Metabolite-Modulated Complex Formation between α-Glycerophosphate Dehydrogenase and Lactate Dehydrogenase†

Huayong Yong, Gerald A. Thomas, and Warner L. Peticolas*

Department of Chemistry, University of Oregon, Eugene, Oregon 97403

Received March 16, 1993; Revised Manuscript Received July 28, 1993®

ABSTRACT: A modified Hummel–Dreyer equilibrium chromatography technique was used to test the hypothesis that NADH induces the molecular association of lactate dehydrogenase (LDH) and α-glycerol-3-phosphate dehydrogenase (α-GDH). In the presence of a very limited NADH concentration, a unique elution profile with a new peak running immediately ahead of a trough at the free α-GDH elution position is obtained. The appearance of this peak–trough profile is physical evidence that reversible association between LDH and α-GDH occurs over a very limited range of free NADH concentrations. The association constant for this complex formation between LDH and α-GDH is estimated to be 2.0 μM^{-1}. With the NADH concentration increased to saturation level, no evidence of binding is observed. Such concentration-dependent behavior suggests that the strong competition between LDH and α-GDH for the limited amount of NADH tends to promote the enzyme–enzyme contact in order to make the most efficient use of the shared metabolite. The experimental results described in this article make a convincing argument for a metabolite-modulated enzyme–enzyme interaction along this metabolic pathway.

It has been proposed that enzymes along a metabolic pathway such as that of glycolysis may pass metabolites directly from the active site of one enzyme to the active site of another without the metabolite first passing into the solution. This phenomenon is referred to as metabolite channeling and is the subject of considerable controversy [for a detailed review, see Ovadi *et al* (1991)]. This mechanism implies the existence of at least a short-lived (dynamic) complex between the two enzymes sharing the common metabolite. A fact that supports this hypothesis is the high concentration of the glycolytic enzymes and the relatively low concentration of glycolytic intermediates in the cell. It is claimed that this may favor the formation of enzyme–enzyme complexes and the functional segregation of metabolites (Srivastava & Bernhard, 1987; Ovadi *et al.*, 1991). Therefore, metabolism, as it occurs *in vivo*, might be more than merely a sequence of the component catalytic reactions that have been characterized *in vitro* using dilute, purified enzymes. Enzyme–metabolite–enzyme interactions could have important consequences for the energetics and kinetics of metabolic reaction sequences (Srere, 1987; Srivastava & Bernhard, 1986). There appeared to be no direct evidence of the enzyme–enzyme complexes along any metabolic pathway in solution, but indirect evidence has been obtained from kinetic studies suggesting the direct transfer of NADH between dehydrogenases under certain conditions (Srivastava & Bernhard, 1985; Ovadi *et al.*, 1991). Kinetic experiments, however, are usully complicated by the extreme rapidity of enzyme-catalyzed reactions; consequently, controversy has arisen concerning the interpretation of the kinetic data (Chock & Gutfreund, 1988; Srivastava *et al.*, 1989; Wu *et al.*, 1991).

As an alternative to kinetic measurements, new approaches are required to search for direct evidence of metabolite-modulated enzyme–enzyme interaction. One approach to studying the possible physical association of two enzymes in their native, unperturbed states is equilibrium gel filtration chromatography. This technique was introduced by Hummel and Dreyer for equilibrium protein–ligand complexes (1962) and has been reviewed by Ackers (1970). Recently, Gegner and Dahlquist (1991) showed that it is possible to study the existence of short-lived protein–protein complexes using the Hummel–Dreyer procedure. In an effort to determine whether complexes are formed between two enzymes sharing a common metabolite, a modification of the Hummel–Dreyer technique based on the procedure of Gegner and Dahlquist (1991) was employed to study the enzyme–enzyme interaction of L-lactate dehydrogenase (LDH;[1] EC 1.1.1.27) and α-glycerol-3-phosphate dehydrogenase (α-GDH; EC 1.1.1.8) as modulated by the presence of the common metabolite, NADH.

Porcine heart LDH and rabbit muscle α-GDH were used in these exploratory studies because this pair of enzymes provides better separation in the gel filtration experiment. The same pair of enzymes has been shown by kinetic methods to transfer NADH directly, presumably through a short-lived ternary complex (Srivastava & Bernhard, 1985). The enzymes LDH and α-GDH catalyze two reversible reactions that are coupled by the passing of the metabolite NADH or NAD^+. LDH catalyzes the reduction of pyruvate by NADH to lactic acid, while α-GDH catalyzes the oxidation of α-glycerol phosphate (GP) by NAD^+ to dihydroxyacetone phosphate (DHAP). These reactions may be represented as a set of coupled, reversible reactions:

$$\text{pyruvate} + \text{NADH} + H^+ \overset{\text{LDH}}{=} \text{lactate} + NAD^+$$

$$\text{GP} + NAD^+ \overset{\alpha\text{-GDH}}{=} \text{DHAP} + \text{NADH} + H^+$$

If channeling is operating in this reaction, the cofactor NADH will be passed from LDH to α-GDH directly and back again, as suggested by Srivastava and Bernhard (1986). During the moment of transfer, the two enzymes must be in contact. Our experiments attempt to obtain physical evidence of such a complex by using the modified Hummel–Dreyer method. The

† Supported by NIH Grant GM15547 and NSF Grant DMB-9003490.
® Abstract published in *Advance ACS Abstracts*, September 15, 1993.

[1] Abbreviations: LDH, L-lactate dehydrogenase; α-GDH, α-glycerol-3-phosphate dehydrogenase; LADH, liver alcohol dehydrogenase.

0006-2960/93/0432-11124$04.00/0

particular advantage of this technique is its high sensitivity to weak, short-lived enzyme–enzyme complexes. The results reported here not only provide physical evidence of α-GDH–NADH–LDH complex formation in the presence of NADH but also show that this enzyme–enzyme interaction is modulated by the concentration (or chemical potential) of the metabolite.

MATERIALS AND METHODS

Preweighed disodium salts of NADH and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO) and were used without further purification. Reagent-grade chemicals and double-distilled water were used to prepare all buffers and solutions. MOPS buffer (50 mM, pH 6.9), containing 100 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 0.02% NaN_3, was used for all experiments. Ammonium sulfate suspensions of porcine heart LDH, rabbit muscle α-GDH, and lyophilized powder of equine liver alcohol dehydrogenase (LADH) (EC 1.1.1.1) were purchased from Sigma and were used without any further purification.

Enzyme Preparations and Assays. Prior to all measurements, the enzymes were dissolved in MOPS buffer, centrifuged, and filtered on a Sephadex G-25 column to remove traces of ammonium sulfate. The concentrations of LDH, α-GDH, and NADH were monitored spectrophotometrically by using extinction coefficients of $\epsilon_{280} = 1.7 \times 10^5$ for LDH, $\epsilon_{280} = 4.78 \times 10^4$ for α-GDH, and $\epsilon_{340} = 6220\ M^{-1}\ cm^{-1}$ for NADH. The protein molecular weights are 144 000 for LDH and 78 000 for α-GDH. LDH was assayed using pyruvate as a substrate, and the rate of decrease in absorbance of NADH at 340 nm was monitored. Samples of the effluent of the Hummel–Dreyer column were collected each minute and subjected to a standard pyruvate assay. From these data, a normalized LDH enzyme activity profile was constructed as a bar graph for each minute of flow in which the sum of the heights of the bars equaled 1.0.

Hummel–Dreyer Chromatography. The gel filtration column used was a high-performance TSK-GEL G3000 SWXL liquid chromatography column of 30 cm × 7.8 mm i.d. equilibrated in MOPS buffer. Typically, a 100-μL injection of sample was pumped through the column at a rate of 0.4 mL/min. The optical density of the eluted solution was monitored at 280 nm with a Pharmacia LKB-optical unit UV-1. All experiments were performed at 4 °C. The enzymes, α-GDH and LDH, were injected into the column individually to establish their normal retention times, which were checked against molecular weight standards. A mixture of the two enzymes was injected into the column to determine whether a strong complex was formed that traveled with a retention time that is shorter than either of the enzymes individually. Reversible weak binding between two enzymes (E1 and E2) is detected by pre-equilibrating and eluting the gel filtration column in a buffer solution containing a uniform concentration of the smaller enzyme (E2). A 100-μL sample injection of E1 plus the buffer concentration of E2 was applied to the column, creating a band that contains both enzymes. If there is complex formation between the two enzymes, the E2 bound to E1 has increased mobility due to the higher mass of the complex, so that the bound E2 leaves that band of the column. Since the remaining concentration of free E2 in this band is now less than the column concentration by the amount bound to E1, a trough is observed in the constant E2 optical absorption at the normal retention time for E2 as determined above. Also as noted above, if the complex has a long lifetime compared to the column elution time, a peak at the short retention time of the combined molecular weights of the two enzymes will appear. However, for short-lived complexes, the retention time falls between that of the lower molecular weight enzyme and that of the higher molecular weight enzyme (Gegner & Dahlquist, 1991), and a band with a retention time shorter than either E1 or E2 is not observed. Using this method, a strong, long-lived complex and a short-lived complex can be easily distinguished. The possible reasons for a complex to be short-lived will be given in the Discussion section; it has to do with the modification of the association by a ligand that can bind to both the enzymes.

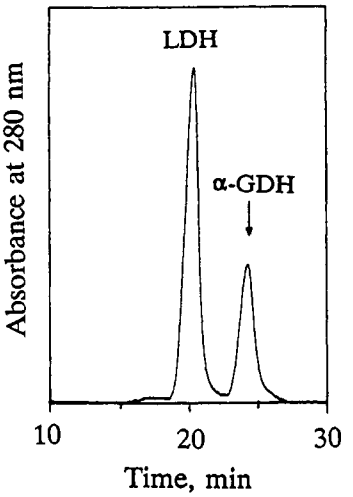

FIGURE 1: Elution profile taken at 280 nm from the gel filtration chromatography of a mixture of the enzymes, LDH, and α-GDH. A solution 1.47 μM in the LDH tetramer, 3.03 μM in the α-GDH dimer, and 0.05 M in MOPS buffer was injected into the column equilibrated in 0.05 M MOPS buffer. The retention times of LDH and α-GDH in the mixture are the same as the times observed when the individual proteins are eluted independently. No peak corresponding to the molecular weight of a 1/1 LDH–α-GDH complex is observed.

RESULTS

In our first set of experiments we searched for a stable, long-lived complex between LDH and α-GDH. We injected both proteins together in the absence of NADH on a column equilibrated with MOPS buffers. As shown in Figure 1, the elution profile obtained is simply the sum of the profiles obtained with LDH and α-GDH individually, *i.e.*, each enzyme runs separately and sharply at its own characteristic retention time determined by its molecular mass. α-GDH consists of two subunits and LDH consists of four subunits, but no evidence is found for the dissociation of these subunits in either protein. There was no evidence from the elution profile of a stable complex α-GDH–LDH at a higher mass than that of LDH. Therefore, if complex formation occurs, it must be in the form of a dynamic or short-lived complex such as that reported between two proteins involved in chemotaxis (Gegner & Dahlquist, 1991).

To determine whether a short-lived complex was present in the absence of NADH, the column was then pre-equilibrated and eluted with the lower molecular weight enzyme, α-GDH, in MOPS buffer. When the solution of LDH and α-GDH is injected into the column, there is a small band on the column that initially contains both LDH and α-GDH, while all of the rest of the column initially contains only α-GDH. As may be seen in Figure 2, a tiny trough is observed at the normal retention time of α-GDH when no NADH is present. Since this binding between LDH and α-GDH is so small, special procedures were taken to obtain a precise measurement of the

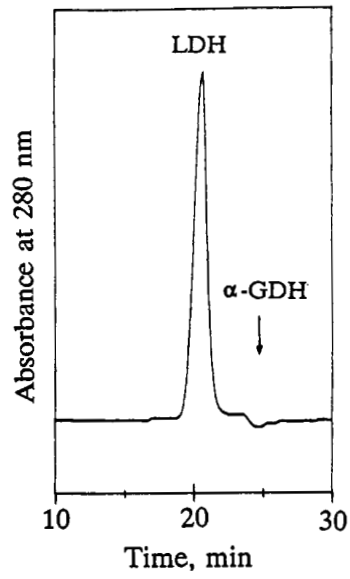

FIGURE 2: Elution profile from Hummel–Dreyer chromatography of LDH and α-GDH in the absence of NADH. A sample 1.57 μM in LDH and 2.94 μM in α-GDH was injected into the column equilibrated in buffer containing 2.88 μM α-GDH (MOPS buffer). A weak trough at the retention time of α-GDH observed. This result is consistent with the temporary binding of α-GDH to LDH. The higher mass of the temporary LDH–α-GDH complex causes it to move out of the band at the α-GDH retention time position, leaving a trough. The α-GDH from the trough is distributed between the α-GDH trough and the LDH peak.

amount of binding. Prior to each experiment, α-GDH blank samples were prepared in a manner to give a zero base line when α-GDH alone was injected into the column against a buffer containing a known concentration of α-GDH. This procedure controls the subtle variation in enzyme concentration that may be induced by the different loading processes of the sample solutions and the equilibrium column solution. The differences between the enzyme concentration in the column and their injected solution necessary to give a flat base line are very small (<0.1 μM out of 1–3 μM). This procedure permits us to determine very weak binding constants in a highly reproducible manner.

Since the injection of LDH plus the α-GDH blank concentration gives a negative trough at the α-GDH elution position, it seems certain that α-GDH is bound to LDH in an amount proportional to the trough area. The absorbance lost must be due to the α-GDH that is missing from the injected band moving more rapidly than the normal α-GDH. This result can only be explained by complex formation that includes the α-GDH as part of an aggregate with a higher mass. The mass balance requires that the α-GDH absorbance that is missing from the trough must be present as a positive absorbance somewhere in the elution profile ahead of the trough. The broadening of the injected band, however, as it travels down the column makes it difficult to measure precisely the free α-GDH concentration in this band—especially for such weak binding as discussed in this article. A series of experiments was carried out in which sample solutions containing a constant LDH concentration plus stepwise-increasing α-GDH concentrations were applied to the column. When the free α-GDH concentration in the sample solution is less than that in the column buffer, a trough is observed at the retention time of α-GDH; when the free α-GDH concentration is greater than the column concentration, a peak is observed.

In order to understand this phenomenon, let us assume that there is no binding between the two proteins. If the injected band contains LDH but no α-GDH, the LDH will run ahead of the α-GDH and a very large trough will appear in the elution profile at the retention time of α-GDH. This trough is "empty" of protein but runs at the velocity of the α-GDH because the α-GDH in the column buffer moves into the back side of the trough and out of the front side of the trough as the α-GDH in the buffer solution is forced down the column. This is the maximum negative trough area that can be obtained and may be set equal to the total α-GDH concentration in the buffer under ideal conditions. Now if the concentration of the α-GDH in the injected band is one-half that in the column buffer, then the trough will have an area one-half that of the area of the empty trough. If the concentration of the α-GDH in the injected band is exactly equal to that in the column buffer, then there will be neither a trough nor a peak but a flat base line. If the concentration of the α-GDH in the injected band is greater than that in the column buffer, a peak will appear. If it is twice that in the column buffer, then the peak will be approximately equal in area to the trough with no α-GDH in the injected band. It is important to note that, if there is no protein–protein association, then both a peak and a trough will never occur in the same elution profile at the elution position of α-GDH. (As we will see later, Figure 6 is an example of the elution profiles obtained when there is no protein–protein binding.)

If binding occurs, the free α-GDH concentration in the injected band is less than the total injection concentration of α-GDH. The α-GDH–LDH complex will move ahead of the free α-GDH, leaving a trough at the α-GDH retention time. (If all of the α-GDH were bound to the LDH, the trough would be same as if no α-GDH were present.) The total concentration of α-GDH in the injected band is $[G_T]_{IB} = [G]_{IB} + [GN]_{IB} + [LNG]_{IB} + [LG]_{IB}$, where we have used L, G, and N for LDH, α-GDH, and NADH, respectively. The total concentration of α-GDH in the column is given by $[G_T]_C = [G]_C + [GN]_C$. The concentration of complexed α-GDH is given by the sum, [LNG] + [LG]. These components will move faster, leaving the free or unbound α-GDH in the injected band on the column. When the unbound α-GDH concentration in the injection band equals the total concentration of α-GDH in the column buffer, *i.e.*, $[G]_{IB} + [GN]_{IB} = [G]_C + [GN]_C$, there will be neither a trough nor a peak at the α-GDH retention time but a flat base line. (There may be, however, an α-GDH peak just ahead of the trough if the α-GDH–LDH complex breaks up at this position on the column and the LDH moves on, resuming its normal mobility.) The amount of bound α-GDH in the injected band is easily calculated as the difference between the total α-GDH concentration injected and the α-GDH concentration in the column buffer. From these data, one can calculate the enzyme–enzyme equilibrium constant.

In light of the above explanation, let us discuss the results of the very weak binding of α-GDH to LDH in the absence of NADH that are shown in Figure 3. Here the binding is so weak that the results are almost those discussed above for the absence of binding. Figure 3 shows a series of elution profiles consisting of plots of the absorbance at 280 nm of the effluent *versus* time. In these experiments, the concentration of the α-GDH in the injected band was varied from zero to about twice the column buffer concentration. The area of the trough or peak at the α-GDH elution position was plotted against the total α-GDH concentration injected. As shown in the inset of Figure 3, an approximately linear relationship was obtained. At the intercept with the zero line where no trough or peak was observed, the free α-GDH concentration

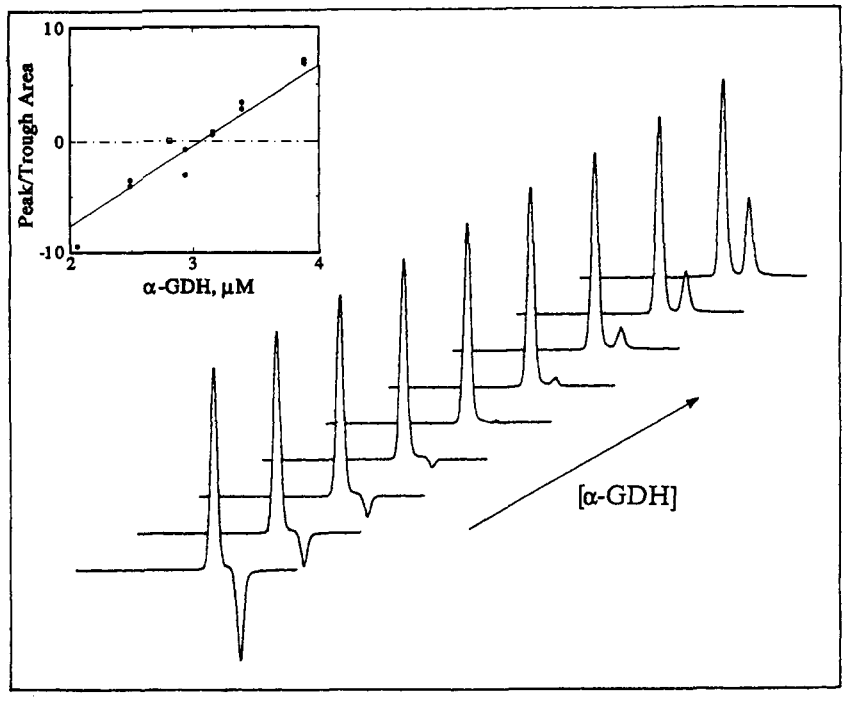

FIGURE 3: Plot of a series of Hummel–Dreyer protein elution profiles for the binding of α-GDH to LDH in the absence of NADH. The column is equilibrated with 2.88 μM α-GDH in MOPS buffer. The concentration of LDH in the injected band is held constant at 1.57 μM, while the various concentrations of α-GDH are 0.0 (bottom plot), 2.05, 2.49, 2.94, 3.16, 3.39, 3.89, 4.63, and 5.92 μm (top plot). The original data are fit to mathematical models for the purposes of data processing and display. However, the original data are used in the calculation. The inset figure shows a plot of the trough or peak areas at the α-GDH elution position as a function of the total concentration of α-GDH in the injected band based on the original data. The α-GDH concentration at which the trough/peak area is zero occurs at a slightly higher concentration than that of the α-GDH in the buffer, denoted by an empty square. From these data, an association constant of 0.04 μM^{-1} is obtained. These data are quite reproducible.

in the sample is equal to the known α-GDH concentration in the column buffer. As the total concentration of α-GDH at this intercept is slightly greater than the column buffer concentration (shown as the empty square on the zero line), it follows that complex formation has occurred and the bound α-GDH was obtained as the difference between the two concentrations. Repeated experiments showed that these results are quite reproducible, even for the small amount of binding that occurs in the absence of NADH. If one assumes a one-to-one binding complex, the apparent association constant is given by

$$K_a = [\alpha\text{-GDH}]_B/[\alpha\text{-GDH}]_\beta[\text{LDH}]_F$$

where $[\alpha\text{-GDH}]_\beta$ is the concentration of α-GDH in the column buffer; the concentration of the bound α-GDH is given by $[\alpha\text{-GDH}]_B = [\alpha\text{-GDH}]_T - [\alpha\text{-GDH}]_\beta$; $[\alpha\text{-GDH}]_T$ is the total concentration of α-GDH in the injected band at the intercept; and $[\text{LDH}]_F$ is the free concentration of LDH, which is given by $[\text{LDH}]_F = [\text{LDH}]_T - [\alpha\text{-GDH}]_B$. In the absence of NADH, K_a was found to be 0.04 ± 0.02 μM^{-1}.

If NADH is being passsed from LDH to α-GDH and back again as suggested by Srivastava and Bernhard (1985) and others (Ovadi *et al.*, 1991), some dependence of the complex formation on NADH concentration might be expected. In particular, the effect might be expected to saturate with NADH concentrations approaching the concentration of the active sites of the two enzymes since there is no longer any need to pass the cofactor back and forth. To test this hypothesis, the above Hummel–Dreyer experiments were repeated at two different [NADH]/[α-GDH] concentration ratios. In these experiments, the column was pre-equilibrated and eluted with a solution containing both α-GDH and NADH in MOPS buffer. The [NADH]/[α-GDH] ratios were either 1/2 or 2. Solutions containing a constant concentration of LDH and a variety of α-GDH and NADH concentrations at the same [NADH]/[α-GDH] ratio as the column buffer were injected onto the column. When the column buffer contained one NADH for every two α-GDH molecules (or, since α-GDH is a dimer, one NADH for every four active α-GDH sites), the elution profiles shown in Figures 4 and 5A were obtained. These seem to be examples of an unusual elution pattern since a positive peak running faster than that of α-GDH was found just in front of the trough at the free α-GDH position, which did not occur when α-GDH–NADH was injected alone. Figure 5B shows that the peak starts to grow at about 2 μM α-GDH injection concentration, while the trough is not filled up until about 4.5 μM. The α-GDH concentration at which the trough area becomes zero occurs at a much higher concentration than the buffer α-GDH concentration. If one assumes that the difference between the total α-GDH concentration of the injected band that gives a zero base line at the position where the α-GDH normally runs and the α-GDH concentration on the column is the concentration of the α-GDH that is bound to the LDH, one obtains an association constant of 2.0 ± 0.6 μM^{-1}.

In order to determine the saturation effect of higher NADH concentrations on enzyme–enzyme binding, a series of elution profiles was obtained using a column buffer and injected band with a NADH concentration twice that of the α-GDH concentration. Neither a trough at the α-GDH retention time nor any new peaks were observed when a sample that contained α-GDH and NADH at the buffer concentrations and LDH was applied to the column. Figure 6 shows a result expected for no binding. The peak does not start to grow until the

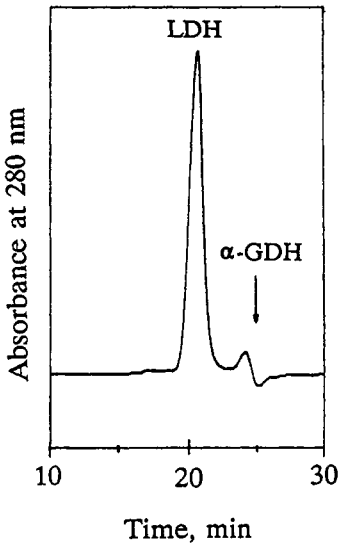

FIGURE 4: Elution profile from Hummel–Dreyer chromatography of LDH and α-GDH in the presence of NADH. Shown in a plot of the absorbance at 280 nm as a function of the retention time from the gel filtration column in which the ratio of [NADH]/[α-GDH] present is 1/2 both in the column buffer and in the injected band. In this experiment the column is equilibrated with 3.12 μM α-GDH and 1.56 μM NADH in MOPS buffer. The total concentrations of LDH, α-GDH, and NADH in the injected band are 1.47, 3.26, and 1.63 μM, respectively. A new peak running slightly ahead and leading the trough at the normal elution position of α-GDH was observed as a result of the unstable binding of α-GDH to LDH in the presence of a limited amount of NADH. (see text).

injected α-GDH concentration is larger than the concentration in the column buffer. The inset in Figure 6 shows the linear plot of the area of the peak or trough of each run at the α-GDH elution position *versus* the total α-GDH concentration in the injected band. As shown in this inset, when the total α-GDH concentration in the injected band exactly equals the column α-GDH concentration, there is neither a trough nor a peak. This intercept with zero area is denoted by a solid square in the inset. This means that $[\alpha\text{-GDH}]_T = [\alpha\text{-GDH}]_\beta$, so that under the condition [NADH]/[α-GDH] = 2/1, no complex is formed between α-GDH and LDH. The fact that NADH at high concentration prevents association of the proteins is a blank or null experiment that supports the existence of α-GDH–LDH association at low NADH concentrations where the trough in the elution pattern is observed.

To better understand the odd elution patterns shown in Figure 4, where a small peak is found at the leading edge of the trough, fractions of the effluent were collected as a function of time. These samples were assayed for LDH enzymatic activity. The results indicate that no LDH was present in the effluent in the region of the α-GDH retention time (Figure 7), nor was an accumulation of NADH within that region detected from measurements of optical absorbance at 340 nm. Therefore, the contribution to the new peak that is eluted slightly faster than the free α-GDH comes only from the α-GDH missing in the trough at the free α-GDH position. (It is inherently difficult to assay for α-GDH due to the strong background contributed by α-GDH in the column buffer.) Again, none of the other fractions were found to contain both LDH and α-GDH above the uniform background, which suggests no stable complex formation, as expected. It might be noted that this behavior is different from that reported by Gegner and Dahlquist (1991) where the absorption intensity lost in the trough appears to be distributed uniformly along a plateau between the front of trough and the trailing edge of the peak of the higher molecular weight protein.

As an additional blank experiment, we carried out parallel experiments for another pair of enzymes, LDH and LADH (which is similar in size to α-GDH), in the absence and presence of NAD^+ or NADH. No detectable evidence of binding was observed under the experimental conditions used, as expected because the stereospecificity of the two enzymes is identical.

DISCUSSION

From the results shown above, we found that, under specialized conditions and in the presence of a very limited range of NADH concentrations, a strange chromatogram with a new peak running immediately ahead of a trough at the free α-GDH elution position is obtained. Enzyme assays and absorption experiments show that this new peak did not come from an accumulation of either LDH or NADH, but consisted entirely of α-GDH that was running slightly ahead of the trough where α-GDH normally runs. The most likely interpretation for this anomalous elution pattern comes from the dependence of the α-GDH–LDH complex on the concentration of the NADH. We have seen that with no NADH there is only very weak binding of the two proteins. When the NADH concentration equals one-half that of α-GDH, a larger trough is observed in the Hummel–Dreyer elution profile. This is very strong evidence that the α-GDH that is missing from the injected band has a higher mobility than that in the column. The only reasonable explanation for this increased mobility of α-GDH is the formation of a higher molecular weight complex with LDH that moves down the column at a rate faster than that of α-GDH alone. The peak leading the α-GDH trough shows that the α-GDH absorbance lost in the trough has been deposited in the peak. The question arises: Why does the complex dissociate as it moves ahead of the injected band? The explanation for this can be found in the fact that, as the NADH concentration gets higher, the α-GDH–LDH complex does not form. In the injected band, the free NADH concentration is much less than in the column concentration because of the binding of NADH to LDH and the α-GDH–LDH complex, neither of which is present in the column. When this complex moves down the column ahead of the α-GDH and NADH in the injected band, it is now in a higher NADH concentration.

We have calculated the concentration of the free NADH both in the injected band and in the column buffer using published equilibrium constants for the binding of NADH to the two proteins. These calculations are shown in the Appendix of this article. The results indicate that the free NADH concentration in the column is 2–3 times that in the injected band depending upon the exact model used, in spite of the fact that the ratio [NADH]/[α-GDH] is always kept at 1/2. We propose that when the α-GDH–LDH complex moves down the column ahead of the injected band, it binds more NADH because the free NADH concentration is higher in the column buffer. With more NADH bound there is no longer a need to share the metabolite between the two enzymes so that the complex dissociates, leaving the α-GDH just at the leading edge of the trough from which it traveled. The LDH continues on its way ahead because of its higher mobility. In this way one accounts for the appearance of the LDH band at its normal retention time: a peak of the α-GDH that comes out just ahead of the trough which runs at the normal retention time of the α-GDH. When the concentration ratio of NADH/α-GDH in the injection band and column is increased, the tendency of complex formation will be reduced for the same reason stated above. At the saturation of α-GDH by NADH, no evidence of binding is observed.

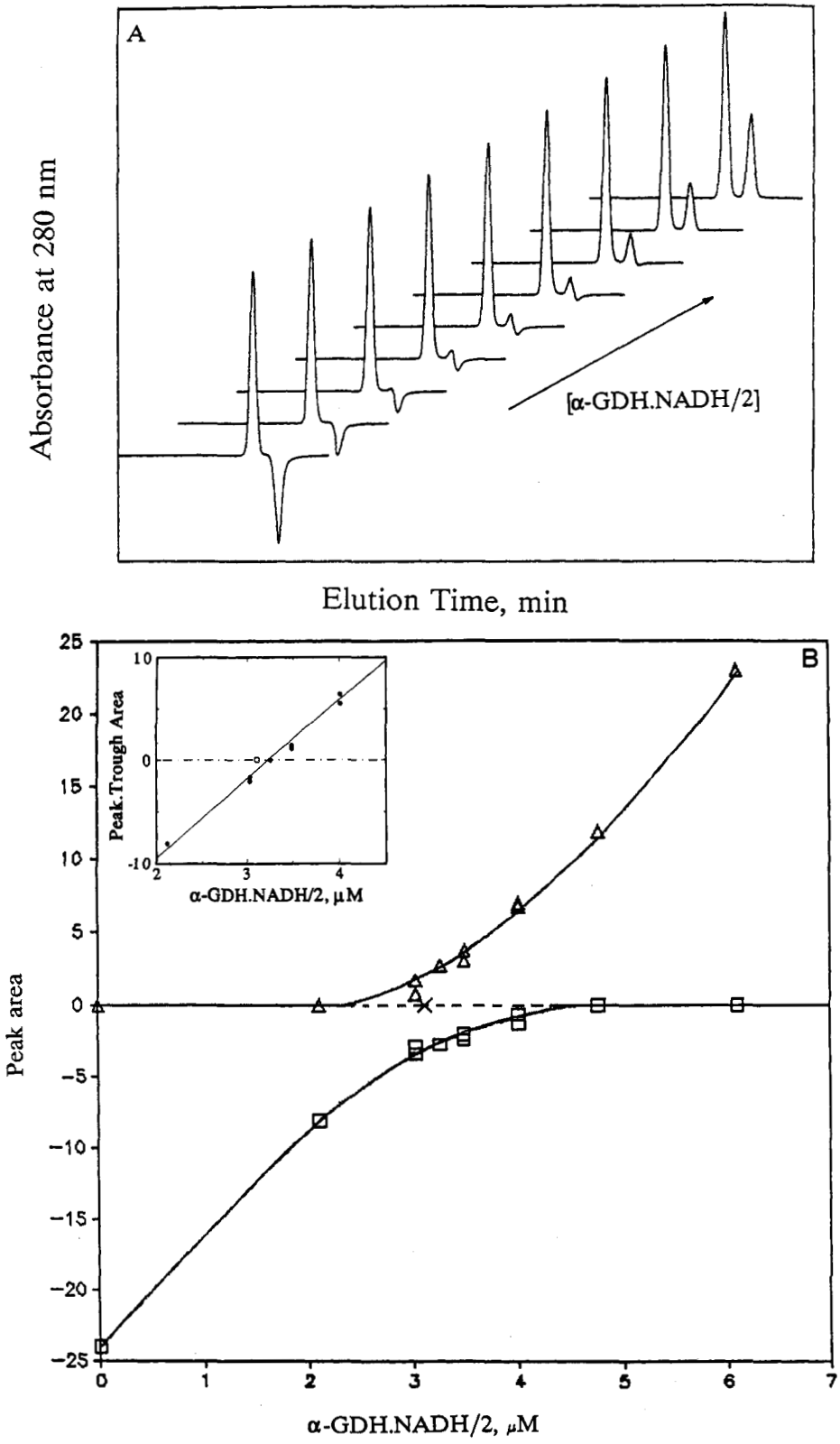

FIGURE 5: (A) Plot of a series of Hummel–Dryer elution profiles. In these experiments the column is equilibrated with 3.12 μM α-GDH and 1.56 μM NADH in MOPS buffer. The concentration of LDH in the injected band is held constant at 1.47 μM while the various concentrations of α-GDH are 0.0 (bottom plot), 2.11, 2.57, 3.03, 3.26, 3.49, 4.01, 4.77, and 6.10 μM (top plot). The [NADH]/[α-GDH] ratio is maintained at 1/2. The original data are fit to a mathematical model for the purposes of data processing and display. However, the original data are used for the calculations. (B) Plot of the trough and peak areas as a function of the total concentration of α-GDH in the injected band based on the same data as in A. Note that between 2 and 3 μM α-GDH the trough area starts to level off to zero and the peak area starts to rise. From about 2 to 4.5 μM there is both a peak and a trough. The addition of the positive area of the peak to the negative area of the trough gives the straight line that would be observed if no NADH were present in the injected band. This shows that the absorbance lost in the trough is due to the α-GDH–LDH–NADH complex that dissociates as it moves along the column into a region of higher free NADH concentration, leaving the α-GDH deposited at the leading edge of the trough. When the concentration of the unbound α-GDH in the injected solution equals that of the column buffer, the trough area is zero. This occurs at a much higher total α-GDH concentration than the α-GDH concentration in the column buffer, which is denoted by ×. If one assumes that the difference between the two concentrations is the concentration of α-GDH that is bound to LDH, one obtains an association constant of 2.0 μM^{-1}.

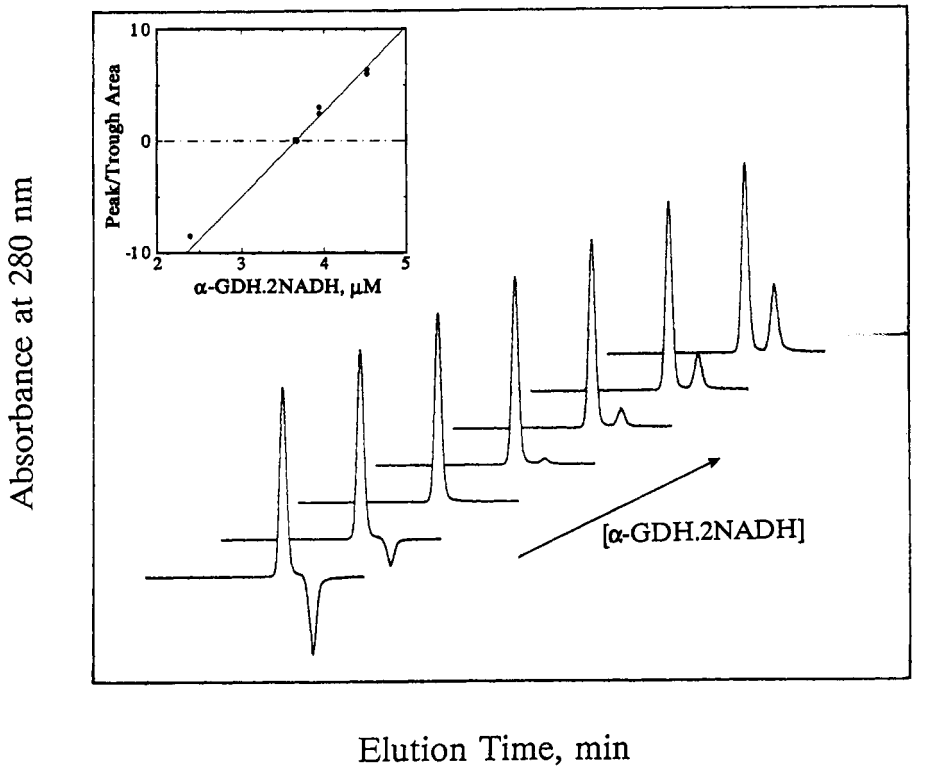

FIGURE 6: Plot of a series of Hummel–Dreyer elution profiles from the gel filtration column when the ratio of [NADH]/[α-GDH] present is 2/1 both in the column buffer and in the injected band. In these experiments the column is equilibrated with 3.67 μM α-GDH and 7.34 μM NADH in MOPS buffer. The concentration of LDH in the injected band is held constant at 1.78 μM while the various concentrations of α-GDH are 0.0 (bottom plot), 2.39, 3.67, 3.94, 4.53, 5.39, and 6.89 μM (top plot). The original data are fit to a mathematical model for the purposes of data processing and display. The original data, however, are used for the calculation. Inset: Plot of the trough or peak area as a function of the total concentration of α-GDH in the injected band based on the same data as the main figure. The α-GDH concentration at which the trough area is zero occurs at exactly the same concentration as the concentration of α-GDH in the buffer, denoted by the solid square. Thus, in an excess of NADH there is no observable α-GDH–LDH binding. (Note that both a peak and a trough never occur in the same elution profile at the elution position of α-GDH when there is no binding.)

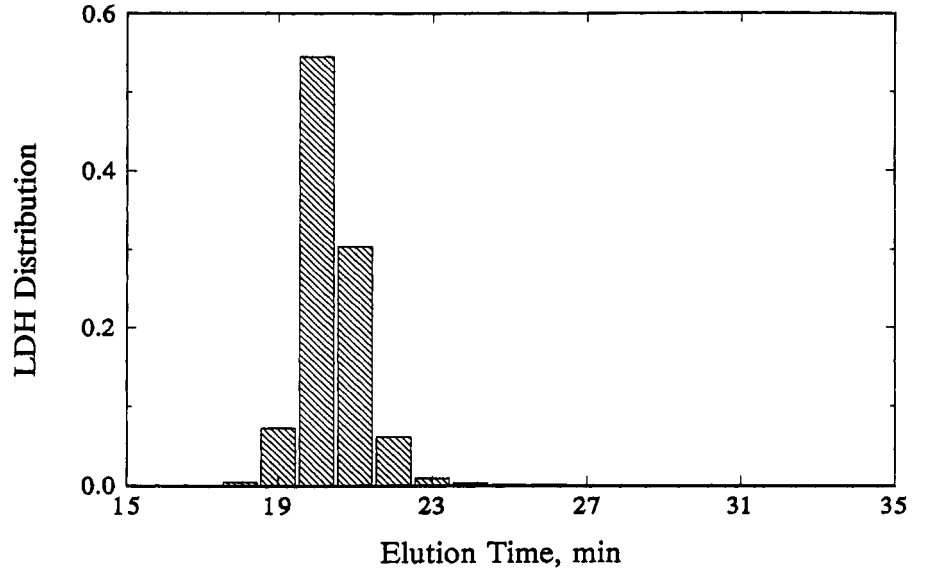

FIGURE 7: Plot of LDH distribution as a function of elution time. A sample of 1.8 μM LDH, 3.4 μM α-GDH, and 1.7 μM NADH was injected into the column equilibrated with 3.2 μM α-GDH and 1.6 μM NADH in MOPS buffer. The effluent fractions were collected and assayed for LDH enzymatic activity. No LDH distribution was found outside the LDH peak area.

One can speculate about the biological relevance of the association of LDH and α-GDH in the presence of a limited amount of NADH. One can assume that there is a strong competition between LDH and α-GDH for the limited amount of NADH, which because of its short supply can only bind to part of the two enzymes. These conditions tend to promote enzyme–enzyme contact in order to make the most efficient use of the shared metabolite. This α-GDH–NADH–LDH complex is probably very unstable, dynamically assembled and disassembled within a very short time scale. When the concentration of NADH is increased, the need for sharing the metabolite no longer exists as each enzyme has access to an adequate supply. In this case the association no longer exists.

The specialized condition required for the competition of NADH here is not strange at all, since the concentrations of the glycolytic enzymes *in vivo* are comparable to or exceed

those of the glycolytic intermediates (Srivastava & Bernhard 1987) and thus are of physiological significance. As indicated by Srivastava and Bernhard (1986), in cells, with its total concentration only a small fraction of the total dehydrogenase binding sites, the distribution of bound NADH among the dehydrogenase must involve some sort of special mechanism, in regard to the commonly high affinity for NADH by all the dehydrogenases. In a certain respect, there is a similarity between the method we used and the "enzyme buffering technique" used in the kinetic approach, in which the experimental conditions are that the concentration of E1 exceeds that of NADH; hence, the concentration of free (aqueous) NADH is exceedingly low. By comparing the observed rate of the E2-catalyzed reaction utilizing NADH with that predicted by the equilibrium concentration of free NADH, either random diffusion or a direct transfer mechanism may be deduced (Srivastava & Bernhard, 1984). As mentioned before, however, the interpretation of these kinetic data is quite controversial. Only with our method does the achievement of direct evidence of enzyme–enzyme interaction become possible.

To summarize the findings: The reproducibility of the data and the procedures used to eliminate experimental artifacts provide confidence in our conclusion. The experimental controls include the evidence that the association that occurs between LDH and α-GDH is quite specific since the control experiment with LADH did not reveal any association with LDH using the same shared metabolite. The chemical identification of the compositions of the peaks by enzyme assays assures us that the first peak is LDH and the second weak peak is only α-GDH. Finally, our association constants of 0.04 ± 0.02 and $2.0 \pm 0.6\ \mu M^{-1}$ for cases of the absence and presence of limited amounts of NADH, respectively, are in excellent agreement with the theoretical predictions of Smolen and Keizer (1990), who have calculated association constants on the order of $1\ \mu M^{-1}$ for complexes between two enzymes and a metabolite—almost exactly the model we have been required to adopt here. The experiment results described in this article suggest that if channeling does occur along a metabolic pathway, it will be highly dependent on the metabolite contribution and possibly may only exist over a short region of metabolite and enzyme concentrations. This concept may help to resolve some of the differences that have been reported in this field (Ovadi *et al.*, 1991).

ACKNOWLEDGMENT

The authors thank the Biotechnology Laboratory for generously providing the column and Professor R. Dahlquist, Dr. D. Graham, and Dr. J. A. Gegner for helpful suggestions and technical assistance. This paper is dedicated to the late Dr. Sidney Bernhard.

APPENDIX: CALCULATION OF FREE NADH CONCENTRATION

Model I. Simplified Model without Considering Ternary Complex Formation.

$$\text{LDH} + \text{NADH} \overset{k_1}{\rightleftarrows} \text{LDH–NADH}$$
$$k_1 = [\text{LDH}][\text{NADH}]/[\text{LDH–NADH}]$$

$$\alpha\text{-GDH} + \text{NADH} \overset{k_2}{\rightleftarrows} \alpha\text{-GDH–NADH}$$
$$k_2 = [\alpha\text{-GDH}][\text{NADH}]/[\alpha\text{-GDH–NADH}]$$

Solve for $[\text{NADH}]^{\text{I}}_{\text{free}}$, the free NADH concentration in the injection band, and

$$\alpha\text{-GDH} + \text{NADH} \overset{k_2}{\rightleftarrows} \alpha\text{-GDH–NADH}$$

Solve for $[\text{NADH}]^{\beta}_{\text{free}}$, the free NADH concentration in the column buffer. LDH and α-GDH represent protein subunits and $k_1 = 0.35\ \mu M$ and $k_2 = 0.88\ \mu M$ are the site dissociation constants for LDH and α-GDH published by Srivastava and Bernhard (1987). On the basis of the experimental data shown in Figure 4, one calculates that $[\text{NADH}]^{\beta}_{\text{free}} > 3[\text{NADH}]^{\text{I}}_{\text{free}}$.

Model II. Considering Simple Ternary Complex Formation.

$$\text{LDH} + \text{NADH} \overset{K_1}{\rightleftarrows} \text{LDH–NADH}$$
$$K_1 = [\text{LDH}][\text{NADH}]/[\text{LDH–NADH}]$$

$$\alpha\text{-GDH} + \text{NADH} \overset{K_2}{\rightleftarrows} \alpha\text{-GDH–NADH}$$
$$K_2 = [\alpha\text{-GDH}][\text{NADH}]/[\alpha\text{-GDH–NADH}]$$

$$\text{LDH–NADH} + \alpha\text{-GDH} \overset{K_3}{\rightleftarrows} \text{LDH–NADH–}\alpha\text{-GDH} \overset{K_4}{\rightleftarrows} \text{LDH} + \alpha\text{-GDH–NADH}$$

Solve for $[\text{NADH}]^{\text{I}}_{\text{free}}$ in the injection band, with LDH and α-GDH representing LDH tetramer and α-GDH dimer, and

$$\alpha\text{-GDH} + \text{NADH} \overset{K_2}{\rightleftarrows} \alpha\text{-GDH–NADH}$$

Solve for $[\text{NADH}]^{\beta}_{\text{free}}$ in the column buffer, with

$$K_1 = k_1/4, \qquad K_2 = k_2/2$$

by assuming the binding sites for NADH are identical and independent. The calculation results based on the experimental data shown in Figure 4 are $[\text{NADH}]^{\beta}_{\text{free}} \geq 3[\text{NADH}]^{\text{I}}_{\text{free}}$.

REFERENCES

Ackers, G. K. (1970) *Adv. Protein Chem. 24*, 343–443.

Chock, P. B., & Gutfreund, H. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8870–8874.

Gegner, J. A., & Dahlquist, F. W. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 750–754.

Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.

Ovadi *et al.* (1991) *J. Theor. Biol. 152*, 1–141.

Smolen, P., & Keizer, J. (1990) *Biophys. Chem. 38*, 241–263.

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., & Bernhard, S. A. (1987) *Annu. Rev. Biophys. Chem. 16*, 175–204.

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.

Wu, X., Gutfreund, H., Lakatos, S., & Chock, P. B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 497–501.