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Direct transfer of NADH between α-glycerol phosphate dehydrogenase and lactate dehydrogenase: Fact or misinterpretation?

(enzyme–enzyme interactions/direct transfer mechanism/coupled enzyme reactions/organization of metabolic pathways)

D. K. SRIVASTAVA*†, P. SMOLEN*‡, G. F. BETTS*§, T. FUKUSHIMA¶, H. O. SPIVEY¶, AND S. A. BERNHARD*‖

*Institute of Molecular Biology, University of Oregon, Eugene, OR 97403; and ¶Department of Biochemistry, Oklahoma State University, Stillwater, OK 74078

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ABSTRACT **Following the criticism by Chock and Gutfreund [Chock, P. B. & Gutfreund, H. (1988) *Proc. Natl. Acad. Sci. USA* 85, 8870–8874], that our proposal of direct transfer of NADH between glycerol-3-phosphate dehydrogenase (α-glycerol phosphate dehydrogenase, α-GDH; EC 1.1.1.8) and L-lactate dehydrogenase (LDH; EC 1.1.1.27) was based on a misinterpretation of the kinetic data, we have reinvestigated the transfer mechanism between this enzyme pair. By using the "enzyme buffering" steady-state kinetic technique [Srivastava, D. K. & Bernhard, S. A. (1984) *Biochemistry* 23, 4538–4545], we examined the mechanism (random diffusion vs. direct transfer) of transfer of NADH between rabbit muscle α-GDH and pig heart LDH. The steady-state data reveal that the LDH–NADH complex and the α-GDH–NADH complex can serve as substrate for the α-GDH-catalyzed reaction and the LDH-catalyzed reaction, respectively. This is consistent with the direct-transfer mechanism and inconsistent with a mechanism in which free NADH is the only competent substrate for either enzyme-catalyzed reaction. The discrepancy between this conclusion and that of Chock and Gutfreund comes from (*i*) their incorrect measurement of the K_m for NADH in the α-GDH-catalyzed reaction, (*ii*) inadequate design and range of the steady-state kinetic experiments, and (*iii*) their qualitative assessment of the prediction of the direct-transfer mechanism. Our transient kinetic measurements for the transfer of NADH from α-GDH to LDH and from LDH to α-GDH show that both are slower than predicted on the basis of free equilibration of NADH through the aqueous environment. The decrease in the rate of equilibration of NADH between α-GDH and LDH provides no support for the random-diffusion mechanism; rather, it suggests a direct interaction between enzymes that modulates the transfer rate of NADH. Thus, contrary to Chock and Gutfreund's conclusion, all our experimental data compel us to propose, once again, that NADH is transferred directly between the sites of α-GDH and LDH.**

In a series of communications over the past 6 years, we have reported that an enzyme-bound metabolite can often serve as a substrate for another enzyme-catalyzed reaction (1, 2). This view is in contrast to the widely held hypothesis that, except for intermediary steps in multienzyme complexes, the substrates and products of enzyme-catalyzed reactions are bound directly from and released directly into the aqueous solution (3). However, since the concentrations of enzymes often exceed the concentrations of their affine metabolites under physiological conditions, the universal application of a free-diffusion mechanism is not clear, and it is a justifiable subject for detailed experimental scrutiny (1, 2). Toward this end, we have distinguished the two extremes of mechanism of metabolite transfer between enzyme pairs that share a common metabolite (Eq. 1):

(*i*) Random diffusion

$$\text{E1–M} \rightleftharpoons \text{E1} + \text{M} + \text{E2} \rightleftharpoons \text{E2–M} \rightarrow \text{products}$$

(*ii*) Direct transfer

$$\text{E1–M} + \text{E2} \rightleftharpoons \text{E1–M–E2} \rightarrow \text{products.} \qquad [1]$$

In the random-diffusion mechanism, the enzyme-bound metabolite first dissociates into the aqueous solvent and then binds at the second enzyme site, whereas in the direct-transfer mechanism, the enzyme-bound metabolite itself serves as a substrate for the second enzyme-catalyzed reaction, through formation of an enzyme–metabolite–enzyme complex.

To probe the alternatives of Eq. 1, we have devised a method that we call the "enzyme buffering method" (4). In this method, we utilize an enzyme (E1), in substrate-like concentration, to buffer a common metabolite (M) such that the concentration of free (aqueous) metabolite is reduced significantly. Under this condition, the rate of a second enzyme (E2)-catalyzed reaction utilizing M can be quantitatively predicted presuming that free metabolite is the only substrate for the E2-catalyzed reaction. If the observed rate of the E2-catalyzed reaction agrees with that predicted by the equilibrium concentration of free M, the transfer mechanism is deduced to be of the random-diffusion type. Rates faster than this prediction are attributed to the direct-transfer mechanism (4).

Our main criterion for distinguishing the two transfer mechanisms of Eq. 1 has been the results of the steady-state experiments. Transient experiments were performed only with those enzyme pairs judged by enzyme-buffering experiments to transfer their metabolites directly. We have reported that the transfer rates between E1 and E2 pairs were either faster or slower than the specific "off" rates of E1–NADH complexes (5).

Among our several demonstrations of the direct-transfer mechanism between cognate enzyme pairs, Chock and Gut-

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Abbreviations: α-GHD, α-glycerol-3-phosphate dehydrogenase; LDH, L-lactate dehydrogenase; DHAP, dihydroxyacetone phosphate.

†To whom reprint requests should be sent at present address: Department of Biochemistry, North Dakota State University, Fargo, ND 58105.
‡Permanent address: The Institute of Theoretical Dynamics, University of California, Davis, CA 95616.
§On leave from: School of Biological Sciences, Queen Mary College, London, E14NS, England.
‖Deceased, July 24, 1988.

freund (6) have specifically challenged the validity of the mechanism for the transfer of NADH between glycerol-3-phosphate dehydrogenase (α-glycerol phosphate dehydrogenase, α-GDH; EC 1.1.1.8) and L-lactate dehydrogenase (LDH; EC 1.1.1.27). In their critique of our work they pointed out an error in our "off" rate measurement of NADH from the α-GDH–NADH complex. We accept that the off rate of NADH is faster than the 9.4 sec^{-1} previously reported (5), and we thank these authors for this clarification. A higher value for the off rate led Chock and Gutfreund to conclude that there is no direct transfer of NADH from α-GDH to LDH. To strengthen their contention, they performed both transient and steady-state kinetic experiments for the transfer of NADH between these enzymes and then claimed that their data are entirely consistent with the random-diffusion mechanism (6). These conclusions have caused us to reinvestigate the mechanism of NADH transfer between these enzymes.

This paper is a joint report from two laboratories that have independently measured relevant parameters needed to differentiate between the random-diffusion and the direct-transfer mechanisms. The data and analysis from these independent investigations are coincident and are different in crucial respects from those of Chock and Gutfreund. As such, they strongly support our previous contention (5, 7) that NADH is directly transferred between α-GDH and LDH.

MATERIALS AND METHODS

Materials. NAD^+ (grade III), the sodium salts of NADH (grade III), pyruvic acid, and EDTA, the lithium salt of dihydroxyacetone phosphate (DHAP), 2-mercaptoethanol, Trizma base, rabbit muscle α-GDH (type I and type X), and rabbit muscle LDH (type II) were obtained from Sigma. Pig heart LDH was obtained from Boehringer Mannheim. Reagent grade chemicals and glass-distilled water were used to prepare all buffers and solutions. Tris·HCl (50 mM) (pH of Trizma base adjusted with dilute HCl), pH 7.5/1 mM EDTA/1 mM 2-mercaptoethanol was used for all experiments, unless stated otherwise.

Methods. All kinetic and thermodynamic experiments were performed at 25°C in the standard Tris·HCl buffer, pH 7.5.

Steady-state kinetic experiments were carried out either on Varian Techtron model 635 or Hitachi 100-80 spectrophotometers or on Hitachi MPF-2A or Perkin–Elmer 650-40 spectrofluorometers (4, 7). Fluorescence titrations were carried out on the same spectrofluorometers (4, 7). One-centimeter-pathlength cuvettes were used for the absorption measurements, except for the K_m determination of NADH in the α-GDH-catalyzed reactions; in the latter experiments, 10-centimeter-pathlength cuvettes were used.

Transient kinetic experiments were performed on a Durrum D-110 single-beam stopped-flow spectrophotometer as described (5) except for two minor modifications. Data collection and analysis were carried out on an IBM XT computer using software developed in the laboratory of Michael F. Dunn at Riverside, CA. In the fluorescence mode, the emission intensity was monitored at all wavelengths above 423 nm, following excitation either at 340 nm or at 360 nm.

Kinetic (K_m and V_{max}) constants and equilibrium dissociation (K_d) constants were obtained from the data by nonlinear regression analyses. The simulated progress curves of NADH transfer between the two enzymes were obtained by numerical integration of the exact rate equation (2) using a program (known as PLOD) written by D. K. Kahner and D. D. Barnett to run on an IBM PC (XT) computer system.

RESULTS

Steady-State Kinetics. The pathways of NADH transfer between pig heart LDH and rabbit muscle α-GDH were investigated by the enzyme-buffering steady-state kinetic technique (4). The K_d values of rabbit muscle α-GDH–NADH and pig heart LDH–NADH were determined by utilizing the difference in fluorescence properties of free NADH vs. NADH bound to either of these enzymes (7). An average of these measurements (in the Oregon laboratory) gave K_d values of 0.8 μM for rabbit muscle α-GDH and 0.51 μM for pig heart LDH. These values are close to our previously reported values for these enzymes (0.88 μM for rabbit muscle α-GDH and 0.35 μM for pig heart LDH) (5). The K_d of LDH–NADH complex was independently checked in the Oklahoma laboratory, both in the absence and in the presence of 0.35 mM DHAP, and a value of 0.8 ± 0.12 μM was obtained.

In our previous work, we reported the steady-state kinetic parameters of several dehydrogenases including rabbit muscle α-GDH (4, 7). Since our K_m for NADH in α-GDH-catalyzed reaction has been challenged by Chock and Gutfreund (6), we redetermined it by two methods: (*i*) a spectrophotometric method utilizing a 10-cm-pathlength cuvette (Fig. 1*A*) and (*ii*) a spectrofluorometric method (Fig. 1*B*). These methods (in the Oregon laboratory) yielded K_m values of 2.7 μM and 2.1 μM, respectively. From our spectrophotometric data, we calculated a k_{cat} value of 57.7 sec^{-1} for the α-GDH-catalyzed reaction in the presence of a saturating concentration of DHAP (1 mM).

The K_m for NADH in the α-GDH-catalyzed reaction has been determined independently for the lyophilized enzyme

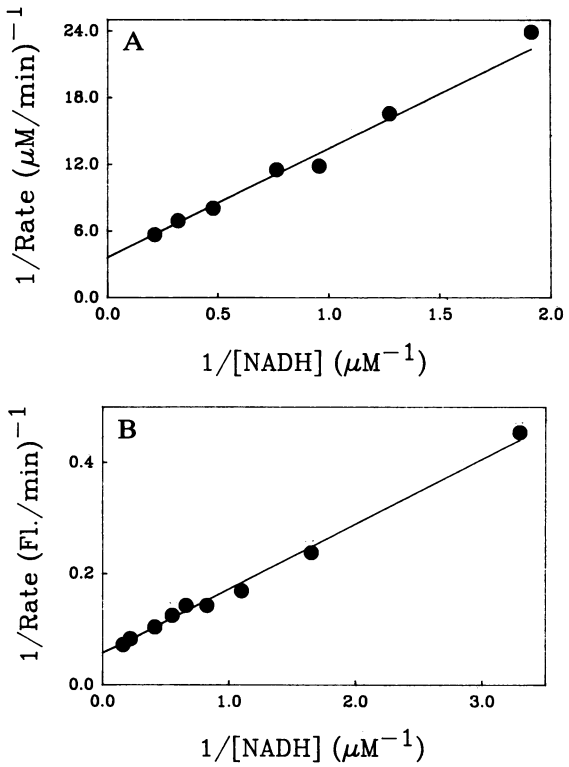

FIG. 1. Double-reciprocal plots of effect of NADH concentration on initial rate of α-GDH-catalyzed reduction of DHAP. (*A*) Results were obtained by measurement of absorption change per min at 340 nm in a 10-cm-pathlength cuvette. Total [α-GDH] = 0.08 nN site (N, mol of site per liter), total [DHAP] = 1 mM. (*B*) Fluorescence emission per min at excitation and emission wavelengths of 340 and 460 nm, respectively. Total [α-GDH] = 0.29 nN site, total [DHAP] = 1 mM. —, Calculated curves; ●, experimental points.

preparation (Sigma type X, used by Chock and Gutfreund) in the Oklahoma laboratory, and a value of 2.0 μM was obtained. These K_m values are in excellent agreement with that reported by us previously (7) but differ at least 5-fold from the K_m of 0.4 μM reported by Chock and Gutfreund (6).

Since we performed the steady-state enzyme-buffering experiments in both directions (see below), we have also determined the kinetic parameters of pig heart LDH-catalyzed reactions. The K_m for NADH is 7.1 ± 0.5 μM, and k_{cat} is 82.8 ± 2 sec^{-1} at a fixed concentration of pyruvate (2 mM). [This work is the first in which we have used pig heart LDH as a catalyst (E2); all our previous experiments with LDH as E2 have involved halibut muscle enzyme.]

Given the kinetic and thermodynamic parameters for these enzymes, we proceeded to examine the mechanisms of transfer of NADH between rabbit muscle α-GDH and pig heart LDH by utilizing the enzyme-buffering technique (4). The results are shown in Table 1. Note that the experimentally observed rates of the E2-catalyzed reaction at various concentrations of E1 are higher than those predicted on the basis of free NADH as the only competent substrate. These results are consistent with a mechanism in which free NADH is not the exclusive substrate for the E2-catalyzed reaction; rather, they are consistent with a mechanism in which E1–NADH can serve as an alternative substrate (a prediction of the direct-transfer mechanism). It is noteworthy that the higher observed rate vis-à-vis the predicted rate (Table 1) is evident irrespective of whether E1 or E2 is either α-GDH or LDH. These findings strengthen our generalization that the direct-transfer mechanism is equally facile in either direction (1, 2).

Transient Kinetics. As acknowledged above, Chock and Gutfreund (6) have detected an error in our determination of the off rate of NADH from α-GDH–NADH. As a consequence, our earlier reported transfer rate of NADH from rabbit muscle α-GDH and halibut muscle LDH (5) is not faster than the true off rate. However, this new finding alone is not sufficient to disprove the direct-transfer mechanism between this enzyme pair.

Table 1. Comparison between observed steady-state rates of S2 reduction by NADH in the presence of E1 and rates predicted on the basis of free NADH as the only competent substrate

		Rate, μM/min	
E1	[Sites], nN	Predicted	Observed
LDH*	8.75	3.56	5.31
	17.5	2.03	4.02
	26.2	1.02	3.14
	35.0	0.65	1.93
	43.7	0.48	1.29
	52.5	0.38	1.45
	61.2	0.31	1.21
	70.0	0.27	1.29
α-GDH†	9.58	2.69	3.34
	19.2	1.78	2.41
	28.8	0.90	2.29
	38.3	0.53	2.09
	47.9	0.37	1.57
	57.5	0.28	1.21
	67.1	0.23	1.00

Thermodynamic and kinetic parameters for individual enzymes for predicting the steady-state kinetic rate are as follows: α-GDH, K_d (NADH) = 0.8 μM, K_m (NADH) = 2.4 μM, V_{max} = 4.75 μM/min; LDH, K_d (NADH) = 0.51 μM, K_m (NADH) = 7.1 μM, V_{max} = 4.2 μM/min.

*E2 = α-GDH at 0.87 nN; S2 = DHAP at 1.10 mM; total NADH = 15.4 μM.

†E2 = LDH at 0.86 nN; S2 = pyruvate at 2.0 mM; total NADH = 21.65 μM.

To complete our investigation for the transient transfer of NADH between rabbit muscle α-GDH and pig heart LDH, we measured the off rates of NADH from these enzymes by the NAD^+-displacement method (5). Because of the difference in fluorescence properties of free NADH vis-à-vis NADH bound to either α-GDH or LDH sites, we could monitor the time-dependent displacement of enzyme-bound NADH by NAD^+. The apparent rate constants calculated from these measurements as a function of NAD^+ concentration are shown in Fig. 2. The extrapolated rate constants at infinite concentration of NAD^+ gave values for the true off-rate constant for NADH of 135 ± 5 sec^{-1} and 95 ± 6 sec^{-1} for rabbit muscle α-GDH–NADH and pig heart LDH–NADH, respectively.

Given the dissociation and the off rate constants of NADH for rabbit muscle α-GDH and pig heart LDH, we can calculate the "on" rates ($k_{on} = k_{off}/K_d$) for these enzymes to be 1.69×10^8 $M^{-1}\cdot sec^{-1}$ and 1.86×10^8 $M^{-1}\cdot sec^{-1}$, respectively. These on and off rate constants can be numerically integrated to predict the rate of equilibration of NADH from E1–NADH to E2 at any concentration of E1, NADH, and E2, presuming that NADH is exclusively transferred via the random-diffusion mechanism (Fig. 3, triangles). The correctness of this prediction can be tested by comparing the experimentally observed rate of equilibration of NADH between rabbit muscle α-GDH and pig heart LDH.

The fluorescence signals used to measure the NADH off rates were also utilized to measure the transient rate of equilibration of NADH when E1–NADH was rapidly mixed with E2. Fig. 3 (circles) summarizes the E2-concentration-dependent observed equilibration rate constant for NADH in both directions—i.e., from α-GDH as E1 to LDH as E2 or

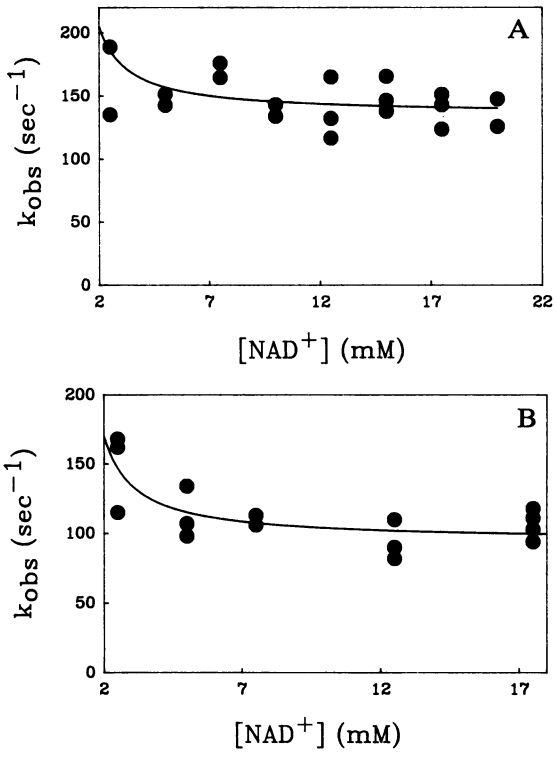

FIG. 2. Off rate constants of NADH for rabbit muscle α-GDH (*A*) and pig heart LDH (*B*). Time-dependent changes in fluorescence of E1–NADH on displacement with various concentrations of NAD^+ were used to calculate apparent first-order rate constants (k_{obs}, ●). Solid lines were calculated for hyperbolic dependence of k_{obs} on NAD^+ concentration and extrapolate at infinite concentration of NAD^+ to give true off rate constants. Enzyme and NADH were at 20 μN and 15 μM, respectively. NAD^+ concentrations shown represent concentration after mixing.

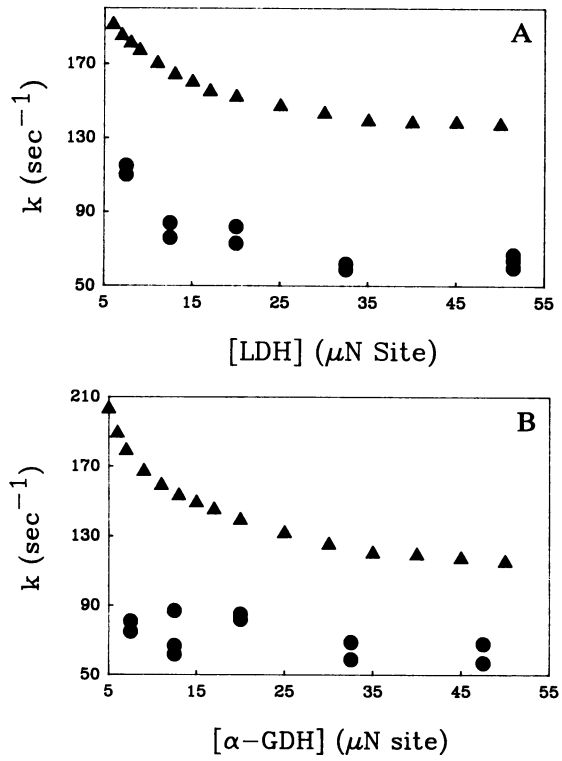

FIG. 3. Equilibration rate constants of E1–NADH with E2 as a function of E2 concentration. Apparent first-order rate constants from the experimental data (●) are compared with those predicted (▲) based on free equilibration of NADH (Eq. 2). The predicted rate constants were obtained by a numerical integration method using the known initial concentrations of E1, NADH, and E2 and the experimentally determined rate constants for these enzymes: α-GDH off rate constant = 135 sec^{-1}, on rate constant = 1.69×10^8 M^{-1}·sec^{-1}; LDH, off rate constant = 95 sec^{-1}, on rate constant = 1.86×10^8 M^{-1}·sec^{-1}. E1 and NADH were at 20 μN and 15 μM, respectively, before mixing. E2 concentrations before mixing were twice those shown. (*A*) E1 = α-GDH, E2 = LDH. (*B*) E1 = LDH, E2 = α-GDH.

from LDH as E1 to α-GDH as E2. Note that the predicted rate constants (triangles) are higher than those obtained experimentally (circles). Furthermore, the equilibration rates are remarkably similar in the two directions. These results cannot be explained simply on the basis of the random-diffusion mechanism. It appears plausible that the transfer of NADH is modulated (impeded) during the course of equilibration, presumably by a direct interaction between these enzymes. We have noted substantial decreases in the transient equilibration rates of NADH between other enzyme pairs that conform to the direct-transfer mechanism (5).

DISCUSSION

The experiments described herein were carried out in response to the criticism by Chock and Gutfreund (6) that our evidence for a direct-transfer mechanism between α-GDH and LDH was in error and involved a misinterpretation of kinetic data. We acknowledge that our off rate estimation of NADH from the α-GDH–NADH complex was in error (5), but this fact does not affect our overall conclusion. A reinvestigation of the transfer mechanism between this enzyme pair compels us to conclude, once again, that NADH is transferred directly between α-GDH and LDH.

We note that the major source of discrepancy between our conclusion and that of Chock and Gutfreund comes from the difference in K_m for NADH in the α-GDH-catalyzed reaction. In contrast to Chock and Gutfreund's measurement of K_m for NADH in the α-GDH-catalyzed reaction, our repeated measurements gave a value of 2.4 μM. We initially suspected that Chock and Gutfreund's lower K_m for NADH (0.4 μM) was due to their use of lyophilized α-GDH powder (Sigma type X), rather than the ammonium sulfate suspension (Sigma type I) used by us (7). But it soon became clear that this was not the case; our determination of K_m from the lyophilized α-GDH powder, exactly under the conditions of Chock and Gutfreund, also gave a value of 2.0 ± 0.2 μM. Furthermore, our K_m is comparable to the K_m values for NADH for rabbit muscle (8, 9) and chicken breast muscle (10). Hence, we have confidence in our value and maintain that Chock and Gutfreund have underestimated the K_m for NADH by a factor of at least 5.

Since our enzyme-buffering technique relies on quantitative estimates of the K_m and K_d values, the prediction would be significantly different for a 5-fold difference in K_m. With their erroneously measured K_m and a presumed K_d for the LDH–NADH complex of 1 μM, Chock and Gutfreund (6) were able to fit their steady-state data to the random-diffusion mechanism. When we reexamined their steady-state data (open squares in figure 5 of ref. 6) with accurately determined K_m and K_d values, it was found to be *inconsistent* with the random-diffusion mechanism, although consistent with the direct-transfer mechanism. We note that Chock and Gutfreund's conclusion relies on only three experimental points. Of these, one point (corresponding to 5 μM NADH) cannot discriminate between the two mechanisms. Even if the direct transfer exists, this high concentration of free NADH will successfully compete with the putative substrate α-GDH–NADH, and the random-diffusion pathway would predominate. The remaining two points are within such a narrow range that there can hardly be a predictable transition from the random-diffusion pathway to the direct-transfer pathway.

Chock and Gutfreund imply (especially in their consideration of the aldolase effects on α-GDH) that inhibition of the E2-catalyzed reaction by addition of E1 contradicts the direct-transfer mechanism. This is an inadequate conception of the kinetics of these systems. We have often observed such inhibitions, not only between dehydrogenases but also between other enzyme pairs where the direct-transfer mechanism is demonstrable (1, 2, 11). An inhibition of the E2-catalyzed rate may be evident both under the direct-transfer and under the random-diffusion pathways. In the random-diffusion mechanism, the E1-dependent inhibition is merely due to sequestration of free NADH at E1 sites, whereas in the direct-transfer mechanism the inhibition is due to one of two reasons: (*i*) competitive inhibition of E1 for the E2-catalyzed utilization of E1-M (see ref. 1) and (*ii*) discrepancy between the kinetic parameters for NADH vs. E1–NADH in the E2-catalyzed reactions; a lower k_{cat} for E1–NADH would result in inhibition of the E2-catalyzed reaction as the concentration of E1 is increased. Such discrepancies can also give erroneous K_m values for the substrates if determined by the enzyme-buffering method. This is because, as the concentration of E1 increases to buffer M, both E1–M and free M are utilized as "competing" substrates in the E2-catalyzed reaction. If the k_{cat} for E1–M is lower than that for free M, the E1-concentration-dependent velocity would exhibit a hyperbolic dependence on calculated free M, with a lower K_m (D.K.S. and G.F.B., unpublished results). Hence, Chock and Gutfreund's belief that the enzyme-buffering experiments can be used to determine K_m values that are low in magnitude, or where product inhibition is a problem, is incorrect.

It should be emphasized that our enzyme-buffering steady-state kinetic experiments do not give positive results for the direct-transfer pathway with all the enzyme pairs (2, 3). With some enzyme pairs we have consistently obtained negative results. For example, among dehydrogenases, we have found that the direct-transfer mechanism is not operative between enzymes of the same chiral specificity (i.e., between A–A or

B–B pairs) (7). We consider that these negative results provide a significant control on our methodology.

Unlike the steady-state kinetic analyses for differentiating the pathways of metabolite transfer (Eq. 1), transient kinetic analyses are complex. Nevertheless, the distinction between the random-diffusion mechanism and the direct-transfer mechanism becomes obvious if the solution-mediated rates are far disparate from the observed rates. On the other hand, if the solution-mediated rates are comparable to the observed rates, a clear-cut distinction between the two mechanisms is impossible without further recourse to numerical integration. A minimal mechanism for the aqueous solution-mediated transfer of NADH from E1–NADH to E2 can be represented by Eq. 2.

$$\text{E1–NADH} \underset{k_{on}\text{E1}}{\overset{k_{off}\text{E1}}{\rightleftharpoons}} \text{E1} + \text{NADH}$$

$$\text{NADH} + \text{E2} \underset{k_{off}\text{E2}}{\overset{k_{on}\text{E2}}{\rightleftharpoons}} \text{E2–NADH} \qquad [2]$$

Whatever the methods used to predict the transfer rate of NADH between E1 and E2, reliable estimates of the individual rate constants of Eq. 2 are essential. An examination of Chock and Gutfreund's criticism of our work reveals that their prediction is based on arbitrary selection of on and off rate constants of NADH for rabbit muscle LDH. These authors presume a K_d for the rabbit muscle LDH–NADH complex to be 3.0 μM, and by further presuming the on rate of NADH to LDH to be 1×10^8 $M^{-1} \cdot sec^{-1}$, calculate the off rate of NADH to be 300 sec^{-1}. Since the prediction of equilibration rate relies on the quantitative parameters of the on and off rate constants for E1 and E2 pairs, a presumption in parameter value may lead to an erroneous conclusion.

In contrast, we have carefully determined these relevant parameters and used them for predicting the equilibration rates between these enzymes. As shown in Fig. 3, the experimentally measured rate constants for E2–NADH formation are invariably lower than those predicted on the basis of the free equilibration model. This is true for the transfer of NADH in either direction—i.e., from α-GDH to LDH and also from LDH to α-GDH. Furthermore the observed rate constants are equal ($\approx$70 sec^{-1}) in the two directions. Thus, the transient equilibration of NADH is not consistent with the random-diffusion mechanism. To explain the disparity between the observed and the predicted rates, one must envision some additional steps contributing to the overall equilibration (relaxation) process. With the precedent of our steady-state kinetic demonstration that NADH is transferred between these enzymes by the direct-transfer pathway, we suggest that these steps are involved in the formation of E1–E2 complex, such that the transfer (direct transfer) rate of NADH is modulated.

All our kinetic data (steady state and transient) presented herein indicate that the transfer of NADH between rabbit muscle α-GDH and pig heart LDH cannot be explained simply on the basis of the random-diffusion mechanism. It can, however, be explained on the basis of the direct-transfer mechanism. This, taken together with our studies with other enzyme pairs, supports the validity of the direct-transfer mechanism between cognate enzyme pairs (1–3, 11).

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