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Direct Transfer of Reduced Nicotinamide Adenine Dinucleotide from Glyceraldehyde-3-phosphate Dehydrogenase to Liver Alcohol Dehydrogenase†

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ABSTRACT: The reduction of benzaldehyde and *p*-nitrobenzaldehyde by NADH, catalyzed by horse liver alcohol dehydrogenase (LADH), has been found to be faster when NADH is bound to glyceraldehyde-3-phosphate dehydrogenase (GPDH) than with free NADH. The rate of reduction of aldehyde substrate with GPDH–NADH follows a Michaelian concentration dependence on GPDH–NADH. The reaction velocity is independent of GPDH concentration when [GPDH] > $[NADH]_{total}$. The K_m for GPDH–NADH is higher than that for free NADH. The reaction velocities in the presence of excess GPDH over NADH cannot be accounted for on the basis of the free NADH concentration arising from dissociation of the GPDH–NADH complex. These observations suggest that transfer of NADH from GPDH to LADH proceeds through the initial formation of a GPDH–NADH–LADH complex. Arguments for a direct enzyme–coenzyme–enzyme transfer mechanism are substantiated and quantitated both by steady-state kinetic studies and by determinations of all of the appropriate enzyme–coenzyme equilibrium dissociation constants. In contrast, over a similar concentration range, the complex lactate dehydrogenase (LDH)–NADH is not a substrate for the LADH-catalyzed reductions. Likewise, the LADH–NADH complex is not a substrate for the LDH-catalyzed reduction of pyruvate.

In a previous paper from this laboratory, Weber & Bernhard (1982) demonstrated that 1,3-diphosphoglycerate is transferred between glyceraldehyde-3-phosphate dehydrogenase (GPDH)[1] and phosphoglycerate kinase (PGK) via direct enzyme–enzyme complex formation. The question arose immediately as to the generality or frequency of occurrence of such direct transfer steps in glycolysis, and in other pathways involving globular cytosolic enzymes which do not form multienzyme complexes.

† From the Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403. *Received January 23, 1984.* This work was supported by grants from the U.S. Public Health Service of the National Institutes of Health (GM 10451-19) and from the National Science Foundation (PCM 801-6249).

[1] Abbreviations: GPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; LADH, liver alcohol dehydrogenase; LDH, lactate dehydrogenase; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

0006-2960/84/0423-4538$01.50/0

The essential question is whether products from a particular enzyme-catalyzed step in the pathway must necessarily dissociate into free aqueous solution before encountering an enzyme involved in the subsequent transformation of metabolite. The distinction can be defined formalistically by the alternative pathways of eq 1a,b.

random diffusion model

$$E_1S_2 \rightleftarrows E_1 + S_2 + E_2 \rightleftarrows E_2S_2 \rightarrow \text{product} \quad (1a)$$

direct transfer model

$$E_1S_2 + E_2 \rightleftarrows E_1{\cdot}S_2{\cdot}E_2 \rightarrow \text{product} \quad (1b)$$

Although an open-minded view is frequently stated regarding which of the two schemes of eq 1 (a or b) is appropriate, the implicit assumption of the diffusion–random association model is usually presented, particularly in discussions of metabolic energy transfer. For example, it is common to present the standard free energies ($\Delta G°$) for the conversion of aqueous substrate to aqueous product for each of the enzyme-catalyzed reactions in the glycolytic pathway [see, for example, the energy metabolism section in standard textbooks, such as Lehninger (1975), Mahler & Cordes (1971), and Metzler (1977)]. These $\Delta G°$ values are (presumably) of relevance to the distribution among intermediates in the metabolic pathway. If transfer of metabolite occurs via the direct enzyme–enzyme pathway, the equilibria in aqueous solution may not be entirely relevant. It has been demonstrated that enzyme-bound substrate/product ratios for a variety of enzymes involved in glycolysis are far different from those predictable on the basis of the aqueous solution equilibrium data (Albery & Knowles, 1976, 1977; Wilkinson & Rose, 1979; Nageshwara RaO et al., 1978, 1979; Huskins et al., 1982).

The distinction between the two mechanisms of eq 1 may also be due to kinetic factors. In some cases, the dissociation of metabolic products from enzyme may be the slowest step in the overall turnover (Bernhard et al., 1970; McFarland & Bernhard, 1972; Wratten & Cleland, 1963, 1965). Thus, a particularly slow turnover may be obviated by the direct transfer of metabolite from one enzyme to another via complex formation as is apparently the case for transfer of 1,3-diphosphoglycerate from PGK to GPDH (Weber & Bernhard, 1982). This is at least in part a consequence of the very high affinity of PGK for 1,3-diphosphoglycerate. The dissociation constant of the enzyme–substrate complex in this case is of the order of 1 nM or less (Huskins et al., 1982; Nageshwara RaO et al., 1978). A simplistic calculation, based on the approximately known second-order rate constant for bimolecular diffusion in aqueous solution (Eigen & DeMaeyer, 1963), predicts a product dissociation velocity of less than 0.1 s^{-1} for the kinase–1,3-diphosphoglycerate complex. This unimolecular rate constant is far slower than the conventional unimolecular turnover numbers ($\sim 10^2$–10^3 s^{-1}) for individual enzymes of the glycolytic pathway. The rate of oxidation of NADH in the coupled reactions involving ATP, 3-phosphoglycerate, and NADH, catalyzed by PGK and GPDH, is approximately 500 s^{-1} at substrate saturation when [PGK] is rate limiting. Since substrates and products do not substantially affect the rate or equilibrium of PGK–1,3-diphosphoglycerate dissociation, it follows that some sort of enzyme–enzyme interaction is involved (Huskins et al., 1982).

In most other enzyme–metabolite systems, the affinity for metabolite is not nearly as high as in the PGK–1,3-diphosphoglycerate system. Dissociation constants of the order of 1 μM allow for dissociation rate constants as large as about 10^2 s^{-1}, values in the same range as the enzyme turnover numbers. Consequently, the rigorous discrimination between the two mechanisms (eq 1) is not as readily detected in other systems as it is with the GPDH–PGK coupled pathway. To overcome the problems consequent on finite enzyme–substrate dissociation constants, we have devised a series of experiments which rely on the complexation of metabolite by a substantial excess of enzyme in order to distinguish aqueous metabolite from enzyme-bound metabolite. We have had to determine equilibrium dissociation constants for enzyme–metabolite complexes in the solvent media in which kinetic experiments are carried out.

This paper deals with the application of such techniques of enzyme buffering of metabolite concentrations. The experiments which we describe involve the transfer of NADH from one enzyme whose substantial concentration exceeds that of the coenzyme to a different enzyme site where catalytic reduction of substrate occurs.

$$NADH + E_2 + S_2 \rightarrow E_2 + NAD^+ + S_2H_2 \quad (2)$$

For example, the reduction of the alcohol dehydrogenase substrate *p*-nitrobenzaldehyde by "catalytic" amounts of LADH ($\sim$1 nM) is monitored in the presence of varying excessive concentrations of GPDH over NADH, as well as in the absence of GPDH. The kinetic results are compared with the results anticipated from the extrapolated aqueous NADH concentration determined from the measured dissociation constant of the GPDH–NADH complex. In addition to GPDH, we have utilized the enzymes liver alcohol dehydrogenase (LADH) and lactate dehydrogenase (LDH) as "buffers" of the free (aqueous) NADH concentration. These three dehydrogenases are available to us in high (substratelike) concentrations.

As will be shown, our results on the transfer of NADH between GPDH and LADH are entirely consistent with the direct transfer mechanism and inconsistent with a mechanism involving transfer via the mediation of the aqueous solvent. These results were not unexpected, since experiments leading to the same conclusions were already carried out by Cori et al. (1950) and by Nygaard & Rutter (1956). We were aware of these papers at the time we initiated these experiments. Substrate, other ligands, and the ionic environment all affect the dissociability of the enzyme–coenzyme complex in ways which were not apparent at the time of the earlier reports [see Harris & Waters (1976), Holbrook et al. (1975), and Brändén et al. (1975) and references cited therein]. Consequently, we have reexamined the dissociability of enzyme–NADH complexes under solvent conditions identical with those utilized for the kinetic transfer experiments. The new experiments reported herein lead to conclusions totally in accord with those presented in the earlier reports. They further provide methodology for investigation of functional enzyme–enzyme interactions among other globular cytosolic enzymes. Some preliminary finding on the limits of the generality of the direct transfer mechanism are reported as well.

Experimental Procedures

Materials

Sodium salts of NAD^+ (grade III), NADH (grade III), pyruvic acid, EDTA, *p*-nitrobenzaldehyde, DL-glyceraldehyde 3-phosphate diethyl acetal monobarium salt (grade I), and 2-mercaptoethanol were obtained from Sigma Chemical Co. Benzaldehyde was from Mallinkrodt Chemical Co. An aqueous solution of DL-glyceraldehyde 3-phosphate was prepared as described by Furfine & Velick (1965). Reagent-grade chemicals and glass-distilled water were used to prepare

all buffers and solutions; 0.05 M tetrasodium pyrophosphate buffer was adjusted to pH 7.5 by the addition of dilute H_2SO_4. This buffer was used as solvent in all experiments reported here.

Enzyme Isolation. All purification steps were carried out at 0–4 °C.

(*A*) *Liver alcohol dehydrogenase* (*LADH*) was obtained from Boehringer Mannheim and was initially purified according to Bernhard et al. (1970). Purified LADH thus obtained was contaminated with LDH activity. The total content, although less than 0.1%, was sufficient to interfere in the experiment which we describe. Therefore, the enzyme was further freed from LDH by chromatography on a DEAE-cellulose column (2.5 × 15 cm), preequilibrated with 5 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM EDTA and 0.35 mM 2-mercaptoethanol. LADH was eluted in the void volume. LDH was retained in the column. Enzyme activity was assayed according to Dalziel (1957). $\epsilon^{0.1\%}_{280nm}$ for LADH was taken as 0.455 (Bonnichsen, 1950) for determining protein concentration.

(*B*) *Glyceraldehyde-3-phosphate dehydrogenase* (*GPDH*) was purified from halibut muscle essentially as described for sturgeon muscle GPDH by Seydoux et al. (1973). In the final purification step (CM-cellulose chromatography), both holo-GPDH (A_{280}/A_{260} = 1.2–1.7) and apo-GPDH (A_{280}/A_{260} > 1.8) were eluted as distinct peaks. Apo-GPDH fractions having $A_{280}/A_{260} \simeq 2.0$ were pooled. Apo-GPDH thus prepared was demonstrated to be contaminated by LDH. The contamination of LDH in the apo-GPDH preparation was ~0.02%, which was removed by chromatography on a DEAE-cellulose column (2.5 × 20 cm), preequilibrated with 5 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM EDTA and 0.35 mM 2-mercaptoethanol. Apo-GPDH was eluted in the void volume. LDH was retained in the column. Apo-GPDH was precipitated by 95% saturation of ammonium sulfate, centrifuged, resuspended in the 50 mM 2-methylimidazole buffer, pH 7.5, and stored at 0–4 °C. Holo-GPDH was further chromatographed on a CM-cellulose column to obtain apo-GPDH. Since LDH activity persisted in this apo-GPDH preparation, it was removed as described above. Enzyme activity was assayed by the method of Ferdinand (1964). $\epsilon^{0.1\%}_{280nm}$ for apo-GPDH was taken as 0.90 for determining protein concentration (Seydoux et al., 1973).

(*C*) *Lactate dehydrogenase* (*LDH*) from halibut muscle was purified by modification of the procedure of Pesce et al. (1967). After the second ammonium sulfate step of their procedure, the enzyme was dialyzed against 5 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM EDTA and 0.35 mM 2-mercaptoethanol. The dialyzed enzyme was applied to a DEAE-cellulose column, preequilibrated with the same buffer, and washed until A_{280} was less than 0.05. A linear gradient of 0–100 mM KCl (in the above buffer) was applied. LDH was eluted from the column in a symmetrical peak. Active enzyme fractions (specific activity 1200–1600 units/mg) were pooled. LDH thus prepared was demonstrated to be contaminated by GPDH. The contamination of GPDH in the LDH preparation was <0.03%, which was removed by chromatography on a CM-cellulose column (2.5 × 15 cm), preequilibrated with 5 mM EDTA, 30 mM KCl, and 1 mM 2-mercaptoethanol, pH 6.5. LDH was eluted in the void volume. GPDH was retained in the column. LDH was precipitated by 95% saturation of ammonium sulfate, centrifuged, resuspended in the 50 mM Tris-HCl buffer, pH, 7.5, containing 0.1 mM EDTA and 0.35 mM 2-mercaptoethanol, and stored at 0–4 °C. Enzyme activity was measured according to Pesce et al. (1967). $\epsilon^{0.1\%}_{280nm}$ for LDH was taken as 1.35 for determining protein concentration (Pesce et al., 1967).

The enzyme site concentration was determined on the basis of molecular weights of 84000 for dimeric LADH (Ehrenberg, 1957), 145000 for tetrameric GPDH (Jaenicke et al., 1968), and 150000 for tetrameric LDH (Pesce et al., 1967).

Methods

Fluorometric Titrations. Fluorometric titrations were carried out at 25 °C in a Hitachi MPF-2A Perkin-Elmer spectrofluorometer equipped with a thermostated cell holder. Small aliquots of titrant (5–10 μL) were added to the enzyme solution in the 50 mM pyrophosphate buffer, pH 7.5 (total volume 2.5 mL). Mixing was accomplished by means of a plastic plunger (Calbiochem).

In these experiments, dissociation constants were determined from the changes in the NADH fluorescence emission at 460 nm for GPDH and LDH, and at 443 nm for LADH. Excitation conditions were as follows: GPDH, 290 nm; LDH and LADH, 340 nm. The difference in fluorescence (Δf) at each addition of NADH was obtained from the total fluorescence of the mixture (enzyme + NADH) and the fluorescence of the individual components (obtained in a parallel control experiment). The fractional saturation of enzyme was calculated from the ratio of $\Delta f/\Delta f_\infty$. Δf_∞ is the maximum fluorescence change at saturating (infinite) concentration of NADH. Dissociation constants of the enzyme–NADH complex were obtained from the "Scatchard plot" of the data (Scatchard, 1949).

Kinetic Experiments. Kinetic experiments were all carried out in a Varian Techtron Model 635 recording spectrophotometer at 25 °C. All dialyzed enzymes used as a "substratelike" concentration were refiltered through a 0.45-μm Millipore filter. The filtered enzymes were incubated with NADH, and with substrate for the catalytic enzyme reaction, in 50 mM pyrophosphate buffer, pH 7.5 at 25 °C. The incubation mixture did not show any detectable loss of absorbance at 340 nm due to either the oxidation of NADH or the formation of "NADH–X" (a covalently modified coenzyme which slowly forms with GPDH at lower pH; Oppenheimer & Kaplan, 1974). The reaction was started by the addition of 10–20 μL of appropriately diluted catalytic enzyme. Mixing was accomplished by means of a plastic plunger. The reference compartment contained all components of the sample except for NADH. The reaction was followed by monitoring the disappearance of NADH as indicated by the loss of absorbance at 340 nm.

For E_1–NADH-dependent E_2-catalyzed reaction, E_1 and NADH were premixed. Aliquots of E_1–NADH were diluted into the same buffer so as to maintain a constant enzyme/coenzyme ratio at various total concentrations. ϵ^{NADH}_{340nm} was taken as 6.22×10^3 M^{-1} cm^{-1} (Horecker & Kornberg, 1948) for determining NADH concentration.

Results

Determination of Enzyme–NADH Dissociation Constants. For the interpretation of the kinetic experiments which we describe below, it is essential to know the enzyme–NADH dissociation constant under the specific conditions of these experiments. We have therefore determined the dissociation constants for the GPDH–NADH, LDH–NADH, and LADH–NADH complexes. In all cases, complex formation was detected by utilizing the fluorescent emission property of NADH when bound to the enzyme. The LDH–NADH complex can be readily detected by excitation of NADH at or near its absorption maximum (340 nm) and by observation

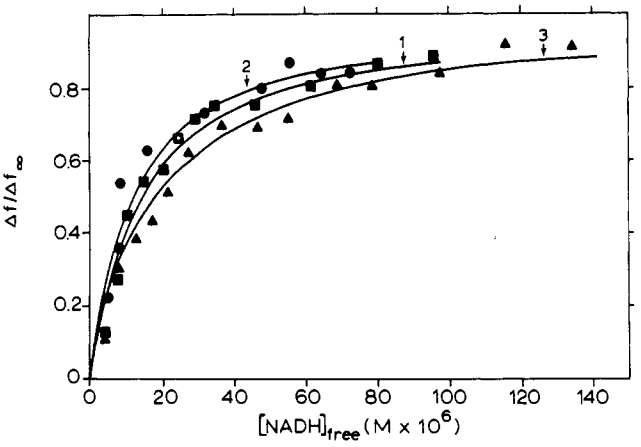

FIGURE 1: Spectrofluorometric titration of dehydrogenases with NADH. Fractional saturation ($\Delta f/\Delta f_\infty$) as a function of free NADH concentration. (1) [LDH–site] = 1.51×10^{-6} M; (2) [LADH–site] = 1.78×10^{-6} M; (3) [GPDH–site] = 1.32×10^{-6} M. Solid lines are calculated on the basis of Michaelian behavior; $K_d = 1.39 \times 10^{-6}$, 1.15×10^{-6}, and 1.8×10^{-6} M, respectively, for curves 1, 2, and 3.

of the fluorescent emission at 460 nm [see Holbrook et al. (1975) and references cited therein]. There is an approximately 40% enhancement of the fluorescent emission over that observed for the coenzyme in aqueous solution. The detected binding of NADH to LDH exhibits a hyperbolic dependence on the free ligand concentration (Figure 1). Similarly, the dissociation constant of LADH–NADH was determined by excitation at 340 nm and observation of the fluorescent emission at 443 nm [see Brändén et al. (1975) and references cited therein]. The binding of NADH to LADH also exhibits a hyperbolic dependence of the free ligand concentration (Figure 1).

To determine the GPDH–NADH dissociation constant, we utilized the fluorescent energy transfer properties of the protein in exciting the bound coenzyme. This is readily achieved by excitation in the 290-nm wavelength region and measurements of the NADH fluorescent emission at 460 nm. Under these wavelength conditions, the emission is due almost exclusively to bound NADH. The interaction of halibut GPDH with NADH thus observed is consistent with an independent site model, as indicated by the hyperbolic saturation with respect to free NADH concentration (Figure 1). We note that the dissociation constants of GPDH–NADH, LDH–NADH, and LADH–NADH are ionic strength dependent. The binding becomes much tighter as the ionic strength is reduced. Due to the availability of large quantities of these enzymes, they can be utilized as an effective buffer of the free NADH concentration, a fact which we shall utilize in subsequent experiments.

Rates of LADH-Catalyzed Reduction of Aldehydes. In these and the following experiments, we compare the "E_2"-catalyzed reduction of substrate (S_2) by NADH in the absence and presence of a molar excess of a second dehydrogenase (E_1). For example, we study the reduction of benzaldehyde and *p*-nitrobenzaldehyde catalyzed by approximate nanomolar concentrations of LADH in the absence and in the presence of greater than micromolar concentrations of GPDH. GPDH does not catalyze the oxidation of these substrates substantially at micromolar concentrations of enzyme, nor does it interact substantially so as to form acyl enzyme.

$$\text{GPDH-NAD}^+ + \text{R—CH=O} \longrightarrow \text{GPDH—C(=O)—R} + \text{NADH} \quad (3)$$

This is in contrast to the reactivity of GPDH toward acetaldehyde. At these high concentrations of GPDH, there is a substantial reaction of GPDH with acetaldehyde according to eq 3. Hence, any reduction of acetaldehyde by LADH would generate oxidized coenzyme (NAD^+) and permit the regeneration of NADH by the pathway of eq 3. At pH 7.5, the rate of reduction of these two aromatic aldehydes is comparable to that for the LADH-catalyzed reduction of acetaldehyde, and the K_m's for the aromatic aldehydes are substantially smaller (D. K. Srivastava and S. A. Bernhard, unpublished results). Thus, the K_m for NADH in the LADH reaction is readily determinable under conditions of aromatic aldehyde saturation. Under such conditions in the absence of any GPDH, the K_m and k_{cat} values for each aromatic aldehyde have been determined as detailed in Table I.

Table I: Kinetic Constants of LADH-Catalyzed Reduction of Aromatic Aldehydes by Free NADH[a]

aromatic aldehyde	K_m^{NADH} ($\times 10^6$ M)	k_{cat} (s^{-1})
benzaldehyde[b]	6.98 ± 0.96	27.2 ± 1.8
p-nitrobenzaldehyde[c]	2.42 ± 0.28	10.2 ± 0.9

[a] In 50 mM pyrophosphate buffer, pH 7.5, at 25 °C. [b] Substrate concentrations of (3.5–4.0) × 10^{-4} M. [c] Substrate concentrations of 8.0 × 10^{-5}–1.0 × 10^{-4} M.

Table II: Comparison of the Observed Rates of LADH-Catalyzed Aldehyde Reduction by NADH with the Calculated Rates due to Free NADH Concentration in the Presence of Excessive GPDH[a]

[GPDH site]$_{total}$ ($\times 10^4$ M)	free [NADH][b] ($\times 10^7$ M)	obsd rate ($\times 10^7$ M/min)	predicted rate[c] ($\times 10^7$ M/min)
In the Presence of Benzaldehyde			
2.60	3.25	8.04	0.41
3.90	2.05	8.76	0.26
4.55	1.75	8.44	0.23
5.20	1.50	8.04	0.19
6.50	1.20	7.56	0.16
In the Presence of *p*-Nitrobenzaldehyde			
1.30	3.20	8.84	0.95
2.60	1.50	9.24	0.47
3.25	1.15	8.64	0.37
3.90	0.95	8.84	0.31
4.55	0.85	8.24	0.28
5.20	0.70	8.44	0.23
5.85	0.65	8.24	0.21

[a] In 50 mM pyrophosphate buffer, pH 7.5 at 25 °C. [b] Calculated from the K_d for GPDH–NADH (1.8×10^{-6} M). $[NADH]_{total}$ was 4.0×10^{-5} and 2.0×10^{-5} M for LADH-catalyzed reduction of benzaldehyde (4.0×10^{-4} M) and *p*-nitrobenzaldehyde (1.0×10^{-4} M), respectively. [c] Calculated from the data given in footnote *b* of this table and the kinetic constants of Table I. $[LADH]_{total}$ was 5.6×10^{-10} and 1.33×10^{-9} M for the cases of benzaldehyde and *p*-nitrobenzaldehyde, respectively.

The free NADH concentration can be buffered by the addition of superstoichiometric concentrations of halibut muscle GPDH. Since the dissociation constants for GPDH–NADH are known, we can calculate the free (aqueous) NADH concentration at any particular total concentration of GPDH and NADH. Such computations are listed in Table II. We can thereby proceed to investigate the role of free NADH vs. GPDH-bound NADH as substrate in the LADH-specific reduction reaction. The effect of free aqueous NADH concentration on the reaction velocity is predictable directly from the parameters listed in Table I, as indicated in the appropriate entries in Table II. The effective binding of NADH to GPDH leads to a prediction of a very slow reaction velocity in the presence of excessive GPDH due to the very low concentration of free NADH. This prediction is contrary to the experimental results listed in Table II. Any reaction velocity in excess of this prediction must arise from the utlization of GPDH–NADH as substrate in the LADH-catalyzed reaction or else

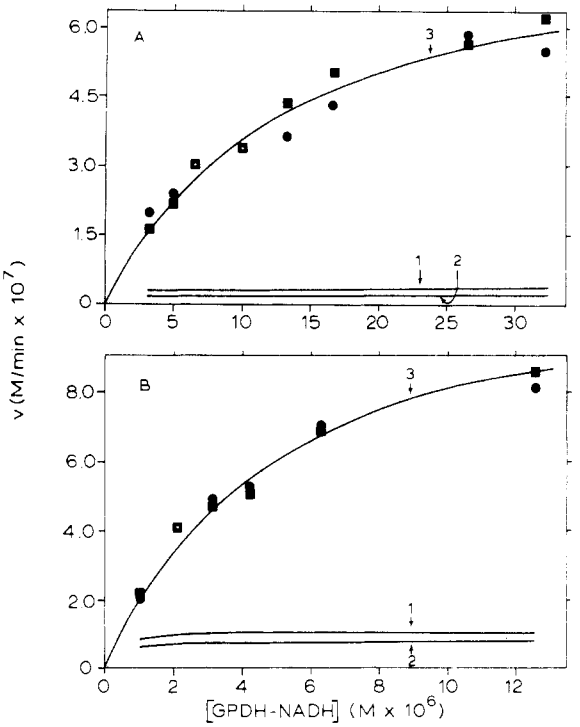

FIGURE 2: Initial rates of LADH-catalyzed aldehyde reduction as as function of GPDH–NADH concentration. (A) S_2 = benzaldehyde (4.0×10^{-4} M); [LADH] is 3.74×10^{-10} M; (■) experimental points for $[GPDH\text{–site}]_{total}/[NADH]_{total} = R = 6.25$; (●) $R = 9.30$. Curves 1 and 2 are the calculated expected velocities for $R = 6.25$ and 9.30, respectively, on the basis that free NADH is the only competent coenzyme species. The solid line of curve 3 is calculated on the basis of Michaelian behavior for GPDH–NADH as a competent coenzyme: $K_m = 1.33 \times 10^{-5}$ M; $k_{cat} = 37.3$ s^{-1}. (B) S_2 = *p*-nitrobenzaldehyde (1.0×10^{-4} M); [LADH] is 1.5×10^{-9} M. Experimental points are for $R = 6.71$ (■) and 9.42 (●). Curves 1 and 2 are calculated on the basis of free NADH as the only competent coenzyme species. Curve 3 is for GPDH–NADH as the competent coenzyme species with $K_m = 4.9 \times 10^{-6}$ M and $k_{cat} = 13.3$ s^{-1}.

must be due to the effect of GPDH interaction with LADH so as to further increase the turnover of LADH substrates.

These alternatives can be distinguished by the effect of still higher concentrations of free GPDH vs. the effect of higher GPDH–NADH complex concentrations on the LADH-catalyzed reaction velocity. Each of these factors can be considered independently from the data listed in Table II. An examination of Table II shows that further excess of free GPDH at a fixed concentration of GPDH–NADH complex has virtually no effect on the rate of the LADH-catalyzed reaction. At saturating aldehyde concentrations, the reaction velocities show a hyperbolic dependence on the concentration of GPDH–NADH (Figure 2). At fixed GPDH/NADH ratios, the LADH-catalyzed reaction rate shows a hyperbolic dependence on the total GPDH concentration (Figure 2A,B). At any fixed GPDH/NADH ratio, the low concentrations of free aqueous NADH, resulting from the buffering by excessive GPDH, are nearly linearly dependent on the total GPDH concentration. Hence, if aqueous NADH were required for the catalyzed reaction, the velocity would be nearly linearly dependent on the GPDH–NADH concentration (Figure 2; curves 1 and 2). When the ratio of excessive GPDH to NADH is changed, the reaction velocity maintains the same hyperbolic dependence on the GPDH–NADH complex (Figure 2) regardless of the extent of excessive GPDH and the low but variable concentration of free NADH. These results argue strongly in favor of the formation of a GPDH–NADH–LADH ternary complex.

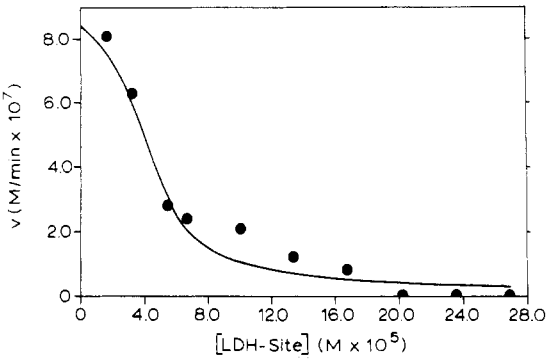

FIGURE 3: Initial rates of LADH-catalyzed reduction of benzaldehyde (3.3×10^{-4} M) by NADH (3.86×10^{-5} M) as a function of LDH concentration: (●) experimental points. The solid line is calculated on the basis of Michaelian behavior in the binding of NADH to LDH (Figure 1) and the known kinetic parameters for free NADH (for LADH–benzaldehyde, Table I) as the only competent coenzyme. [LADH] = 4.5×10^{-10} M.

An extrapolation to infinite GPDH–NADH concentration and to infinite aromatic aldehyde substrate concentration gives a k_{cat} values of 37.3 and 13.3 s^{-1} for turnover of the quaternary complexes involving benzaldehyde and *p*-nitrobenzaldehyde, respectively. These values are approximately 30% greater than the k_{cat}'s for the turnover of these substrates in the absence of GPDH. An analysis of the reaction velocity data as a function of the concentration of the GPDH–NADH complex yields K_m values for GPDH–NADH of 13.3 and 4.9 μM in the benzaldehyde and *p*-nitrobenzaldehyde reductions, respectively. These values should be compared with the somewhat lower K_m values for the same reactions with aqueous NADH (Table I). It is noteworthy that all of these K_m values are considerably larger than the equilibrium dissociation constant for the enzyme–coenzyme binary complex.

We wanted to study the rate of the GPDH-catalyzed reduction of 1,3-diphosphoglycerate using LADH–NADH as the reductant. We are, however, unable to perform this experiment satisfactorily at this time because the product of the GPDH-catalyzed reaction, glyceraldehyde 3-phosphate, is a substrate, albeit a poor one for LADH. Since we utilize approximately 10^4–10^5 molar excesses of the carrier enzyme (E_1) to the "catalytic" enzyme (E_2), the further reutilization of glyceraldehyde 3-phosphate in the LADH system becomes a major contribution to the overall reaction velocity.

We were initially frustrated in some of the experiments we report herein due to another feature of the skewed ratio of carrier to catalytic enzyme concentration. Trace impurities of the catalytic enzyme in the carrier enzyme preparation can make very substantial contributions to the overall catalyzed reaction velocity. For this reason, it was necessary to prepare NADH carrier enzymes, free from catalytic enzymes, to less than the 10^4–10^5-fold level dictated by the enzyme ratios. In all cases, it was found that this criterion of purity could be reached by repeated column chromatography of the isolated enzyme proteins.

To test the generality of the direct transfer mechanism, we have studied the transfer of NADH between LADH and LDH. The rates of LADH-catalyzed reduction of benzaldehyde by LDH–NADH and the LDH-catalyzed reduction of pyruvate by LADH–NADH are both slow. Utilizing LDH as an NADH buffer, we can compare the rate of benzaldehyde reduction with that predicted for free (aqueous) NADH as the only competent coenzyme substrate. As can be seen in Figure 3, there does not appear to be any contribution to this LADH-catalyzed reaction other than from NADH in aqueous solution. Similarly, when LADH was utilized as an NADH

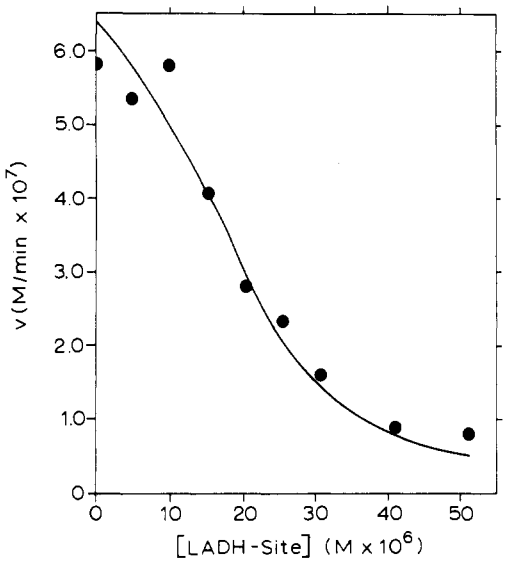

FIGURE 4: Initial rates of LDH-catalyzed reduction of pyruvate (2.0×10^{-3} M) by NADH (2.44×10^{-5} M) as a function of LADH concentration: (●) experimental points. The solid line is calculated on the basis of Michaelian behavior in the binding of NADH to LADH (Figure 1) and the known kinetic parameters for free NADH in the LDH-catalyzed pyruvate reduction ($K_m^{NADH} = 1.92 \times 10^{-5}$ M, $k_{cat} = 3.24 \times 10^{-3}$ s^{-1}). [LDH] = 2.36×10^{-11} M.

buffer, the rate of LDH-catalyzed reduction of pyruvate also appears to be due exclusively to the free (aqueous) NADH (Figure 4).

Discussion

The results regarding the transfer of NADH from GPDH–NADH to the site of catalysis of aromatic aldehyde reduction in LADH lead us to the following conclusions. (1) The pathway involves the direct transfer of NADH from GPDH to LADH, without the intermediate dissociation of NADH into the aqueous environment. Under the conditions we describe, the aqueous concentration of NADH is never sufficient to allow for the rapid rate of reduction of aromatic aldehyde substrates via LADH catalysis. (2) In the presence of sufficiently high GPDH–NADH concentrations and high substrate concentration (S_2), catalytic quantities of LADH (nanomolar concentrations) become saturated with GPDH–NADH and substrate (S_2), as is indicated by the attainment of a maximal rate of substrate reduction. It is tempting to equate this saturation in reaction velocity with a stoichiometric formation of the GPDH–NADH–LADH complex. It may indeed be the case that at saturation in E_1–NADH such a ternary E_1–NADH–E_2 complex is the major LADH component. However, we are aware that there are other plausible mechanism for attaining saturation in steady-state reaction velocity which may never result in appreciable ligation of E_2 to E_1-containing species. Consider the extreme example of direct transfer of coenzyme without the formation of any stoichiometrically detectable E_1–E_2 complex (eq 4):

$$E_1\text{–NADH} + E_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_1 + \text{NADH–}E_2 \xrightarrow[k_{cat}]{S_2} E_2 + P_2 \quad (4)$$

Derivation of the steady-state turnover rate according to the model of eq 4, under the conditions $[E_1] \geq [E_1\text{–NADH}] >>> [E_2]$, yields the steady-state velocity expression of eq 5:

$$v = \frac{V_{max}[E_1\text{–NADH}]}{[E_1\text{–NADH}] + (k_{-1}[E_1] + k_{cat})/k_1} \quad (5)$$

Equation 5 is analogous to the Michaelis–Menten equation except that in this case the rates of substrate (NADH) on and off E_2 are each dependent on the concentration of E_1 components. The on rate for NADH to E_2 depends on the excessive concentration of E_1–NADH, whereas the reverse rate depends on the concentration of unliganded E_1. In all of the experiments which we have described, the concentrations of these two E_1 components are substantial. The concentration of E_1 must exceed the concentration of NADH sufficiently to reduce the free NADH concentration, well below the K_m for NADH, so as to substantially reduce the rate of turnover via free NADH. The dependence of reaction velocity on E_1 or E_1–NADH must, minimally, start from the high enzyme concentration required to lower the free NADH concentration. In the experiments which we describe, $[E_1]$ ($[E_1]_{total}/[E_1\text{–NADH}]$) varies from about 6 to 30. Equation 5 predicts hyperbolic saturation in E_1–NADH at any fixed concentration of unliganded E_1, a prediction consistent with our observations. However, in order for the reaction velocity to be *independent* of the unliganded E_1 concentration, it follows that the rate of transfer of NADH to E_2 must exceed the reverse transfer rate back to E_1–NADH. We find this inequality in reaction velocity in the two directions (eq 4) difficult to accept. Since E_1 and NADH are of comparable concentrations, it is difficult to formulate a mechanism for the distinction in rate especially since the thermodynamic stabilities of E_1–NADH and E_2–NADH are comparable. The distinction between the forward and reverse rates could be made more plausible if E_1–NADH were required for the obligatory formation of the E_1–NADH–E_2 complex in order to transfer to occur as is given in eq 6:

$$E_1\text{–NADH} + E_2 \rightleftharpoons E_1\text{–NADH–}E_2 \rightleftharpoons E_1 + E_2\text{–NADH} \xrightarrow{S_2} \text{product} \quad (6)$$

It is interesting to note that if the formation of an E_1–NADH–E_2 complex were limited in rate by diffusion, that a limiting value of K_m could be predicted from the ratio of k_{cat} to k_{on} by assuming that ligands leave the enzyme–enzyme complex most rapidly by way of chemical transformation. The predicted ratios for k_{cat}/k_{on} are $37.3/(2\text{–}6) \times 10^7$ μM and $13.3/(2\text{–}6) \times 10^7$ μM for benzaldehyde and *p*-nitrobenzaldehyde, respectively. Any breakdown of the E_1–NADH–E_2 complex to yield unreacted aqueous ligands would cause an increase in the predicted value of K_m (a lowering of the apparent affinity for coenzyme). The observed K_m's are larger than the predicted limit but not by very much. The relative K_m's for the two substrates are indeed quantitatively correlated with the two k_{cat}'s for the reaction, indicating a sizable contribution of the turnover rate to K_m. Circumstantial evidence appears to corroborate the notion that saturation of reaction velocity with increasing E_1–NADH concentration is the result of complex formation with E_2 but the evidence is not totally compelling to us at this time.

It might appear, intuitively, that the well-known high affinity of GPDH for NAD^+ [see Harris & Waters (1976) and references cited therein] as compared with NADH plays an important role in the acceleration in V_{max} observed in the presence of GPDH. This consideration might be particularly important in our experiments because the excessive concentration of GPDH over NADH ensures that virtually all of the NAD^+ formed as a consequence of reaction would be bound to "tight-binding" NAD^+ sites (Seydoux et al., 1973; Kelemen et al., 1975). In order for the V_{max} to be 30% accelerated, it would follow from this argument alone that the steady-state ratio of product complex (E_2–NAD^+–alcohol) concentration to total E_2 concentration is increased by 30%. This is a highly unlikely situation, since the enzyme–substrate complex is virtually all in the E–NAD^+–alcohol form (Jacob et al., 1974;

McFarland & Chu, 1975; Plapp et al., 1978; Anderson & Dahlquist, 1980) in the absence of GPDH. Moreover, if GPDH were acting as a buffer of the steady-state free NADH concentration, the rate enhancement must arise from the inhibition of turnover by the readsorption of NAD^+. Thus, the net rate constant for turnover in the absence of GPDH should contain contributions from both the dissociation and association rates for E_2–NAD^+, whereas in the presence of GPDH, only the desorption rate is relevant. Thus, different effects would be anticipated for the rate enhancement in the case of substrates with different V_{max} values, since the rates of desorption of both the coenzyme and product are product dependent (Dunn et al., 1979). This is not the case. Both benzaldehyde and *p*-nitrobenzaldehyde are identically influenced in V_{max} by the presence of GPDH. Thus, we do not find a satisfactory explanation for the rate enhancement other than by the direct interaction of GPDH with LADH and substrates.

In this paper, we have emphasized the methodology for discriminating reaction pathways (eq 1a,b) by utilizing GPDH as the coenzyme donor and LADH as the catalyst for the NADH-dependent reduction of substrate. Under different solvent conditions, we have shown that the transfer of NADH from LDH to catalytic GPDH and from GPDH to catalytic LDH also proceeds via the same direct transfer mechanism (Srivastava & Bernhard, 1984). We have been unable to experimentally test the reverse transfer of NADH from LADH to GPDH substrate because the product of the GPDH-catalyzed reaction (glyceraldehyde 3-phosphate) is itself a substrate for the LADH reaction at the high concentrations of LADH required for the mechanistic discrimination. Interestingly, and as a hindsight control, we have demonstrated that LADH and LDH do not act as carriers of NADH to the opposite dehydrogenase. LADH–NADH is not a substrate for the LDH-catalyzed pyruvate reduction. Similarly, LDH–NADH is not a substrate for the LADH-catalyzed reduction of benzaldehyde.

The inability of apo-GPDH to inhibit the LADH-catalyzed reaction of GPDH–NADH with aldehydes suggests that the coenzyme plays an important role in the recognition of cognate enzyme species. Two distinct mechanisms are immediately obvious: (1) The coenzyme itself serves as a recognition determinant while bound to the carrier enzyme. (2) The coenzyme ligand causes a transition in the carrier protein conformation so as to provide a protein recognition site for the cognate enzyme. Each of these mechanisms has some plausibility. An A-side dehydrogenase presumably is tightly interacted with protein on the B face. As such, the A face could be recognized by a B-side dehydrogenase which presumably interacts tightly with the accessible A face. On this basis, the nicotinamide ring itself would provide the site for protein–protein recognition provided that the complex is between an A and a B dehydrogenase. A qualitatively similar mechanism was suggested by Cardon & Boyer (1982) to explain more rapid NADH production in the GPDH-catalyzed reaction of glyceraldehyde 3-phosphate with NAD^+ in the presence of LDH.

A cursory examination of the three-dimensional structures of glyceraldehyde-3-phosphate dehydrogenase (holo-NAD^+ enzyme) (Buehner et al., 1974; Moras et al., 1975), LADH (holo-ADP-ribose and NADH structures) (Brändén et al., 1975; Eklund et al., 1981), and LDH (holo-NAD^+ structure) (Holbrook et al., 1975) indicates that the bound coenzymes are not readily accessible to other polypeptides. It would appear that some conformational change is required in order for enzyme–enzyme recognition to be possible. A detailed analysis of potential interactions via model building or computer graphics must await more refined structures for GPDH, and LADH–NADH. Recent studies on the structure of LADH suggest that all specific ligands effect some degree of conformational change in the enzyme structure (Eklund et al., 1981, 1982; Cedergren-Zeppezauer et al., 1982). It is noteworthy that apo-PGK is not an effective competitor of 1,3-phosphoglycerate transfer from PGK to GPDH (Weber & Bernhard, 1982). In this case, the 1,3-diphosphoglycerate substrate is believed to close the hinge separating two domains of the kinase structure (Banks et al., 1979; Pickover et al., 1979). If so, this provides a mechanism for ligand-induced protein conformational change and consequent enzyme–enzyme recognition. Detailed structural analysis of any of these glycolytic enzyme–enzyme complexes is at this time impossible.

Our results with LDH–GPDH interactions suggest that pure protein–protein interactions play an important role in complex formation between these two halibut muscle enzyme species (Srivastava & Bernhard, 1984). These results as well as the higher affinity between GPDH and PGK from the same cell type (Weber & Bernhard, 1982) suggest that the interactions are species specific and hence protein (rather than substrate) in nature. However, we have very little data regarding species specificity.

In a separate paper, we present more extensive evidence that the transfer of NADH between dehydrogenases occurs only *between* A and B dehydrogenase sites (*not* between two A or two B dehydrogenases). The potential for direct transfer is in no other way dependent on the specific dehydrogenase pair (Srivastava & Bernhard, 1984).

Acknowledgments

We appreciate discussions with Dr. Paul Boyer and Harvey Penefsky, who called our attention to the original references suggesting direct transfer between dehydrogenases. One of the original authors, Dr. William Rutter, provided us with great encouragement to further pursue this problem. We have benefited from discussions with Dr. Jon P. Weber, who was responsible for the initial discovery of functional complex formation between GPDH and phosphoglycerate kinase. Carl Steifbold was of great assistance in the isolation of highly purified lactate and alcohol dehydrogenase.

Registry No. GPDH, 9001-50-7; LADH, 9031-72-5; NADH, 58-68-4; benzaldehyde, 100-52-7; *p*-nitrobenzaldehyde, 555-16-8.

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