Mechanism of Transfer of Reduced Nicotinamide Adenine Dinucleotide among Dehydrogenases†

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ABSTRACT: The pathway for the transfer of NADH from one dehydrogenase (E_1) to another dehydrogenase (E_2) has been investigated by studying the E_2-catalyzed reduction of S_2 by NADH. The experimental conditions are that the concentration of E_1 exceeds that of NADH, which in turn is very much greater than E_2; hence, the concentration of free (aqueous) NADH is exceedingly low. The rate of reduction of S_2 will hence be very slow if unliganded aqueous NADH is required for the E_2-catalyzed reaction. Our results with eight dehydrogenases are entirely consistent with the direct transfer of NADH between E_1 and E_2 whenever the two enzymes transfer hydrogen via opposite faces (A and B) of the nicotinamide ring. Whenever the two enzymes are both A or both B, NADH transfer occurs only via the aqueous solvent. Some mechanistic inferences and their possible physiological significance are discussed.

In previous papers from this laboratory (Weber & Bernhard, 1982; Srivastava & Bernhard, 1984), the transfer of metabolite from one enzyme, where it is a bound product, to another enzyme, where it is a reactant, has been demonstrated to proceed via the direct transfer of metabolite in an enzyme–substrate–enzyme complex. This was first established for the transfer of metabolite between phosphoglycerate kinase (PGK)[1] and glyceraldehyde-3-phosphate dehydrogenase (GPDH). The exceedingly tight binding of 1,3-diphosphoglycerate (DPG, $K_d < 10$ nM) to the kinase (Nageshwara RaO et al., 1978; Scope, 1978; Huskins et al., 1982) precludes dissociation and random diffusion as a mechanism for the transfer of metabolite. The rapid overall turnover number for the coupled two-enzyme system far exceeds the maximal rate of dissociation of the PGK–DPG complex (Weber & Bernhard, 1982). In a recent paper (Srivastava & Bernhard, 1984), the transfer of NADH from GPDH to liver alcohol dehydrogenase (LADH) was also demonstrated to proceed via the direct transfer mechanism. In the latter case, the direct transfer mechanism was established by lowering the free NADH concentration by buffering with excessive concentrations of E_1, a noncatalytic dehydrogenase in the E_2-catalyzed reduction of S_2. This method of enzyme saturation of substrate (coenzyme) is a generally valid method for investigating the mechanism of coupled enzyme reactions. It involves the use of a minute amount of an S_2-specific catalyst (E_2) to probe the following alternate mechanisms: (1) the direct transfer of NADH from E_1–NADH to E_2; (2) the dissociation of E_1–NADH followed by random diffusion of NADH to E_2. The method depends, however, on the availability of highly purified enzyme (E_1) as the carrier of metabolite, since even minute traces of the limiting catalytic enzyme (E_2) as contaminant in the highly excessive E_1 can lead to misinterpretation of the kinetic results.

The "high" concentration of E_1 which we utilize is in actuality low or comparable to the physiological concentration of glycolytic dehydrogenases (e.g., LDH and GPDH) (Ottaway & Mowbray, 1977). Since saturation of the enzyme site is determined by the concentration of the excessive reactant, our results, which sometimes demonstrate direct transfer of NADH (see below), may likely be relevant to the physiological metabolic pathway. Wherever direct transfer is demonstrable, the concentration of free metabolites in aqueous solution may be nearly irrelevant to the bioenergetics of the metabolic sequence. It should be noted that the concentrations of all the three-carbon glycolytic intermediates, as well as of NADH, are always less than the molar concentration of specific enzyme sites to which they are bound (see Sols & Marco, 1970, Ottaway & Mowbray, 1977, and references cited therein). The enzyme site concentrations are invariably larger than, or comparable to, the dissociation constant of the enzyme–substrate complex [see Bloch et al. (1971) and references cited therein].

In this paper, we describe experiments which probe the mechanism of NADH transfer among diverse dehydrogenases. We examine the NADH-bound complexes (E_1–NADH) of glyceraldehyde-3-phosphate dehydrogenase (GPDH), lactate dehydrogenase (LDH), α-glycerolphosphate dehydrogenase (α-GDH), and horse liver alcohol dehydrogenase (LADH) as potential donors of NADH in the E_2-catalyzed NADH-dependent reduction of S_2. E_2 in these instances represents one of these above-mentioned enzymes or one of four other dehydrogenases available to us in catalytic rather than "substratelike" quantities. In this way, we intend to probe the generality of the direct transfer mechanism which we have previously established as valid in a limited number of cases (Weber & Bernhard, 1982; Srivastava & Bernhard, 1984).

We have already reported that some E_1–NADH complexes do not serve as competent coenzyme carriers for an E_2-catalyzed reduction of S_2. For example, LADH-bound NADH is not a competent substrate for the NADH-dependent reduction of pyruvate catalyzed by LDH (Srivastava & Bernhard, 1984). In this report, we attempt to interpret en-

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[1] Abbreviations: GPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; LADH, liver alcohol dehydrogenase; LDH, lactate dehydrogenase; α-GDH, α-glycerolphosphate dehydrogenase; G-3-P, glyceraldehyde 3-phosphate; Gly-3-P, glycerol 3-phosphate; 3PG, 3-phosphoglycerate; DPG, 1,3-diphosphoglycerate; DHAP, dihydroxyacetone phosphate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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zyme–enzyme interactions, or the lack thereof, in terms of molecular mechanistic principles. We present herein clear-cut evidence both *for* and *against* the direct transfer mechanism, depending on the specific dehydrogenases involved as NADH carrier (E_1–NADH) and catalyst (E_2). The distinctions in mechanism appear to have a clear-cut structural basis and may be of direct relevance to the organization of reaction sequences in the metabolic pathway.

Experimental Procedures

Materials

Sodium salts of NAD^+ (grade III), NADH (grade III), pyruvic acid, and EDTA, the lithium salt of dihydroxyacetone phosphate, DL-glyceraldehyde 3-phosphate diethyl acetal monobarium salt (grade I), 2-mercaptoethanol, *cis*-oxaloacetic acid (grade I), oxidized glutathione, and D-fructose were obtained from Sigma Chemical Co. and were used without further purification. 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. Tris buffer (50 mM) (pH of Trizma base adjusted with dilute HCl), pH 7.5, containing 0.1 mM EDTA and 0.35 mM 2-mercaptoethanol was used for all experiments, unless stated otherwise.

Preparation of 1,3-Diphosphoglycerate. 1,3-Diphosphoglycerate (DPG) was prepared from glyceraldehyde 3-phosphate (G-3-P) according to Furfine & Velick (1965). The thus prepared crude mixture was suspended in 50 mM imidazole buffer, pH 7.4, and centrifuged, and the pH was readjusted to 7.4 with dilute NaOH. The above solution was immediately applied on an A-25 DEAE-Sephadex column (2 × 15 cm) preequilibrated with the above buffer at 0–4 °C. The column was vigorously washed with approximately 200 mL of buffer. DPG was eluted from the column by a linear gradient of 0–600 mM KCl. Peak fractions of DPG were collected and stored at −80 °C. It was thawed at 0 °C immediately before use. The DPG thus purified did not contain any detectable amount of G-3-P, pyruvate, lactate, NAD^+, or NADH.

Enzymes. Halibut muscle enzymes, glyceraldehyde-3-phosphate dehydrogenase (GPDH), and lactate dehydrogenase (LDH) were prepared and assayed as described earlier (Srivastava & Bernhard, 1984). Horse liver alcohol dehydrogenase (LADH) was obtained from Boehringer Manheim and was further purified and assayed as described earlier (Srivastava & Bernhard, 1984).

Rabbit muscle α-glycerolphosphate dehydrogenase (α-GDH, type I), porcine heart cytoplasmic malate dehydrogenase, yeast glutathione reductase (type III), sheep liver sorbitol dehydrogenase, and alanine dehydrogenase from *Bacillus subtilis* were obtained from Sigma Chemical Co. These dehydrogenases were used without any further purification. Since α-GDH was used as an NADH buffer in some of the experiments described here, we precisely assayed enzyme activity and determined protein concentrations according to Grazi et al. (1974). The enzyme site concentrations of α-GDH were determined on the basis of the molecular weight of dimeric enzyme as 78 000 (Van Eys et al., 1959).

Methods

Tris-HCl (50 mM), pH 7.5, containing 0.1 mM EDTA and 0.35 mM 2-mercaptoethanol was used as a solvent in all fluorometric titrations and steady-state kinetic experiments, except in experiments involving LADH. Various components of the standard buffer solvent are inhibitory toward LADH catalysis. In the experiments involving LADH, the solvent was 50 mM pyrophosphate, pH 7.5.

Table I: Summary of Thermodynamic and Kinetic Parameters for NADH Ligation to Three Dehydrogenases[a]

enzyme	K_d^{NADH} (×10⁷ M)	K_m^{NADH} (×10⁶ M)	k_{cat} (×10⁻² s^{-1})
GPDH	3.5 ± 0.5	5.57 ± 0.4[b]	2.15 ± 0.21[b]
LDH	2.1 ± 0.4	13.43 ± 2.5[c]	25.78 ± 1.93[c]
α-GDH	8.8 ± 1.2	2.74 ± 0.5[d]	1.55 ± 0.25[d]

[a] In 50 mM Tris-HCl buffer, pH 7.5 at 25 °C. [b] In the presence of (8.0–10.0) × 10^{-5} M DPG. [c] In the presence of 2.0 × 10^{-3} M pyruvate. [d] In the presence of 5.0 × 10^{-4} M DHAP.

All NADH buffering enzymes (E_1) were dialyzed against appropriate buffers and filtered through a 0.45-μm Millipore filter.

Fluorometric Titrations and Kinetic Experiments. Fluorometric titrations were carried out in standard Tris-HCl buffer, pH 7.5 at 25 °C, in a Hitachi MPF-2A Perkin-Elmer spectrofluorometer as described earlier (Srivastava & Bernhard, 1984). The dissociation constants for E_1–NADH were determined from changes in the NADH fluorescence emission at 460 nm. Excitation conditions were as follows: GPDH, 290 nm; LDH and α-GDH, 340 nm.

Steady-state kinetic experiments were carried out in a Varian Techtron Model 635 recording spectrophotometer at 25 °C under appropriate buffer conditions, essentially as described earlier (Srivastava & Bernhard, 1984).

Results

All of our kinetic experiments reported herein can be generalized to the scheme of eq 1. In each experiment, one

$$E_1\text{–NADH} + S_2 \xrightarrow{E_2} E_1\text{–NAD}^+ + P_2 \qquad (1)$$

dehydrogenase is utilized in high substratelike concentration as a potential carrier of substrate quantities of NADH (E_1–NADH) to participate in the specific reduction reaction catalyzed by nanomolar quantities of a different dehydrogenase (E_2), for example, the LDH-catalyzed reduction of pyruvate by NADH in the presence of a molar excess of GPDH over NADH. In every reaction we consider, NADH free in solution is a competent substrate (coenzyme) for carrying out the specific catalyzed reduction. Therefore, the kinetic alternatives are either of the following: (1) Addition of a stoichiometric excess of E_1 over NADH would reduce the free aqueous NADH concentration to a low buffered value. Thus, the rate of reduction of S_2 catalyzed by E_2 is greatly reduced according to known Michaelian parameters for the E_2-catalyzed reaction. (2) The carrier dehydrogenase–NADH complex (E_1–NADH) may itself be a substrate in the E_2-catalyzed reduction of S_2. If E_1–NADH is utilized with comparable efficiency to the aqueous NADH, the direct transfer mechanism may be apparent due to the very large excess of E_1–NADH over unliganded NADH. To make such a distinction in the mechanism, we must have precise estimates of the dissociation equilibrium constants for E_1–NADH and we must know the Michaelian parameters, K_m (for NADH) and V_{max} for each E_2-catalyzed reaction. The equilibrium dissociation constants (K_d) and K_m for NADH are both solvent dependent. Therefore, we have determined these parameters, as well as V_{max}, in a particular buffer solvent, as listed in Table I.

All dissociation constants were determined from fluorescence emission measurements. For the enzymes LDH, LADH, and α-GDH, the substantial increase in a coenzyme fluorescence upon binding to the enzyme could be monitored as a signal of formation of the complex (see Experimental Procedures). In the case of GPDH, we utilized excitation at 290 nm to

Table II: Comparison of the Observed Rates of S_2 Reduction in the Presence of E_1 with Those Predicted on the Basis of Aqueous NADH as the Only Competent Coenzyme[a]

[E_1 site] ($\times 10^4$ M)	[E_2] ($\times 10^{10}$ M)	fixed concn (M)	predicted rate[b] ($\times 10^7$ M/min)	obsd rate ($\times 10^7$ M/min)
		E_1 = GPDH		
2.20	LDH = 0.29	[NADH]$_t$ = 1.34×10^{-4}	1.68	10.45
2.48		[pyruvate] = 2×10^{-3}	1.31	8.84
2.76			1.06	6.03
2.87			1.00	5.47
2.98			0.93	4.82
		E_1 = LDH		
0.71	GPDH = 1.08	[NADH]$_t$ = 4.95×10^{-5}	1.10	8.04
0.85		[DPG] = 9.0×10^{-5}	0.69	7.88
1.13			0.39	6.91
1.41			0.30	6.03
1.70			0.21	5.15
		E_1 = LDH		
0.42	α-GDH = 1.38	[NADH]$_t$ = 3.26×10^{-5}	2.45	6.83
0.57		[DHAP] = 5.0×10^{-4}	1.19	6.29
0.85			0.58	4.34
1.13			0.39	3.85
1.41			0.30	3.22
		E_1 = GPDH		
0.16	α-GDH = 2.11	[NADH]$_t$ = 2.25×10^{-5}	15.38	14.47
0.32		[DHAP] = 5.0×10^{-4}	5.71	6.59
0.40			3.43	2.73
0.47			2.44	2.41
0.63			1.52	1.21
		E_1 = α-GDH		
0.11	GPDH = 1.08	[NADH]$_t$ = 4.95×10^{-5}	21.92	16.88
0.22		[DPG] = 7.0×10^{-5}	13.55	8.44
0.33			6.70	6.43
0.44			4.17	4.50
0.56			2.98	3.21
0.67			2.34	1.61

[a] In 50 mM Tris-HCl buffer, pH 7.5 at 25 °C. [b] Based on the aqueous NADH concentration calculated from the appropriate dissociation constants of E_1–NADH and kinetic parameters for the E_2-catalyzed reaction (Table I).

monitor coenzyme emission at 460 nm. The low-wavelength excitation results in some direct excitation of the coenzyme and a substantial excitation due to energy transfer from the protein to the bound coenzyme. Dissociation constants were determined as described in another paper (Srivastava & Bernhard, 1984) except that the solvent in these experiments is 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 mM EDTA and 0.35 mM 2-mercaptoethanol.

K_m for NADH and V_{max} were determined at or near substrate (S_2) saturation from the strictly Michaelian dependence of the steady-state reaction velocity on NADH concentration.

Steady-state kinetic experiments in the presence of excessive E_1 over NADH were carried out similarly to the kinetic experiments in the absence of E_1 (see above). Since the carrier enzyme (E_1) is present in nearly 10^6-fold molar excess over the catalytic enzyme (E_2), it is necessary to check that any particular carrier enzyme preparation be free from E_2 contamination at a level $<<10^{-6}$. The extent of impurity in the carrier enzyme can be quantitatively assayed even at exceedingly low extents of contamination.

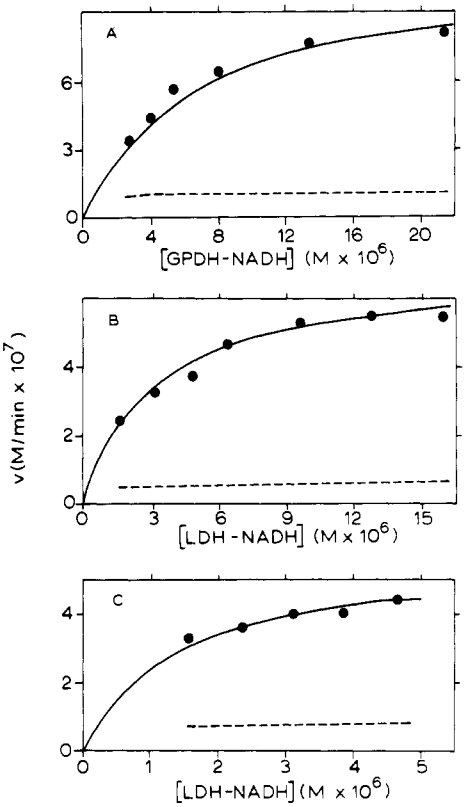


FIGURE 1: Initial rates of E_2-catalyzed reduction of S_2 as a function of E_1–NADH concentrations at a fixed ratio of [E_1]/[NADH]$_t$. (●) represents observed values. The solid lines are calculated from the kinetic parameters of Table III. The dashed lines are calculated on the basis of free NADH as the only competent coenzyme substrate. (A) E_1 = GPDH, E_2 = LDH (2.86×10^{-11} M), [E_1]$_t$/[NADH]$_t$ = 2.02, [pyruvate] = 2.0×10^{-3} M. (B) E_1 = LDH, E_2 = GPDH (1.08×10^{-10} M), [E_1]$_t$/[NADH]$_t$ = 1.71, [DPG] = 9.0×10^{-5} M. (C) E_1 = LDH, E_2 = α-GDH (1.38×10^{-10} M), [E_1]$_t$/[NADH]$_t$ = 2.02, [DHAP] = 5.0×10^{-4} M.

The expectations of reaction velocity in E_1-buffered NADH, assuming that free NADH is the only competent coenzyme substrate, can be determined from the parameters listed in Table I. According to this mechanism, the reaction velocity will be given from the Michaelian parameters and the calculated concentrations of free (unliganded) NADH. As can be seen from the experimental velocities summarized in Table II, results fall into two distinct classes. Either the velocity of reduction is that predicted from the dissociation of the E_1–NADH complex (a very slow reduction rate in all such cases) or the velocities are so much faster that the direct transfer mechanism must be operative.

Wherever the direct transfer mechanism is evident from the velocity studies (Table II), we have further investigated the reaction by studying the effect of E_1–NADH concentration on the reaction velocity. Typical results are shown in Figure 1. In every instance, saturation in the reaction velocity at high E_1–NADH concentration is observable. The reaction velocities appear to follow a Michaelian dependence on the E_1–NADH concentration. Accordingly, we have calculated K_m and V_{max} for the formation and reaction of the "enzyme–coenzyme–enzyme" complex. These results are summarized in Table III.

The effect of additional (superstoichiometric) amounts of the carrier dehydrogenase on the steady-state reaction velocity is also apparent from the data of Table II. In some instances, very little effect of apoenzyme is observable, whereas in others there is considerable inhibition by excess E_1. For example,

Table III: Summary of the Kinetic Constants for the Steady-State Reduction of S_2 by E_1–NADH[a]

E_1–NADH	E_2	$K_m^{E_1\text{-NADH}}$ ($\times 10^6$ M)	k_{cat} ($\times 10^{-2}$ s^{-1})
GPDH–NADH	LDH	5.88 ± 1.21[b]	6.21 ± 1.32[b]
LDH–NADH	GPDH	2.60 ± 0.45[c]	0.99 ± 0.22[c]
LDH–NADH	α-GDH	1.21 ± 0.36[d]	0.66 ± 0.14[d]

[a] In 50 mM Tris-HCl buffer, pH 7.5 at 25 °C. [b] In the presence of 2.0×10^{-3} M pyruvate. [c] In the presence of 9.0×10^{-5} M DPG. [d] In the presence of 5.0×10^{-4} M DHAP.

it is interesting to note that apo-GPDH inhibits the LDH-catalyzed reduction of pyruvate by GPDH–NADH, even at concentrations of apo-GPDH which are comparable to that of the GPDH–NADH complex (Table II). At still higher ratios of apo-GPDH to NADH-bound GPDH, the inhibition increases nearly linearly (data not shown). In contrast, very little inhibition by apo-LDH is observable in the GPDH-catalyzed reduction of DPG by LDH–NADH: Note that in this instance (Table II) the ratio of apo-LDH to NADH-bound LDH can vary by a factor of 5 without substantial change in the rate of reduction. Mechanisms for such discrimination are discussed in the following section.

In a more cursory manner, we have carried out similar kinetic experiments using GPDH or LADH as NADH buffers and a variety of commercially available dehydrogenases as catalysts (E_2). Although the equilibrium dissociation and kinetic parameters for these latter dehydrogenases have not been specifically determined in the solvent which we utilized, the rate data (Table IV) clearly fall into two classes. Sometimes the observed rates are slow as would be anticipated on the basis of the requirement for free NADH, and sometimes the rates in the presence of excess E_1 resemble those in the absence of E_1. It should be noted that in all these experiments (Table IV), the buffered concentration of free NADH is approximately the same, regardless of whether E_1 is LADH or GPDH. According to the mechanism which allows only free NADH to participate in the E_2-catalyzed reduction, the reaction velocity observed in these experiments should be independent of whether E_1 is GPDH or LADH. The results listed in Table IV clearly contradict these expectations.

At this time, we have found difficulty in carrying out kinetic NADH transfer experiments in *both* directions with other dehydrogenase pairs for the following reasons. (1) Substrates (S_2) frequently inactivate or inhibit E_1; a common occurrence is the precipitation of E_1 by S_2. (2) The high concentration of the E_1 species frequently contains levels of contaminant with E_2 activity which are substantial relative to the low concentration of the purified E_2 added. (3) The product (P_2) of one dehydrogenase sometimes reacts with E_1–NADH (Eq 2). (4) We have only a limited quantity of some of the dehydrogenases listed in Table IV.

$$\text{LADH–NADH} \xrightarrow{\text{GPDH}} \text{LADH–NAD}^+ + \text{G-3-P} \xrightarrow{\text{LADH–NADH}} \text{Gly-3-P} \quad (2)$$

Discussion

An examination of the dissociation constants for E_1–NADH among the dehydrogenases we have investigated reveals that all these enzymes have approximately the same affinity for NADH (within an order of magnitude). Nevertheless, the ability of a particular E_1–NADH to serve as a substrate in a different dehydrogenase (E_2)-catalyzed reaction is clearly divided into two groups (Tables II and IV). We cannot ascribe the ability to transfer NADH to any specific enzyme–coenzyme dissociation equilibrium.

From the results thus far reported (Tables II and IV), we offer a hypothesis regarding the ability of a dehydrogenase to transfer NADH directly to another dehydrogenase without intervention of the solvent environment. From the classic work of Westheimer and his collaborators and from many additional studies (Fisher et al., 1953; Simon & Kraus, 1976; You et al., 1978; You 1982), we know that NAD^+-dependent dehydrogenases are always stereospecific for the particular C_4 H which is transferred from the nicotinamide ring to the substrate. Thus, all such dehydrogenases can be classified as either A or B depending on the chirality of the C_4 H which is transferred. In this limited survey, our results show that A dehydrogenases transfer NADH directly only to B dehydrogenase catalyzed reactions and B dehydrogenases transfer NADH direct only to A dehydrogenase catalyzed reactions; A–A transfer and B–B transfer are not allowed.

One may inquire as to the physical nature of the recognition between E_1–NADH and E_2. Is the recognition primarily between the "exposed face" of the bound nicotinamide ring and E_2, or are E_1–E_2 protein-specific interactions largely responsible for the recognition? We note that wherever E_1–NADH–E_2 transfer is possible, the K_m for E_1–NADH is similar to, but approximately 1 order of magnitude larger than, the dissociation constant for E_1–NADH. We note that the reduction of DPG by GPDH and LDH–NADH is not substantially inhibited by the presence of comparable or excessive concentrations of apo-LDH. Likewise, the LDH–NADH

Table IV: Comparison of the Rates of S_2 Reduction by NADH in the Presence of E_1 and in Its Absence

E_1	E_2[c]	$[S_2]$[d] ($\times 10^3$ M)	rate in the absence of E_1 ($\times 10^6$ M/min)	rate in the presence of E_1 ($\times 10^6$ M/min)	stereochemistry of E_2
LADH[a]	malate dehydrogenase	oxaloacetate = 2.0 NH_4Cl = 5.0	2.32	<0.10	A
	alanine dehydrogenase	pyruvate = 2.0 NH_4Cl = 5.0	1.69	<0.10	A
	sorbitol dehydrogenase	fructose = 5.0	7.07	<0.10	A
	glutathione reductase	glutathione = 5.0	1.28	0.82	B
GPDH[b]	malate dehydrogenase	oxaloacetate = 2.0 NH_4Cl = 5.0	3.46	1.23	A
	alanine dehydrogenase	pyruvate = 2.0 NH_4Cl = 5.0	1.45	1.29	A
	sorbitol dehydrogenase	fructose = 5.0	7.07	2.89	A
	glutathione reductase	glutathione = 5.0	1.42	<0.10	B

[a] In 50 mM pyrophosphate buffer, pH 7.5; $[\text{LADH site}]_t = 1.47 \times 10^{-4}$ M, K_d for LADH–NADH = 1.15×10^{-6} M (Srivastava & Bernhard, 1984). [b] In 50 mM Tris-HCl buffer, pH 7.5; $[\text{GPDH site}]_t = 8.2 \times 10^{-5}$ M. [c] $E_2 \simeq 10^{-3}$ unit in assay mixture. [d] $[\text{NADH}]_t = 2.45 \times 10^{-5}$ M in each case.

reaction with dihydroxyacetone phosphate in α-GDH catalysis is not inhibited by substantial concentrations of apo-LDH. These results seem to argue in favor of a coenzyme role in the recognition. In contrast, the reduction of pyruvate by GPDH–NADH, catalyzed by LDH, shows very substantial apo-GPDH inhibition, an argument that NADH recognition, or NADH-induced conformational change, is not required for A–B dehydrogenase interaction. Alternatively, the recognition may be signaled by some universal complementary A and B surface protein structure.

The fact that LDH–NADH is specifically recognized by GPDH–DPG, whereas GPDH–NADH is recognized no better than apo-GPDH by LDH–pyruvate, argues that specific ligation affects the recognition. In the GPDH-catalyzed reduction of DPG, it is virtually certain that the stoichiometrically principle component of the GPDH system is the 3-phosphoglyceroyl enzyme (Trentham, 1971; Seydoux et al., 1976; Kellershohn & Seydoux, 1979; Duggleby & Dennis, 1974; Seydoux & Bernhard, 1974). The rate of acyl enzyme formation is diffusion controlled, whereas the rate of acyl enzyme reduction (at NADH saturation) is only of the order of 100 s^{-1} (Kellershohn & Seydoux, 1979). Other reports from this laboratory have stressed the conformational transition which occurs upon change in ligation of GPDH with NAD^+ vs. NADH (Schwendiman et al., 1976; Bernhard & Malhotra, 1974; Malhotra & Bernhard, 1968). These conformational states affect the chemistry of the acyl enzyme; the NADH-induced conformation allows reduction, whereas the NAD^+-induced conformation allows only phosphorolysis (Malhotra & Bernhard, 1968, 1973, 1981; Schwendiman et al., 1976; Bernhard & Malhotra, 1974). Perhaps the two different product acceptor enzymes (LDH and PGK) each recognize one of the two alternate conformations of acyl-GPDH. Consequently, the recognition, and the transfer of NADH from GPDH to LDH, must occur via the interaction of the acyl-GPDH–NADH complex with LDH. In the normal direction of glycolysis, the acyl-GPDH–NADH complex is the species generated after the oxidative step of reaction of G-3-P with NAD^+.

$$\text{GPDH–NAD}^+ + \text{G-3-P} \rightarrow \text{acyl-GPDH–NADH} \quad (3)$$

The subsequent phosphorolysis of the acyl enzyme requires the presence of bound NAD^+ at the acyl site (Seydoux & Bernhard, 1974; Malhotra & Bernhard, 1973, 1981):

$$\text{acyl-GPDH–NAD}^+ + P_i \rightarrow \text{acyl-P} + \text{GPDH–NAD}^+ \quad (4)$$

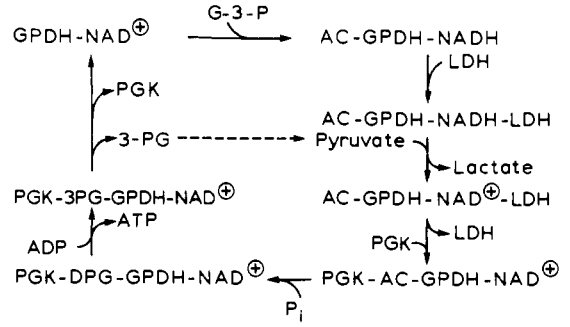
Hence, NAD^+ must replace NADH at any acyl site in order to phosphorolyze the acyl enzyme, a necessary step in the subsequent phosphoglycerate kinase catalyzed generation of ATP:

$$\text{acyl-P} + \text{ADP} \rightarrow \text{3-PG} + \text{ATP} \quad (5)$$

It is particularly interesting to note that apo-PGK is not a substantial inhibitor of the direct transfer of DPG from PGK–DPG to GPDH–NAD^+ (Weber & Bernhard, 1982) even though saturation in the rate of DPG transfer from DPG is demonstrable. Thus, it would appear possible that a coordinated synthesis of one molecule of ATP is coupled sequentially via GPDH to the reduction of one molecule of pyruvate to lactate (Scheme I).

Note that in Scheme I, we present no detailed mechanism for the transfer of metabolite, other than that metabolite transfer occurs within a cognate enzyme–enzyme complex. We are at present studying the detailed mechanism in the case of DPG transfer and shall report on these findings shortly. Elsewhere, we are ignorant of the detailed mechanism of

Scheme I



metabolite or coenzyme transfer. Scheme I is based on the experimental results we present in this paper and elsewhere (Srivastava & Bernhard, 1984; Weber & Bernhard, 1982). Suggestive evidence has been presented by others that particular glycolytic systems form functional aggregates (Ovadi et al., 1978; Strapazon & Steck, 1976; Yu & Steck, 1975; MacGregor et al., 1980). We have not considered higher order enzyme aggregates in Scheme I, but only the binary enzyme–enzyme interactions which we have detected.

The saturation effect of E_1–NADH concentration on the rate of the E_2-catalyzed reaction are suggestive of the formation of substantial E_1–NADH–E_2 complex during the course of catalysis, a speculation not inconsistent with the above-mentioned suggestions for enzyme–enzyme interactions. However, as we have previously noted (Srivastava & Bernhard, 1984), the saturation phenomenon is not a sufficient argument for the demonstration of substantial complex formation. Any kinetic circumstance which leads to a mirror-stoichiometric buildup of E_2–NADH–S_2 complex during steady-state turnover will lead to such a saturation phenomenon. Nevertheless, we are investigating the possibility of substantial complex formation via physical techniques designed to detect specific protein–protein aggregations. We shall report on such experiments subsequently.

The results we present herein, as well as our earlier results with the GPDH–PGK system (Weber & Bernhard, 1982), all show a greater degree of organization than might have been previously anticipated on the basis of the in vitro activities of the individual soluble glycolytic enzymes. One consequence of the direct transfer mechanism is the irrelevance of the aqueous solvation of metabolites to the energetics of the metabolic pathway. These changes in solvation often result in large changes in free energy as compared with the free-energy change accompanying chemical reaction at the enzyme site. As becomes ever more evident, equilibrium constants for reactions at the enzyme surface (under conditions of substrate and product saturation) are near unity under near-physiological conditions (Nageshwara RaO et al., 1978, 1979; Huskins et al., 1982; Albery & Knowles, 1976, 1977; Wilkinson & Rose, 1979; Nambiar et al., 1983). The transfers we observe (when we observe them) are comparably facile in both directions. Hence, the distribution of NADH-bound sites must be near unity in the cognate enzyme–NADH–enzyme complex. It has recently been postulated that the redox potentials of NADH in the syn vs. the anti conformation are intrinsically different (Benner, 1982; Nambiar et al., 1983). On the basis of the aqueous solution equilibria, Benner has argued that a substantial fraction of the free-energy compensation, which leads to internal equilibrium constants near unity for the redox reaction, is contributed by the syn vs. anti specificity of binding rather than by compensatory protein–coenzyme complementary interactions. An examination of the dissociation constants for enzyme–NADH complex (Table I) reveals that all of these

dissociation constants are essentially the same to within ±0.4 kcal/mol. Hence, the equilibrium constant of unity for the transfer of NADH between cognate sites in the E_1–E_2 complex is not unanticipated. If, as has been argued by Benner, the role of A vs. B dehydrogenases is to adjust the redox potential so as to maintain the bound internal equilibrium between reactants and products near unity (Benner, 1982; Nambiar et al., 1983), our results suggest that a major part of the free-energy compensation is already provided in the binary E–NADH complexes. The hypothesis that A and B dehydrogenases differ in the range of compensation for the external aqueous redox potential of the reactants has recently been challenged by Oppenheimer (1984).

In any event, the correlation of direct transfer with A–B pairs vs. random transfer through the solvent with A–A or B–B pairs appears clear. At this time, we are unable to propose a convincing teleological argument for the phenomenon, either according to the arguments of Benner (S. A. Benner, personal communication) or by considerations of sequences of metabolic reductions and oxidations involving NAD^+-dependent enzymes. We do note that the NADH synthesized in the initial oxidation of G-3-P is not utilizable for the biosynthesis of phospholipids until substantial glycolysis has taken place so as to generate the cognate LDH–NADH complex. Our own ignorance of the specific sequences of hydrogenations and dehydrogenations elsewhere in metabolism utilizing nicotinamide coenzyme limits our speculation as to the physiological relevance of our findings.

Conclusion

NADH is transferred from one dehydrogenase binding site to another via an E_1–NADH–E_2 complex, when, and only when, the chirality of stereospecific C_4 H transfer is opposite in the two dehydrogenases. Glycolytic metabolism is not the exclusive consequence of a mixture of independently acting soluble enzymes and substrates; some of the properties of glycolytic systems cannot be derived from a detailed knowledge of the kinetics and thermodynamics of the individual enzyme-catalyzed processes.

Registry No. NADH, 58-68-4; GPDH, 9001-50-7; LDH, 9001-60-9; α-GDH, 9075-65-4; LADH, 9031-72-5; dehydrogenase, 9035-82-9; malate dehydrogenase, 9001-64-3; alanine dehydrogenase, 9029-06-5; sorbitol dehydrogenase, 9028-21-1; glutathione reductase, 9001-48-3.

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