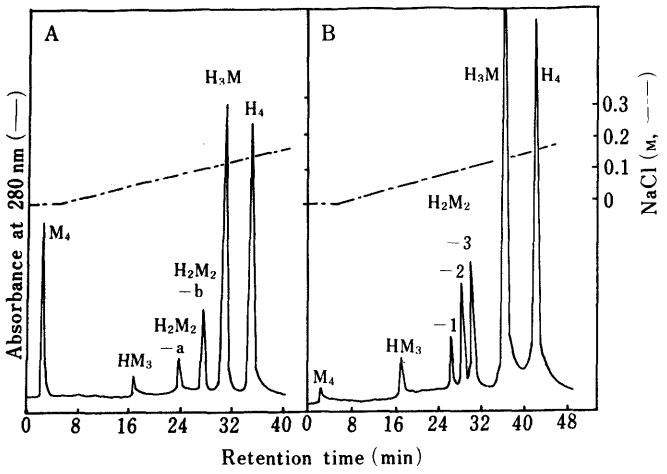

Fig. 1. Chromatogram of LDH Isoenzymes on HPLC

LDHs purified from heart of bovine (A) and rat (B) were dialyzed against 25 mM Tris–HCl buffer (pH 8.0) and used as sample for HPLC. Samples were analyzed as in Methods. The absorbance at 280 nm was monitored.

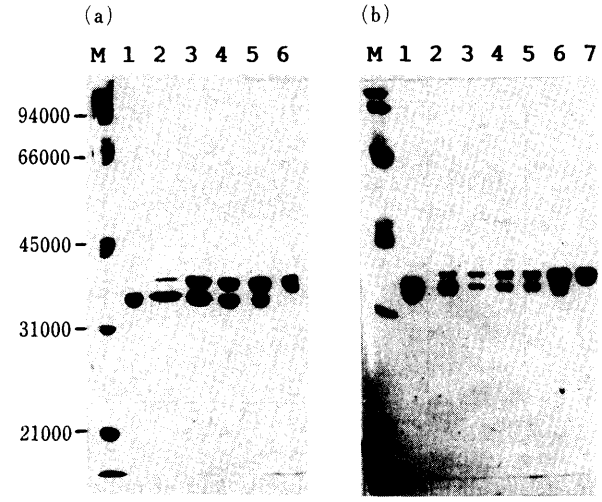

Fig. 2. Identification of LDH Isoenzyme Fractions Separated by HPLC

Respective isoenzyme fractions of bovine (a) and rat (b) LDHs on HPLC were analyzed by SDS-PAGE. a: In bovine LDH isoenzymes lanes 1 to 6, respectively, are peaks with the retention times of 2, 16, 23, 27, 30 and 35 min on HPLC. b: In rat LDH isoenzymes lanes 1 to 7, respectively, are peaks with retention times of 2, 17, 26, 28, 30, 36 and 41 min on HPLC. M indicates molecular weight markers.

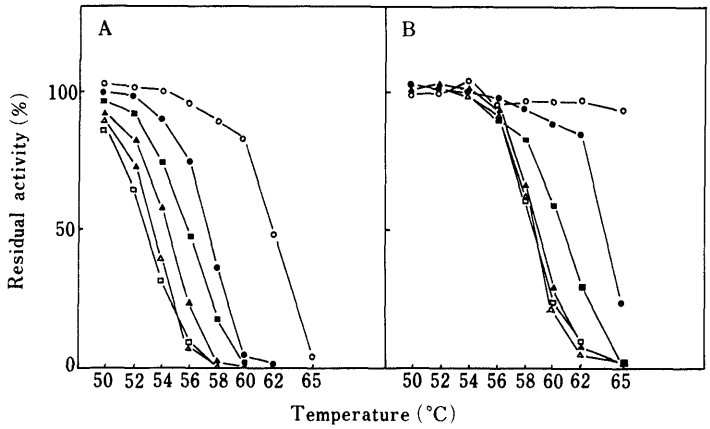

Fig. 3. Heat Stability of Bovine LDH Isoenzymes

Enzyme samples in the absence (A) and presence (B) of 0.5 mM NADH were heated at various temperatures for 30 min, then immediately cooled in an ice-bath. The activity was expressed as percent of original value for each isoenzyme. ○, H_4; ●, H_3M; □, H_2M_2-a; ■, H_2M_2-b; △, HM_3; ▲, M_4.

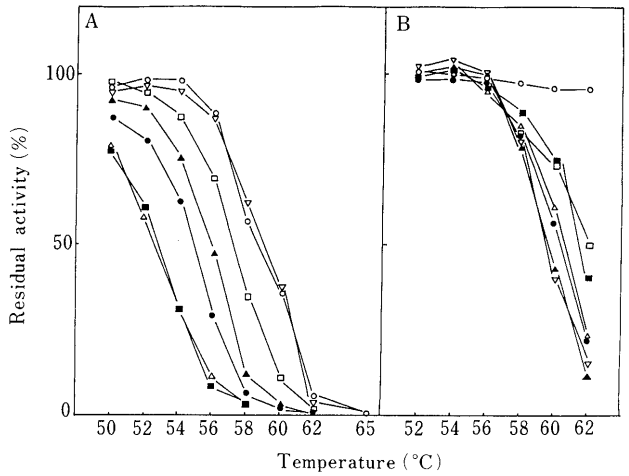

Fig. 4. Heat Stability of Rat LDH Isoenzymes

Enzyme samples in the absence (A) and presence (B) of 0.5 mM NADH were heated at various temperatures for 30 min, then immediately cooled in an ice-bath. The activity was expressed as percent of original value for each isoenzyme. ○, H_4; ●, H_3M; □, H_2M_2-1; ■, H_2M_2-2; △, H_2M_2-3; ▲, HM_3; ▽, M_4.

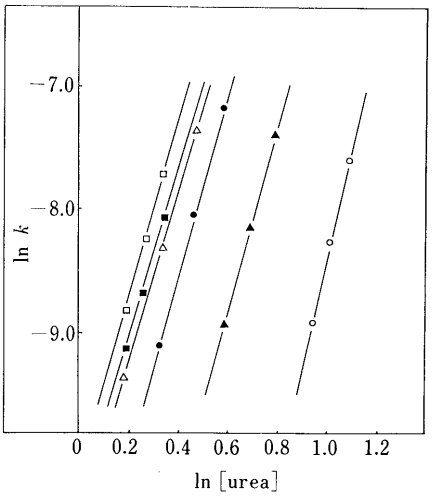

Fig. 5. Relationship between Inactivation Rate of Bovine LDH Isoenzyme and Urea Concentration

The k is the first order rate constant (s^{-1}) obtained from inactivation kinetics of isoenzymes at various concentrations of urea.[17] Symbols are the same as in Fig. 3.

is observed on PAGE[12] and HPLC.[3] We also observed multiple peaks of H_2M_2 in LDHs obtained from various bovine and rat tissues on HPLC and examined the differences in physicochemical properties among isoenzymes by analyses of heat stability, inactivation rate of urea and electrophoretic mobility. The respective isoenzymes of bovine and rat LDHs were isolated by HPLC and used for subsequent experiments.

Bovine LDH isoenzymes in the presence or absence of 0.5 mM NADH were heated for 30 min at various temperatures and the changes in their residual activities are shown in Fig. 3. The heat stability of isoenzymes in the absence of NADH decreased in the order of $H_4 > H_3M > H_2M_2$-b $> M_4 > HM_3 > H_2M_2$-a, while that in the presence of 0.5 mM NADH decreased in the order of $H_4 > H_3M > H_2M_2$-b $> M_4 = HM_3 = H_2M_2$-a. The heat stabilities of all isoenzymes increased in the presence of NADH and the residual activity of H_4 was about 90% at 65 °C. In the same manner, the heat stability of rat LDH isoenzymes in the

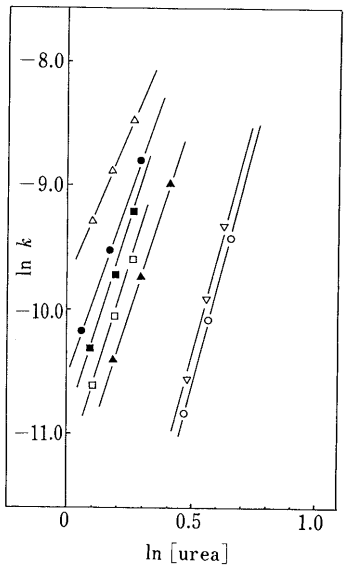

Fig. 6. Relationship between Inactivation Rate of Rat LDH Isoenzyme and Urea Concentration

The k is the first order rate constant (s^{-1}) obtained from inactivation kinetics of isoenzymes at various concentrations of urea.[17] Symbols are the same as in Fig. 4.

absence of NADH decreased in the order $M_4 = H_4 > H_2M_2$-1 $> HM_3 > H_3M > H_2M_2$-2 $= H_2M_2$-3, while that in the presence of 0.5 mM NADH decreased in the order of $H_4 > M_4 = H_2M_2$-1 $HM_3 = H_2M_2$-3 $> H_3M = H_2M_2$-2 (Fig. 4). In the presence of 0.5 mM NADH, the stabilization of all isoenzymes and the marked stabilization of H_4 were very similar to the results obtained for bovine LDH isoenzymes.

The urea inactivation of LDH isoenzymes was shown from the relationship between inactivation rate and urea concentration (Figs. 5 and 6). The urea stability of bovine LDH isoenzymes decreased in the order $H_4 > M_4 > H_3M > HM_3 > H_2M_2$-b $> H_2M_2$-a, while that of rat LDH isoenzymes decreased in the order $H_4 > M_4 > HM_3 > H_2M_2$-1 $> H_2M_2$-2 $> H_3M > H_2M_2$-3.

From these results, the differences in physicochemical properties among isoenzymes of bovine or rat LDHs can be summarized as follows. In bovine LDH isoenzymes, 1) the stabilities of isoenzymes between heat and urea treatments do not always agree: urea-stable M_4 is less heat-stable than H_2M_2-b and H_3M; 2) H_2M_2-b is always more heat- and urea-stable than H_2M_2-a; 3) H_3M is always more heat- and urea-stable than HM_3; 4) heat and urea stabilities of H_4 are superior to those of M_4. In rat LDH isoenzymes, 1) heat and urea stabilities of H_4 are similar to those of M_4; 2) H_2M_2-1 is always more heat- and urea-stable than H_2M_2-2 and -3; 3) H_2M_2-3 is less urea-stable than H_2M_2-2; 4) HM_3 is always more heat- and urea-stable than H_3M.

Figure 7 shows the relative mobilities of LDH isoenzymes on polyacrylamide gel electrophoresis. The electrophoretic patterns of the respective bovine and rat isoenzymes are shown in Fig. 7A. Because rat M_4 is a more basic protein with an isoelectric point of 8.8 than bovine M_4 (pI = 8.3), it is not incorporated into the separating gel with pH 8.3 and therefore not observed. Bovine H_2M_2-a and -b, respectively, showed a single band with different mobilities, the H_2M_2-a band being more anodal than H_2M_2-b (Fig. 7B). On the other hand, the electrophoretic patterns of the

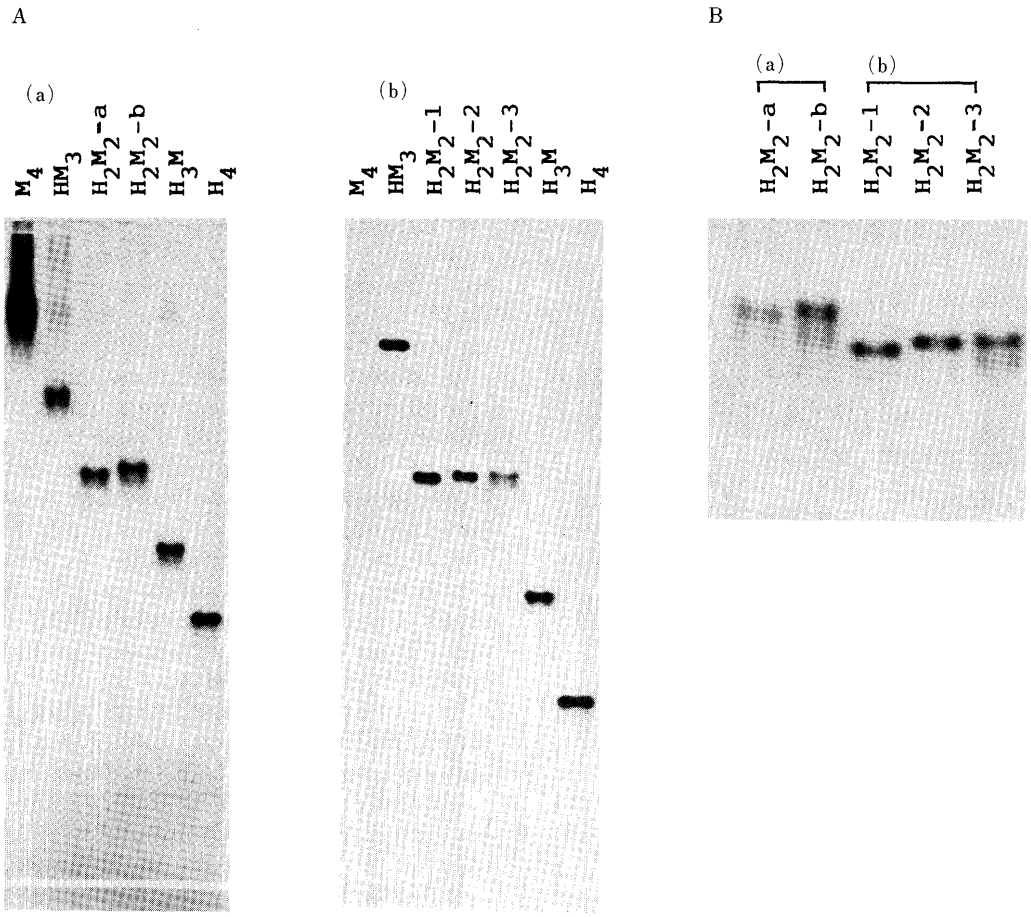

Fig. 7. Electrophoresis of LDH Isoenzymes

Bovine (a) and rat LDH (b) isoenzymes separated by HPLC were pooled and 2.5 μl each of solution containing about 5 U/ml was subjected to polyacrylamide gel electrophoresis (separating gel with pH 8.3) at 15 mA for 4 h (A). The electrophoresis (separating gel with pH 8.0) of multiple H_2M_2 fractions was carried out with a constant current of 15 mA for 5.5 h (B).

three rat H_2M_2 peaks showed slight differences in their relative mobilities and the decreasing mobility towards the anodal side was in the order H_2M_2-1, -2 and -3. Although H_2M_2-3 is contained in a minor band migrating just at the anodal side of H_2M_2-1, we cannot explain the production of two bands in this study.

Production of Multiple H_2M_2 Forms upon Hybridization of H_4 and M_4 To clarify the existence of multiple H_2M_2 forms, hybridization experiments of H_4 and M_4 obtained from rat and/or bovine were performed according to the freeze-thaw procedure, and then HPLC was used for the separation of isoenzymes from the hybridization mixture. The hybridization mixture of bovine H_4 and bovine M_4 was separated into six isoenzyme peaks, including two H_2M_2 peaks with the same retention times as those of bovine LDHs shown in Fig. 1A; that of rat H_4 and rat M_4 was also separated into seven isoenzyme peaks, including three H_2M_2 peaks with the same retention times as those of rat LDHs shown in Fig. 1B (Fig. 8A and B). These observations demonstrated that the hybridization of H_4 and M_4 from the same animal reproduced the formation of all isoenzymes including multiple H_2M_2. The hybridization of rat H_4 (or M_4) and bovine M_4 (or H_4) obtained from different rat and bovine specimens, in contrast, produced hybrid isoenzymes (HM_3, H_2M_2 and H_3M) with different retention times, H_2M_2 being split into two peaks (Fig. 8C and D). These results indicate that the formation of three H_2M_2 isomers on hybridization is achieved only by the combination of rat H_4 and rat M_4.

Discussion

LDH is a typical 222-tetramer of point-group symmetry and therefore each subunit has three different types of contact generated by the molecular P, Q and R axes.[22] Levitzki found that H_2M_2 split into two sub-bands during electrophoresis of porcine LDH isoenzymes obtained by hybridization based on reversible acid denaturation, and proposed three possible geometrical structures consisting of pp-, qq- and rr-contact homodimers.[13] Nagamine and Okochi also found that H_2M_2 in normal human and other animal sera split into two sub-bands on polyacrylamide gel electrophoresis, and they postulated that two types of tetrameric arrangement (*cis* and *trans*) are possible only in H_2M_2.[12] Thus, two different hypotheses have been proposed from the splitting of H_2M_2 into two sub-bands on electrophoresis, this point of whether the third band of the three possible geometrical isomers of H_2M_2 is actually present or not therefore needs further clarification. We found that H_2M_2 in rat LDH isoenzymes was separated into three peaks by HPLC and that these peaks showed different physicochemical properties on analysis of heat stability, inactivation rate of urea and electrophoretic mobility.

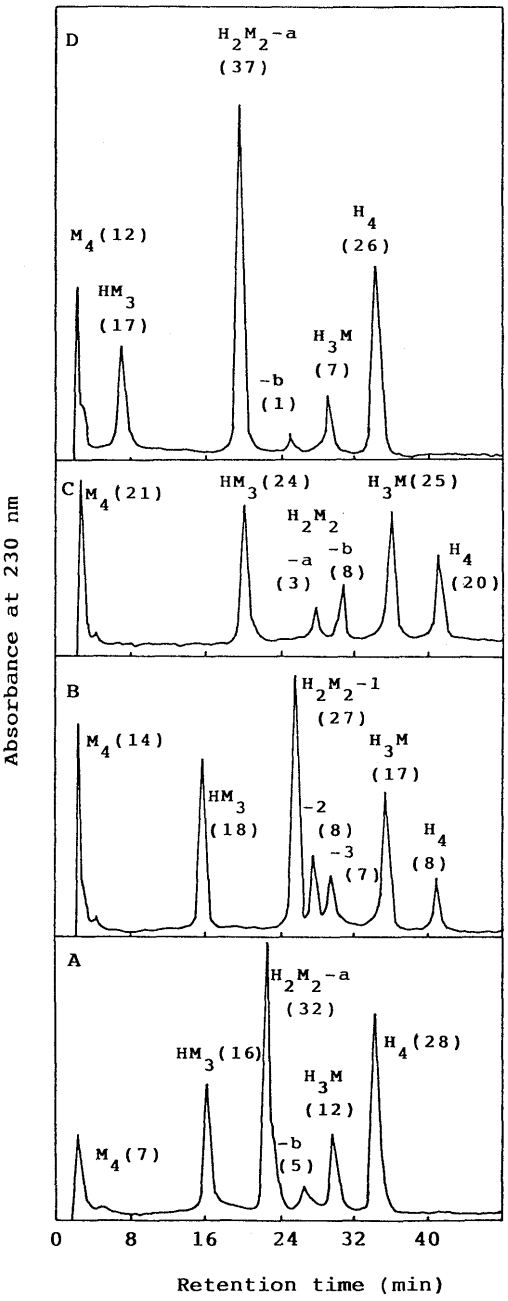

Fig. 8. Chromatogram of LDH Isoenzymes from the Hybridization Mixture on HPLC

The combinations of H_4 and M_4 on hybridization are as follows: A, bovine H_4 and bovine M_4; B, rat H_4 and rat M_4; C, rat H_4 and bovine M_4; D, bovine H_4 and rat M_4. Numbers in parentheses are the percent of each isoenzyme toward total isoenzyme areas. The hybridization mixtures were analyzed by HPLC as in Fig. 1. The absorbance at 230 nm was monitored.

H_2M_2 obtained upon hybridization of rat H_4 and rat M_4 is split into three peaks by HPLC (Fig. 8B), like those of rat LDHs purified from various tissues (Fig. 1B), supporting the existence of three H_2M_2 subforms having different molecular structures. The hybridizations of bovine H_4 and bovine M_4, of rat H_4 and bovine M_4 and of bovine H_4 and rat M_4, always result in the splitting of H_2M_2 into two peaks by HPLC (Fig. 8A, C and D). The most straightforward explanation of the multiplicity of H_2M_2, which can be split by HPLC into two or three peaks, is as follows: The multiple forms of H_2M_2 are based on three possible geometrical structures. This hypothesis was proposed by Levitzki, who postulated that the association of pp-, qq- and rr-contact homodimers results in three H_2M_2 isomers.[13] However, Müller and Klein reported that the hybrids of LDH from chicken heart and porcine heart are formed by the association of homo- and heterodimers based on Q-axis contact.[14] The behavior of amino acid residues in the subunit–subunit contact area is strikingly different for three differing types of contact generated by the molecular P-, Q- and R-axes.[23] The Q-axis contact surface has more amino acid residues for contact and these residues are far more conserved than in the P- and R-axis contact surfaces,[24] suggesting that the Q-axis contact results in more stable and constant interactions between homo- or heterodimers than the other two contacts. Indeed, the Q-axis contact is observed in the soluble malate dehydrogenase dimer.[25] Therefore, we postulate the formation of three H_2M_2 subforms by a modification of Levitzki's hypothesis based on the three possible geometrical isomers (Chart 1). This is that all three different dimers of HH, MM and HM are formed by Q-axis contacts of H and M monomer subunits and that five tetrameric LDH isoenzymes then result from possible combination of the three different dimers. Accordingly, H_2M_2 permits three geometrical isomers of one isomer based on the association of HH- and MM-homodimers (II type), and of two isomers based on the association of two HM-heterodimers (I and III types).

There are two different interpretations with respect to heat denaturation of LDH isoenzymes: the stability of the tetramer is determined by the destruction of the more unstable dimer[14] or by the survival of the more stable dimer.[26] We analyzed the heat stabilities of bovine and rat isoenzymes on the basis of the former interpretation. The heat stability of bovine LDH isoenzymes decreased in the order $H_4 > H_3M > H_2M_2\text{-b} > M_4 > HM_3 > H_2M_2\text{-a}$, while that of rat LDH isoenzymes decreased in the order $M_4 = H_4 > H_2M_2\text{-1} > HM_3 > H_3M > H_2M_2\text{-2} = H_2M_2\text{-3}$ (Figs. 3 and 4). From these results, the stabilities of bovine and rat dimers follow the orders HH > HM > MM and MM $\geqq$ HH > HM, respectively. If the heat stability of LDH isoenzymes is determined by the destruction of the more unstable dimer, the theoretical heat stabilities of bovine and rat LDH isoenzymes, respectively, would decrease in the order of H_4 (HH-HH) > H_3M (HH-HM) = H_2M_2 (HM-HM) > M_4 (MM-MM) = HM_3 (MM-HM) = H_2M_2 (HH-MM), and of $M_4 \geqq H_4 = H_2M_2$ (HH-MM) > $HM_3 = H_3M = H_2M_2$ (HM-HM). Thus, the experimental orders for the heat stabilities of bovine and rat LDH isoenzymes exhibit a good similarity to their theoretical orders, while the theoretical order of the heat stability of bovine LDH isoenzymes based on the latter interpretation (the more stable dimer) does not agree with the theoretical order ($H_4 = H_3M = H_2M_2$ (HH-MM) > H_2M_2 (HM-HM) = $HM_3 > M_4$). Accordingly, the geometrical structure of each H_2M_2 peak on HPLC can be assumed from the heat stability of LDH isoenzymes determined by the destruction of the more unstable dimer: bovine H_2M_2-a and -b, respectively, are HH-MM (II type) and HM-HM (I and/or III types). Rat H_2M_2-1, -2 and -3, respectively, are HH-MM (II type), HM-HM (I or III types) and HM-HM (III or I types).

On the basis of our hypothesis, the occurrence of three possible geometrical isomers of H_2M_2 indicates that each subunit has three stable contacts generated by the molecular

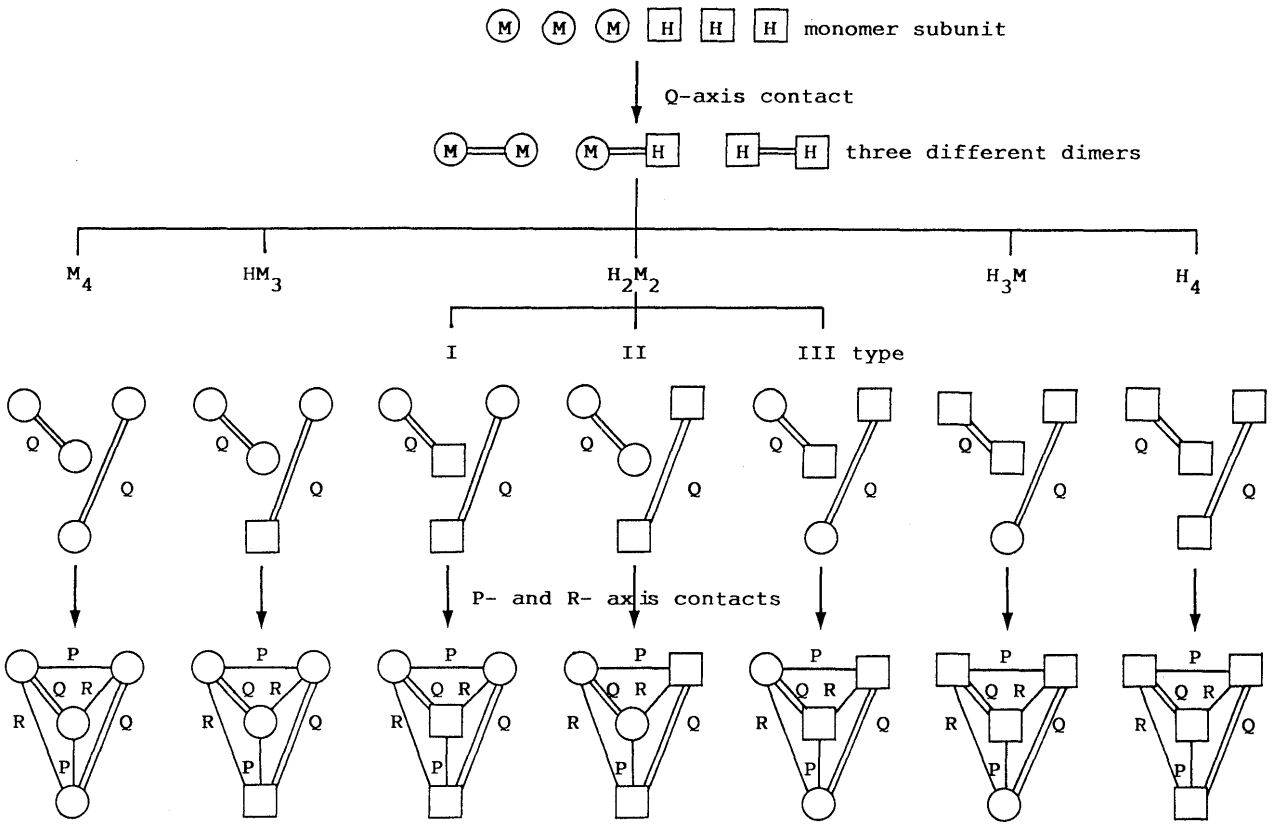

Chart 1. Formation of LDH Isoenzymes Based on the P-, Q- and R-Axis Contacts among Three Different Dimers

P-, Q- and R-axes (Chart 1). Therefore, the specificity of rat LDH subunits resulting in three H_2M_2 isomers was examined by two species of contact rates using the quantitative analysis of isoenzymes in hybridization experiments shown in Fig. 8 (Chart 2). The first contact rate is the formation of three different dimers (HH-, MM- and HM-dimers) based on Q-axis contact and was calculated from the quantity of their dimers constituting five tetrameric isoenzymes (Chart 2A). The second contact rate is the formation of three hybrid tetramers (HM_3, H_2M_2 and H_3M) based on P- and R-axis contacts and was calculated from the quantity of HM-dimer associated with three different dimers (Chart 2B). The geometrical structures of H_2M_2 split into two or three peaks on HPLC can be assumed from the heat stability of isoenzymes described above in Discussion. Following the hybridization of rat H_4 and rat M_4, all HH-, MM- and HM-dimers were present in nearly equal quantities and, further, all associations of HM-dimer with the three different dimers on the formation of hybrid tetramers (HM_3, H_2M_2 and H_3M) also proceeded at the same rate, indicating that P- and R-axis contacts between homo- or heterodimers are stable. On the other hand, the three hybridization experiments resulting in two H_2M_2 isomers can be summarized as follows. The formation of three hybrid tetramers (HM_3, H_2M_2 and H_3M) did not proceed at the same rate, even if all three dimers were formed in equal quantities (in hybridization of rat H_4 and bovine M_4). In addition, the association rate of HM-dimer with the same dimer was always lower than those with the other two dimers (HH- and MM-dimers). These observations suggest that the Q-axis contact between H and M subunits resulting in two H_2M_2 isomers induces the distortion of its

(A) dimers	hybridization experiments I bovine H_4 /bovine M_4 (%)	II rat H_4 /rat M_4 (%)	III rat H_4 /bovine M_4 (%)	IV bovine H_4 /rat M_4 (%)
MM	33[a] (3)[c]	37 (1)	33 (1)	39 (3)
HH	50 (5)	30 (1)	32 (1)	48 (4)
HM	19 (2)	33 (1)	35 (1)	13 (1)

(B) hybrid tetramers	hybridization experiments I bovine H_4 /bovine M_4 (%)	II rat H_4 /rat M_4 (%)	III rat H_4 /bovine M_4 (%)	IV bovine H_4 /rat M_4 (%)
HM_3	48[b] (3)[c]	36 (1)	40 (2)	68 (11)
H_3M	36 (2)	34 (1)	41 (2)	26 (4)
H_2M_2	16 (1)	30 (1)	19 (1)	6 (1)

Chart 2. The Association Rates between Subunits (A) or Dimers (B) on the Formation of Hybrid Tetramers Assumed from the Hybridization Experiments

As shown in Chart 1, three different dimers (HH, MM and HM) are the basic units forming tetrameric isoenzymes. From the quantitative analysis of the hybridization experiments shown in Fig. 8, the quantities of the three different dimers were calculated (A). The dimer structures of multiple H_2M_2 peaks on HPLC are assumed from the heat stability of isoenzymes described in Discussion: bovine M_4/bovine H_4 (H_2M_2-a = HH–MM and H_2M_2-b = HM–HM); rat H_4/rat M_4 (H_2M_2-1 = HH–MM, H_2M_2-2 and -3 = HM–HM); rat H_4/bovine M_4 (H_2M_2-a and -b = HM–HM); bovine H_4/rat M_4 (H_2M_2-a = HH–MM and H_2M_2-b = HM–HM). Further, the quantities of HM-dimer associated with three different dimers in the formation of hybrid tetramers (HM_3, H_3M and H_2M_2) were calculated from the results shown in Fig. 8B.

a) Values are the percent of each dimer toward total isoenzymes areas.
b) Values are the percent of HM-dimer toward total HM-dimer areas.
c) Numbers in parentheses are the nearest integers.

dimer structure and thus the P- and R-axis contacts in H_2M_2 become particularly unstable. This may then allow the occurrence of three possible geometrical isomers of H_2M_2 when both subunits of H and M have equal affinities at the P-, Q- and R-axis contact surfaces.

Similarly, the splitting of bovine into two peaks on HPLC may reflect the distortion of its dimer structure based on the association of H and M subunits and subsequently indicate the absence of a third peak as unstable interaction of P- or R-axis contacts in H_2M_2. Some data support this speculation. The heat and urea stabilities of isoenzymes indicated more marked differences in physicochemical properties between bovine H_4 and bovine M_4 than those between rat H_4 and M_4; furthermore, bovine H_4 was more urea-stable than rat H_4, rat M_4 and bovine M_4 showed similar urea stabilities, indicating that bovine H subunit has the different physicochemical properties from the other three kinds of subunits. As shown in Chart 2B, the association rates of HM-dimer with three different dimers suggest that bovine H subunit rather than M subunit of bovine or rat induces a large distortion of the HM-dimer structure which inhibits a stable interaction in H_2M_2.

There is apparently a discrepancy between the elution order of multiple H_2M_2 peaks on HPLC with an anion-exchange column (Figs. 1A and 2) and the relative mobilities of the same H_2M_2 peaks with PAGE (Fig. 7B). Based on the chromatographic elution pattern of bovine LDH (Fig. 1A), since H_2M_2-a is eluted before H_2M_2-b by HPLC, H_2M_2-a has a less negative charge than H_2M_2-b. However, H_2M_2-a has more anodal mobility than H_2M_2-b on electrophoresis (Fig. 7B). Rat H_2M_2 showed the same behavior. This conflicting behavior of multiple H_2M_2 peaks on the two analyses was observed[12] and can be related to the molecular sieving effect of polyacrylamide gel electrophoresis, suggesting the conformational change of respective H_2M_2 isomers. The induction of conformational change of tetrameric LDH is caused by the incorporation of NADH into LDH molecule, and this results in the subsequent formation of more stable interaction among subunits (Figs. 3 and 4). Similarly, the incorporation of NAD, NADH and pyruvate into LDH molecule results in the increase of renaturation rate and amount from acid denaturation.[13] These observations suggest that the stable contacts among subunits in LDH molecule are caused by the conformational change of its tetrameric structure which is induced by the incorporation of ligands such as NADH, NAD and pyruvate, and that hetero tetramers without ligands become particularly unstable causing distortion of the HM-heterodimer structure.

As described above, some results may explain the speculation based on the conformational change of tetrameric LDH structure. However, from the data obtained in this study, it is difficult for us to explain theoretically the occurrence of two of three H_2M_2 isomers.

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