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Quaternary Structure, Subunit Activity, and in Vitro Association of Porcine Mitochondrial Malic Dehydrogenase†

Rainer Jaenicke,* Rainer Rudolph, and Ingrid Heider

ABSTRACT: The native, dimeric quaternary structure of porcine mitochondrial malic dehydrogenase (m-MDH) remains unchanged upon dilution in the concentration range from 114 to 0.2 μg/mL, as shown by gel chromatography in 0.2 M phosphate buffer, pH 7.6, at 20 °C. In the given concentration range the enzyme can be reassociated and reactivated after dissociation and denaturation at acid pH, as well as in 6 M urea or 6 M guanidine hydrochloride. Removal of the denaturants and separation of inactive "wrong aggregates" lead back to a final yield of about 60% of renatured enzyme. Reactivated m-MDH is found to be indistinguishable from the native enzyme as far as quaternary structure, enzymatic activity, Michaelis constants, and spectral properties are concerned. The concentration dependence of the rate of reactivation and the sigmoidicity of the kinetic profiles may be described by a consecutive first-order transconformation and second-order association reaction. One set of rate constants, $k_1 = 6.5 \times 10^{-4}$ s^{-1} and $k_2 = 3 \times 10^4$ M^{-1} s^{-1} (20 °C), is sufficient to describe the kinetics of reconstitution, independent of the mode of denaturation used to disrupt the native structure of the enzyme. This implies (1) that there exists a common, folded monomeric intermediate which is formed prior to the rate-limiting reactions described by k_1 and k_2 and (2) that this monomer does not show full enzymatic activity. The presence of NAD^+ does not influence the kinetics of reactivation, when one uses the apoenzyme as a reference.

The acquisition of the native three-dimensional structure of proteins as the "kinetically accessible minimum of the potential energy" is an intrinsic property of the polypeptide chain which is determined by kinetic and thermodynamic factors (Wetlaufer & Ristow, 1973). In the case of oligomeric enzymes, composed of two or more subunits, it is of interest to know whether the isolated chains are able to maintain the enzymatically active tertiary structure, i.e., whether or not intersubunit interactions are required to attain the native overall configuration of the molecule. For a number of oligomeric enzymes, isolated monomers have been reported to be accessible at low enzyme concentrations; however, most oligomers are dissociable only by strong denaturants such as guanidine hydrochloride or urea or by extremes of pH. Under these conditions the isolated subunits do not show catalytic activity because dissociation is accompanied by deactivation and a more or less pronounced loss of secondary and tertiary structure. In general all three processes, dissociation, denaturation, and deactivation, are found to be reversible. Therefore, under certain conditions structured monomers are accessible as intermediates during reconstitution. To characterize their properties regarding the correlation of folding, association, and catalytic function, the kinetics of reactivation and renaturation can be applied (Jaenicke, 1978a).

Previous investigations on the reconstitution of some tetrameric dehydrogenases have proved this approach to be useful (Jaenicke, 1974, 1978b; Rudolph, 1977). However, in the case of the tetrameric enzymes no clear-cut information regarding the functional properties of association intermediates was obtained because the dimer or tetramer could equally well be considered the active species fitting the time course of reconstitution in a quantitative way (Jaenicke & Rudolph, 1977; Hermann et al., 1979).

In order to provide an unambiguous correlation between the state of association and the processes of folding and reactivation, a *dimeric* enzyme has been investigated in the present study. Since there is a close relationship regarding the tertiary structure and the spatial arrangement of the subunits in the tetrameric lactic dehydrogenases (which were studied in detail in previous experiments) and in dimeric malic dehydrogenase, the latter enzyme was chosen to elucidate the mechanism of reconstitution. Besides the expected deeper understanding of the general mechanism of folding and association which may be deduced from the close evolutionary relationship of the two enzymes, there are two aspects of interest in a comparative study of LDH[1] and MDH.

While in the case of LDH earlier findings indicating concentration dependent dissociation were clearly disproved, recent reports for MDH strongly suggest monomers to predominate under the conditions of the enzymatic test. However, this observation is still controversial. Reconstitution experiments are expected to shed light on this problem.

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[1] Abbreviations used: m-MDH, pig heart mitochondrial malic dehydrogenase; LDH (LDH-H_4 and LDH-M_4), lactic dehydrogenase (H_4 and M_4 refer to the isoenzymes from heart and skeletal muscle, respectively); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NAD, nicotinamide adenine dinucleotide; *c*, enzyme concentration; ϑ_i, denatured state; D and D′, monomeric intermediate states; N′, dimeric intermediate state; N and N*, dimeric native and renatured states; DTE, dithioerythritol; oxac, oxaloacetate.

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One further question concerns the importance of small ligands as nucleating effectors in the process of structure formation. For both enzymes mentioned before, coenzymes or substrates were reported to increase the rate of reconstitution. A careful analysis has shown, however, that there is no significant effect of NAD^+ or NADH on the reactivation kinetics of the heart and muscle isoenzymes of LDH. Whether this finding can be generalized may be answered only based on additional experiments with other enzymes.

Materials and Methods

Porcine mitochondrial MDM, NADH, and NAD^+ were purchased from Boehringer (Mannheim), dithioerythritol was from Roth (Karlsruhe), and ultrapure guanidine hydrochloride and urea were supplied by Schwarz/Mann (Orangeburg, NY). All other reagents were A-grade substances from Merck (Darmstadt); quartz twice-distilled water was used throughout.

Stock solutions of the enzyme (~3 mg/mL) were prepared by repeated dialysis at 4 °C against 0.2 M phosphate buffer, pH 7.6, containing 1 mM EDTA and 1 mM dithioerythritol. Enzyme activity was measured in 0.2 M phosphate buffer, pH 7.6, plus 0.5 mM oxaloacetate and 0.2 mM NADH, by use of a recording Eppendorf spectrophotometer thermostated at 25 °C. The specific activity of the native enzyme was 1040 IU/mg.

Enzyme concentrations were calculated from $A_{280}^{1\%} = 2.5$ (Gregory et al., 1971); this figure holds for the native and for the renatured enzyme when the Lowry method is used for comparison. Molar concentrations are based on a subunit molecular weight of 35000 (cf. Thorne & Kaplan, 1963).

Gel filtration was performed at 20 °C by use of a Sephadex G-75 superfine column (1.6 × 80 cm) equilibrated with 0.2 M phosphate buffer, pH 7.6, plus 1 mM EDTA and 1 mM dithioerythritol. Appropriate proteins of known molecular weight were used to calibrate the column.

Elution profiles with varying protein concentrations were analyzed by measuring fluorescence emission and enzymatic activity. Concentrations of the eluted proteins were determined by fluorescence.

Deactivation and dissociation of the native enzyme were achieved at 20 °C by 0.1–3-h incubation after dilution (1:10) with 1 M glycine/H_3PO_4 buffer, pH 2.3, plus 1 mM EDTA and 1 mM dithioerythritol, or by incubation in 0.2 M phosphate buffer, pH 7.6, plus 10 mM EDTA and 10 mM dithioerythritol in the presence of 6 M guanidine hydrochloride or 6 M urea.

For reactivation and reassociation, the denaturation mixtures were diluted with 0.2 M phosphate buffer, final pH 7.6, in the presence of 10 mM EDTA and 10 mM dithioerythritol; in some experiments 10 mM NAD^+ was present in the reactivation buffer. The degree of saturation for the reactivated enzyme with NAD^+ was calculated on the basis of the dissociation constants reported for native enzyme, $K_{D,NAD^+} = 480$ μM (Holbrook & Wolfe, 1972).

The kinetics of reactivation were analyzed by taking aliquots at defined times, the optical tests being performed as described. The recovery of native fluorescence was measured in a Hitachi Perkin-Elmer MPF 44A spectrophotometer ($\lambda_{exc} = 275$ nm; $\lambda_{em} = 315$ nm).

To further characterize the reactivated enzyme, solutions were concentrated in an Amicon diaflo with PM 10 filters. The renaturation mixture contained high aggregates which were separated from the reactivated dimers by Millipore filtration and gel filtration on Sephadex G-75 superfine. Saturation characteristics of native and reactivated m-MDM were measured for oxaloacetate and NADH; K_M values were derived by double-reciprocal linearization. Absolute fluorescence spectra of native, reactivated, and denatured enzyme were measured by use of the Perkin-Elmer "corrected spectra computer". Sedimentation velocity runs were performed in an analytical ultracentrifuge (Beckman; Model E) with a high-sensitivity photoelectrical scanning system.

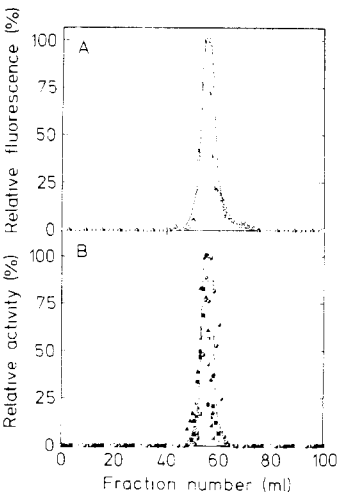

FIGURE 1: Molecular weight of native m-MDH, determined by gel filtration at various enzyme concentrations. For experimental details see Materials and Methods. Concentrations of the eluted enzymes (μg/mL) were determined by fluorescence: (○) 114; (△) 60; (□) 28; (◑) 6; (▲) 3; (◪) 1.4; (●) 0.7; (▲) 0.4; (■) 0.2. (A) Elution profiles analyzed by fluorescence. $\lambda_{exc} = 275$ nm; $\lambda_{em} = 315$ nm. (B) Elution profiles analyzed by enzymatic activity.

Results

Equilibrium Measurements

The analysis of the reconstitution of oligomeric enzymes is based on the assumption that the initial and final states, i.e., the native and renatured enzyme and the denatured one, belong to homogeneous oligomers or monomers, respectively. In order to perform significant kinetic analyses, the enzymes under nondenaturing conditions must retain their native quaternary structure even at very low concentrations. In the case of malic dehydrogenase (m-MDH), analysis of the intrinsic fluorescence as a function of concentration does not indicate changes of the quaternary structure of the enzyme even at concentrations as low as ~3 μg/mL = 0.1 μM. As shown in Figure 1, this indirect evidence is directly proven by column chromatography, when we analyzed the fractions for total protein and specific activity by fluorescence and activity measurements. In the concentration range 100–0.2 μg/mL, only one species belonging to the native dimer of $M_r = 70000$ could be detected by either method. Frieden et al. (1978), in an attempt to detect changes of the native quaternary structure in the same concentration range by means of fast kinetics, were equally unable to confirm reports claiming MDH to show concentration-dependent dissociation. Therefore, it is obvious that under the given experimental conditions m-MDH retains its dimeric structure without changing its catalytic properties at enzyme concentrations: $c \geq 0.2$ μg/mL. It is well-known that m-MDH can be easily dissociated into inactive, denaturated subunits by treatment with acid, urea, or guanidine hydrochloride (Murphey et al., 1967).

In the present experiments acidification to pH 2.3 or addition of 6 M guanidine hydrochloride was found to cause the almost instantaneous loss of activity, while in the presence of 6 M urea this inactivation reaction was comparatively slow. After 50-min incubation in 6 M urea there was still a residual activity of 3% left. The profiles of the pH dependences of

Table I: Characterization of m-MDH in Its Native, Refolded, and Dissociated States

state of the enzyme	$s_{20,w}$ (S)	M_r[a] × 10^{-3}	act. (IU/mg)	$K_{M,oxac}$ (μM)	$K_{M,NADH}$ (μM)	fluorescence (%)
native dimers (pH 7.6)	3.48	61.4	1040	41	77	100
renatured dimers (pH 7.6)	nd[b]	69.7	1130	39	76	99.7
dissociated monomers (pH 2.3)	2.44	nd	0			230

[a] Determined by gel chromatography. [b] nd, not determined.

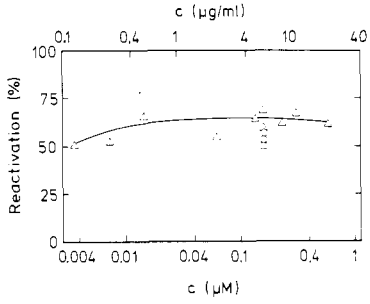

FIGURE 2: Effect of enzyme concentration on the yield of reactivation of m-MDH after deactivation at 20 °C (calculated relative to the initial enzymatic activity before denaturation). Reactivation at 20 °C was initiated by dilution with 0.2 M phosphate buffer, pH 7.6, plus 10 mM EDTA plus 10 mM DTE. Reactivation time was up to 290 h. Deactivation by 5-min incubation in 1 M glycine/H_3PO_4, pH 2.3, 1 mM EDTA, 1 mM DTE (△), or 0.2 M phosphate buffer, pH 7.6, 10 mM EDTA, 10 mM DTE plus 6 M guanidine hydrochloride (□), or by 0.8–3-h incubation in 0.2 M phosphate buffer, pH 7.6, 10 mM EDTA, 10 mM DTE plus 6 M urea (○).

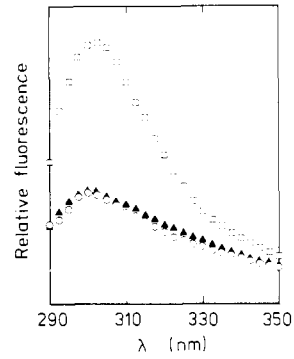

FIGURE 3: Fluorescence emission of native, denatured, and reactivated m-MDH. Fluorescence measurements (20 °C) at c = 10 μg/mL and λ_{exc} = 275 nm of native (▲) and renatured (○) m-MDH in 0.2 M phosphate buffer, pH 7.6, 1 mM EDTA, 1 mM DTE, and of denatured monomers (□) in 1 M glycine/H_3PO_4, pH 2.3, 1 mM EDTA, 1 mM DTE.

dissociation, deactivation, and denaturation were analyzed by Hodges et al. (1977) and by Thorne & Kaplan (1963). The respective reverse reactions have not been analyzed; therefore the occurrence of a range of "hysteresis" where unfolded monomers are unstable and tend to polymerize can only be deduced from the incompleteness of reconstitution (cf. Jaenicke & Rudolph, 1977; Rudolph et al., 1977b).

As shown in Figure 2, the yield of reactivation does not strongly depend either on enzyme concentration or on the mode of denaturation. Concerning the concentration dependence, a similar behavior was observed for the reactivation of LDH-M_4 (Rudolph & Jaenicke, 1976), as well as yeast GAPDH (Rudolph et al., 1977a).

The significance of in vitro reassociation experiments depends on the identity of the native enzyme with the enzyme after reassociation. To compare renatured m-MDH with the native enzyme (N), the renatured dimers (N*) had to be separated from native higher aggregates by Millipore filtration and gel chromatography. As shown by their respective molecular weights both the native and the renatured proteins are dimeric (Table I). As taken from Figure 3, there are no significant differences between the fluorescence spectra of the native and reassociated dimers. The fluorescence of denatured monomers is drastically increased compared to that of N or N*. As shown in Table I and Figure 4, the reassociated dimer is not only fully active but shows the same saturation characteristics for oxaloacetate and NADH as the native enzyme; the respective K_M values, determined by double-recipocal linearization, are the same within the limits of error (3%).

Kinetic Measurements

The analysis of the initial product of the in vitro reassociation proves N* to be indistinguishable from the enzyme in its native state (N* = N). Therefore, reactivation kinetics can be used to analyze the correlation between the state of association and the catalytic function of the enzyme.

The reconstitution of the dimeric enzyme must include first-order transconformation reactions and the second-order

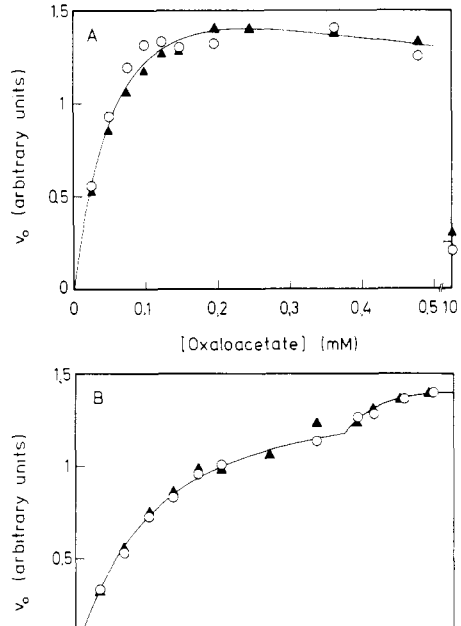

FIGURE 4: Saturation characteristics of native and reactivated m-MDH with substrate and coenzyme. Enzymatic test of native (▲) and reactivated (○) enzyme at c = 0.017 μg/mL (0.5 nM), 25 °C. (A) Saturation characteristics for oxaloacetate. Enzymatic test in the presence of 0.2 mM NADH. Determination of $K_{M,oxac}$ by double-reciprocal linearization gives $K_{M,oxac}$ = 41 μM for the native and $K_{M,oxac}$ = 39 μM for the reactivated enzyme. (B) Saturation characteristics for NADH. Enzymatic test in the presence of 0.5 mM oxaloacetate. Determination of $K_{M,NADH}$ by double-reciprocal linearization gives $K_{M,NADH}$ = 77 μM for the native and $K_{M,NADH}$ = 76 μM for the reactivated enzyme.

association of the folded monomers to the native dimeric enzyme. From the foregoing equilibrium data, it is evident that the reconstitution takes place under essentially irreversible conditions; e.g., changing of the pH from 2.3 to 7.6 leads to

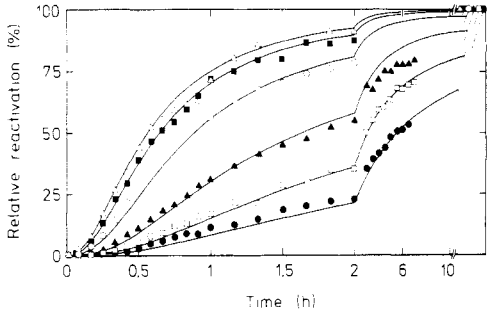

FIGURE 5: Kinetics of reactivation of m-MDH after 5-min deactivation in 1 M glycine/H_3PO_4, pH 2.3, 1 mM EDTA, 1 mM DTE, at 20 °C, in 0.2 M phosphate buffer, pH 7.6, 10 mM EDTA, 10 mM DTE at varying enzyme concentrations (nM): (△) 143; (■) 88; (○) 35; (▲) 10; (□) 4; and (●) 2. Reactivation was calculated relative to final values, determined after a reactivation time of up to 290 h. Solid lines are calculated according to an irreversible uni-bimolecular mechanism with $k_1 = 6.5 \times 10^{-4}$ s^{-1} and $k_2 = 3 \times 10^4$ M^{-1} s^{-1}.

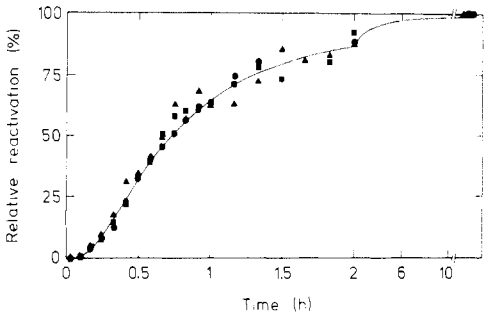

FIGURE 6: Kinetics of reactivation of m-MDH after deactivation in various denaturants. Denaturation at 20 °C by 5-min incubation in 1 M glycine/H_3PO_4, pH 2.3, 1 mM EDTA, 1 mM DTE (●) or 0.2 M phosphate buffer, pH 7.6, 10 mM EDTA, 10 mM DTE, plus 6 M guanidine hydrochloride (■), or by 50-min incubation in 0.2 M phosphate buffer, pH 7.6, 10 mM EDTA, 10 mM DTE, plus 6 M urea (▲). In the case of urea denaturation 3% residual activity was observed after 50-min deactivation; the reactivation relaxations were corrected for this value. Reactivation in 0.2 M phosphate buffer, pH 7.6, 10 mM EDTA, 10 mM DTE, 20 °C, at $c = 60$ nM. Reactivation was calculated relative to the final values of reactivation, determined after a reactivation time of up to 120 h. Solid lines are calculated according to an irreversible uni-bimolecular mechanism with the rate constants derived from the reactivation kinetics after acid dissociation (cf. Figure 5).

a shift of the equilibrium D ⇌ N toward the native dimer. As a consequence, at least some of the single elementary steps in the transition D → N must proceed in an irreversible fashion so that the reverse reaction can be neglected in the kinetic analysis. For the given reason the following irreversible consecutive transconformation–association processes have to be considered as a minimum scheme:

$$2D \xrightarrow[\text{uni}]{k_1} 2D' \xrightarrow[\text{bi}]{k_2} N' \xrightarrow[\text{uni}]{k_1'} N^* \qquad (1)$$

Whether or not the isolated monomers possess intrinsic enzymatic activity may be easily determined when we quantitatively analyze the time course of the reactivation reaction. If the reactivation turns out to depend on the bimolecular association reaction, the isolated monomers (D or D′) cannot be active. Association-dependent structural changes, which might be essential for the recovery of full enzymatic activity, are expected to affect certain conformational parameters, such as the intrinsic protein fluorescence. In this context, it is important to know whether the varying degree of denaturation after dissociation at acid pH or in the presence of high concentrations of guanidine hydrochloride influences the kinetics of reactivation (cf. Gerschitz et al., 1977; Rudolph et al., 1977b). Furthermore, it is of considerable interest to determine the effect of the coenzymes on the time course of reactivation in order to evaluate the nucleating power of specific ligands considered to be important in the acquisition of the native three-dimensional structure (cf. Teipel & Koshland, 1971).

Reactivation. Recovery of enzymatic activity after acid dissociation was monitored by sampling aliquots at defined time intervals. The sigmoidal kinetic traces (Figure 5) indicate that the reactivation reaction cannot be determined by only one single rate-limiting reaction. As illustrated by eq 1, two alternative, consecutive reaction sequences involving two rate-limiting steps may be considered to describe the observed kinetic behavior. Because of the concentration dependence of the reactivation rate, a bimolecular process must be involved (Figure 5).

Therefore, another alternative comprising two consecutive first-order reactions can be excluded. On the basis of the present data, it is not possible to decide which of the two alternative reactions

$$2D \xrightarrow[\text{uni}]{k_1} 2D' \xrightarrow[\text{bi}]{k_2} N' \qquad (2)$$

$$2D' \xrightarrow[\text{bi}]{k_2} N' \xrightarrow[\text{uni}]{k_1'} N^* \qquad (3)$$

does in fact underly the kinetic traces given in Figure 5. As in the case of the heart isoenzyme of LDH (Rudolph et al., 1977b) or yeast GAPDH (Rudolph et al., 1977a), the sigmoidal reactivation relaxations of m-MDH can be easily fitted by an irreversible two-step consecutive reaction of the uni-bimolecular type according to eq 2, with D′ as a hypothetical inactive intermediate. The second alternative, according to eq 3, which cannot be excluded would implicate an inactive dimeric intermediate (N′). In the mathematical approximation used to analyze eq 2 or 3 (Chien, 1948), irreversibility only implies that the reverse reactions have rate constants much below those holding for the forward reactions. As shown by the agreement of the calculated full lines with the experimental points in Figure 5, one set of kinetic constants, $k_1 = 6.5 \times 10^{-4}$ s^{-1} and $k_2 = 3 \times 10^4$ M^{-1} s^{-1}, is sufficient to describe the reactivation profiles over the entire range of enzyme concentrations applied ($c = 2$–143 nM). To describe both the sigmoidicity and the concentration dependence of the reactivation profiles, both constants had to be in a defined range. Within the limits of error, the same set of rate constants described the kinetics of reactivation of m-MDH after dissociation in all of the different denaturants applied: pH 2.3, 6 M urea, and 6 M guanidine hydrochloride (Figure 6).

As illustrated in Figure 7, the presence of saturating amounts of oxidized coenzyme, NAD^+, has no influence on the time course of reactivation.

Renaturation. As shown in Table I, the intrinsic fluorescence of m-MDH is considerably increased upon acid denaturation. A similar increase in protein fluorescence has been previously reported for the denaturation in 6.5 M urea (Thorne & Kaplan, 1963). The time course of the fluorescence changes during renaturation reveals a complex profile (Figure 8): after an initial fast decrease a further slow decrease is observed. The profiles of the slow relaxations are sigmoidal; their rates depend on enzyme concentration in a way comparable to the rates of reactivation. Obviously, association is essential for both reactivation and renaturation.

Discussion

Recent investigations on the reconstitution of oligomeric enzymes have shown that the kinetic analysis of reactivation

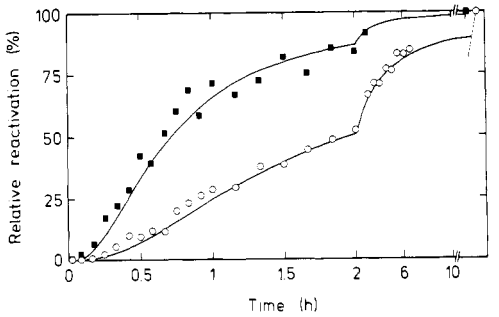

FIGURE 7: Kinetics of reactivation of m-MDH in the presence of 10 mM NAD^+. Denaturation as described in Figure 5. Reactivation in 0.2 M phosphate buffer, pH 7.6, 10 mM EDTA, 10 mM DTE, at 20 °C; enzyme concentrations were 65 nM (■) and 7.4 nM (○). At this concentrations native m-MDH is 95% saturated with NAD^+ (Holbrook & Wolfe, 1972). Reactivation was calculated relative to final values, determined after a reactivation time of up to 72 h. Solid lines are calculated according to an irreversible uni-bimolecular mechanism with the rate constants derived from the reactivation kinetics in the absence of coenzyme (cf. Figure 5).

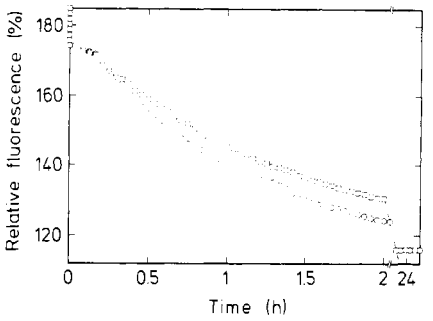

FIGURE 8: Recovery of native fluorescence of m-MDH after denaturation in 1 M glycine/H_3PO_4, pH 2.3, 1 mM EDTA, 1 mM DTE, at 20 °C. Renaturation in 0.2 M phosphate buffer, pH 7.6, 10 mM EDTA, 10 mM DTE (20 °C) at varying enzyme concentrations (nM): (○) 211 and (□) 111. Final values of renaturation were determined after 24 h. Fluorescence emission at λ_{em} = 315 nm (λ_{exc} = 275 nm) was calculated relative to the native enzyme (100%).

and renaturation is a powerful tool in analysis of the correlation of folding, association, and enzymatic function (Jaenicke, 1978). The rationale of this approach is based on the fact that during renaturation denatured monomers must fold to structured intermediates which are able to recognize identical association partners. If these monomeric intermediates show full enzymatic activity, reactivation must be independent of the following association reactions. If, on the other hand, these structured monomers are inactive, association reactions must be rate limiting in the process of reactivation, at least in a certain concentration range. By use of this concept, previous investigations proved the monomers of some tetrameric dehydrogenases to be enzymatically inactive while the subunits of aldolase were shown to be partially active (cf. Jaenicke, 1978). In all cases investigated so far, the reactivation is characterized by complex kinetics because of the influence of transconformation reactions besides the obligatory rate-limiting association reaction. As a first approximation, in general, a consecutive uni-bimolecular process turned out to be sufficient to describe the time- and concentration-dependent profiles. However, in the case of the tetrameric enzymes it remained undecided whether the active species formed in this rate-limiting association process was a dimeric intermediate of the final tetramer (Hermann et al., 1979). The present data apply to an enzyme which is dimeric in its native state so that this ambiguity no longer exists. In the given context, observations from comparative X-ray studies (Rossmann et al., 1975) are of importance, which clearly established that there is a close structural relationship between MDH and the above-mentioned tetrameric dehydrogenases. On the basis of its tertiary structure, MDH may in fact be considered a half molecule of LDH or GAPDH (Rossmann et al., 1975). According to Rossmann's *P*, *Q*, and *R* coordinates (Rossmann et al., 1973), s-MDH, LDH, and GAPDH represent "*Q*-axis dimers". In the case of the two tetrameric dehydrogenases, the *Q*-axis dimers are considered to be associated "head-to-head" in the case of GAPDH and "tail-to-tail" in the case of LDH. Therefore, analysis of the formation of the *Q*-axis dimers for MDH might give some indication as to whether the reassociation reactions which are rate limiting for the reactivation of LDH and GAPDH belong to one and the same reaction, namely, the association of monomers.

It is generally accepted that under normal physiological conditions mitochondrial and cytoplasmic MDH from mammalian sources is dimeric with a molecular weight in the range of 60 000–70 000 (Thorne & Kaplan, 1963; Murphey et al., 1967). Recent reports, however, suggest that the enzymes undergo concentration-dependent dissociation to the monomer (Cassman & King, 1972; Koren & Hammes, 1975; Shore & Chakrabarti, 1976; Bleile et al., 1977; Wood et al., 1978). Investigations by Frieden et al. (1978) have shown that a variety of kinetic properties of m-MDH do not depend on protein concentration. This would imply that association, if it occurs, is unrelated to the kinetic characteristics of the active enzyme, which means that the isolated monomers should be fully active. Careful analyses of the molecular weight prove that m-MDH does not dissociate at concentrations as low as 0.2 μg/mL (Figure 1; cf. Frieden & Fernandez-Sousa, 1975). Therefore the active enzyme investigated by Frieden et al. (1978) using kinetic and activity transport measurements may equally be considered the dimer. As far as the present reactivation experiments are concerned, the conclusion has to be drawn that under the given experimental conditions m-MDH does not dissociate in the range of concentrations where the reactivation measurements were performed.

One further prerequisite of significant studies refers to the identity of the enzyme in its native and renatured state. After separation of inactive aggregates from the dimeric main product of reconstitution (~70% yield; cf. Figure 2), it was shown that the reactivated enzyme consists of active dimers, indistinguishable from the active enzyme regarding its physicochemical and enzymological properties (Figures 3 and 4 and Table I). Similar results were reported previously regarding the electrophoretic and immunological parameters of native and renatured MDH (Chilson et al., 1965, 1966). Conflicting observations (Teipel & Koshland, 1971) may be explained by the fact that separation of aggregates was omitted in these studies.

With regard to the reactivation kinetics of pig heart m-MDH, it has been previously shown that the respective profiles are sigmoidal (Chilson et al., 1965, 1966) and do not obey simple first- or second-order kinetics (Teipel & Koshland, 1971). The quantitative analysis of the present kinetic data (Figure 5) reveals that the reactivation of m-MDH closely resembles the one reported for the heart isoenzymes of LDH (Rudolph et al., 1976) or for yeast GAPDH (Rudolph et al., 1977a). For the given enzymes the reactivation relaxations may be adequately described by an irreversible consecutive uni-bimolecular reaction sequence. The alternative bi-unimolecular mechanism which requires a more complex mathematical fitting program has not been analyzed, because it can not be decided on the basis of the present data which of the two mechanisms holds true. In both alternatives the

monomer–dimer association ($2D' \rightarrow N'$) is a necessary prerequisite for the reactivation of the enzyme. The similarity of the second-order rate constant for the reactivation of m-MDH ($k_2 = 3 \times 10^4$ M^{-1} s^{-1} at 20 °C) to the corresponding second-order rate constants observed for LDH-M_4 ($k_2 = 2.3 \times 10^4$ M^{-1} s^{-1} at 20 °C), LDH-H_4 ($k_2 = 0.5 \times 10^4$ M^{-1} s^{-1} at 20 °C), and yeast GAPDH ($k_2 = 4.5 \times 10^4$ M^{-1} s^{-1} at 15 °C) may be considered to indicate that, in the case of all the tetrameric dehydrogenases under concern, the formation of the *Q*-axis dimer is rate limiting.

In the given context it should be mentioned that the reactivation of a dimeric MDH, isolated from halophilic bacteria, after dissociation at low ionic strength, obeys second-order kinetics too, except for a small "delay phase"; the rate constants [$k_2 = (0.8–2.8) \times 10^4$ M^{-1} s^{-1} at 30 °C, depending on the salt concentration] are again comparable to those mentioned before. According to the rate-limiting bimolecular reaction involved in the reactivation of porcine mitochondrial MDH, the isolated monomers must be enzymatically inactive. The question of whether there is a structural basis for stable (and active) monomers has been previously discussed on the basis of the known three-dimensional structure without a clear-cut answer (Banaszak & Bradshaw, 1975).

It is most suggestive to assume that the hypothetical conformational differences between the MDH subunits in their dimeric and monomeric form, which were postulated by Banaszak, are responsible for the observed inactivity of the isolated monomers. As mentioned, the quantitative description of the reactivation by one unimolecular and one bimolecular rate constant is independent of the denaturant applied to dissociate and deactivate the enzyme (Figure 6). Since the various modes of denaturation leading to the different states, ϑ_i, differ widely regarding the secondary and tertiary structure of the subunits (cf. Gerschitz et al., 1977), the unfolded species ϑ_i must fold in fast reactions to a common intermediate D. These fast reactions ($\vartheta_i \rightarrow D$), which may underly the fast initial phase in the fluorescence changes illustrated in Figure 8, can perhaps be correlated to the formation of nucleation centers (Karplus & Weaver, 1976).

Once the intermediate state D has been reacted, the folding and association pathway is obligatory. Similar results were obtained for aldolase (Gerschitz et al., 1977), LDH (Rudolph et al., 1977b), and yeast GAPDH (H. Krebs, R. Rudolph, and R. Jaenicke, unpublished experiments).

According to the foregoing results, the reaction scheme given in eq 3 has to be modified according to

$$2\vartheta_i \xrightarrow{k_2} 2D \xrightarrow{k_1} 2D' \xrightarrow{k_2} N' \xrightarrow{k_1'} N^* \qquad (4)$$

In this scheme k_i describes the fast nucleation reactions; whether k_1 or k_1' is the rate-limiting transconformation reaction cannot be decided yet.

Similar to earlier observations for LDH-H_4 (Rudolph et al., 1977c), no influence of NAD^+ on the rate of reactivation could be detected for m-MDH (Figure 7); obviously, the coenzyme does not play a significant role in the folding of the enzyme.

In summary, we may conclude that in the case of m-MDH, like LDH and GAPDH, no additional information beyond that contained in the amino acid sequence is required to reconstitute the enzyme after complete unfolding and dissociation. In general, association turns out to be a necessary prerequisite for the acquisition of the native three-dimensional structure as well as full enzymatic activity. The intramolecular interactions between the residues of the isolated chains direct the folding to form intermediate structures which are able to recognize the correct partners of association. Obviously, the primary function of the nascent or refolding subunit of an oligomeric enzyme is its own self-assembly. As suggested by their lack of activity and by the differences in their physical properties, the subunits in the stage of association are still imperfect regarding their three-dimensional structure in the final state of the native quaternary structure. The acquisition of this final structure depends on intrachain *and* interchain interactions.

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Fluorescence Studies of the Pyruvate Dehydrogenase Multienzyme Complex from *Escherichia coli*†

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ABSTRACT: Reduced lipoic acids on the pyruvate dehydrogenase multienzyme complex from *Escherichia coli* have been preferentially labeled by making use of the differential reactivity of lipoic acids in different environments. The lipoic acids have been labeled with *N*-ethylmaleimide, *N*-(3-pyrene)maleimide (MalPy), and *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide (DDPM). As the extent of labeling of the enzyme with MalPy increases, the fluorescence quantum yields decrease, the fluorescence polarization increases, and the emission spectrum changes in a manner indicating increased excimer formation. These results suggest that MalPy on different lipoic acids interact strongly and that some lipoic acids are very close to other lipoic acids. Energy-transfer measurements between MalPy (energy donor) and DDPM (energy acceptor) on different lipoic acids indicate that the average distance between donor and acceptor increases from 24 to 33 Å as the ratio of donors to acceptors increases. Energy-transfer measurements between MalPy (energy donor) on lipoic acids in different environments and FAD (energy acceptor) at the catalytic site of the lipoamide dehydrogenase enzyme indicate an intermolecular distance varying from 23 to >47 Å, depending on the particular lipoic acids labeled. Furthermore, the MalPy-labeled lipoic acids appear to move away from the FAD and aggregate with each other as the extent of labeling of the enzyme with MalPy increases. Energy-transfer measurements between thiochrome diphosphate (energy donor) located at the catalytic site of the pyruvate decarboxylase enzyme and DDPM (energy acceptor) labeled lipoic acids indicate little energy transfer with a variety of labeled derivatives; the intermolecular distances calculated range from 38 to >45 Å. These results are consistent with a mechanism involving multiple mobile lipoic acids which transfer acetyl groups and electrons between the three catalytic sites and adjacent lipoic acids.

The pyruvate dehydrogenase multienzyme complex from *Escherichia coli* consists of three enzymes which catalyze the overall reaction (Koike et al., 1960)

$$\text{pyruvate} + \text{NAD}^+ + \text{CoA} \rightarrow \text{acetyl-CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \quad (1)$$

The multienzyme complex is composed of a central core transacetylase (E_2)[1] to which a thiamine pyrophosphate requiring decarboxylase (E_1) and a lipoamide dehydrogenase flavoprotein (E_3) are bound (cf. Reed, 1974). The central core enzyme contains two covalently bound lysyllipoic acids per polypeptide chain (Danson & Perham, 1976; Shepherd & Hammes, 1977; Speckhard et al., 1977). The subunit stoichiometry of the multienzyme complex is controversial: models in which the ratio of E_1:E_2:E_3 is 24:24:12 (Reed et al., 1975) and 24–48:24:24 have been proposed (Bates et al., 1975). The molecular mechanism for the overall enzyme reaction also is not yet clear. The simplest mechanism proposed is that a single lipoic acid residue transfers the acetyl group by rotating between the three catalytic sites (Reed, 1974). This mechanism requires all of the catalytic sites to be within a radius of 14 Å. This possibility is inconsistent with resonance energy-transfer measurements: the intermolecular distances between specific binding sites and/or specific fluorescent labels indicate the catalytic sites of E_1, E_2, and E_3 and the disulfides of the lipoic acids are considerably further apart than 28 Å on the average (Shepherd & Hammes, 1976; Shepherd et al., 1976; Moe et al., 1974). However, an alternative mechanism in which multiple lipoic acids participate both to transfer the acetyl group and to oxidize the reduced lipoic acid is consistent with the distances measured (Shepherd & Hammes, 1977; Collins & Reed, 1977; Bates et al., 1977). Recent measurements have suggested that such interactions are possible and are part of the normal catalytic mechanism (Angelides & Hammes, 1978; Frey et al., 1978).

The work presented here extends previous measurements of the distances between lipoic acids and between lipoic acids and the catalytic sites on E_1 and E_3. The time course of the labeling of lipoic acid with maleimides is complex and suggests the lipoic acids within a given enzyme complex exist in several different environments (Angelides & Hammes, 1978). Because of this differential reactivity of the lipoic acids, lipoic acids in different environments can be labeled preferentially. The distances between these preferentially labeled lipoic acids and the catalytic sites of E_1 and E_3 vary significantly with the particular lipoic acids labeled. Some lipoic acids are quite close to the catalytic site of E_3 (~23 Å), some are within 38 Å of the catalytic site of E_1, and some are not very close to either (>45 Å). In addition the distance between lipoic acids in an

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[1] Abbreviations used: E_1, pyruvate decarboxylase; E_2, dihydrolipoyl transacetylase; E_3, lipoamide dehydrogenase; TPP, thiamine pyrophosphate; MalPy, *N*-(3-pyrene)maleimide; MalNEt, *N*-ethylmaleimide; DDPM, *N*-[4-(dimethylamino)-3,5-dinitrophenyl]maleimide.

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