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Some Kinetic Characteristics of Immobilized Protomers and Native Dimers of Mitochondrial Malate Dehydrogenase: An Examination of the Enzyme Mechanism†

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ABSTRACT: Some kinetic characteristics of immobilized native mitochondrial malate dehydrogenase dimers and immobilized protomers, prepared by direct immobilization under conditions yielding complete dissociation without substantial unfolding, were compared to those of native soluble enzyme. Enzyme was covalently immobilized to derivatized porous glass by using a technique which permitted subsequent release of bound enzyme with 0.2 M hydroxylamine at room temperature and pH 7. Kinetic properties of enzyme released from both immobilized dimers and protomers were the same as those for native soluble enzyme, indicating that the immobilization reaction per se did not affect the structure. Both immobilized native dimers and the immobilized protomers exhibited activity with a pH dependence similar to that of native soluble enzyme. The effects of diffusional inhibition were demonstrated for both forms of the immobilized enzyme, especially for the NADH → NAD^+ reaction direction. Intrinsic Michaelis constants of both immobilized forms, obtained by extrapolation of apparent values, were similar to those of the soluble enzyme. Furthermore, the effects of inhibitors and effectors with the immobilized forms were the same as those with native soluble enzyme. For example, substrate inhibition was observed with oxalacetate, the inhibitor hydroxymalonate was competitive with ketomalonate and uncompetitive with L-malate, and inhibition was observed with citrate in the NADH → NAD^+ direction. Thus, immobilization did not appear to suppress the conformational equilibria of either protomers or dimers. More significantly, the kinetic characteristics of the immobilized protomer were indistinguishable from those of the dimer. Hence, a reciprocating mechanism involving subunit interactions cannot be invoked to explain the allosteric behavior of this dimeric enzyme. Rather, the results are consistent with an equilibrium between two conformers as proposed by Mullinax et al. [Mullinax, T. R., Mock, J. N., McEvily, A. J., & Harrison, J. H. (1982) *J. Biol. Chem. 257*, 13233–13239], one of which preferentially binds citrate and NAD^+ while the other binds NADH.

Catalysis of reactions by enzymes attached to a surface may deviate in kinetic behavior from that observed for enzymes in solution, due both to substrate, product, or effector molecules' partitioning between the bulk phase and the surface and to diffusional limitations (Engasser & Horvath, 1973, 1974a–c; Goldstein, 1976; Taylor & Swaisgood, 1981; Cho & Swaisgood, 1974). If the enzyme has been chemically attached by covalent linkage, the immobilization reaction itself can also affect activity (Ollis & Datta, 1976; Mosbach, 1980; Bickerstaff, 1980). However, failure to consider the above effects can limit the information derived and possibly result in misinterpretations. Therefore, in this study, the effects of diffusional factors and the chemistry of immobilization were examined in an investigation of immobilized protomers and dimers of malate dehydrogenase (MDH).[1]

Malate dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) is obtained as a dimer of two identical 35000-dalton subunits. MDH subunits immobilized on Sepharose have been shown to be active (Jurgensen et al., 1981). The kinetic properties of immobilized protomers and dimers were further

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[1] Abbreviations: MDH, malate dehydrogenase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; OAA, oxalacetate; Tris, tris(hydroxymethyl)aminomethane; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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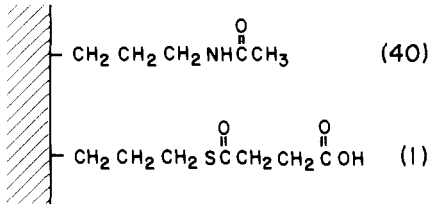

FIGURE 1: Surface derivative prepared on controlled-pore glass beads. The numbers in parentheses indicate the molar ratio of the two reagents used in the silanization mixture.

examined and compared in this study to more fully delineate the role of subunit interactions in the function of this enzyme.

Materials and Methods

Materials. Porcine heart mitochondrial MDH was a generous gift of Dr. John Harrison of The University of North Carolina, Chapel Hill, NC (this enzyme preparation is hereafter abbreviated as UNCCH-MDH). The enzyme was received as an ammonium sulfate suspension and was dialyzed at 0–5 °C overnight against two changes of 50 mM sodium phosphate, pH 7.6, containing 2 mM DTT and 1 mM EDTA, and the solution was clarified by centrifuging at 9000 rpm for 10 min prior to use. Porcine heart mitochondrial MDH was also purchased as an ammonium sulfate suspension from Sigma Chemical Co. (St. Louis, MO) and prepared for use as described for UNCCH-MDH. Controlled-pore glass beads (120–200 mesh, 73-nm pore diameter), AMP (type II), oxalacetic acid, ketomalonic acid, L-malic acid, hydroxymalonic acid, thenoyltrifluoroacetone, NAD^+ (grade III), NADH (grade III), and dithiothreitol (DTT) were also obtained from Sigma. Other chemicals, of reagent grade, were purchased from Fisher Scientific (Raleigh, NC).

Methods. Soluble enzyme was assayed in both reaction directions by measuring the ΔA_{340} upon addition of enzyme to a 2-mL reaction mixture containing 50 mM sodium phosphate, pH 7.6, 2 mM DTT, 1 mM EDTA, and the specified concentrations of substrate and coenzyme. The immobilized enzyme was assayed similarly by using a microrecirculation reactor system and flow rates of 16 mL/min or greater to minimize external diffusion effects (Taylor & Swaisgood, 1980). The amount of immobilized catalyst used was always low enough to give less than 2% per pass conversion of substrates to products (Ford et al., 1972; Taylor & Swaisgood, 1980). Kinetic parameters were evaluated from direct linear plots as described by Cornish-Bowden & Eisenthal (1978).

An acetamidopropyl/(succinylthio)propyl glass derivative (see Figure 1) was prepared by mixing (γ-aminopropyl)triethoxysilane and (γ-mercaptopropyl)triethoxysilane in the ratio 40:1 for the glass silanization reaction (DuVal et al., 1984). Both amino and thiol groups were acetylated with acetic anhydride. The thio ester was then cleaved by using 1 M hydroxylamine, and the formation of thiol groups was monitored by reaction with DTNB (DuVal et al., 1984). The regenerated thiol groups were then succinylated with succinic anhydride (DuVal et al., 1984).

Enzyme was immobilized by using a sequential method of activation of the immobilized carboxyl groups with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), followed by reaction of the *O*-acylisourea groups with enzyme amino groups (Janolino & Swaisgood, 1982). Dimers were immobilized by recirculation of 0.1–10 mg/mL solutions of MDH in 50 mM sodium phosphate, pH 7.6, containing 1 mM EDTA, 2 mM DTT, 10 mM L-malate, and 10 mM AMP. Since MDH reversibly dissociates without extensive unfolding at pH 5 (Wood et al., 1981), protomers were immobilized by recirculating 0.1–1.0 mg/mL solutions of MDH in 50 mM sodium phosphate, pH 4.8, containing 1 mM EDTA and 1 mM DTT. AMP and malate were not included since their binding favors dimer formation (Bleile et al., 1977). Following 3-h reaction at 0–5 °C, the enzyme solution was drained, and the beads were washed in a fixed-bed configuration with 200 mL of 50 mM sodium phosphate, pH 7.6, containing 1 mM EDTA and 2 mM DTT.

Released enzyme was collected from 3–4 g of beads packed in a 1.4 × 14 cm column. Ten milliliters of 50 mM sodium phosphate, pH 7.6, containing 2 mM DTT, 1 mM EDTA, and 0.2 M hydroxylamine was applied, allowed to quickly (ca. 30 s) drain through the matrix, and stopped with roughly 0.5 mL still remaining above the porous bead column. Under these conditions, the release reaction was allowed to continue for 16–24 h at room temperature. The released protein was collected by washing the column every 10 min for 1.5 h with 2 mL of 50 mM sodium phosphate, pH 7.6, containing 1 mM EDTA and 2 mM DTT. All washes were pooled.

Table I: Michaelis Constants for Soluble (Native) and Released Malate Dehydrogenase in pH 7.6 Phosphate

enzyme[a]	K_M^{NADH} (μM)	K_M^{OAA} (μM)	$K_M^{NAD^+}$ (mM)	K_M^{malate} (mM)
commercial MDH	38	18	0.23	0.37
UNCCH-MDH	31	15	0.11	0.42
released commercial dimer	40	34	0.27	0.45
released UNCCH dimer	31	17	0.11	0.43
released UNCCH promoter (expt 1)	18	12	0.15	0.36
released UNCCH protomer (expt 2)	34	14	0.15	0.31

[a] Note that the designations "dimer" and "protomer" represent the forms immobilized, not necessarily the form assayed. The released enzyme had been covalently immobilized on (succinylthio)propyl/acetamidopropyl glass and was released by treatment with 0.2 M hydroxylamine at 24–26 °C in 50 mM sodium phosphate, pH 7.5, containing 2 mM DTT and 1 mM EDTA. Michaelis constants were determined from secondary plots by using the method of Cornish-Bowden & Eisenthal (1978).

Results

The Michaelis constants obtained for native porcine heart MDH are listed in Table I. These values are similar to those of soluble enzyme for NADH and oxalacetate in phosphate buffer at pH 7.5, as reported by Jurgensen et al. (1981), and for NAD^+ and malate in Tris buffer at pH 8.0, as reported by Raval & Wolfe (1962a,b). However, there appears to be some difference in kinetic behavior observed in phosphate as opposed to Tris buffers. For example, we found the values for K_M^{NADH} and K_M^{OAA} obtained in Tris buffer at pH 7.6 to be significantly lower than those observed in phosphate. In addition, the inhibition of the enzyme in these buffers at pH 7.6 by thenoyltrifluoroacetone in the NADH → NAD^+ direction, with NADH as the variable substrate, was competitive in phosphate buffer and noncompetitive in Tris buffer. Since the enzyme appears to be more stable in phosphate buffers (T. R. Mullinax, personal communication), phosphate buffer at pH 7.6 was used throughout the remainder of this study.

Porcine heart MDH prepared in the research laboratory (UNCCH-MDH) dissociates to give stable subunits at pH 5.0 in 50 mM sodium phosphate (Wood et al., 1981). However, we found that the commercial enzyme was not completely dissociated, as judged by sedimentation velocity experiments, until a pH of 4.0 or lower was attained. Furthermore, gel

Table II: Kinetic Parameters of Immobilized Commercial Malate Dehydrogenase before and after Release of a Portion of the Immobilized Enzyme

	immobilized MDH before release			remaining immobilized MDH after partial release		
prepn[a]	V'_m (ΔA_{340} min^{-1} g^{-1})	$K_M^{NAD^+}$ (mM)	K_M^{malate} (mM)	V'_m (ΔA_{340} min^{-1} g^{-1})	$K_M^{NAD^+}$ (mM)	K_M^{malate} (mM)
1	3.72	0.35	0.52	1.75	0.46	0.61
2	3.24	0.61	0.93	2.34	0.79	1.27

[a] Enzyme was immobilized on (succinylthio)propyl/acetamidopropyl glass and released by treatment with 0.2 M hydroxylamine in 50 mM sodium phosphate, pH 7.5, containing 2 mM DTT and 1 mM EDTA at 24–26 °C. The immobilized enzyme was assayed by using the microcirculation reactor system described by Taylor & Swaisgood (1980).

filtration, sedimentation velocity, and activity measurements indicated that acid-dissociated commercial malate dehydrogenase tended to aggregate and lose activity. Consequently, the UNCCH-MDH preparation was used for preparation of immobilized subunits.

The possibility that immobilization of the protomer at pH 4.8 could have resulted in its being "locked" into an inactive conformation in the neutral and alkaline pH range was eliminated by examining the activity as a function of pH. Data in Figure 2 show that the pH dependence of the enzymic activity of immobilized protomers and that of immobilized native enzyme were essentially identical and were similar to that of native, soluble MDH. The observed differences in the pH–activity profiles for immobilized and soluble enzyme preparations most likely result from the effects of pore diffusion. Since the reaction in the NADH → NAD$^+$ direction is severely diffusion limited (see below), increasing inhibition by increasing proton concentration is offset by decreasing degrees of diffusion inhibition; consequently, the observed activity does not decline as sharply. On the other hand, product inhibition is enhanced by diffusional limitations (Engasser & Horvath, 1974b), which may be the overriding factor on the alkaline pH side of the optimum, resulting in the smaller difference between the soluble and immobilized enzyme pH profiles on the alkaline side.

The Michaelis constants for soluble MDH, both that which is native and that which is released from covalently immobilized forms by treatment with 0.2 M hydroxylamine, are compared in Table I. These kinetic parameters do not appear to have been significantly altered by either the form of the enzyme immobilized or the chemistry of the immobilization reaction.

Apparent Michaelis constants for the immobilized enzyme, obtained in the NADH → NAD$^+$ direction (both κ^{OAA} and κ^{NADH}), exhibited curvilinear dependence on the amount of enzyme loading on the matrix. These results dramatically illustrate the effect of pore diffusion limitations on the apparent kinetics. Moreover, data for the immobilized protomer and data for the dimer fell on the same curves. As expected from theoretical predictions (Engasser & Horvath, 1973; Goldstein, 1976), values for the Michaelis constants approach that for soluble enzyme at low levels of loading.

For the case of pore diffusion limitation, the apparent Michaelis constant, κ, is related to the K_M for the soluble enzyme by an effectiveness factor, ϵ, in the first-order region ([S] < K_M) according to the relationship (Engasser & Horvath, 1973; Swaisgood et al., 1976)

$$\kappa = K_M/\epsilon \qquad (1)$$

where

$$\epsilon = \frac{3}{\phi}\left(\frac{1}{\tanh \phi} - \frac{1}{\phi}\right)$$

and

$$\phi = R(V'_m/D_eK_M)^{1/2}$$

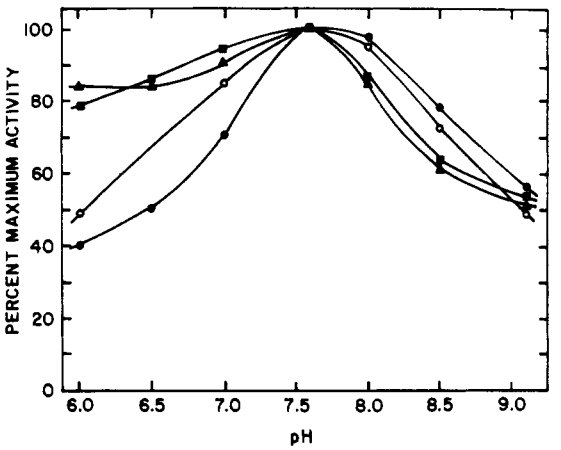

FIGURE 2: Observed activity as a function of pH for immobilized protomers and native dimers, and for native soluble MDH. Activity was assayed in the NADH → NAD$^+$ direction using an assay cocktail containing 50 mM sodium phosphate, 1 mM EDTA, 2 mM DTT, 0.1 mM NADH, and 0.1 mM oxalacetate. Immobilized enzyme was assayed by using a microrecirculation reactor system (Taylor & Swaisgood, 1980) and a 2-mL reaction solution. Soluble enzyme assays were initiated by adding 20 μL of enzyme solution to 2 mL of substrate solution in a quartz cuvette. All assays were at room temperature. (▲) Immobilized protomer; (■) immobilized native dimer; (●) soluble enzyme; (○) data from Ravel & Wolfe (1968b).

where R is the radius of the porous particle, V'_m is the maximum rate per unit volume of porous catalyst, and D_e is the diffusivity of the substrate in the catalyst bead. Thus, defining a constant, $B \equiv (R^2/D_eK_M)^{1/2}$, one obtains

$$\kappa = (K_M/3)B\sqrt{V'_m}\left(\frac{1}{\tanh B\sqrt{V'_m} - 1/B\sqrt{V'_m}}\right)$$

Numerical calculations show that κ is roughly linear with $B\sqrt{V'_m}$, deviating from linearity only slightly below $\phi = 4$. When the apparent Michaelis constants for immobilized MDH for OAA are plotted as a function of $\sqrt{V'_m}$, linear relationships are observed as shown in Figure 3. Since $\phi \rightarrow 1$ as $V'_m \rightarrow 0$, the extrapolated value for κ should approximate K_M; thus, with this approximation, $K_M^{OAA} = 18$ μM. Similar treatment for NADH as the variable substrate yields $K_M^{NADH} = 64$ μM.

The effects of pore diffusion limitation in the NAD$^+$ → NADH direction should be much smaller since the K_M values and corresponding substrate concentrations are larger and the reaction velocities are smaller. As shown by data for two immobilized MDH preparations given in Table II, this is indeed the case as the K_M values are nearly the same as those for the soluble enzyme. Furthermore, the apparent K_M values do not change substantially following release of part of the enzyme from the catalyst particle. In fact, if the slight increase in values is real, it is in the opposite direction from that expected to result from decreasing diffusional inhibition. It could be due to a preferential release of enzyme from the outer volume of the catalyst particle or to preferential release of a subpopulation (e.g., molecules attached by a single bond) from

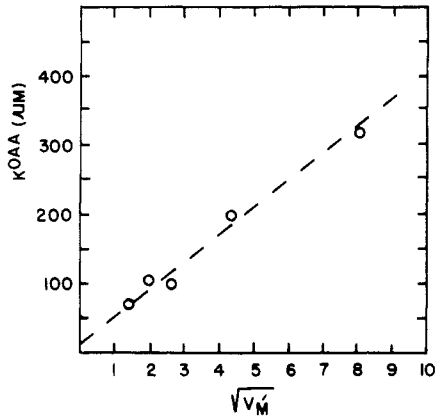

FIGURE 3: Dependence of the apparent Michaelis constants for oxalacetate on the square root of the maximum velocity per unit volume of catalyst particle. The dashed line represents the linear fit for oxalacetate, correlation = 0.991.

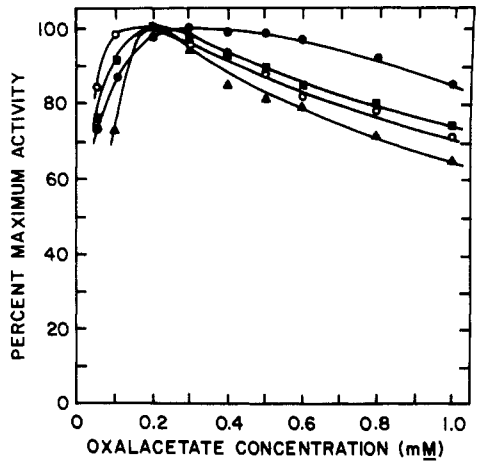

FIGURE 4: Substrate inhibition of soluble and immobilized forms of MDH by oxalacetate. Native dimers or protomers were immobilized on acetamidopropyl/(succinylthio)propyl (40:1) porous glass beads and assayed at room temperature using a microcirculation reactor system with 2 mL of substrate solution containing 50 mM sodium phosphate, pH 7.6, 2 mM DTT, 1 mM EDTA, 0.1 mM NADH, and the indicated oxalacetate concentration. (▲) Soluble enzyme; (●) 14.8 ΔA_{340} min^{-1} (g of immobilized dimer)$^{-1}$; (■) 0.26 ΔA_{340} min^{-1} (g of immobilized dimer)$^{-1}$; (○) immobilized protomer.

a heterogeneous population of immobilized molecules having slightly different K_M values.

Native soluble MDH exhibits substrate inhibition with oxalacetate (Bernstein et al., 1978; also see Figure 4). The immobilized native dimer displayed a similar inhibition pattern as shown in Figure 4. Again, however, the effect of diffusion limitation can be noted since inhibition becomes increasingly less pronounced as the enzyme loading of the catalyst particle increases; this is as had been predicted theoretically by Engasser & Horvath (1974a,c). Furthermore, the immobilized protomer exhibited a substrate inhibition similar to that shown by immobilized dimer.

Citrate inhibits the reaction catalyzed by mitochondrial MDH in the NADH → NAD$^+$ direction, presumably by binding to a regulatory site (Mullinax et al., 1982). Both immobilized native MDH and immobilized protomers exhibited similar citrate inhibition as illustrated by the data in Figure 5. As observed with inhibition by oxalacetate, the apparent inhibition by citrate was partly suppressed by increasing levels of immobilized catalyst activity. Citrate inhibition of immobilized promoters also has been observed in Harrison's laboratory (T. R. Mullinax, personal communication).

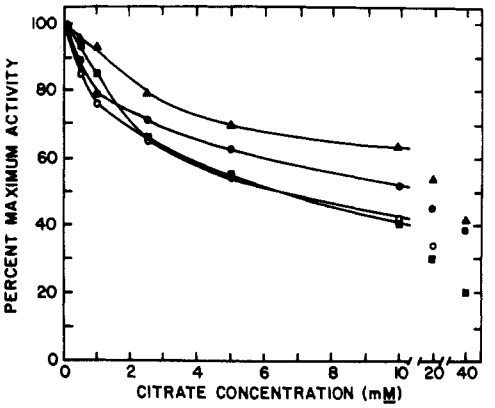

FIGURE 5: Inhibition of immobilized forms of MDH by citrate and the effect of enzyme loading of the catalyst particle on the inhibition of immobilized dimers. Protomers or native dimers were immobilized on acetamidopropyl/(succininylthio)propyl (40:1) porous glass beads and assayed at room temperature by using a microrecirculation reactor system with 2 mL of substrate solution containing 50 mM sodium phosphate, pH 7.6, 2 mM DTT, 1 mM EDTA, 0.1 mM NADH, and 0.1 mM oxalacetate: (▲) 8.40, (●) 2.24, and (■) 0.50 ΔA_{340} min^{-1} (g of immobilized dimer)$^{-1}$; (○) immobilized protomer.

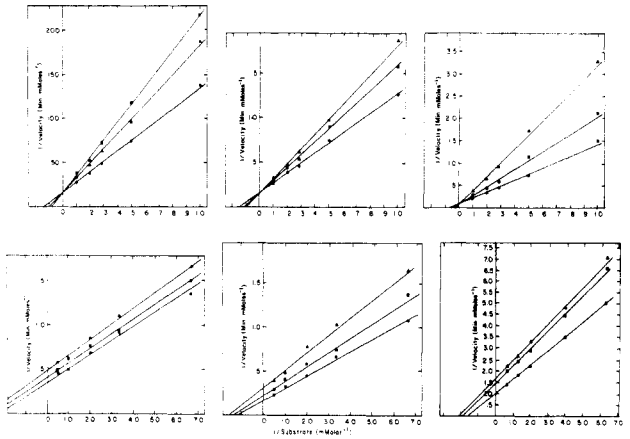

FIGURE 6: Hydroxymalonate inhibition of soluble native MDH, immobilized native dimers, and immobilized protomers. The UNCCH-MDH preparation was used for these studies. The top three panels, from left to right, represent the inhibition patterns obtained with double-reciprocal plots of data from the reduction of ketomalonate catalyzed by soluble MDH, immobilized native dimers, and immobilized protomers, respectively. All assays contained 0.1 mM NADH, 50 mM sodium phosphate, pH 7.6, 2 mM DTT, and 0.1 mM EDTA. The hydroxymalonate concentrations were the following: (top left) (●) 0, (▲) 0.2, and (■) 0.4 mM; (top middle) (●) 0, (■) 0.067, and (▲) 0.33 mM; (top right) (●) 0, (■) 0.5, (▲) and 2.0 mM. The lower three panels, from left to right, represent the inhibition patterns obtained with double-reciprocal plots of data from the oxidation of malate catalyzed by soluble MDH, immobilized native dimers, and immobilized protomers, respectively. The soluble enzyme was assayed with 1.5 mM NAD$^+$ and the immobilized forms with 1.0 mM NAD$^+$ in the buffer described. The hydroxymalonate concentrations were the following: (lower left) (●) 0, (■) 0.25, and (▲) 0.5 mM; (lower middle) (●) 0, (■) 0.83, and (▲) 1.67 mM; (lower right) (●) 0, (■) 0.8, and (▲) 4.0 mM.

The inhibition patterns produced by hydroxymalonate were examined in both directions with the native soluble enzyme and with both immobilized forms. To minimize the pore diffusion effect for the immobilized enzymes in the NADH → NAD$^+$ direction, a low level of enzyme loading was used (κ^{NADH} = 90 μM and κ^{OAA} = 60 μM) and ketomalonate was examined as the variable substrate, rather than oxalacetate, since its higher K_M gives slower reaction velocity. In all cases, competitive inhibition was observed with respect to ketomalonate, while the pattern with malate as the variable substrate appeared to be uncompetitive (Figure 6). Similar

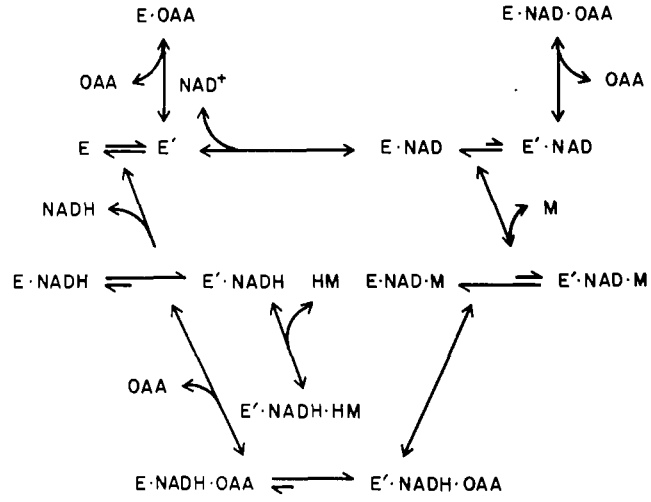

FIGURE 7: Schematic representation of the proposed catalytic mechanism for MDH involving two forms (conformers), one which preferentially binds NAD^+ (E) and the other which preferentially binds NADH (E'). M, malate; HM, hydroxymalonate.

observations have been reported previously for the soluble enzyme (Harada & Wolfe, 1968a).

Discussion

A question of critical importance to understanding of the contribution of subunit interactions to the structure and mechanism of catalysis by oligomeric enzymes is whether or not protomers exhibit activity (Chan, 1970; Bickerstaff, 1980). Observation of "normal" activity and allosteric regulation of immobilized MDH protomers would rule out the mechanism proposed by Harada & Wolfe (1968b). In the only other reported study of immobilized forms of MDH, immobilized protomers were obtained from immobilized dimers by dissociation (Jurgensen et al., 1981). In the present study, a different approach was tested, viz., direct immobilization of protomers under conditions which produce complete dissociation without substantial unfolding of the polypeptide chain structure.

Since the method of immobilization used permitted subsequent release of the covalently immobilized enzyme by treatment with hydroxylamine under very mild conditions, the effect of the immobilization reaction per se could also be examined. Thus, cleavage of the thio ester linkage should leave a succinyl group attached to the amino group which had linked the enzyme to the derivatized surface. The fact that the released enzyme, whether it came from immobilized native dimers or immobilized protomers, exhibited the same kinetic parameters as did the native soluble MDH strongly argues that the immobilization reaction did not significantly affect its structure.

When the effects of pore diffusion are taken into account, the Michaelis constants for both immobilized native dimers and immobilized protomers are similar to those of the native soluble enzyme. In their studies of dimers and protomers immobilized on Sepharose, Jurgensen et al. (1981) observed Michaelis constants which were considerably larger than those of the soluble enzyme. The K_M values were particularly large for immobilized enzyme in the NADH → NAD^+ direction, and the activity vs. pH profile was flat. Although such observations could be explained by the effects of pore diffusion, the authors argued for restrictions of conformational mobility as the cause. Nonetheless, in our case, there was less deviation of the kinetic behavior of immobilized forms from that of native soluble enzyme (e.g., the activity vs. pH profile), and merely effects of pore diffusional limitations are sufficient to account for the observed differences.

Not only did the immobilized forms, the dimer and, more significantly, the protomer, exhibit K_M values similar to those of native soluble enzyme but also similar inhibition patterns were obtained with substrate analogues and effectors. Thus, substrate inhibition by oxalacetate was observed with the immobilized protomer as were competitive inhibition by hydroxymalonate with ketomalonate as substrate and uncompetitive inhibition with this inhibitor when L-malate was the substrate. Consequently, subunit interactions in a reciprocating mechanism (Harada & Wolfe, 1968b) cannot be invoked to explain the kinetic behavior of this dimeric enzyme.

Mullinax et al. (1982) described the regulation of mitochondrial MDH by citrate. They proposed that such regulation was accomplished by the binding of citrate to an allosteric site, therby shifting the equilibrium ratio between the two conformers. Although an effect on the equilibrium between two conformers by subunit interactions would not be ruled out by the data presented here, similar effects of citrate on the activity of both immobilized dimers *and protomers* demonstrate that MDH subunits are independently capable of allosteric regulation. Our findings support the proposed equilibrium between conformers suggested by Mullinax et al. (1982) as an explanation of the kinetic properties of MDH. The proposed mechanism is outlined in Figure 7. Hydroxymalonate binds to the E′ conformer, which also binds NADH preferentially, and thus inhibits uncompetitively with respect to malate but competitively with ketomalonate. Such preferential binding would explain the interaction between NADH and hydroxymalonate binding sites and the increase in affinity for hydroxymalonate in the presence of NADH, as first noted by Harada & Wolfe (1968a). On the other hand, citrate binds preferentially to the E conformer, which also binds NAD^+ preferentially and thus inhibits the reaction in the NADH → NAD^+ direction (Mullinax et al., 1982). Likewise, if ketomalonate shifts the equilibrium toward the E conformer, the reaction rate will be slower as compared to that for oxalacetate. Finally, substrate inhibition by oxalacetate may result from binding to the E conformer, competing for the site which binds citrate (Mullinax et al., 1982).

Kinetic data presented here also experimentally confirm the theoretically predicted (Engasser & Horvath, 1973, 1974a–c) effects of diffusional inhibition on reaction rates. Since many enzymes are actually associated with surfaces of intracellular structures in vivo, the modulation of their activities by inhibitors and effectors will undoubtedly be different from that observed in solution.

Registry No. MDH, 9001-64-3; NADH, 58-68-4; NAD, 53-84-9; OAA, 328-42-7; L-malic acid, 97-67-6; hydroxymalonic acid, 80-69-3; ketomalonic acid, 473-90-5; citric acid, 77-92-9.

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Stereochemical Course of Hydrolysis of DNA by Exonuclease I from *Escherichia coli*†

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ABSTRACT: Exonuclease I has been purified from an overproducing strain of *Escherichia coli* K12 [Prasher, D. C., Conarro, L., & Kushner, S. R. (1983) *J. Biol. Chem. 258*, 6340–6343]. The enzyme hydrolyzes deoxyribonucleic acids that contain chiral phosphorothioate diester linkages, and the stereochemical course of the reaction is inversion of configuration at phosphorus. This result is most consistent with hydrolysis occurring via the direct attack of water on a phosphorothioate diester rather than through the intermediacy of a covalent nucleotidyl-enzyme intermediate. This finding represents the first example of a processive exonuclease whose stereochemical pathway has been determined.

Exonuclease I (EC 3.1.4.25) from *Escherichia coli* is a processive 3′→5′ exonuclease that acts specifically on single-stranded DNA. Early studies by Lehman & Nussbaum (1964) showed that the enzyme catalyzes the release of 5′-mononucleotides from the 3′ termini of single-stranded DNA, degrading the chains to 5′-terminal dimers that are inert to further hydrolysis. Thomas & Olivera (1978) subsequently showed that exonuclease I is highly processive in that an enzyme molecule will bind to and degrade a single polydeoxyribonucleotide chain to near completion before reacting with another molecule of nucleic acid. More recently, Kushner and co-workers (Prasher et al., 1983) constructed a strain of *E. coli* that overproduces exonuclease I between 140 and 400 times the level found in wild-type cells. These workers purified the enzyme to near homogeneity and found that the enzyme is a monomer of molecular weight 55 000.

A mechanistic study of exonuclease I must address both the catalytic mechanism of phosphodiester hydrolysis and the protein–nucleic acid interactions that allow the enzyme to hydrolyze successive nucleotides without dissociating from the nucleic acid chain. A necessary initial step in such an investigation is the determination of whether the enzymatic reaction proceeds by direct attack of water on a phosphodiester or via a double displacement mechanism in which a covalent nucleotidyl-enzyme intermediate is formed and subsequently hydrolyzed. The research described in this paper addresses this question via a stereochemical analysis of the reaction of exonuclease I with deoxyribonucleic acids that contain chiral phosphorothioate internucleotide linkages.

Experimental Procedures

Materials. Strain SK 4258 of *E. coli* K12 was a gift of Dr. Sidney Kushner. Poly[d(T_s-A)][1] and pdTp$_s$dApdTp$_s$dA were synthesized as described by Brody & Frey (1981). pdTpdApdTpdA, calf intestine alkaline phosphatase, and T4 polynucleotide kinase were purchased from Pharmacia P-L Biochemicals. [γ-^{32}P]ATP, calf thymus terminal transferase, *E. coli* [^{3}H]DNA, and the Klenow fragment of DNA polymerase I were purchased from New England Nuclear. [^{3}H]-dATP was purchased from Schwarz/Mann. Bio-Gel P-10 was purchased from Bio-Rad. Salmon sperm DNA, adenylate kinase, pyruvate kinase, and yeast inorganic pyrophosphatase were purchased from Sigma.

Enzyme Purification and Assay. Exonuclease I was purified from an overproducing strain of *Escherichia coli* K12 (SK

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