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Mechanism of Transfer of Reduced Nicotinamide Adenine Dinucleotide among Dehydrogenases. Transfer Rates and Equilibria with Enzyme–Enzyme Complexes†

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ABSTRACT: The direct transfer of NADH between A–B pairs of dehydrogenases and also the dissociation of NADH from individual E–NADH complexes have been investigated by transient stopped-flow kinetic techniques. Such A–B transfers of NADH occur without the intermediate dissociation of coenzyme into the aqueous solvent environment [Srivastava, D. K., & Bernhard, S. A. (1985) *Biochemistry 24*, 623–628]. The equilibrium distributions of limiting NADH among aqueous solvent and A and B dehydrogenase sites have also been determined. At sufficiently high but realizable concentrations of dehydrogenases, both the transfer rate and the equilibrium distribution of bound NADH are virtually independent of the excessive enzyme concentrations; at excessive E_2 concentration, substantial NADH is bound to the E_1 site. These results further substantiate earlier kinetic arguments for the preferential formation of an E_A–NADH–E_B complex, within which coenzyme is directly transferred between sites. The unimolecular specific rates of coenzyme transfer from site to site are nearly invariant among different A–B dehydrogenase pairs. The equilibrium constants for the distribution of coenzyme within the E_A·E_B complexes are near unity. At high $[E_2]$ and for $[E_2] > [E_1] > [NADH]$, E_1–NADH·E_2 and E_1·NADH–E_2 are virtually the only coenzyme-contained species. In contrast to the nearly invariant unimolecular NADH transfer rates within E_A·E_B complexes, unimolecular specific rates of dissociation of NADH from E–NADH into aqueous solution are highly variable. The specific rate of coenzyme transfer within the E_A·E_B complex can be either substantially greater than or substantially less than the rate of coenzyme dissociation from E_1–NADH into the aqueous environment. Thus, enzyme–enzyme interactions within the A–B pairs modulate the rate of coenzyme transfer.

In previous communications from this laboratory, we have shown that the utilization of NADH, in the form of the E_1–NADH complex, can proceed at velocities very much greater than that predicted from the known dissociation constant of E_1–NADH and the Michaelian parameters for the E_2-catalyzed hydrogenation of S_2 by the available aqueous NADH (Srivastava & Bernhard, 1984, 1985) (eq 1). This

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

enhanced velocity via direct transfer is operative whenever the

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two dehydrogenases catalyze reactions with opposite chiral specificity for the C_4 hydrogen of NADH (Srivastava & Bernhard, 1985). Molecular graphic analyses of the juxtaposed pair of dehydrogenases of known structure and opposing chiral specificities provide strong suggestive evidence as to the direct transfer mechanism (Srivastava et al., 1985).

In this paper, we examine the transfer of NADH more directly by transient kinetic studies. We measure the rate of dissociation of coenzyme (NADH) from the enzyme site (E_1) into the aqueous medium. Does this E_1–NADH dissociation limit the rate of transfer of NADH between E_1 and E_2?

In order to obtain meaningful information from such experiments, it is necessary that both the coenzyme dissociation rate and the transient rate of transfer be resolvable on the "stopped-flow" kinetic time scale. As shown, this is the case for a variety of dehydrogenases which we have already shown to follow the direct transfer pathway in steady-state kinetic studies (Srivastava & Bernhard, 1985, 1986a; Bernhard & Srivastava, 1986). The results presented herein further substantiate our conclusions regarding direct transfer of NADH among dehydrogenases of opposite chiral specificity (Srivastava & Bernhard, 1985; Srivastava et al., 1985).

In every dehydrogenase of known structure, two protein conformational alternatives are invariably demonstrable (Branden & Eklund, 1980; Grau, 1982; Janin & Wodak, 1983). These protein conformations are the predominant structures observed for the crystalline "apo"- and "holo"enzyme species (White et al., 1976; Murthy et al., 1980; Eklund et al., 1981). Coenzymes, bound in the holoenzyme conformation, are not accessible to the outside solvent environment, whereas the coenzyme bound in the apoenzyme conformation is freely accessible to solvent environment.

In this paper, we interpret the transient transfer data structurally in terms of the apo–holo conformational transition. A major finding presented herein is that the rate of transfer of NADH within an $E_A \cdot E_B$ complex is constant, independent of the specific function of either E_A or E_B. This constancy is in contrast to the variable rates of coenzyme dissociation from individual enzymes, indicating a modulation of the coenzyme transfer rate by specific enzyme–enzyme interaction.

Materials and Methods

Materials. Sodium salts of NAD^+ (grade III), NADH (grade III), pyruvic acid, acetoacetylcoenzyme A, and ethylenediaminetetraacetic acid (EDTA),[1] the lithium salt of dihydroxyacetone phosphate, 2-mercaptoethanol, and *cis*-oxaloacetic acid (grade I) were obtained from Sigma Chemical Co. and were used without further purification. Reagent-grade chemicals and glass-distilled water were used to prepare all buffers and solutions.

Tris-HCl Buffer. The pH of 50 mM Trizma base was adjusted to pH 7.5 with dilute HCl. The solvent contained 0.1 mM EDTA and 0.35 mM 2-mercaptoethanol. This buffered solvent was used for all experiments, unless otherwise stated.

Enzymes. Halibut muscle enzymes, glyceraldehyde-3-phosphate dehydrogenase (GPDH), and lactate dehydrogenase (LDH) were purified and assayed as described earlier (Srivastava & Bernhard, 1984). Specific activities of halibut muscle GPDH and halibut muscle LDH were 400 and 1000 units/mg, respectively, in our standard assay conditions (Srivastava & Bernhard, 1984). These specific activities are higher than any previously reported values for these enzymes from other sources. "Scatchard plots" (Scatchard, 1949) for the titration of each of the enzyme sites with NADH gave a stoichiometry of 1.0 NADH per enzyme subunit and adhered strictly to the linearity uniquely predicted on the basis of noninteracting homogeneous enzyme sites.

Rabbit muscle α-glycerolphosphate dehydrogenase (αGDH) (type I), bovine heart lactate dehydrogenase (type III), and porcine heart cytoplasmic malate dehydrogenase (MDH) were obtained from Sigma Chemical Co. Pig heart lactate dehydrogenase was from Boehringer Mannheim. Specific activities of αGDH, bovine heart LDH, and pig heart LDH were 104, 206, and 280 units/mg, respectively, in our standard 50 mM Tris-HCl buffer, pH 7.5 at 25 °C. The highest specific activities for these enzymes reported under more or less identical conditions are as follows: αGDH, 116.2 units/mg (Fondy et al., 1968); bovine heart LDH, 318 units/mg (Pesce et al., 1967); and pig heart LDH, 278 units/mg (Eventoff et al., 1974). Unlike our halibut muscle LDH preparation, the commercially prepared LDH's show a decrease of about 20% in the number of NADH binding sites per subunit by Scatchard plot analyses (Scatchard, 1949). A crystalline suspension of pig heart 3-hydroxybutyryl-CoA dehydrogenase (HBDH) was received as a gift from Dr. L. Banaszak.

Methods. All measurements were carried out at 25 °C in the standard Tris-HCl buffer. Enzymes utilized in the following experiments were extensively dialyzed against this buffer and concentrated by utilizing an Amicon ultrafiltration apparatus.

(*A*) *Fluorescence intensity measurements* were carried out as described in earlier communications (Srivastava & Bernhard, 1984, 1985), when only a single dehydrogenase was preincubated with NADH.

(*B*) *Enzyme–NADH Fluorescence Spectral Measurements.* Fluorescence spectra of individual E–NADH were all determined at an excess of enzyme sites over NADH. Excitation spectra were determined from the intensity of fluorescence emission at 460 nm (emission slit width 7.0 mm, excitation slit width 3.5 mm). Emission spectra were determined by excitation at either 340 or 360 nm (emission slit width 7.0 mm, excitation slit width 3.5 mm). NADH concentration was invariably 8.6 μM.

In experiments involving the distribution of NADH among LDH, αGDH, and the solvent, both excitation and emission fluorescence techniques were utilized. The concentration of αGDH was invariably fixed at 12.92 μN (in sites), and total [NADH] was 8.6 μM. LDH concentration was varied between 0 and 64.2 μN. The spectra of NADH (8.6 μM) and of LDH–NADH ([LDH] = 12.8 μN, [NADH] = 8.6 μM) were determined as reference controls.

(*C*) *Rapid transient kinetic studies* were carried out with a Durrum D-110 single-beam stopped-flow spectrophotometer, equipped for both adsorption and fluorescence emission optical detection. In the fluorescence detection mode, the photomultiplier tube was aligned at 90° angle with respect to the incident beam. A high-energy xenon source was used for excitation. The photomultiplier tube output voltage during the rapid reaction course was accessed to an on-line Varian 620i computer. The instrument has a dead time of about 3–5 ms. The data collection was started even before the dead time of the instrument.

For NADH dissociation rate studies, 0.2 mL of enzyme-bound NADH ([E] > [NADH] > $K_d^{E\text{-NADH}}$) was rapidly

[1] Abbreviations: GPDH, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; αGDH, α-glycerolphosphate dehydrogenase; HBDH, 3-hydroxybutyryl-CoA dehydrogenase; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

Table I: Summary of the Equilibrium Dissociation Constant and the Specific "Off-Rate" Constant of E–NADH Complexes[a]

enzyme	$K_d^{E\text{-}NADH}$ (μM)	$k_{off}^{E\text{-}NADH}$ (s^{-1})
halibut muscle GPDH	0.35 ± 0.05	>350
rabbit muscle αGDH	0.88 ± 0.12	9.4 ± 2.8
halibut muscle LDH	0.21 ± 0.04	~245
bovine heart LDH	0.29 ± 0.05	120 ± 20
pig heart LDH	0.35 ± 0.05	40.8 ± 8.6
horse liver ADH	1.15 ± 0.22	9.9 ± 2.3
pig heart S-MDH	~0.45	>300
pig heart HBDH		13.2 ± 3.4

[a] Determined in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 0.35 mM 2-mercaptoethanol. k_d and k_{off} for LADH–NADH were determined in 50 mM phosphate buffer, pH 7.5.

mixed with an equal volume of excessive NAD^+ (15–20 mM). The decrease in fluorescence emission intensity with time was monitored at all wavelengths above 408 nm by utilizing a 408-nm cutoff filter.

(*D*) *NADH transfer* between enzymes was carried out as follows: 0.2 mL of E_1–NADH ([E_1] > [NADH]) was rapidly mixed with an equal volume of excessive E_2 in the stopped-flow apparatus. Fluorescence excitation was at 360 nm (340 nm when GPDH was present). Changes in fluorescence emission were followed at wavelengths greater than 408 nm.

(*E*) *Data Analysis.* The fractional ligation of limiting NADH to mixtures of αGDH and LDH was calculated from ΔF at 460 nm (emission spectra) or at 370 nm (excitation spectra). The total change in fluorescence for the complete transfer of NADH from αGDH to LDH was determined by fluorescence measurement of the two individual enzyme–NADH spectra at identical limiting NADH concentrations. In all experiments, the high concentration of enzyme precluded an appreciable concentration of aqueous NADH.

(*F*) *Transient Kinetic Analysis.* Transient rate constants were all calculated by assuming a single exponential decay rate law (see Results). All kinetic data reported herein adhered strictly to a first-order decay rate law as judged both by graphic analyses of the entire progress curve after the dead time of the instrument and by estimation of the first-order rate constant following 3–4 half-life decays.

Results

The rate constants for dissociation of NADH from a variety of dehydrogenases, as well as the equilibrium dissociation constants for the same enzyme–NADH complexes, have been reported on extensively (Branden et al., 1975; Holbrook et al., 1975; Harris & Waters, 1976; Keleti et al., 1977; Whitaker et al., 1974; del Rosario & Hammes, 1971). Nevertheless, since these rates and equilibria are dependent both on the solvent environment and on the particular species from which a dehydrogenase has been derived, we have undertaken a new study of transient dissociation rates, as well as E–NADH dissociation equilibria. Our study deals with enzyme species and solvents for which we have already verified the direct transfer mechanism; these enzymes can transfer NADH directly to others according to the A–B transfer rule (Srivastava & Bernhard, 1985). Values for the equilibrium dissociation constant (K_d) and for the specific "off-rate" constants (k_{off}) for E–NADH are contained in Table I. The kinetic data for NADH dissociation adhered strictly to a first-order decay rate law (see Materials and Methods). The strict first-order dependence precludes the possibility of heterogeneity in enzyme sites for any of these enzymes as regards NADH binding. The calculated off-rate constant (k_{off}) in no case varied with variation in the range of NAD^+ concentration utilized for NADH displacement. From k_{off} and K_d values, we can straightforwardly calculate the highly variable second-order "on rates" (k_{on}). These second-order rate constants range between an order of magnitude lower than, to 2 orders of magnitude higher than, the usually assumed aqueous diffusion limit of 1×10^8 M^{-1} s^{-1} (Hammes, 1982). These, perhaps surprising, results are derivable in instances where the determination of k_{off} is not limited by instrument resolution (e.g., halibut muscle LDH and αGDH, as well as in the case of GPDH where only a minimal k_{off} can be estimated). The fast on rates are not unique to our dehydrogenase NADH studies. Similarly, high bimolecular diffusion rates have been reported for the interaction of oppositely charged small or multiple-charged molecules (Eigen & DeMaeyer, 1963).

Two features are obvious from a comparison of K_d and k_{off} among the various dehydrogenases. (1) The K_d values for E–NADH are nearly invariant (K_d varies by less than a factor of 2 among the group). On the contrary, (2) rather large differences exist among the various k_{off} values. The range of determinable k_{off} values is limited by our inability to resolve some of the faster off rates with our stopped-flow apparatus. The range of variation of k_{off} spans at least 2 orders of magnitude among this group of dehydrogenases.

In some instances, the unimolecular k_{off} values are substantially smaller than the steady-state catalytic turnover numbers for E–NADH and specific substrate. This is not necessarily surprising, since the avidity of binding of coenzyme product (NAD^+) is usually far weaker than that for NADH [for reviews, see Branden et al. (1975), Holbrook et al. (1975), and Banaszak & Bradshaw (1975)]. The slow E–NADH off rates do pose a problem when the direct transfer mechanism is operative, i.e., when E^1–NADH is a competent substrate for the E_2-catalyzed reaction. For example, we have previously shown that α-glycerolphosphate dehydrogenase (αGDH)–NADH complex is itself a substrate for the reduction of pyruvate catalyzed by lactate dehydrogenase (LDH) (Srivastava & Bernhard, 1985; Bernhard & Srivastava, 1986). From Michaelian kinetic studies, we have established that k_{cat} for the LDH-catalyzed hydrogenation of pyruvate by αGDH–NADH is approximately 50 s^{-1}, a value more than 5-fold greater than the measured specific off rate for αGDH–NADH into aqueous solution (see Table I).

In an attempt to solve this seeming paradox, we have carried out stopped-flow transient experiments involving the transfer of NADH from αGDH to LDH. The rate of transfer of NADH from αGDH to an excess of LDH was measured under conditions such that almost all the NADH is αGDH bound initially, and αGDH or LDH bound at equilibrium. This transient coenzyme exchange experiment can be readily tracked due to the difference in the fluorescence emission spectrum of NADH when bound to αGDH vs. LDH (Figure 1). For comparison, we include in Figure 1 the transient kinetic data for the dissociation of αGDH–NADH into aqueous solution, as monitored by the decrease in αGDH–NADH fluorescence emission accompanying the displacement of NADH by NAD^+. Note the greater than 1 order of magnitude disparity in the time regimes for the two experiments.

The faster transient rate of transfer of NADH between E_1 and E_2 is straightforward evidence for the direct transfer mechanism. Thus, the disparity noted between k_{off} for αGDH–NADH and k_{trans} for coenzyme transfer from αGDH to LDH must be a consequence of the effect of the LDH interaction on the process of desorption of NADH from the αGDH site.

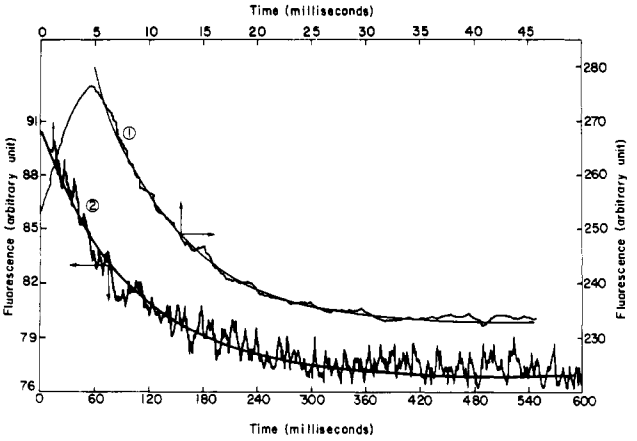

FIGURE 1: Transient rates of NADH transfer. The rate of transfer of NADH from αGDH–NADH to halibut muscle LDH (curve 1) is compared with the NADH dissociation rate from αGDH into aqueous solution (curve 2). Theoretical (smooth) curves are the single-exponential fit to the experimental data. Configuration and concentrations of reactants are shown for each curve. Curve 1: αGDH (24.56 μN), NADH (16.24 μM), + LDH (50.18 μN); curve 2: αGDH (12.32 μN), NADH (11.4 μM), + NAD^+ (20 mM).

The differences between absorption and fluorescence emission spectra of NADH, when bound to LDH vs. αGDH, are not unique to this A–B pair (Figure 2). Qualitatively, the same sorts of spectral changes occur when spectra of NADH bound to any "B" vs. "A" dehydrogenases are compared (Fisher et al., 1969; Velick, 1958). Via these spectral differences, we have been able to monitor the transient rate of transfer of NADH between a variety of A–B pairs of dehydrogenases under concentration conditions similar to those described above. In all cases, the progress of the transfer follows a first-order kinetic rate law. A summary of the derived transient first-order rate constants (k_{trans}) is listed in Table II.

Note the similarity in magnitudes for all of the transfer rate constants. These similarities are often in contrast to the substantial disparity in k_{off} for the dissociation of E–NADH into aqueous solvent (Table I). These latter constants (k_{off}) are sometimes larger, and sometimes smaller, than the k_{trans} (compare Tables I and II). These results indicate a strong modulating influence of enzyme–enzyme interactions on the transfer rates.

In some cases cited in Table II, we have determined k_{trans} at varying excessive concentrations of E_2 (the acceptor enzyme which is always present in excess over E_1) (Table III). Note in all of these cases the invariance of the experimentally derived first-order rate constant to the concentration of excessive E_2. Thus, the rate constant derived from the experimental data appears to be a true unimolecular rate constant: it must involve transfer of coenzyme within an enzyme–enzyme complex. The similar magnitudes in total amplitudes of fluorescence change starting with E_A–NADH and E_B or with E_B–NADH and E_A indicate similar rates in both directions. For example, the total fluorescence changes due to NADH transfer from halibut to

Table II: Summary of the Transfer of NADH (k_{trans}) between Pairs of Dehydrogenases of Opposite Chiral Specificity[a]

E_1	E_2	k_{trans} (s^{-1})
αGDH[b]	(H.M.) LDH	142 ± 21
(H.M.) LDH	αGDH	172 ± 22
(H.M.) LDH	GPDH	180 ± 25
GPDH	(H.M.) LDH	232 ± 28
(P.H.) LDH	GPDH	140 ± 18
GPDH	(P.H.) LDH	220 ± 21

[a] In 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 0.35 mM 2-mercaptoethanol. [b] αGDH and GPDH are invariably from rabbit muscle and halibut muscle, respectively. LDH's are either from halibut muscle (H.M.) or from pig heart (P.H.).

Table III: Transient Rate Constants for the Transfer of NADH (k_{trans}) at Different E_2 Concentrations[a]

$[E_1]$ (μN/site)	[NADH] (μM)	$[E_2]$ (μN/site)	k_{trans} (s^{-1})
αGDH		(H.M.) LDH	
24.6	16.2	50.2	132.5
24.6	16.2	65.4	153.4
24.6	16.2	75.3	146.0
24.6	16.2	100.4	136.2
(H.M.) LDH		αGDH	
23.4	16.2	49.1	196.6
23.4	16.2	73.7	168.4
23.4	16.2	85.9	169.4
(H.M.) LDH		GPDH	
16.8	11.7	27.7	176.4
16.8	11.7	55.5	193.1
(P.H.) LDH		GPDH	
19.9	13.1	49.4	153.4
19.9	13.1	74.1	138.7
GPDH		(P.H.) LDH	
19.0	13.1	30.0	241.5
19.0	13.1	60.0	221.6

[a] Under conditions and specifications similar to those of Table II.

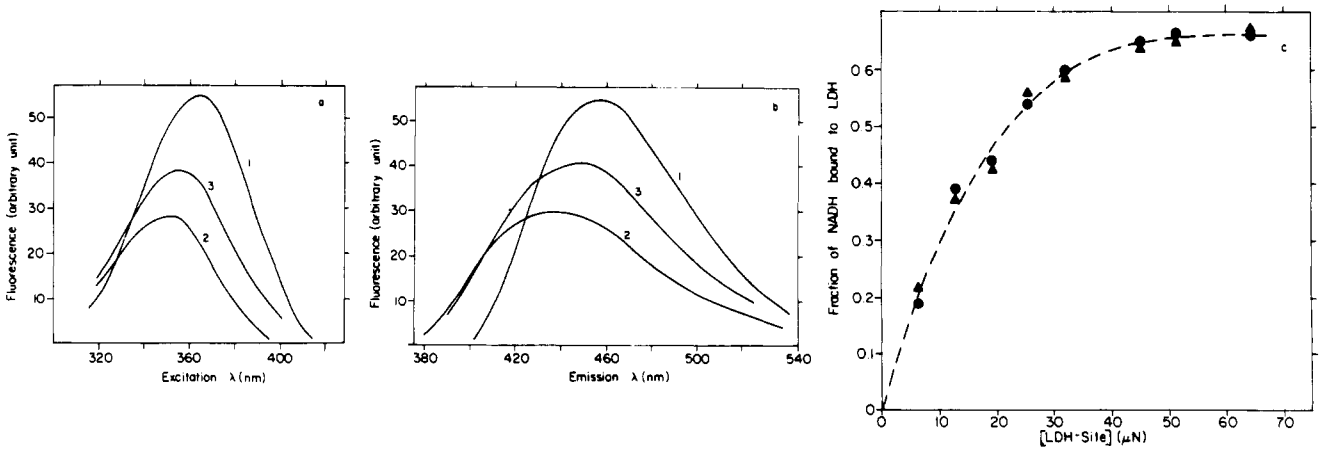

FIGURE 2: Interaction of NADH with αGDH, LDH, and a mixture of the two enzymes. Fluorescence excitation (a) and emission (b) spectra of NADH (8.6 μM) in an excess of (1) αGDH (12.92 μN), (2) LDH (12.84 μN), and (3) an enzyme mixture containing 12.92 μN αGDH and 38.52 μN LDH. Panel c shows the extent of ligation of limiting NADH to LDH [from excitation spectra (▲) and from emission spectra (●)] as a function of the LDH concentrations at constant αGDH concentration. Details are given under Materials and Methods.

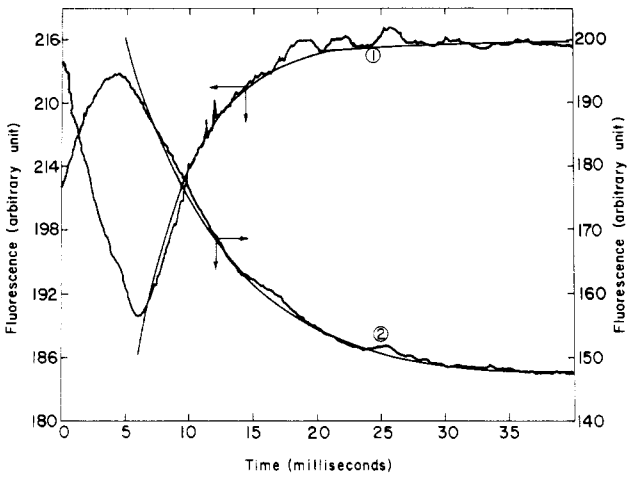

FIGURE 3: Transient kinetic progress curve for the transfer of NADH from (1) halibut GPDH to pig heart LDH and from (2) pig heart LDH to halibut GPDH. Concentrations of GPDH and LDH were 19.0 and 60 μN for curve 1 and 54.2 and 19.9 μN for curve 2, respectively. [NADH] was invariably 13.1 μM. Theoretical (smooth) curves are the single-exponential fit to the experimental data. The total changes in fluorescence calculated by extrapolation of single exponential plots to zero time are 112.62 and 103.00 for curves 1 and 2, respectively.

pig heart LDH are similar to the transfer of NADH from pig heart LDH to halibut GPDH (Figure 3).

The above-noted kinetic transfer experiments led us to examine some interesting equilibrium properties regarding the distribution of coenzyme among enzyme sites. Because of the spectral differences between coenzyme ligated to A vs. B dehydrogenases, these equilibrium distributions are sometimes readily measurable. The equilibrium distribution of NADH between αGDH and LDH was studied as a function of increasing LDH concentration (Figure 2); [LDH] > [αGDH] > [NADH]. Note the saturation of the change in NADH fluorescence on excessive LDH concentration. This result would be readily explainable if all of the NADH were bound to LDH sites at fluorescence saturation. This explanation seems inappropriate since the observed spectrum at saturating [LDH] is the NADH emission spectrum anticipated for a nearly equal mixture of αGDH–NADH and LDH–NADH, if the change in fluorescence emission accompanying transfer of NADH within the complex is the same as the change accompanying NADH transfer between the individual enzymes. On the basis of the affinities of the individual enzymes for NADH (Table I), and assuming only individual E–NADH species, approximately 5% of the limiting NADH would be anticipated to be bound to the αGDH site at the extreme molar excess of LDH over αGDH. These results constitute definitive evidence for the formation of a stable E_1–NADH–E_2 complex (see Discussion).

We should note that many more experiments (of the type described in the determination of k_{trans}) were carried out than are reported herein. The unreported experiments are a consequence of the often erratic formation of aggregates and precipitates upon mixing relatively concentrated solutions of the two cognate enzymes. It is of interest to note that these same dehydrogenases in comparable or higher concentrations do not precipitate in the complex cytosolic milieu. Moreover, the individual enzymes are soluble in the solvent we utilize at far higher concentrations. Such aggregation has prevented our reporting on the equilibrium titration properties of E_a–NADH with E_b other than the case of αGDH and LDH. Such complications are not a factor in the steady-state kinetic experiments we have reported on previously, where comparably high concentrations of one of the enzymes (E_1) are utilized together with minute concentrations of E_2 (Srivastava & Bernhard, 1984, 1985).

Discussion

The high concentrations of protein in the cytoplasm, coupled with the generally lesser concentrations of substrates and coenzymes, ensure that the direct transfer of coenzyme via an E_1·E_2 complex will be the preferred pathway in vivo, provided E_1 and E_2 are dehydrogenases of opposite conformational specificity (A vs. B) for NADH (Srivastava & Bernhard, 1986a; Bernhard & Srivastava, 1986). The results presented herein further demonstrate the formation of NADH-dependent E_A·E_B complexes. Two types of evidence are crucial toward this conclusion: (1) the invariance of k_{trans} to E_2 concentrations (Table III) and (2) the invariance of the distribution of NADH between E_A and E_B sites at varying excessive concentrations of E_A (in particular, when E_A is LDH and E_B is αGDH) (Figure 2). The equilibrium titration of αGDH–NADH with an excess of LDH leads to an estimated distribution of 65% of the NADH ligated to LDH at LDH saturation. This calculation is based on the assumption that the spectra of NADH when bound to αGDH and to LDH in the E_A·E_B complex are the same as in the individual E–NADH complexes. At present, we have no evidence to substantiate or negate this assumption. The conclusions based on kinetics presented herein are not dependent on the quantitative details of this assumption. Quantitative conclusions regarding the equilibrium distribution of NADH between the two sites of the complex are, however, dependent on the assumed amplitude of the difference in fluorescence emission of NADH accompanying the transfer within the complex. The arguments that the amplitudes of signal changes accompanying NADH transfer within the complex are similar in the forward and reverse directions, and follow the same unimolecular rate law, rule out the possibility that NADH is localized to predominantly one site within the E_1·E_2 complex at equilibrium.

The distribution of NADH among water, LDH, and αGDH does not appear to depend on the molar excess of LDH used in the titration, indicating that within the range of LDH concentrations used NADH is more tightly bound to the LDH·αGDH complex than to either of the individual enzymes. Otherwise, the proportion of LDH-bound NADH would continue to increase with increasing [LDH] until all NADH was LDH bound. The saturation of NADH distribution between αGDH and LDH at excessive concentrations of LDH ensures the formation of the αGDH–NADH–LDH complex.

The magnitudes of the rate constants for dissociation of the individual E–NADH into water are substantially variable, despite a near equality in the magnitudes of the equilibrium dissociation constants (Table I). Therefore, whatever factors slow the rate of coenzyme dissociation, they must act in a similar manner to decelerate the bimolecular rate of enzyme association with aqueous NADH. In the transfer of NADH within the E_A·E_B complex, the most notable feature of the first-order relaxation rate constant (k_{trans}) is its insensitivity to the magnitude of the rate constant for dissociation of E–NADH into aqueous solution. The magnitudes of k_{trans} are nearly equal for all direct transfer processes; regardless of the particular A–B pair, enzyme–enzyme interactions within the E_A–E_B complex modulate the rate of NADH transfer. Over the range of dehydrogenases reported in Table II, there is less than a factor of 2 variation in k_{trans}.

In a few instances, the rate of direct transfer of NADH from E_1 to E_2 exceeds the rate at which E–NADH dissociates into aqueous solution. This phenomenon is illustrated in Figure

1 for the case of NADH transfer from αGDH to LDH. In this particular instance, the specific relaxation rate constant for NADH transfer within the αGDH–LDH complex exceeds k_{off} by more than an order of magnitude. The experimentally derived relaxation rate constant (k_{trans}) is the sum of the two microscopic rate constants for forward and reverse NADH transfer between the two sites (eq 2). As discussed below,

$$\alpha\text{-GDH-NADH} \cdot \text{LDH} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \alpha\text{-GDH} \cdot \text{NADH-LDH} \qquad (2)$$

$$k_{trans} = k_f + k_r$$

these two microscopic rate parameters must be nearly equal. Thus, there is a nearly 8-fold increase in the rate of dissociation of NADH from αGDH within the complex as compared with the rate of dissociation of NADH from αGDH into aqueous solution. Such phenomenology provides further evidence for the direct transfer process. In other instances, the magnitude of k_{off} can substantially exceed that for k_{trans}. For example, the k_{off} for GPDH–NADH is too large to be determined by our stopped-flow transient techniques ($k_{off} > 350\ s^{-1}$). Nevertheless, k_{trans} for transfer between GPDH and LDH is readily determinable. Moreover, the k_{trans} so determined is the same regardless of the source of the LDH (halibut muscle or pig heart).

In this regard, it is interesting to note that the k_{trans} for the apparent reverse process (LDH–NADH + GPDH → LDH + GPDH–NADH) is, within experimental error, the same as that determined for the reverse transfer (see Table II and Figure 3). This relationship between forward and reverse transfer rate constants, as expected, suggests that the transfer occurs within the $E_A \cdot E_B$ complex exclusively. Under such conditions (i.e., at the rapid saturation of E_1–NADH with E_2), the relaxation time for the transfer process is given by eq 3.

$$\begin{array}{ccc} E_1\text{-NADH} + E_2 & \rightleftharpoons & E_1\text{-NADH} \cdot E_2 \\ & & k_f \downarrow \uparrow k_r \\ & & E_1 \cdot \text{NADH-}E_2 \rightleftharpoons E_1 + E_2\text{-NADH} \end{array} \qquad (3)$$

$$1/\tau = k_f + k_r,\ k_f = k_r$$

$$\therefore 1/\tau = 2k_f$$

The relaxation time for the reverse process is, of course, exactly the same. Indeed, wherever we have measured both the transfer of NADH from E_A to E_B and the reverse transfer from E_B to E_A, the derived first-order rate constants are identical, as illustrated in Table II. These results preclude the formation of any stoichiometrically substantial E–NADH binary complex despite the large excess of E_2 concentration over E_1 and NADH concentrations. In these kinetic experiments, and also in the equilibrium experiment involving αGDH and LDH, the molar excess of E_2 over E_1 has been as great as 5-fold. Thus, the affinity of $E_1 \cdot E_2$ for NADH must likewise be at least an order of magnitude greater than that for E_2; the inability to detect any change at high molar excesses of E_2 suggests that the affinity constant for NADH is much more than an order of magnitude greater in the ternary (E_1–NADH–E_2) complex than in the binary (E_1–NADH or E_2–NADH) complex. The apparent increase in affinity of the $E_1 \cdot E_2$ complex for NADH must simply reflect the stabilization resulting from E_2 binding to E_1–NADH.

It is interesting to note the identical equilibrium dissociation constants for the three species of LDH–NADH complex into the aqueous environment (Table I). The invariance of these equilibrium constants coupled with the invariance of k_{trans} for the relaxation of the distribution of NADH within the $E_A \cdot E_B$ complex argues strongly in favor of a very similar environment for NADH within the LDH binding site regardless of the enzyme species. What must be variable among the three enzyme species are the transient mechanistic details by which coenzyme leaves and enters the site to and from the aqueous environment. Whatever the differences are in transient mechanistic details among these three enzyme species, they appear to be irrelevant to the mechanism of coenzyme transfer within the $E_A \cdot E_B$ complex.

No other class of proteins has been investigated as extensively as the dehydrogenases in regard to their three-dimensional structure and the influence of specific ligands on these structures (Janin & Wodak, 1983). In every dehydrogenase for which three-dimensional structures of the apo- and holoenzymes have been determined, two discernibly different protein conformations have been reported (Buehner et al., 1974; Moras et al., 1975; Murthy et al., 1980; Eklund et al., 1981, 1982; Grau et al., 1981; White et al., 1976; Cedergren-Zeppezauer et al., 1982). These conformations depend on the presence or absence of bound coenzyme in the protein crystals. There is no crystallographic evidence for the existence of an apo conformation in the presence of bound coenzyme, or for the holo conformation in the absence of bound coenzyme, suggesting that the protein conformation is virtually entirely ligand dependent. In the holoenzyme conformation, the coenzyme (NAD^+) is not accessible to the outside solvent environment. On the other hand, in the apo conformation, coenzyme in the external environment is accessible to those residues of the protein responsible for coenzyme binding in the holoprotein conformation (Grau, 1982; Janin & Wodak, 1983; Srivastava et al., 1985). On the basis of these crystallographic studies of wet dehydrogenase crystals, we postulate that only two protein conformations are available to dehydrogenases in solution. In order for bound coenzyme to exit the site, it is thus obligatory for the enzyme protein to undergo a holo → apo transition. Accepting for the ligand-induced two-state hypothesis, this coenzyme-bound apoprotein can have only transient significance in the overall process of transfer of coenzyme between the holoenzyme and the external solvent (eq 4).

$$E^{(holo)}\text{-NADH} \rightleftharpoons E^{(apo)}\text{-NADH} \rightleftharpoons E^{(apo)} + \text{NADH} \qquad (4)$$

We note, once again, the near equality of equilibrium dissociation constants for E–NADH in contrast to the diversity of magnitudes of k_{off} among the various dehydrogenases. If the intermediate, apo-E–NADH, has little stoichiometric significance, the stoichiometrically substantial intermediates are holo-E–NADH and apo-E (eq 4). The rate of exit, as well as the rate of entry, of coenzyme is determined by the relative instability of the transient species, apo-E–NADH. Hence, the common magnitude for K_d among dehydrogenases must reflect a common free-energy difference between holo-E–NADH and apo-E.

The results we present herein demonstrate that the affinity for NADH is much greater at E_A and E_B sites within the $E_A \cdot E_B$ complex than it is for coenzyme within the individual enzyme sites. Thus, even at substantially excessive concentrations of E_2 over E_1, there is no apparent tendency toward the exclusive formation of E_2–NADH complexes. Presumably, the interaction of holo-E_1–NADH with apo-E_2 provides further stabilization for the holo-E–NADH complex.

In the case of αGDH·LDH, equilibrium measurements demonstrate that NADH is distributed nearly equally between the A and B sites in the $E_A \cdot E_B$ complex. In the transient

kinetic experiments, the derived unimolecular rate constant for the transfer of NADH is the sum of the microscopic forward and reverse rate constants for coenzyme transfer (eq 2 and 3). Note that the experimental k_{trans} values exhibit only small variations with particular A–B enzyme pairs. Such near equality in k_{trans} indicates that at least one of the two rate parameters (k_f and k_r) is comparable in magnitude to k_{trans} (eq 2 and 3) in the αGDH·LDH system. Wherever data are available regarding transfer in both the forward and reverse directions, the fluorescence changes are always similar regardless of the particular cognate E_A·E_B pair (Figure 3). Hence, it follows that k_{trans} in every case must be the sum of k_f and k_r of comparable magnitudes. Thus, to within less than an order of magnitude, the intrinsic microscopic rate constants for the transfer of NADH within the E_A·E_B complex are all the same.

These distinctions between the variable k_{off} and the nearly constant k_f and k_r indicate a special biological selective factor for transfer of NADH among E_A·E_B pairs of dehydrogenases. A perusal of the various NAD^+-dependent dehydrogenases of known three-dimensional structure reveals no common underlying structural principle leading to these equal rate and equilibrium parameters among the various enzymes. Note that both charged residues and specific catalytic moieties in the near vicinity of the coenzyme binding regions are variable among these dehydrogenases. For example, different dehydrogenases contain active-site Zn^{2+} or –SH or positively charged R groups in the near vicinity of the nicotinamide residue (Branden & Eklund, 1980; Grau, 1982). Therefore, the equal transfer rates and equilibria must arise from a complexity of individual enzyme–coenzyme interactions and from common mechanisms by which cognate enzyme interactions influence these parameters (Srivastava & Bernhard, 1986b). Presumably, the equality in transfer parameters among the various cognate pairs arises via natural selection rather than via a unique change in interaction accompanying each coenzyme transfer between cognate enzymes. Hence, selection appears to be based on both the kinetic and thermodynamic equal facility for coenzyme transfer between the E_A and E_B sites of the E_A·E_B complex.

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