Biochem. J. (1991) 278, 875–881 (Printed in Great Britain)

Re-evaluation of the glycerol-3-phosphate dehydrogenase/L-lactate dehydrogenase enzyme system

Evidence against the direct transfer of NADH between active sites

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An investigation of the direct transfer of metabolites from rabbit muscle L-lactate dehydrogenase (LDH, EC 1.1.1.27) to glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.1.8) revealed discrepancies between theoretical predictions and experimental results. Measurements of the GPDH reaction rate at a fixed NADH concentration and in the presence of increasing LDH concentrations gave experimental results similar to those previously obtained by Srivastava, Smolen, Betts, Fukushima, Spivey & Bernhard [(1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6464–6468]. However, a mathematical solution of the direct-transfer-mechanism equations as described by Srivastava *et al.* (1989) showed that the direct-transfer model did not adequately describe the experimental behaviour of the reaction rate at increasing LDH concentrations. In addition, experiments designed to measure the formation of an $LDH_4 \cdot NADH \cdot GPDH_2$ complex, predicted by the direct-transfer model, indicated that no significant formation of tertiary complex occurred. An examination of other kinetic models, developed to describe the LDH/GPDH/NADH system better, revealed that the experimental results may be best explained by assuming that free NADH, and not $E_1 \cdot NADH$, is the sole substrate for GPDH. These results suggest that direct transfer of NADH between rabbit muscle LDH and GPDH does not occur *in vitro*.

INTRODUCTION

Over the past several years, evidence in support of an apparent direct transfer of nicotinamide coenzyme between active sites of NADH-requiring enzymes has been presented (Weber & Bernhard, 1982; Srivastava & Bernhard, 1984, 1985; Srivastava *et al.*, 1989) and extensively reviewed (Srivastava & Bernhard, 1986; Srere, 1987). These studies presented evidence that NADH may be transferred directly between enzyme active sites based on data obtained at high concentrations of A-faced and B-faced dehydrogenase enzymes. The experiments were performed by adding micromolar quantities of an enzyme (E_1) that functioned only to decrease the free NADH concentration. In these experiments E_1 was catalytically inactive because the reaction medium lacked its second substrate. The velocity of a second NADH-utilizing dehydrogenase enzyme (E_2) was then measured at increasing E_1 concentrations and at a fixed NADH concentration. By using this simple system, it was possible to predict the velocity of the E_2 reaction when the K_d for the E_1–NADH binding reaction and the K_m and $V_{max.}$ for the E_2 reaction were known. The predicted velocity was calculated assuming that the E_1–NADH interaction was at equilibrium and that the E_2 reaction could be described by the general rate equation:

$$v_{obs.} = d[NAD^+]/dt = V_{max.,E2} \cdot [NADH]_{free}/(K_{m,E2} + [NADH]_{free}) \quad (1)$$

with $[NADH]_{free}$ calculated from total NADH and E_1 concentrations. Measurements with several pairs of A-faced and B-faced dehydrogenase enzymes showed that the velocities observed at high E_1 concentrations were greater than the velocities predicted by eqn. (1) (Srivastava & Bernhard, 1984, 1985; Srivastava *et al.*, 1989). The discrepancy between observed and expected results suggested to those authors that the $E_1 \cdot NADH$ complex could transfer NADH directly to E_2 without dilution into the surrounding solvent, i.e. that $E_1 \cdot NADH$ acted as an E_2 substrate. Such a direct-transfer mechanism would functionally increase the NADH concentration available for reaction, giving observed velocities that were higher than those predicted from the free NADH concentration alone. The direct-transfer mechanism was further supported by the observation that higher rates were not observed when pairs of only A-faced or pairs of only B-faced enzymes were used. This latter observation suggested that the transfer mechanism was stereospecific, and was expected if NADH could not re-orient during transfer from E_1 to E_2.

The major criticism of the Direct-Transfer Model is the fact that the observed E_2 velocities decrease significantly in the presence of increasing E_1 concentrations. If $E_1 \cdot NADH$ is a viable substrate for E_2 (as predicted by the Direct-Transfer Model), increasing E_1 concentrations should not appreciably affect the overall rate of E_2; total substrate should remain constant because total substrate $= [NADH]_{free} + [E_1 \cdot NADH]$. Thus, if the E_2 K_m value for $E_1 \cdot NADH$ and the $k_{cat.}$ for the $E_1 \cdot NADH \cdot E_2$ complex are comparable with those of the free enzyme (as reported by Srivastava & Bernhard, 1985), then the observed velocity should not decrease but remain nearly constant. Because the observed velocity decreased by 5-fold (Srivastava & Bernhard, 1985; Srivastava *et al.*, 1989), we believe that other mechanisms may account for the discrepancies in the observed velocities.

The present paper examines several kinetic mechanisms in an attempt to describe completely the velocity-versus-$[E_1]$ profile previously reported for the glycerol-2-phosphate dehydrogenase/L-lactate dehydrogenase system (Srivastava & Bernhard, 1985; Srivastava *et al.*, 1989). We also attempted to characterize the enzyme complexes predicted by the various kinetic mechan-

Abbreviations used: GPDH, glycerol-3-phosphate dehydrogenase; $GPDH_2$, dimeric GPDH; DHAP, dihydroxyacetone phosphate; LDH, L-lactate dehydrogenase; LDH_4, tetrameric LDH.

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isms in an effort to resolve the different models experimentally. We conclude that the observed velocity in the presence of increasing concentrations of E_1 was best described by a model that did not incorporate the assumption of direct transfer between E_1 and E_2.

EXPERIMENTAL

Materials

Glycerol-3-phosphate dehydrogenase (GPDH, EC 1.1.1.8; type X) and L-lactate dehydrogenase (LDH, EC 1.1.1.27; type XI) from rabbit muscle, Trizma base, EDTA and 2-mercaptoethanol were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sepharose S-200 was purchased from Pharmacia (Uppsala, Sweden), Bio-Gel P-150 was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and NADH (grade II) was purchased from Boehringer–Mannheim (Montreal, P.Q., Canada). The buffer used for all experiments was 50 mM-Tris/HCl buffer, pH 7.4, containing 0.1 mM-EDTA and 1 mM-2-mercaptoethanol (basic buffer).

Methods

Column chromatography. Two different column regimes were followed. Chromatography on dissociating columns was performed as described by Dixon (1976) with a 0.36 cm × 24 cm (2.5 ml) column of Bio-Gel P-150. The column matrix totally excluded tetrameric LDH (LDH_4), as determined from its elution position relative to Blue Dextran sulphate. Samples of LDH_4, dimeric GPDH ($GPDH_2$) or an $LDH_4/GPDH_2$ mixture were loaded on to columns equilibrated in either basic buffer or basic buffer plus 40 μM-NADH at 21 °C. Chromatography on associating columns was performed as described by Ackers (1975) with a 0.36 cm × 24 cm (2.5 ml) column of Sepharose S-200. A sample of an $LDH_4/GPDH_2$ mixture was loaded on to a column equilibrated in basic buffer plus 20 μM-NADH at 21 °C. All columns were developed in the same buffer as that used to equilibrate them. Elution velocities were approx. 40 μl/min at 21 °C. Elution profiles were monitored by following enzyme activity at 340 nm in the presence of 0.15 mM-NADH and either 1 mM-dihydroxyacetone phosphate (DHAP) (for GPDH) or 2 mM-pyruvate (for LDH). Enzyme concentrations were calculated by using specific activities of 350 units (μmol/min)/mg and 300 units (μmol/min)/mg for LDH_4 and $GPDH_2$ respectively (Barman, 1969) and M_r values of 140000 and 78000 for LDH_4 and $GPDH_2$ respectively (Darnall & Klotz, 1975).

Kinetic measurements. K_m values for NADH were determined fluorimetrically in basic buffer with excitation and emission wavelengths set to 340 nm and 460 nm respectively. When required, pyruvate was present at 2 mM (for LDH) and DHAP at 1 mM (for GPDH). The LDH K_d value for NADH was determined kinetically as described in the Results section. GPDH activity (in the presence of increasing LDH_4 concentrations) was measured spectrophotometrically at 340 nm (Pye–Unicam SP8-100 instrument) in basic buffer plus 1 mM-DHAP. All kinetic measurements were performed at 21 °C.

Statistical methods. Kinetic constants were determined by using a modified Gauss–Newton non-linear least-squares-regression algorithm (Gallant, 1982). Applicability of the model to the data was tested by two non-parametric methods: a Sign test for a random number of positive and negative residuals and a Wald–Wolfowitz runs test to test whether the signs of the residuals occurred in a random sequence (Swed & Eisenhart, 1943; Miller & Miller, 1988). Scatter plots of the residuals were also examined by eye for discernible patterns. Best-fit values are reported ±1 S.D. determined from the regression correlation matrix in accordance with Gallant (1982).

THEORY

The five independent kinetic models presented below were developed to describe a system containing two different enzymes that bind NADH. However, these models can also be used to describe other enzyme pairs that bind a common metabolite. The use of these equations requires that both enzymes catalyse two substrate reactions. This enables the experimenter to control enzyme activity via selective addition of the non-NADH substrates. In our experiments the second substrate for the second enzyme (E_2) is present so that the velocity of the reaction can be defined by eqn. (1) with $V_{max.}$ defined as $k_{cat.,E2}\cdot[E_2]_{total}$. Under our conditions the second substrate for the first enzyme (E_1) was absent, so that addition of E_1 served only to bind NADH, decreasing the free NADH concentration according to eqn. (2):

$$K_{d,E1}^{NADH} = [E_1]_{free}\cdot[NADH]_{free}/[E_1\cdot NADH] \quad (2)$$

All the models presented below also assume that the GPDH reaction can be adequately described by eqn. (1). This assumption is valid, since initial-rate studies demonstrated that GPDH obeys an Ordered Bi Bi mechanism (Young & Pace, 1958; Black, 1966; Lee & Craine, 1971). The initial-velocity expression for the Ordered Bi Bi mechanism can be rewritten to express velocity in terms of the free NADH concentration, the DHAP concentration and the dissociation and kinetic constants for substrate binding:

$$v_{obs.} = V_{max.,GPDH}\cdot[NADH]_{free}/([NADH]_{free}+K_{m,GPDH}) \quad (3)$$

where $V_{max.,GPDH} = k_{cat.}\cdot[E_2]_{total}/(1+\delta)$, $K_{m,GPDH} = (K_{m,GPDH}^{NADH} + \delta\cdot K_{d,GPDH}^{NADH})/(1+\delta)$ and $\delta = K_{m,GPDH}^{DHAP}/[DHAP]$. In eqn. (3), $K_{m,GPDH}^{DHAP}$ and $K_{m,GPDH}^{NADH}$ represent the GPDH Michaelis constants for DHAP and NADH respectively, and $K_{d,GPDH}^{NADH}$ represents the GPDH dissociation constant for NADH (Cleland, 1963*a*,*b*; Segal, 1975).

In the models presented below all kinetic constants refer to E_2 unless otherwise indicated. For example, $K_{d,E1}^{NADH}$ is the dissociation constant of E_1 for NADH whereas K_m^{NADH} is the K_m value of E_2 for NADH.

1. Equilibrium Model

This model assumes that E_1 competes directly with E_2 for NADH, diminishing the velocity of the reaction catalysed by E_2 by decreasing its available substrate. This system can be solved by assuming that eqn. (2) is at equilibrium and that E_2 obeys a Michaelis–Menten kinetic mechanism. The concentration of E_1-bound NADH can then be calculated directly from the dissociation constant of E_1 for NADH ($K_{d,E1}^{NADH}$; eqn. 2) and the total concentrations of NADH and E_1 according to eqn. (4):

$$0 = [E_1\cdot NADH]^2 - [E_1\cdot NADH]\cdot(K_{d,E1}^{NADH} + [NADH]_{total} + [E_1]_{total}) + [NADH]_{total}\cdot[E_1]_{total} \quad (4)$$

The observed velocity can then be calculated from eqn. (3) by using the concentration of $E_1\cdot NADH$ from eqn. (4) and the relationship: $[NADH]_{free} = [E_1]_{total} - [E_1\cdot NADH]$.

2. Direct-Transfer Model

Similarly to the Equilibrium Model, the Direct-Transfer Model assumes that the reaction of eqn. (2) is in equilibrium, so that

$[NADH]_{free}$ and $[E_1 \cdot NADH]$ can be obtained from eqn. (4). However, in the Direct-Transfer Model, the $E_1 \cdot NADH$ complex can transfer NADH directly to E_2 so that $E_1 \cdot NADH$ is also a substrate for E_2. The direct transfer of NADH from E_1 to E_2 is thought to account for the 'extra' velocity observed in the presence of high concentrations of E_1:

$$E_{2,free} + NADH_{free} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} E_2 \cdot NADH \overset{k_{+3}}{\rightarrow} \text{products} \quad (5)$$

$$E_{2,free} + E_1 \cdot NADH \underset{k_{-4}}{\overset{k_{+4}}{\rightleftharpoons}} E_2 \cdot NADH \cdot E_1 \overset{k_{+5}}{\rightarrow} \text{products} \quad (6)$$

At steady state, eqns. (5) and (6) can be resolved to give the steady-state velocity, $v_{obs.}$:

$$v_{obs.} = \frac{k_{+3} \cdot [E_2]_{total} \cdot [NADH]_{free}}{[NADH]_{free} + K_m^{NADH}(1 + [E_1 \cdot NADH]/K_m^{E_1 \cdot NADH})} + \frac{k_{+5} \cdot [E_2]_{total} \cdot [E_1 \cdot NADH]}{[E_1 \cdot NADH] + K_m^{E_1 \cdot NADH}(1 + [NADH]_{free}/K_m^{NADH})} \quad (7)$$

In eqn. (7) $K_m^{NADH} = (k_{-2} + k_{+3})/k_{+2}$ and $K_m^{E_1 \cdot NADH} = (k_{-4} + k_{+5})/k_{+4}$. The values of $[E_1 \cdot NADH]$ and $[NADH]_{free}$ are obtained directly from eqn. (4). Differences in the 'off' constants for $E_1 \cdot NAD^+$ and NAD^+ dissociation from E_2 (see Chock & Gutfreund, 1988) are incorporated into the catalytic constants k_{+3} and k_{+5}.

3. Enzyme-Binding Model

As in the previous two models, we assume that E_1 and NADH are in rapid equilibrium so that eqn. (4) gives the correct solution concentration of free NADH. The Enzyme-Binding Model also assumes formation of an $E_1 \cdot E_2$ complex with kinetic parameters different from those of the free E_2 enzyme and a dissociation constant defined by:

$$K_d^{E^1 \cdot E^2} = [E_1]_{free} \cdot [E_2]_{free}/[E_1 \cdot E_2] \quad (8)$$

In order to obtain a simple equation that describes the observed velocity as a function of increasing E_1 concentrations, the dissociation constant for the interaction must be small enough so that $[E_2]_{total} \approx [E_1 \cdot E_2]$ under the conditions of these experiments. This latter assumption is supported by measurements of observed velocity at increasing concentrations of E_1 (Srivastava & Bernhard, 1984, 1985; Srivastava *et al.*, 1989; the Results section of this paper), which suggest that, if an $E_1 \cdot E_2$ complex existed, its $K_d^{E_1 \cdot E_2}$ value would be approx. 0.1 μM. This assumption allows one to express the observed velocity as:

$$v_{obs.} = k_{+3}' \cdot [E_1 \cdot E_2] \cdot [NADH]_{free}/([NADH]_{free} + K_m^{NADH'}) \quad (9)$$

where k_{+3}' and $K_m^{NADH'}$ are the $k_{cat.}$ and K_m values for E_2 when bound in the $E_1 \cdot E_2$ complex.

4. Steady-State Model

If one does not assume that the E_1–NADH interaction is at equilibrium, the kinetic expression for eqn. (2):

$$E_{1,free} + NADH_{free} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E_1 \cdot NADH \quad (10)$$

and the reactions of eqns. (5) and (6) can be solved for the observed velocity in the steady state. This gives eqn. (3) once again, with $[NADH]_{free}$ calculated by using eqn. (4). Eqn. (4) can be used to calculate $[NADH]_{free}$ because, under the conditions of our experiments, $[NADH]_{total} \approx [NADH]_{free} + [E_1 \cdot NADH]$ ($[E_2 \cdot NADH] \ll [NADH]_{total}$). The Steady-State Model therefore predicts kinetic behaviour identical with that of the Equilibrium Model.

5. Inhibition Model

If $E_1 \cdot NADH$ and free E_1 compete directly for the E_2 substrate-binding site, then direct transfer of NADH coenzyme may occur, but the reaction rate would be inhibited in the presence of high concentrations of E_1. The kinetic equations for this system may be solved by including a reaction for the equilibrium binding of free E_1 to E_2 to give an inhibited $E_1 \cdot E_2'$ complex according to:

$$K_i = [E_2]_{free} \cdot [E_1]_{free}/[E_1 \cdot E_2'] \quad (11)$$

Solving eqns. (5), (6) and (11) simultaneously gives:

$$v_{obs.} = \frac{k_{+3} \cdot [E_2]_{total} \cdot [NADH]_{free}}{[NADH]_{free} + K_m^{NADH}(1 + [E_1]_{free}/K_i + [E_1 \cdot NADH]/K_m^{E_1 \cdot NADH})} + \frac{k_{+5} \cdot [E_2]_{total} \cdot [E_1 \cdot NADH]}{[E_1 \cdot NADH] + K_m^{E_1 \cdot NADH}(1 + [E_1]_{free}/K_i + [NADH]_{free}/K_m^{NADH})} \quad (12)$$

RESULTS

In order to investigate the possibility of a direct transfer of NADH between dehydrogenase active sites, we employed a system containing NADH and rabbit muscle LDH and GPDH. Kinetic measurements on this system were obtained under two different conditions: (i) by omitting pyruvate from the reaction cuvette and including 1 mM-DHAP or (ii) by omitting DHAP from the reaction cuvette and including 2 mM-pyruvate. Under condition (i) LDH functioned only to bind free NADH, so that the reaction velocity was equal to the GPDH turnover rate (following the nomenclature used in the Theory section we can designate E_1 = LDH and E_2 = GPDH). When condition (ii) was employed, the reverse was true: E_1 = GPDH and E_2 = LDH. Binding experiments designed to determine the dissociation constant for $E_1 \cdot E_2$ complexes under conditions identical with those of the kinetic experiments were also performed in the presence and in the absence of NADH but with mixtures that did not contain either DHAP or pyruvate to ensure a constant NADH concentration throughout the columns. For the purposes of these experiments LDH and GPDH are treated as non-divisible entities with four and two NADH-binding sites/molecule respectively. This assumption is valid under the conditions of our experiments because neither LDH nor GPDH would dissociate to any appreciable extent (Holbrook *et al.*, 1975).

Measurement of LDH and GPDH kinetic parameters

Before examination of the kinetic effects of increasing concentrations of LDH on the observed GPDH kinetic rate,

relevant LDH and GPDH kinetic parameters were determined. These measurements gave a GPDH K_m value for NADH of 2.3 ± 0.2 μM in the presence of 1 mM-DHAP, and an LDH K_d value for NADH for 1.93 ± 0.3 μM. The $K_{d,LDH}^{NADH}$ value was determined from plots of initial velocity versus substrate concentrations (with those of NADH and pyruvate varied) assuming that LDH obeys an Ordered Bi Bi mechanism with NADH as the first substrate (Holbrook *et al.*, 1975).

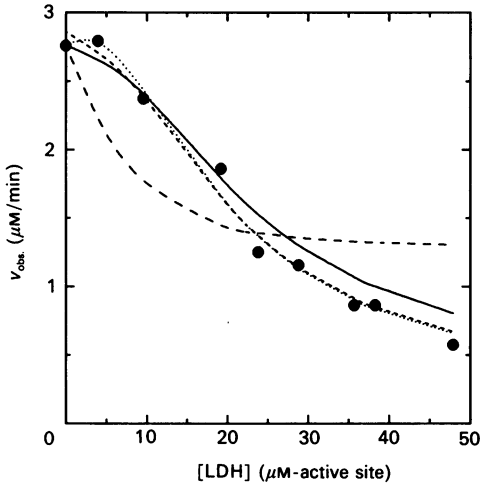

Fig. 1. Effect of increasing LDH_4 concentration on the GPDH reaction velocity: comparison of five models

GPDH velocity was measured as a function of increasing LDH_4 concentrations at 21 °C in basic buffer (●). Total volume was 1 ml, and NADH concentration was fixed at 14.5 μM. Reactions were initiated by the addition of 0.8 nM-GPDH and represent the means of two runs. The individual lines represent theoretical predictions of the relationship between observed velocity and added LDH subunit concentration (expressed in terms of active sites) based on the five models outlined in the Theory section: ——, Equilibrium Model (1) and Steady-State Model (4); – – –, Direct-Transfer Model (2); ------, Enzyme-Binding Model (3); ·····, Inhibition Model (5). Constants used for the evaluation of the theoretical lines are presented in Table 1.

Table 1. Values of kinetic constants used to construct the theoretical predictions shown in Fig. 1

$[NADH]_{total} = 14.5$ μM and $[GPDH_2]_{total} = 0.80$ nM. Data were obtained as follows: [a]present paper; [b]from Srivastava & Bernhard (1985); [c]from non-linear least-squares regression of the data from Fig. 1 to the appropriate model. Note that the sequence of residuals and the number of positive and negative residuals were non-random for Model 2. The large standard errors for the regression parameters of Model 5 indicate either (i) that not enough data are available to obtain accurate estimates of these values or (ii) that Model 5 is not a good descriptor of the data of Fig. 1.

Model no.	LDH–NADH interaction K_d^{NADH} (μM)	GPDH–substrate interaction K_m (μM)	$k_{cat.}$ (s^{-1})
1	1.93[a]	$K_m^{NADH} = 2.3$[a]	$k_{+3} = 66.7$[a]
2	1.93	$K_m^{NADH} = 2.3$ $K_m^{LDH \cdot NADH} = 1.21$[b]	$k_{+3} = 66.7$ $k_{+5} = 28.4$[b]
3	1.93	$K_m^{NADH'} = 0.79 \pm 0.08$[c]	$k_{+3}' = 59.8 \pm 1.6$[c]
4	1.93	$K_m^{NADH} = 2.3$	$k_{+3} = 66.7$
5	1.93	$K_m^{LDH \cdot NADH} = 0.053 \pm 1.8$[c] $K_i = 0.029 \pm 0.75$[c]	$k_{+3} = 66.7$ $k_{+5} = 76.6 \pm 7.9$[c]

Kinetic experiments with E_2 = GPDH

Fig. 1 presents the results of an experiment designed to determine the kinetic effect of increasing concentrations of LDH on the observed GPDH velocity. These results were obtained by adding increasing concentrations of LDH to a fixed concentration of GPDH in the presence of 14.5 μM-NADH in a fashion analogous to that of previous studies (Srivastava & Bernhard, 1984, 1985; Srivastava *et al.*, 1989). In agreement with these studies, the observed velocity decreased as a function of increasing LDH concentrations. Under these conditions, then, LDH apparently inhibited the reaction velocity by decreasing the free concentration of NADH.

The data of Fig. 1 also permitted a test of the different models developed to describe the kinetic behaviour of GPDH at increasing concentrations of LDH. The curves of Fig. 1 represent the theoretical predictions of the various models outlined in the Theory section, constructed by using the parameters outlined in Table 1. It is immediately obvious that the Direct-Transfer Model did not adequately describe the data. The large discrepancy between experimental results (●) and theoretical predictions (– – –) indicated that the kinetic parameters measured *in vitro* were inadequate in predicting GPDH kinetic behaviour at higher LDH concentrations. On the other hand, Models 1, 3, 4 and 5 all appeared to describe adequately the experimental data with the use of the parameters listed in Table 1. Three of these models (1, 3 and 4) assume that $NADH_{free}$ is the only GPDH substrate, with the Enzyme-Binding Model (3) requiring formation of a complex between GPDH and LDH before the catalytic step. The Inhibition Model (5) predicts direct transfer of NADH between LDH and GPDH but requires that free LDH competitively inhibits LDH·NADH binding to GPDH. Since these models could not be resolved by using the kinetic system of Fig. 1, it was necessary to perform other kinetic experiments as well as enzyme–enzyme-binding experiments to determine their validity.

Binding experiments

The Direct-Transfer Model, the Enzyme-Binding Model and the Inhibition Model predict the existence of complexes between LDH and GPDH. Consideration of the data of Fig. 1 allows one to estimate the K_d value for formation of an $LDH_4 \cdot GPDH_2$ complex if it exists. If more than 90% of the total $GPDH_2$ is bound to LDH_4 when $[LDH_4] = 1$ μM and $[GPDH_2] = 0.8$ nM, one can calculate a $K_d^{E_1 \cdot E_2}$ value of 0.1 μM from eqn. (13):

$$K_d^{E_1 \cdot E_2} = [E_1]_{total}^{(F-1)} + [E_2]_{total}(1 - 1/F) \quad (13)$$

where F represents the fraction of $GPDH_2$ bound in the complex. In the case of the Inhibition Model, regression of the data in Fig. 1 to eqn. (12) indicated that the K_i for the $LDH_4 \cdot GPDH_2$ complex should be approx. 25 nM (see Table 1).

Fig. 2 shows the results of experiments designed to determine the dissociation constant for the interaction between LDH_4 and $GPDH_2$ in the presence and in the absence of NADH. The data of Fig. 2(*b*), obtained by using the dissociating-column methodology in the absence of NADH, indicate that no $LDH_4 \cdot GPDH_2$ complex exists under the conditions of Fig. 1. This was illustrated by : (i) a very close correspondence between the ○ symbols (experimental data) and the —— line (free $GPDH_2$ elution profile from Fig. 2*a*) in Fig. 2(*b*), and (ii) a lack of correlation between the ○ symbols and the ····· line of Fig. 2(*b*). The ····· line represents the expected $GPDH_2$ distribution assuming a 1:1 complex between LDH_4 and $GPDH_2$ exists with a dissociation constant of 1 μM (Dixon, 1976).

Figs. 2(*c*) and 3 present the results of experiments designed to test the existence of an $LDH_4 \cdot NADH \cdot GPDH_2$ complex

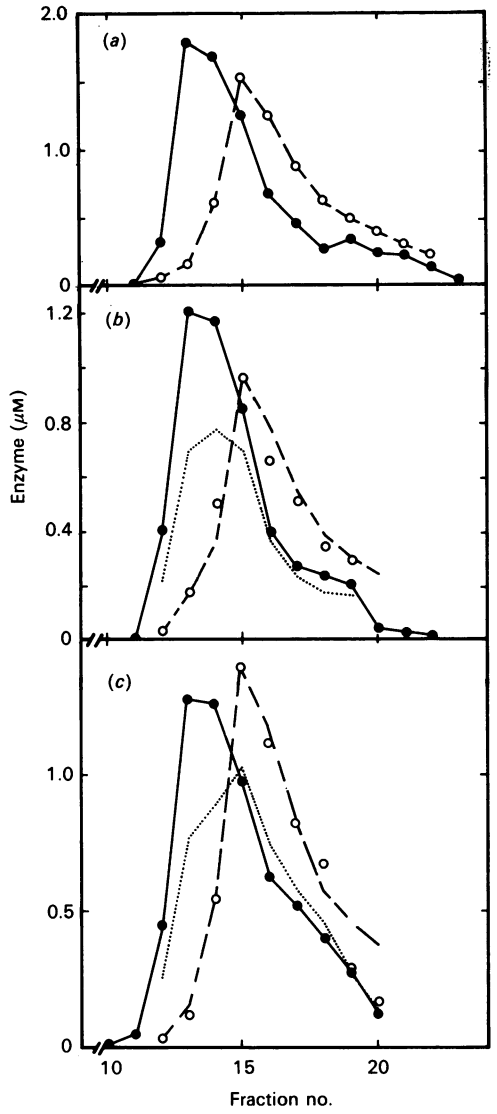

Fig. 2. Measurement of the binding interaction between LDH_4 and GPDH: determinations in the presence and in the absence of NADH

A 30 μl portion of either 27 μM-LDH_4 or 24 μM-$GPDH_2$ (*a*) or a 30 μl portion of 27 μM-LDH_4 plus 24 μM-$GPDH_2$ (*b* and *c*) was loaded on to a 0.36 cm × 24 cm (2.5 ml) column of Bio-Gel P-150. Columns were developed in either basic buffer (*a* and *b*) or basic buffer plus 40 μM-NADH (*c*). Fractions of 80 μl volume were collected. Column velocities were approx. 40 μl/min at 21 °C. ● symbols represent LDH_4 concentrations and ○ symbols represent $GPDH_2$ concentrations measured in each fraction. ——, Elution profile of free $GPDH_2$. ·····, Distribution expected assuming that a $GPDH_2 \cdot LDH_4$ or a $GPDH_2 \cdot NADH_4 \cdot LDH_4$ complex exists with a dissociation constant of 1 μM (Dixon, 1976).

predicted by the Direct-Transfer Model and the Inhibition Model. In Fig. 2(*c*) a mixture of LDH_4, NADH and $GPDH_2$ was chromatographed on a Bio-Gel P-150 column with the use of dissociating-column methodology to determine if the presence of NADH promoted formation of a complex between LDH_4 and $GPDH_2$. As was the case for Fig. 2(*b*), a very close correspondence between experimental data (○) and the elution profile for free $GPDH_2$ (——) indicated that a complex between $GPDH_2$ and $LDH_4 \cdot NADH_4$ was not formed under experimental conditions identical with those of Fig. 1. The —— curve of Fig. 2(*c*) was also demonstrably different from the elution profile predicted for a 1:1 interaction between $GPDH_2$ and $LDH_4 \cdot NADH_4$

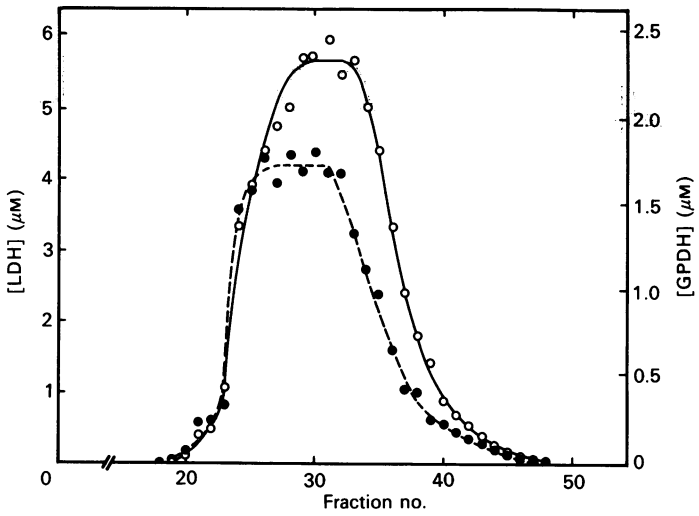

Fig. 3. Measurement of GPDH–NADH–LDH_4 interaction by the use of Sepharose S-200 column chromatography

A 1.3 ml portion of 4.2 μM-LDH_4 plus 2.4 μM-GPDH was loaded on to a 0.36 cm × 24 cm (2.5 ml) column of Sepharose S-200. The Figure shows the concentration of either LDH_4 (●) or GPDH (○) in each fraction. The column was developed in basic buffer plus 20 μM-NADH at 21 °C. The column velocity was approx. 40 μl/min. Fractions of 40 μl volume were collected.

or between LDH_4 and $GPDH_2 \cdot NADH_2$ (····· in Fig. 2*c*) calculated assuming a dissociation constant of 1.0 μM (see Dixon, 1976).

The associating-column methodology was also used to measure possible interactions between $GPDH_2$ and $LDH_4 \cdot NADH_4$ or between LDH_4 and $GPDH_2 \cdot NADH_4$. In Fig. 3 a 1.5 ml sample of 4.2 μM-LDH_4 plus 2.4 μM-$GPDH_2$ was loaded on to a 2.5 ml Sepharose S-200 column in the presence of 20 μM-NADH. The column methodology followed that used to determine interactions between non-identical protein subunits (Nichol & Winzor, 1964; Gilbert & Kellett, 1971; Ackers, 1975). The profiles of Fig. 3 are indicative of a non-interacting enzyme system; the elution pattern did not show a leading or trailing step, which is characteristic of interacting proteins (Nichol & Winzor, 1964; Gilbert & Kellett, 1971). If one assumes a K_d value of 1 μM and a ratio of 4 molecules of $GPDH_2$ bound per molecule of LDH_4 one can calculate that 53% of the $GPDH_2$ should be bound to LDH_4 under the conditions of Fig. 3. Alternatively, if one assumes that $GPDH_2$ binding to one LDH·NADH subunit inhibits binding to other subunits on the same LDH_4 tetramer (steric-hindrance assumption) then one can calculate that 71% of the $GPDH_2$ should be bound to LDH_4. The percentage increases in the latter case because a greater proportion of LDH_4 active sites, as compared with LDH subunit active sites, would be occupied by NADH. The data of Fig. 3 demonstrate that virtually 100% of the $GPDH_2$ was free under our conditions.

Note that the theoretical binding curves of Fig. 2 were calculated with the use of a K_d value of 1 μM. These curves thus represent calculations for a complex with an affinity 10-fold lower than that predicted from experimental data for the Enzyme-Binding Model, an affinity equivalent to that for the Direct-Transfer Model (Srivastava & Bernhard, 1985) or an affinity 20-fold lower than that predicted from the Inhibition Model. Note also that calculations of column profiles with the use of other equations developed for this purpose (Nimmo & Bauermeister, 1978) predict elution profiles similar to the theoretical predictions of Fig. 2.

Table 2. Effect of increasing concentrations of GPDH on the LDH_4 K_m value for NADH

Concentration of GPDH is expressed in μmol of dimeric enzyme/l. Percentage of LDH_4 bound was calculated by using eqn. (9) and by assuming a 1:1 stoichiometric complex between $GPDH_2$ and LDH_4 and a $K_d^{LDH \cdot GPDH}$ value of 1 μM for the $GPDH \cdot LDH_4$ complex. K_m values were determined in the presence of 2 mM-pyruvate, and are corrected to account for the binding of NADH to GPDH by using a K_d^{NADH} value for the GPDH·NADH complex of 0.66 μM (Chock & Gutfreund, 1988).

$[GPDH]_{total}$[a] (μM)	Percentage of LDH bound if $K_d^{E_1 \cdot E_2} = 1.0$ μM	Measured LDH K_m value for NADH (μM)
0	0	6.74±1.9
0.28	34	7.98±2.4
1.71	63	6.85±0.9

Table 3. Non-linear least-squares-regression fit of the Direct-Transfer Model to the data of Srivastava *et al.* (1989)

The sequence of residuals was non-random when the Direct-Transfer Model (2) with fixed k_{+3} was used as the regression model. The large standard errors in this case reflect the fact that this model is a poor descriptor of the data in Fig. 4.

Model no.	Fixed value	Regression value	Line on Fig. 4
1	None	$k_{+3} = 81 \pm 9.3\ s^{-1}$	None
1	$k_{+3} = 45.5\ s^{-1}$	None	——
2	$k_{+3} = 45.4\ s^{-1}$	$k_{+5} = 2.2 \times 10^4 \pm 751 \times 10^4\ s^{-1}$ $K_m^{LDH \cdot NADH} = 0.23 \pm 76.1$ M	– – – –
2	None	$k_{+3} = 70.3 \pm 5.3\ s^{-1}$ $k_{+5} = 9.5 \times 10^6 \pm 3.3 \times 10^6\ s^{-1}$ $K_m^{LDH \cdot NADH} = 146 \pm 4$ M	·····

Kinetic experiments with E_2 = LDH

The Enzyme-Binding Model predicts that both GPDH and LDH kinetic constants should be perturbed by formation of an $LDH_4 \cdot GPDH_2$ complex. This prediction comes from previous data of $v_{obs.}$ measured at various GPDH concentrations performed with E_2 = LDH and E_1 = GPDH (Srivastava *et al.*, 1989). It is therefore possible to quantify the possible formation of $LDH_4 \cdot GPDH_2$ complex by monitoring expected changes in the LDH K_m value for NADH. Regression of the data of Srivastava *et al.* (1989) in terms of the Enzyme-Binding Model suggested that the LDH K_m value for NADH should decrease from 7.1 μM to 0.7 μM if LDH was bound in a complex. Table 2 presents measurements of the LDH K_m value for NADH at increasing GPDH concentrations. The results show no change in the K_m value up to a GPDH concentration of 1.71 μM even though calculations predict that 63% of the total LDH_4 should be complexed to $GPDH_2$ assuming a K_d value of 1 μM and a 1:1 $GPDH_2/LDH_4$ ratio.

DISCUSSION

A comparison between the experimental data of the present paper and the theoretical predictions derived from the five kinetic models revealed discrepancies between expected and observed results. These discrepancies were especially evident when the Direct-Transfer Model was used to describe the GPDH reaction velocity in the presence of increasing concentrations of LDH. Specifically, the Direct-Transfer Model (i) did not accurately predict the changes in observed GPDH velocity at increasing LDH_4 concentrations (Fig. 1) and (ii) predicted an $LDH_4 \cdot NADH \cdot GPDH_2$ complex that was not detected under experimental conditions that readily demonstrated the kinetic effect (Figs. 2*c* and 3). It may be argued that Table 1 was not an accurate test of the Direct-Transfer Model because we assumed that the kinetic constants previously determined for LDH by Srivastava & Bernhard (1985) applied to our system. This point may be addressed by using non-linear regression to find best-fit values for $K_m^{LDH \cdot NADH}$ and k_{+5} of eqn. (7). Using the fixed values of K_m^{NADH} and k_{+3} shown in Table 1 we could obtain $K_m^{LDH \cdot NADH} = 55.7 \pm 66$ μM and $k_{+5} = 1.8 \times 10^{-8} \pm 1.3 \times 10^{-8}\ s^{-1}$. The $K_m^{LDH \cdot NADH}$ value is indicative of a very poor affinity of $LDH_4 \cdot NADH_4$ for $GPDH_2$ and is approx. 50-fold higher than the $K_m^{LDH \cdot NADH}$ value of 1.21 μM previously reported (Srivastava & Bernhard, 1985) and 25-fold higher than the K_m^{NADH} measured for the free enzyme (Table 1). The extremely low k_{+5} value (10^8-fold lower than the $k_{cat.}$ value for the free enzyme; Table 1) indicates that the kinetic $LDH_4 \cdot NADH \cdot GPDH_2$ complex, if it exists, is catalytically inactive under our conditions. Note that the standard errors of these estimates are much larger than the estimates themselves, further indicating that the model is not a good descriptor of the data.

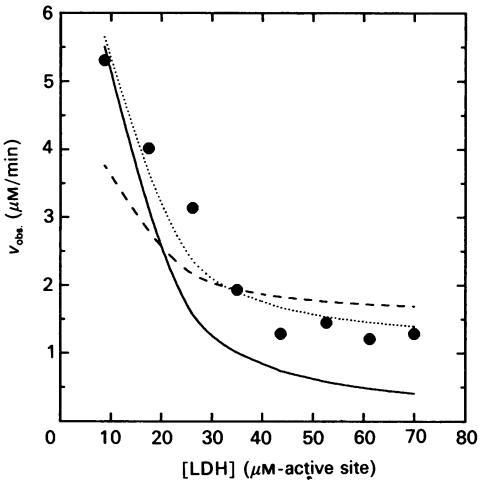

Fig. 4. Analysis of data obtained from Srivastava *et al.* (1989): fit of data to the Equilibrium Model and the Direct-Transfer Model

●, Observed GPDH velocity is plotted as a function of the total number of LDH active sites. Data, from Table 1 of Srivastava *et al.* (1989), were fitted to the Equilibrium Model (——) or the Direct-Transfer Model (– – – –, with fixed k_{+3}; ·····, with varied k_{+3}) with the use of the values reported in Table 3.

It may also be argued that our analysis of the Direct-Transfer Model must be confined to the $LDH_4/GPDH_2$ enzyme pair of the present study. However, a similar conclusion was reached when the data of Srivastava *et al.* (1989) were used to evaluate the Equilibrium Model and the Direct-Transfer Model. Table 3 presents the results of non-linear-regression analysis of these data and Fig. 4 illustrates the regression lines graphically. Note that neither the Equilibrium Model nor the Direct-Transfer Model applies to the data of Srivastava *et al.* (1989). In the case of the Equilibrium Model, the theoretical curve was generated by using the values reported by Srivastava *et al.* (1989) (see —— in

Fig. 4). It is possible to obtain a closer correspondence between observed and theoretical predictions if k_{+3} is not held constant at its reported value (see Table 3). In the case of the Direct-Transfer Model, the ---- curve (Fig. 4) was constructed by using the regression values of Table 3 by holding k_{+3} at a fixed value. Because of the striking disagreement between the experimental data and the Direct-Transfer Model when k_{+3} was fixed, the data were re-fitted assuming that the k_{+3} value reported in Table 1 of Srivastava *et al.* (1989) was incorrect. This curve (variable k_{+3} of Fig. 4) gave a much closer agreement between theoretical prediction and experimental data. However, the k_{+5} and $K_m^{LDH\cdot NADH}$ values obtained from this regression, as well as the standard errors associated with them, are still extremely large (Table 3), indicating that this model does not provide a useful physical description of the reaction in the presence of increasing LDH concentrations. In the absence of any data to the contrary, the assumption that the reported k_{+3} value is incorrect cannot be justified. However, by demonstrating the manipulations required to make the theoretical curves of Fig. 4 correspond to the experimental data, we illuminate the fact that both the Equilibrium Model and the Direct-Transfer Model are poor descriptors of the experimental data.

Binding studies in the present paper also failed to confirm the experimental predictions of the Direct-Transfer Model. Measurement of $LDH_4\cdot NADH_4$ affinity for $GPDH_2$ showed that no tertiary complex existed under conditions that clearly showed a kinetic effect. The absence of a complex was demonstrated by comparing the elution profile for free GPDH with that predicted assuming a 1:1 $GPDH_2\cdot(LDH_4\cdot NADH_4)$ complex with a dissociation constant of 1 μM. A value for the $LDH_4\cdot NADH\cdot GPDH_2$ dissociation constant was estimated from measurements of the GPDH K_m value for $LDH_4\cdot NADH_4$ obtained by Srivastava & Bernhard (1985) and probably represents an upper limit of the true K_d value. The theoretical curve of Fig. 2(*c*) (·····) therefore shows the elution profile expected for a complex with the lowest affinity.

The failure of the Direct-Transfer Model to account for the kinetic and binding data stimulated us to generate other models in an attempt to account for the experimental results. Four such models were developed: the Equilibrium Model, the Enzyme-Binding Model, the Steady-State Model and the Inhibition Model. It was immediately obvious that both the Equilibrium Model and the Steady-State Model adequately predicted the kinetic behaviour of $v_{obs.}$ in Fig. 1. The derivation of these models assumed (i) that LDH_4 acts only to decrease the free NADH concentration and (ii) that $NADH_{free}$ is the only GPDH substrate. The good agreement between theoretical and experimental results demonstrated that it was not necessary to assume a direct transfer of NADH between enzyme active sites to account for the decrease in observed velocity at higher LDH_4 concentrations. The results of the present paper are also in good agreement with the data of Chock & Gutfreund (1988), who indicated that free diffusion of NADH from LDH_4 to GPDH could account for the observed velocities. Tests of the Enzyme-Binding Model and Inhibition Model showed that they also adequately predicted the experimental data (Fig. 1). However, in order to prove these models, it was necessary to demonstrate the existence of $LDH_4\cdot GPDH_2$ or $LDH_4\cdot NADH\cdot GPDH_2$ complexes under the conditions of Fig. 1. The failure of the column regimes to detect complex-formation probably indicates that these latter two models do not apply to the rabbit muscle $GPDH_2$/NADH/LDH_4 system of the present paper.

Finally, it is obvious that kinetic observation of the direct-transfer phenomenon rests primarily on the method used to quantify the LDH_4 concentration in these experiments. Overestimating the LDH_4 concentration would result in a higher actual free NADH concentration (as compared with the calculated value) and give a result consistent with a direct-transfer mechanism. Underestimating the LDH_4 concentration would give the opposite effect: an apparent inhibition of GPDH by increasing LDH_4 concentrations would result. In the absence of a definitive active-site titrant, one must rely either on spectrophotometric analysis of purified enzyme solutions (Chock & Gutfreund, 1988) or on specific-activity measurements of purified enzymes. In the present paper, we used the specific activities for LDH_4 and GPDH reported in the literature to ensure that we were calculating the concentration of functional active sites. By using this method, it can be shown that possible errors in active-site calculations, which may result from less active enzyme preparations, would lead to overestimation of the true LDH_4 concentration. Our method for calculating [LDH_4] should allow us to remove one possible source of error from our calculations.

We thank J. M. Storey for overcoming her fear of equations and proof-reading the typescript. The work was supported by a grant from the Natural Sciences and Engineering Council of Canada to K.B.S.

REFERENCES

Ackers, G. K. (1975) in The Proteins, 3rd edn. (Neurath, H. & Hill, R. L., eds.), vol. 1, pp. 1–94, Academic Press, New York
Barman, T. E. (1969) Enzyme Handbook, vol. 1, pp. 31–48, Springer-Verlag, New York
Black, W. J. (1966) Can. J. Biochem. **44**, 1301–1317
Chock, P. B. & Gutfreund, H. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 8870–8874
Cleland, W. W. (1963*a*) Biochim. Biophys. Acta **67**, 104–132
Cleland, W. W. (1983*b*) Biochim. Biophys. Acta **67**, 173–187
Darnall, D. W. & Klotz, I. M. (1975) Arch. Biochem. Biophys. **166**, 651–682
Dixon, H. B. F. (1976) Biochem. J. **159**, 161–162
Gallant, A. R. (1982) in Nonlinear Regression Analysis and its Applications (Bates, D. M. & Watts, D. G., eds.), pp. 1–52, John Wiley and Sons, Toronto
Gilbert, G. A. & Kellett, G. L. (1971) J. Biol. Chem. **246**, 6079–6086
Holbrook, J. J., Liljas, A., Steindel, S. J. & Rossmann, M. G. (1975) Enzymes 3rd Ed. **11**, 191–298
Lee, Y.-P. & Craine, J. E. (1971) J. Biol. Chem. **246**, 7616–7622
Miller, J. C. & Miller, J. N. (1988) Statistics for Analytical Chemistry, 2nd edn., pp. 137–162, John Wiley and Sons, Toronto
Nichol, L. W. & Winzor, D. J. (1964) J. Phys. Chem. **9**, 2455–2463
Nimmo, I. A. & Bauermeister, A. (1978) Biochem. J. **169**, 437–440
Segal, I. H. (1975) Enzyme Kinetics: Behaviour and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, pp. 560–574, John Wiley and Sons, New York
Srere, P. A. (1987) Annu. Rev. Biochem. **56**, 89–124
Srivastava, D. K. & Bernhard, S. A. (1984) Biochemistry **23**, 4538–4545
Srivastava, D. K. & Bernhard, S. A. (1985) Biochemistry **24**, 623–628
Srivastava, D. K. & Bernhard, S. A. (1986) Curr. Top. Cell. Regul. **28**, 1–68
Srivastava, D. K., Smolen, P., Betts, G. F., Fukushima, T., Spivey, H. O. & Bernhard, S. A. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 6464–6468
Swed, F. S. & Eisenhart, C. (1943) Ann. Math. Stat. **14**, 66–87
Weber, J. P. & Bernhard, S. A. (1982) Biochemistry **21**, 4189–4194
Young, H. L. & Pace, N. (1958) Arch. Biochem. Biophys. **75**, 125–141

Received 5 November 1990/25 March 1991; accepted 3 April 1991