Physical and Chemical Properties of Reversibly Inactivated Lactate Dehydrogenases

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SUMMARY

The effect of reversible inactivation on the physical and chemical properties of dogfish M4 and chicken heart H4 lactate dehydrogenases has been studied. Reduced diphosphopyridine nucleotide was found to have a significant effect on the rate of recovery of the enzymatic activity, but not on the overall recovery. Optical rotatory dispersion, circular dichroism, fluorescence, and immunological studies have shown structural differences between the native and reassociated enzyme. Further evidence for the difference in conformational structure was obtained from the pattern of reactivity of sulfhydryl groups of the reactivated enzyme. The native enzyme was also found to be more stable to heat and urea denaturation than the reassociated form. Pyruvate inhibition, and the catalytic activity with coenzyme analogues, was found to be different for the native and the reassociated enzyme. These results indicate the possibility that a conformational difference exists between the native and the reassociated enzyme for both dogfish M4 and chicken H4 lactate dehydrogenase. Studies on the lactate dehydrogenase re-associated in the presence of reduced DPN showed structural characteristics which are closer to the native dehydrogenase than the enzyme which was reassociated in the absence of the reduced coenzyme. Incubation of the enzyme with DPNH after reassociation does not convert the reassociated enzyme to a structure similar to that of the native enzyme. These studies suggest a possible role of the reduced coenzyme in the folding of the peptide chain or in the subunit interaction and in the attainment of the native conformation of the enzyme.

Previous reports (2, 3) from this laboratory have described the rates of dissociation and reassociation of several pyridine nucleotide dehydrogenases following treatment with guanidine hydrochloride, urea, or acidic pH. The dissociation and reassociation of these enzymes are affected by the presence of pyridine nucleotide coenzymes. Evidence has been obtained that an intermediate, active form of lactate dehydrogenase was present during the reactivation process. This raised the question of whether the unfolded peptide chain returns to the exact conformation of the native protein upon renaturation, or whether other factors are essential for the proper folding.

The three-dimensional structure of a protein in solution is usually thought to be largely determined by the amino acid sequence; it is also presumed to be the configuration at which the total free energy of the system is at a minimum (4, 5). These hypotheses are based upon experiments with several enzymes which are capable of undergoing reversible denaturation, with restoration of catalytic activity. Goldberger and Epstein (6) have studied egg white lysozyme after reversible inactivation and found the renatured products to be physically and chemically indistinguishable from the native protein. Kitto, Stolzenbach, and Kaplan (7), however, after reversible denaturation of mitochondrial malate dehydrogenase observed a form of the enzyme which is not identical with the untreated protein. Also, from the work of Pfamm and Beychok (8) it was clearly shown that pancreatic ribonuclease refolds to a conformation that is different from that of the native enzyme upon reduction and subsequent oxidation of its disulfide bonds.

Dogfish muscle (M4) lactate dehydrogenase is an enzyme which is presently being investigated for its three-dimensional (9, 10) as well as its primary (11) structure. The lactate dehydrogenases do not contain disulfide bonds (12), hence the possibility of a previously determined three-dimensional conformation, because of the formation of thermodynamically favored S—S— bonds, as found in ribonuclease and insulin (5), is not a factor in the lactate dehydrogenase structure. For these reasons the dogfish muscle lactate dehydrogenase appeared to be an ideal model system for the study of structural relationships with respect to the dissociation and reassociation phenomena. This report deals with an investigation of the effects of the reversible inactivation by lithium chloride on the physical and catalytic properties of the reassociated dogfish M4 lactate dehydrogenases, unless otherwise stated.

EXPERIMENTAL PROCEDURE

Materials

Pyridine nucleotides and their analogues1 were obtained from

1 The abbreviations used are: (AcPy)DPN+ and (AcPy)DPNH, acetyl pyridine diphosphopyridine nucleotide and its reduced form; (De)DPN+ and (De)DPNH, hypoxanthine analogue of...
P-I. Biochemicals. Sodium pyruvate was purchased from Nutritional Biochemicals. β-Mercuraptoethanol was a product of Distillation Products Industries. p-Hydroxymercuribenzoate was purchased from Sigma. Sephadex was obtained from Pharmacia. Lithium chloride was a reagent grade product from Fisher. The cellulose acetate strips, used in electrophoresis, were purchased from Oxoid Consolidated Laboratories, Inc., Chicago Heights, Illinois and cellogel strips were obtained from Chemtron (Via Gustavo, Modena 24, Milano, Italy). Amido black was purchased from Allied Chemical, Morristown, New Jersey.

**Methods**

**Measurement of Enzyme Activity**—Enzyme activity was measured by the method of Pesce et al. (13), with a Zeiss Spectrophotometer model PMQ II. Protein determinations were carried out according to the method of Lowry et al. (14). Enzyme Preparation—Dogfish M₄ and chicken H₄ lactic dehydrogenase were prepared and crystallized in accordance with the method described by Pesce et al. (15). In the case of the dogfish M₄ lactic dehydrogenase a minor contaminant was removed by Sephadex G-100 column chromatography. The enzyme was eluted from the column with a 0.05 M Tris-HCl buffer, pH 7.5. The enzyme was then recrystallized from 50% saturated ammonium sulfate.

Enzyme preparations were considered pure when (a) a constant specific enzymatic activity across the peak was obtained upon elution from a column, (b) a symmetrical single peak was observed in the analytical ultracentrifuge during sedimentation analysis, and (c) a single protein band was found upon electrophoresis on cellulose acetate or cellogel strips at two different pH values (7.0 and 8.5). Enzyme sample prepared according to this procedure is a pure protein and does not contain any bound DPNH or DPN⁺.

Dissociation of Lactate Dehydrogenases—Lactate dehydrogenase dissociation by lithium chloride was carried out with a modified method of Chilson et al. The β-mercaptoethanol concentration in the reassociation medium was 0.05 M instead of 0.1 M. After 90 min of reassociation at room temperature, reassociation was continued by the following procedures. The sample was dialyzed against 0.05 M Tris-HCl buffer, pH 7.5, at room temperature for 1 hour; further dialysis was carried out overnight in a 4° room, with several changes of buffer. Both modifications do not interfere with the over-all recovery of the enzymatic activity; this finding corresponds to that reported by Chilson et al. (3).

Reassociated enzyme solutions were concentrated in an Aminco Pressure Concentrator, fitted with a Diaflo Filter No. UM3. Concentrated enzyme solutions were centrifuged to remove insoluble material; the supernatant was then passed through a Sephadex G-100 column. The major protein peak was used for the reported studies. Enzyme which was reassociated in the presence of DPNH, was incubated with a sufficient amount of pyruvate to convert the DPNH to DPN⁺, since the latter has a lower binding constant. This procedure was followed by dialysis and gel filtration on a Sephadex G-100 column in order to remove all salts and the oxidized coenzyme. Control samples were subjected to a similar treatment except for the dissociation step and such treatment did not affect the properties of the native enzyme.

Electrophoresis—Routine electrophoresis was carried out at 250 volts, 10 ma for 1 hour at 4° (with cellulose acetate or cellogel strips) with 0.05 M Tris-HCl buffer, pH 7.5, as the buffering medium. Localization of enzyme on the electrophoresis strips was carried out according to the method of Fine and Costello (16). Amido black was used as 1.0% solution in 10% acetic acid for protein staining. Destaining of the electrophoresis strips was accomplished by the repeated washing in a mixture of methanol-water-acetic acid in a ratio of 5:5:1.

**Immunological Technique and Stability Studies**—Quantitative microcomplement fixation was carried out according to the method described by Levine (17). For stability studies, enzyme samples were diluted with a solution of 1% bovine serum albumin in 0.05 M Tris-HCl buffer, pH 7.5, in order to prevent any enzyme denaturation caused by low protein concentration. Samples were also diluted to give equal enzyme activity per unit volume.

**Physical Measurements**—Optical rotatory dispersion and circular dichroism spectra were obtained with a Cary model 60 spectropolarimeter, equipped with an attachment for CD. CD spectra were also obtained with a Jasco-Durrum spectropolarimeter model J-10. Fluorescence studies were carried out on a Zeiss spectrophotofluorometer, model AFM-4C, and polarization of fluorescence studies were made with a polarization spectrophotofluorometer, built according to the specifications of Weber and Bablouzian (18). Stopped flow experiments were performed in an Amino-Morrow Stopped Flow Apparatus, equipped with an Amino Grating Monochrometer and a Tektronix Storage Oscilloscope type R564B. Ultracentrifugal analysis was performed with a Spinco analytical centrifuge equipped with a photoelectric scanner. Titration of the sulfhydrl groups with HMB was carried out as described by Boyer (19), by measuring the increase in optical density at 250 nm. Fluorescent measurements of the coenzyme binding were made according to the method of McKay and Kaplan (20).

**RESULTS**

Effect of Reassociation on Sedimentation Coefficient—In order to assess the effects of the reassociation on the tetrameric structure of the enzyme, we determined the sedimentation coefficients (s₂₀,W) for the native as well as the reassociated proteins. The results of this investigation are summarized in Table I. The data
show that the $\theta_{0,w}$ value for the reassociated protein is not significantly different from that of the native enzyme. These findings indicate that following the refolding, the peptide chains have the capacity to form the tetramer. The data also suggest that the tetrameric form is thermodynamically the most stable form of the reassociated enzyme.

Effect of Reassociation of Reactivity of Sulphydryl Groups—Fondy et al. (21), have reported that the sulphydryl groups of lactate dehydrogenases from different species are not identical in their reactivity towards HMB. They found that dogfish M₄ lactate dehydrogenase contains four sulphydryl groups that react rapidly with HMB without affecting the catalytic activity of the enzyme. Prolonged treatment with HMB results in the binding of another four sulphydryl groups, which are essential for the catalytic activity. The latter sulphydryl groups react more slowly with HMB; their rate of reaction may be followed spectrophotometrically (19) as well as by the loss in enzymatic activity (21).

Fig. 1 compares the reactivity of the sulphydryl groups toward HMB in the reassociated enzyme as compared to that of the native enzyme. The results indicate that the essential slowly reacting sulphydryl groups in the native protein have become more reactive toward HMB after dissociation and reassociation of the protein. This may indicate that the essential sulphydryl groups in the reassociated enzyme are more "exposed" to an interaction with HMB than those in the native protein, or that other factors that affect the reactivity of these sulphydryl groups have, in some way, been altered by the dissociation-reassociation process.

Effect of Reassociation on Protein Fluorescence—Teale (22) has demonstrated that structural changes in proteins may produce changes in the quantum yield of the tryptophan fluorescence. A decrease in the tryptophan fluorescence of lactate dehydrogenases has been observed as the result of binding the reduced coenzymes (20). Fig. 2 indicates the changes in fluorescence that can be observed when the dogfish lactate dehydrogenase is treated with increasing amounts of (AcPy)DPNH. The data show that the changes in fluorescence yield as well as the transfer of fluorescence energy from the enzyme to the coenzyme as measured by the yield at 450 nm are only about 50% for the reassociated enzyme when compared to the changes observed with the native protein. This decrease in the transfer of energy resonance may indicate a less effective binding between the enzyme and the coenzyme, which may be the result of some loss of native protein structure (Drand, Everse, and Kaplan (23)).

The binding of coenzymes to apoenzymes has also been studied with the use of polarization fluorometry (24, 25). This technique may also yield information concerning the strength of the bonding. Thus, it seemed reasonable to study the effects on the polarization of fluorescence, which may occur when increasing amounts of (AcPy)DPNH are added to the reassociated lactate dehydrogenase, as compared to those obtained with the native enzyme.

If the observed decrease in protein fluorescence in the reassociated form (Fig. 2) was actually caused by a loss of native structure, one would expect a less rigid conformation and therefore more freedom of rotation (low rotational relaxation time), which would in turn lead to a low degree of polarization. Fig. 3 shows the polarization of fluorescence ($\phi$) of both the native and the reassociated enzyme as a function of increasing concentrations of (AcPy)DPNH. The polarization of the fluorescence observed with the reassociated apoenzyme is considerably less than that found with the native protein. This suggests, thus, that a more open conformation may exist, at least in certain parts of the reassociated lactate dehydrogenase, in comparison to the native enzyme.

The polarization of the reduced coenzyme fluorescence is the sum of the contributions of the free and bound forms of the coenzyme (25). Since the bound form has a much higher rotational relaxation time (giving a higher degree of polarization) than the free form, it seems probable that the binding of the coenzyme to the reassociated apoenzyme might be not as strong as it is to the native enzyme and hence a lower polarization should be observed.
Results presented in Fig. 3 indicate that the polarization of the fluorescence of the (AcPy)DPNH is significantly lower upon interacting with the reassociated catalyst.

**Rates of Protein Fluorescence and Catalytic Activity Recoveries upon Reassociation of Dogfish Muscle Lactate Dehydrogenase—Chilson et al. (3) have demonstrated that after the reversible denaturation of the chicken H4 lactate dehydrogenase with 8 M urea, the recovery of fluorescence paralleled the recovery of enzymatic activity. We have investigated the loss and the recovery of the protein fluorescence, as well as the enzymatic activity of dogfish M4 lactate dehydrogenase upon the reversible denaturation with lithium chloride.** Fig. 4 shows the results of such an experiment as measured by the stopped flow technique. Although the enzymatic activity is completely lost within 2 min, the protein fluorescence does not decrease below 20% of the original value upon denaturation. Upon reassociation, the recovery of the protein fluorescence precedes the recovery of the enzymatic activity. This may suggest that there is some attainment of tertiary and possibly quaternary structure before the reassociated protein becomes enzymatically active. It is worth noting that the recovery of the fluorescence does not exceed 60% of the original value, which is in agreement with the data presented in Fig. 2. The results shown in Fig. 4 indicate that the recovery of the total enzymatic activity is about 70 to 80%, whereas the recovery of the protein fluorescence is only 50 to 60%. This may indicate a subtle difference in the folding of the reassociated enzyme as compared to the native form.
Effect of Reassociation on Optical Rotatory Properties of Lactate Dehydrogenase—In order to obtain further information about the conformation of the reassociated dogfish $M_4$ lactate dehydrogenase, we measured the optical rotatory dispersion and circular dichroism spectra. The ORD spectra are shown in Fig. 5. It is clear that the specific rotation ($\alpha$) is different for the two proteins, and that the rotation is significantly less for the reassociated enzyme. It is also worth noting that the cross-over point, where ($\alpha$) is equal to zero, and the depth of the negative cotton effect at 233 nm, both of which are related to the degree of $\beta$ structure (26, 27), are also different for the native and reassociated enzymes. Circular dichroism spectra were significantly different for the renatured enzyme when compared to the untreated dogfish lactate dehydrogenase. [0], which is proportional to the helical content (28) of the renatured enzyme was somewhat less than that of the native (71%).

Effect of Reassociation on Immunological Properties of Dogfish $M_4$ Lactate Dehydrogenase—Several reports have used the immunological technique to detect subtle differences in protein structure (17, 29–31). In the work of Kitto, Wasserman, and Kaplan (32) it was shown that the reactivity of the reversibly denatured mitochondrial malate dehydrogenase with rabbit antibody prepared against the native enzyme was altered.

Fig. 6 shows the reaction of rabbit antisera prepared against native dogfish $M_4$ lactate dehydrogenase with the native enzyme as well as with the renatured enzyme. The lateral as well as the vertical shift suggests (17) the existence of structural differences between the native and reassociated protein.

Effect of Reassociation on Enzymatic Activity—In order to determine whether or not the structural changes, produced by the dissociation and reassociation, altered the enzymatic properties, coenzyme analogues were used to probe for possible differences in the kinetic constants (33). Table II summarizes the results of such experiments. Although no striking differences could be detected in the specific activities, some differences are clearly demonstrable in the ratio of activities with the various analogues and particularly in the direction of lactate oxidation (Table II). The rate of DPNH oxidation by pyruvate may be significantly lower with the reassociated than with the native enzyme, in contrast to the rate of DPN$^+$ reduction by lactate.

The profile of enzymatic activity with increasing pyruvate concentration was determined for the dogfish $M_4$ lactate dehydrogenase, in the native as well as in the reassociated form. Since

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**Table II**

<table>
<thead>
<tr>
<th></th>
<th>Native</th>
<th>Reassociated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity micromoles DPNH per mg protein per min</td>
<td>0.41</td>
<td>0.36</td>
</tr>
<tr>
<td>Specific activity micromoles DPN$^+$ per mg protein per min</td>
<td>0.12</td>
<td>0.09</td>
</tr>
<tr>
<td>DPNH: (AcPy)DPNH</td>
<td>1.26</td>
<td>1.35</td>
</tr>
<tr>
<td>DPNH: (De)DPNH</td>
<td>1.86</td>
<td>1.38</td>
</tr>
<tr>
<td>DPN$^+$: (De)DPN$^+$</td>
<td>3.72</td>
<td>2.36</td>
</tr>
<tr>
<td>DPN$^+$: (TN)DPN$^+$</td>
<td>5.94</td>
<td>7.65</td>
</tr>
<tr>
<td>TPN$^+$: (PyAld)DPN$^+$</td>
<td>6.78</td>
<td>4.82</td>
</tr>
<tr>
<td>DPN$^+$: (PyAld) (De)DPN$^+$</td>
<td>13.45</td>
<td>30.5</td>
</tr>
</tbody>
</table>

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Fig. 7. Profile of enzymatic activity with increasing pyruvate concentration. $A$, native and reassociated dogfish lactate dehydrogenase ($M_4$); $B$, native and reassociated chicken heart lactate dehydrogenase ($H_4$). Buffer for assay was 0.05 M Tris-HCl, pH 7.5. Untreated enzymes, $\circ$—$\circ$; reactivated enzymes, $\triangle$—$\triangle$. Enzymes were reactivated after denaturation with 5.3 M lithium chloride.

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The profile of enzymatic activity with increasing pyruvate concentration was determined for the dogfish $M_4$ lactate dehydrogenase, in the native as well as in the reassociated form. Since
Reversible Inactivation of Lactate Dehydrogenases

Reversible Inactivation of Lactate Dehydrogenases

I I I I I I I I 0
1 2 3 4 5 6 7 8 9 10
PYRUVATE X 10 MOLAR

FIG. 8. Effect of previous incubation at 37° for 45 min on the pyruvate inhibition of native and reassociated chicken heart (H4) lactate dehydrogenase. Enzyme solution (6.1 X 10^7) in 0.05 M Tris-HCl, pH 7.5, was used for both the native and reassociated protein. O---O, native chicken heart (H4) lactate dehydrogenase; after incubation; A--A, reactivated chicken heart (Hd) lactate dehydrogenase; A- - -A, reactivated chicken heart (Hc) lactate dehydrogenase after incubation.

Pyruvate inhibition is more pronounced in the H4 lactate dehydrogenases, a comparison was also made with the chicken heart (H4) lactate dehydrogenase. The results of this study are shown in Fig. 7. It appears that the reversible dissociation has affected the degree of pyruvate interaction with both the chicken H4 and the dogfish M4 enzymes. Upon reassociation of the chicken H4 lactate dehydrogenase, the pyruvate inhibition is about 50% of that for the native enzyme. These catalytic differences may be a reflection of changes in protein structure and consequently a decreased coenzyme binding as suggested from data shown previously in Figs. 2, 3, and 5.

Epstein and Schechter (34) have postulated that conformational changes that are produced in an enzyme by dissociation and reassociation should be stable and “not readily interconverted” and that the differences in free energy between conformations are not readily overcome. To test whether the differences observed in Fig. 7b are caused by a less stable conformer, the native and reassociated enzymes were incubated at 37° for 45 min. No changes in the substrate inhibition properties were observed after the incubation (Fig. 8). This suggests that upon reassociation the enzyme refolds to a conformation that is thermodynamically stable at 37° and which is different from the conformation of the native enzyme.

The reassociated enzyme gave no significant differences in the K_m values for pyruvate, lactate, DPN+, and DPNH, as compared to the native enzyme.

Effect of Reassociation on Stability of Lactate Dehydrogenase—In view of the results presented here and from the argument presented by Epstein and Schechter (34), we assumed that denaturation studies on the native and the reversibly denatured enzyme may be of further assistance in the understanding of the nature of the protein refolding. According to Epstein, Goldberger, and Anfinsen (5), the conformation of a protein depends solely on the amino acid sequence of the peptide chain. Therefore, one would expect no difference in the stability between a native and a reassembled protein. Fig. 9a shows the stability of the native and reassociated dogfish M4 lactate dehydrogenases at elevated temperatures and in urea. The results indicate that the reassociated enzyme is significantly less stable than the native enzyme under these conditions. Similar results were obtained from a study of the heat stability of the native and reassociated chicken H4 lactate dehydrogenase (Fig. 9b).

Effect of Presence of DPNH during Reassociation on Properties of Renatured Lactate Dehydrogenase—Chilson et al. (3) have observed that the rate of recovery of enzymatic activity of chicken H4 lactate dehydrogenase after dissociation in 8 M urea is influenced by the presence of DPNH in the reassociation media. Similar results were obtained for dogfish M4 lactate dehydrogenase after dissociation with 5.3 M lithium chloride (Fig. 10). The data indicate that the initial rate of reassociation as measured by the recovery of enzymatic activity, is almost doubled in the presence of DPNH.
A comparison of the properties of the native lactate dehydrogenase with those of the enzyme which has been reassociated in the presence and absence of DPNH was carried out. Fig. 11 shows that the presence of DPNH during reassociation increases the thermal stability of the reassociated chicken $H_4$ as well as that of reassociated dogfish $M_4$ lactate dehydrogenase. This would seem to indicate that the presence of DPNH in the reassociation media brings about a more stable conformation than that attained by the enzyme which is reactivated in the absence of DPNH.

Fig. 12 shows the degree of helicity of the native enzyme as well as that of the enzymes which were reassociated in the presence and absence of DPNH; the figure indicates that the structure of the enzyme that was reassociated in the presence of DPNH is closer to that of the native lactate dehydrogenase.

**DISCUSSION**

Several reports have indicated that during reversible denaturation of a protein, the presence of the prosthetic group or the substrate in the media causes an increase in the rate of refolding of the peptide chain during reactivation. Such a view is illustrated by the addition of heme to globin (35) and malate to urea-denatured fumarase (36). The results presented in this communication indicate a similar effect of DPNH with lactate dehydrogenase. Chilson et al. (3) working with chicken $H_4$ lactate dehydrogenase...
dehydrogenase suggested the possibility of an equilibrium state between folded and urea-denatured polypeptide chains, and proposed that the presence of the cofactor affects the rate of recovery but not the equilibrium. Results presented in this paper support such a hypothesis. The data in Fig. 10 indicate that the same amount of activity is ultimately recovered whether or not DPNH is present, as in the case of dogfish M₄ lactate dehydrogenase. In the early stages of reassociation of the dogfish M₄ enzyme, however, the initial rate of recovery of activity in the presence of DPNH is almost twice that observed in the absence of DPNH, maximum activity being reached considerably later in the absence of the cofactor.

Steiner and Edelhoch (37) and Edelhoch and Steiner (38) have pointed out that the close proximity of tryptophan to tyrosine can affect the fluorescence yield of a protein. Bränd et al. (23) have shown that the loss of native structure in alcohol and lactate dehydrogenases causes a sharp decrease in the fluorescence yield of these enzymes. The same authors have also suggested that the native structure of an enzyme is essential for the binding of the coenzyme. The decrease in coenzyme-binding capacity of the lactate dehydrogenase (Fig. 2), and the decrease in the fluorescence quantum yield (Figs. 2 and 4) after the reversible denaturation of dogfish M₄ lactate dehydrogenase, could be attributed to a subtle loss in the native structure, possibly leading to a separation of tryptophan and tyrosine residues, that would cause a decrease in energy transfer by resonance. Data presented in Figs. 5 and 12 show that upon the reversible denaturation of dogfish M₄ lactate dehydrogenase a significant change in the structure is observed for the reassociated protein as compared to the untreated enzyme. Such findings support the hypothesis that some change in native structure occurs upon dissociation and reassociation of the dogfish M₄ lactate dehydrogenase and that the coenzyme affects the reassociation. We have also found that the properties of the sample completely reassociated in the absence of the coenzyme does not revert back to properties similar to those of the native upon prolonged incubation with DPNH.

Givol et al. (39) and Anfinsen (40) have postulated that the attainment of a three-dimensional structure of a protein is governed solely by the information present in the amino acid sequence of the polypeptide chain (thermodynamic hypothesis), and not through cellular control. It would seem, however, from the work presented here, that upon dissociation and refolding of the peptide chain, structural changes do result, which are less pronounced when the coenzyme is included in the reassociation media (Fig. 12). In addition, if the three-dimensional structure is governed only by the amino acid sequence, one would expect that, upon refolding, the peptide chain would return to the stable structure it possessed prior to denaturation. The results, presented in Figs. 9 and 11 show that the reassociated enzyme, although thermodynamically stable, differs from the native protein in its stability toward urea and heat denaturation. Previous work of Kitto et al. (7) and that of Braginski et al. (41), show the essentiality of such comparisons of the physical properties of the native and reassociated enzymes.

If the in vivo folding of a polypeptide chain depends solely on information contained in the amino acids sequence, and if we accept that the in vivo renaturation is a good model for the study of this folding, then the time required for the folding in vivo and in vitro should be identical. It is generally agreed that the time required for biosynthesis of active protein is in the order of seconds to minutes (5). However, it was shown that the orderly folding of the dissociated RNase is much more prolonged, with 50% reactivation being achieved only after 6 hours (42). The addition of the microsomal disulfide interchange enzyme and other cellular components to the reaction media of RNase, significantly reduce the reaction time (43). Although the reassociation of lactate dehydrogenase does not require as much time as RNase, it is definitely clear from Fig. 10 that the refolding and reassociation of this enzyme requires more time in vitro than that postulated for the in vivo synthesis and folding (5). It is also clear from Fig. 10 that in the presence of DPNH the time required for maximum refolding in vitro is considerably decreased.

Berson and Yalou (44) have shown that, although sperm whale and pig insulin have an identical amino acid sequence, certain antisera can distinguish between the two proteins. The authors (44) concluded that in addition to the amino acid sequence there exists a cellular control on the precise folding of a protein and on the determination of its three-dimensional conformation.

A conformational change in one of several antigenic determinants may alter the complementary nature between the antigen and its antibody. This can be sufficient to alter the complement fixation curve (17). The data presented in Fig. 6 show the difference in the interaction of the rabbit antisera, prepared against native dogfish M₄ lactate dehydrogenase, with the native as well as the renatured enzyme. The decrease in the extent of complement fixation as well as the shift of the peak of fixation toward higher protein concentration for the renatured enzyme may indicate a decreased antigen-antibody aggregation and lower affinity to each other because of altered complementarity (17).

Deal (45) has demonstrated the absolute requirement for DPNH in the reassociation media of glyceraldehyde 3-phosphate dehydrogenase. The work presented in this paper also indicates that the inclusion of the coenzyme in the reassociation media of lactate dehydrogenases causes the protein to refold into a structure whose properties more closely resemble those of the native protein. Blethen and Kaplan (46) have also demonstrated the necessity for adding arginine in the reactivating media of denatured arginine kinase. This has been interpreted (47) to mean that "the substrate, by binding to the unfolded polypeptide chain, helps to direct the enzyme into a conformation which is catalytically active . . . or by allowing the proper association of subunits."

Another role which has been postulated (49) for the coenzyme or the substrate is the regulation of the enzyme level in vivo by protecting the enzyme from its degradative pathway, by inducing a more stable conformation. A classical example for this role is the action of tryptophan on the enzyme tryptophan pyrrole.
It has been shown that tryptophan prevents the degradation of the tryptophan pyrrolase by converting the apoenzyme into an active holoenzyme which is resistant to proteolytic attack (48–50).

It is our view that the amino acid sequence certainly is the determining factor in producing structures possessing catalytic activity. The effect of specific modification of amino acids on the reassociation of lactate dehydrogenase shows the uniqueness of the sequence in determining structure. However, our results, as well as others, indicate the possibility of formation of several closely related structures on reassociation in vitro.

Previous reports (44–48) and the data presented in this paper suggest the importance of the cell constituents in achieving the proper conformation of an enzyme. The present report indicates that the amino acid sequence of lactate dehydrogenase is not the sole determinant of its higher order structure. Cell constituents such as DPNH may play a role in the in vivo folding and the assembly of the subunits of this enzyme.

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* While this manuscript was in preparation, Teipel and Kosland have published supporting evidence (61) for a different structure of lactate dehydrogenase as result of reversible denaturation.

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