# Functional Asymmetry of Tetrameric Glyceraldehyde-3-phosphate Dehydrogenase in the Transient Kinetics of Reductive Dephosphorylation of 1,3-Diphosphoglycerate†

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ABSTRACT: The transient kinetics of the reductive dephosphorylation of 1,3-diphosphoglycerate catalyzed by sturgeon glyceraldehyde-3-phosphate dehydrogenase has been studied. When the reaction is initiated with the unacylated, coenzyme free apoenzyme, a well-defined lag phase is observed. This lag phase is invariant with respect to the enzyme and NADH concentrations but shows a hyperbolic dependence with respect to the concentration of 1,3-diphosphoglycerate in the reaction mixture. Direct spectrophotometric observations of the acylation of apoglyceraldehyde-3-phosphate dehydrogenase by 1,3-diphosphoglycerate indicate that this process is relatively slow and shows saturation kinetics with respect to the substrate. This acylation reaction is strongly activated by $NAD^+$ which is a product of the overall reaction. The occurrence of a positive lag phase can be explained on the basis of these observations. Furthermore, this lag phase disappears upon preincubation of the enzyme with $NAD^+$ or 1,3-diphosphoglycerate. However, a new transient is observed when more than *two* of the *four* active sites are preacylated by the substrate or saturated with $NAD^+$. This transient appears as a pseudoburst or negative lag phase. It is shown that the final steady state of the system is not dependent on the initial state of the enzyme. It is proposed that this negative lag phase arises from the slow and irreversible transition from a first steady state involving the fully acylated enzyme into a second steady state which corresponds to the catalytic cycling of a "half of the sites" diacylated enzyme species. The turnover rate of the first steady state is about twice as fast as the second one. This functional pairwise asymmetry of the enzyme oligomer is discussed in relation to other anticooperative properties of the enzyme tetramer.

Muscle glyceraldehyde-3-phosphate dehydrogenases from various species exhibit anticooperative properties which have been exhaustively described over the past 10 years. The anticooperative behavior of tetrameric Glyc-3-P-dehydrogenase[1] is apparent through two related but distinct phenomena. (1) Negative cooperativity in the binding of the coenzyme $NAD^+$ to the apoenzyme. In the case of sturgeon Glyc-3-P-dehydrogenase, the $NAD^+$ binding isotherm can be resolved into two distinct classes of two binding sites each (Seydoux et al., 1973; Kelemen et al., 1975; Long & Dahlquist, 1977). (2) Half-site reactivity with the substrate analogue β-(2-furyl)acryloyl phosphate (Malhotra & Bernhard, 1968, 1973) and various alkylating reagents (Levitzki, 1974).

These two phenomena have been discussed within the framework of the general, induced model (Levitzki & Koshland, 1976) and the preexistent asymmetry model of Bernhard & Mac Quarrie (1973). Both models postulate the occurrence of asymmetrical quaternary structures of the enzyme tetramer which are energetically favored over symmetrical states. Arguments in favor of each model have been discussed elsewhere (Seydoux et al., 1974; Levitzki & Koshland, 1976).

Although the physiological significance of these anticooperative phenomena has given rise to various speculations (Levitzki & Koshland, 1976; Schwendimann et al., 1976), recent observations have raised doubt about the involvement of anticooperative phenomena in the physiological reaction of Glyc-3-P-dehydrogenase. These observations can be summarized as follows. (1) The four active sites of the enzyme oligomer were found equivalent in various reactions of the lobster and sturgeon enzymes with physiological substrates (Trentham, 1971). Noteworthily, all four active sites of the enzyme can be acylated by 1,3-$P_2$Glyc or Glyc-3-P in the presence of an excess of $NAD^+$ (Trentham, 1971; Seydoux et al., 1973; Kelemen et al., 1975). (2) The binding of $NAD^+$ to the 3-phosphoglycerol enzyme, a physiologically important reaction intermediate (Bloch et al., 1971), is not anticooperative in the case of the sturgeon enzyme (Seydoux et al., 1976).

In view of these contradictory observations, we analyze in the present paper the pre-steady-state kinetics of the reductive dephosphorylation of 1,3-$P_2$Glyc catalyzed by sturgeon Glyc-3-P-dehydrogenase. According to previous studies (Trentham, 1971; Duggleby & Dennis, 1974; Seydoux et al., 1976), this two substrates–three products reaction proceeds via two consecutive steps.

$$\underset{}{ECH_2SH} + \underset{\text{1,3-}P_2\text{Glyc}}{RCOOPO_3^{2-}} \rightleftharpoons \underset{\text{acyl enzyme}}{ECH_2SCOR} + HPO_4^{2-} \quad (1a)$$

$$ECH_2SCOR + NADH + H^+ \rightleftharpoons ECH_2SH + \underset{\text{D-Glyc-3-P}}{RCHO} + NAD^+ \quad (1b)$$

The first step (eq 1a) corresponds to the acylation of an essential cysteine by 1,3-$P_2$Glyc. In the absence of an excess of orthophosphate as acceptor, this reaction is nearly irreversible (Seydoux et al., 1973). The second step (eq 1b) corresponds to the reductive deacylation of the thioacyl enzyme, which becomes nearly irreversible in the presence of a sufficient excess of NADH. As we shall demonstrate, the effect of the preincubation of the enzyme with 1,3-$P_2$Glyc and $NAD^+$ on the transient kinetics of this reaction indicates that a half-acylated species predominates under steady-state conditions. This lead us to the conclusion that functional asymmetry of the enzyme oligomer is also apparent in the

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[1] Abbreviations used: Glyc-3-P-dehydrogenase, D-glyceraldehyde-3-phosphate dehydrogenase; Glyc-3-P, glyceraldehyde 3-phosphate; 1,3-$P_2$Glyc, 1,3-diphosphoglycerate.

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reaction of Glyc-3-P-dehydrogenase with its physiological substrates.

## Experimental Section

*Reagents.* DL-Glyceraldehyde-3-phosphate, $NAD^+$, and NADH (grade III) were purchased from Sigma Chemical Co. 1,3-$P_2$Glyc was prepared, purified, and assayed as previously described (Furfine & Velick, 1965). Fractions of this substrate (up to 3 mM) were frozen and stored in liquid nitrogen. 1,3-$P_2$Glyc can be kept in these conditions for several weeks with only negligible hydrolysis.

*Enzyme.* Highly purified sturgeon muscle Glyc-3-P-dehydrogenase was prepared by a procedure similar to that described by Seydoux et al. (1973). The specific activities and $A_{280}/A_{260}$ ratio of the enzyme were never below 300 units and 2.05, respectively. The contamination of the apoenzyme by $NAD^+$ was found previously to be less than 0.3% (Seydoux et al., 1973).

*Stopped-Flow Measurements.* Kinetic studies reported herein were carried out with a single beam absorbance stopped-flow apparatus, Durrum Gibson Model 13002. Two distinct observation chambers were used, one for the kinetic studies of the reductive dephosphorylation of 1,3-$P_2$Glyc (1.95-cm optical path) and the other for direct acylation studies of the apoenzyme by 1,3-$P_2$Glyc (1.75-cm optical path). The dead time of the instrument was about 3 ms. Monochromatic light was obtained from a 12-V 50-W quartz–tungsten–iodine lamp (Osram) at wavelengths > 330 nm or a deuterium lamp at wavelengths < 330 nm and a grating monochromator, Bausch et Lomb (type 500 mm serie 210UB), with a slit aperture of 2 mm (i.e., 4-nm dispersion).

A Tektronix oscilloscope, type RM 564, and a Sefram Servotrace type PE recorder were used for the analogic recordings of the signal. Kinetic records were generally made over a 0.2–2-s time range with a time constant of 1–2 ms.

All experiments were carried out in Tris–sulfonate (10 mM), KCl (0.1 M), and EDTA (1 mM) standard buffer, pH 7.0. In order to facilitate the observation of transient phases and to avoid partial hydrolysis of the substrate, the stopped-flow apparatus was thermostated at 10 °C.

*Data Acquisition.* The analogic output of the photomultiplier (EMI 9558QA) of the Durrum stopped-flow apparatus was first transformed into logarithmic form and then, after analogic to digital conversion, stored in the memory of a Model 802 Biomation transient recorder. The kinetic data (1024 points of 8 bits each) were then transferred into a Wang 2200B minicomputer through a Wang 2250 interface. This simple data acquisition system allows numerical averaging of several kinetic runs (from 4 to 7 in practice) and substraction of the base line. This permits the elimination of a substantial part of the signal perturbation which occurs after stopping of the flow. Hence, the resolution of the progress curves can be considerably improved, and this makes possible the detection of very small changes in absorbance (i.e., in the order of $2 \times 10^{-4}$ absorbance units at 370 nm with a time constant of 2 ms).

*Numerical Treatment of the Kinetic Data.* Kinetic data were analyzed with iterative curve fitting procedures programmed in the BASIC version of the Wang 2200B calculator. Two independent methods were used for a quantitative evaluation of the lag time. (1) The progress curve was fitted to eq 2, which relates the observed changes in absorbance ($\Delta A$)

$$\Delta A = at + b(e^{-kt} - 1) \qquad (2)$$

to a first-order change in activity (exponential term) and a zero-order term. The parameter $k$ is the first-order rate constant associated with the transient process, and the observed

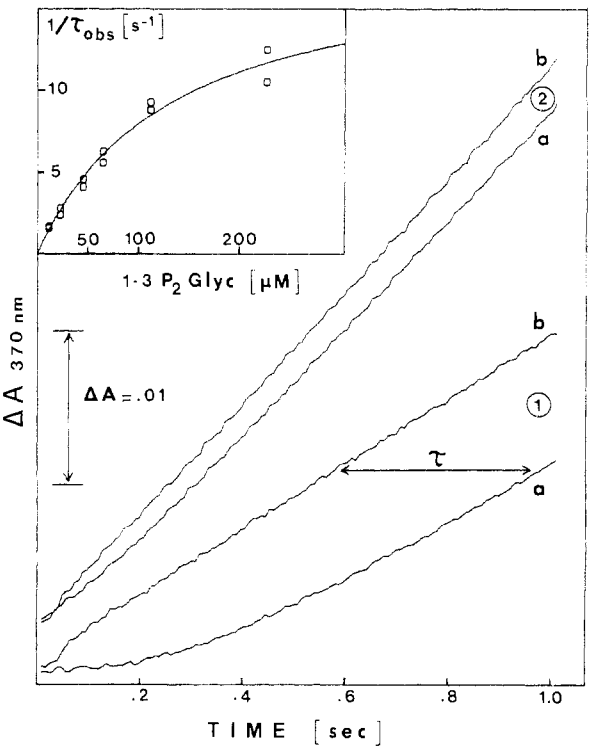



FIGURE 1: Kinetics of the Glyc-3-P-dehydrogenase-catalyzed reductive dephosphorylation of 1,3-$P_2$Glyc. Dependence of the lag phase of the reaction of Glyc-3-P-dehydrogenase with respect to the 1,3-$P_2$Glyc concentration in the reaction mixture. The reaction mixture contained, in standard Tris–sulfonate buffer, 50 μM NADH, 22 μM (1) or 224 μM (2) 1,3-$P_2$Glyc, and 0.06 μM apo-Glyc-3-P-dehydrogenase (a) or the enzyme at the same concentration, preincubated with 0.18 μM 1,3-$P_2$Glyc (b). One syringe contained 1,3-$P_2$Glyc and NADH and the other contained the enzyme. The actual lag time $\tau$, as defined in the experimental section, is indicated by the arrow. The insert shows the hyperbolic dependence of the lag time ($\tau_{obsd}$) with respect to the final concentration of 1,3-$P_2$Glyc.

lag time $\tau_{obsd}$ is given by the ratio $b/a$. This method was used only when the progress curve showed sufficient linear portions after the transient phase (i.e., under conditions where the 1,3-$P_2$Glyc concentration was significantly larger than the $K_m$ for this substrate). (2) When the curvature of the progress curve was important after the transient phase (i.e., under conditions where 1,3-$P_2$Glyc < $K_m$), the progress curve was fitted to a polynomial and compared to a reference curve showing no lag phase. This reference curve was obtained upon preincubation of the enzyme with about 2 equiv of 1,3-$P_2$Glyc. For several absorbance values, the time difference between the two progress curves (i.e., the actual lag time) was estimated numerically and extrapolated to infinite reaction time. These two methods, when used conjointly, were found to give consistent estimates of the lag time ($\tau_{obsd}$) within ±10%.

## Results

*Lag Phase in the Reductive Dephosphorylation of 1,3-$P_2$Glyc.* When the apoenzyme is mixed in the stopped-flow apparatus with a large excess of NADH and 1,3-$P_2$Glyc, the oxidation of NADH monitored at 370 nm shows a well resolved lag phase before reaching a steady state (see Figure 1). This lag phase was found unchanged when the enzyme concentration was increased by a factor of 2.5. Thus, no enzyme-dependent association–dissociation processes are involved in this phenomenon. The lag phase was also found invariant with respect to NADH concentration which was varied from 20 to 100 μM. This result is not surprising, however, since both apoenzyme and acyl enzyme are nearly saturated by NADH in this concentration range (Kelemen et

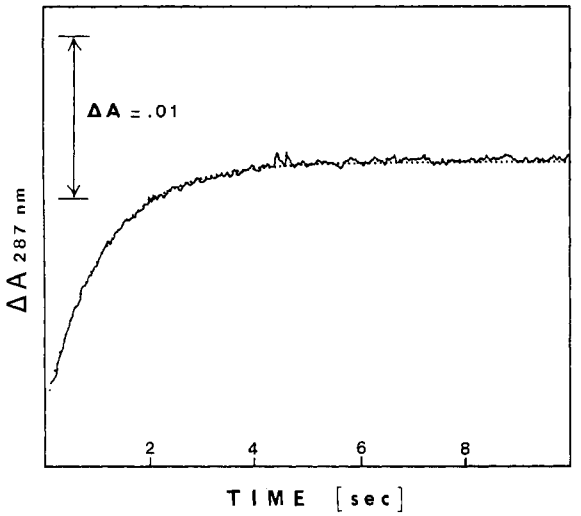



FIGURE 2: Acylation of apo-Glyc-3-P-dehydrogenase by 1,3-$P_2$Glyc as monitored by the change in absorbance at 287 nm. The dashed curve is fitted according to a pseudo-first-order process (rate constant = 0.91 $s^{-1}$). The reaction mixture contained 26 $\mu$M 1,3-$P_2$Glyc and 3.95 $\mu$M apo-Glyc-3-P-dehydrogenase in standard Tris–sulfonate buffer.

al., 1975; Seydoux et al., 1976).

*Dependence of the Lag Phase with Respect to the 1,3-$P_2$Glyc Concentration.* As shown in Figure 1, the lag time decreases when the final concentration of 1,3-$P_2$Glyc is increased in the reaction mixture. A quantitative estimation of the lag time can be obtained by comparison of the progress curves with a reference kinetics in which the reaction has been initiated with partially acylated Glyc-3-P-dehydrogenase (see below). As illustrated in the insert of Figure 1, the lag time exhibits a hyperbolic dependence with respect to the final concentration of 1,3-$P_2$Glyc. This indicates that the acylation of the enzyme by 1,3-$P_2$Glyc is involved in this phenomenon.

*Acylation of the Apo-Glyc-3-P-dehydrogenase by 1,3-$P_2$Glyc.* Since the lag phase seems to involve a 1,3-$P_2$Glyc-dependent process, the kinetics of the reaction of apoenzyme with this substrate has been investigated. The reaction of the four sites of apo-Glyc-3-P-dehydrogenase with an excess of 1,3-$P_2$Glyc has been monitored spectrophotometrically at 287 nm. This wavelength corresponds to the maximum of the difference spectrum which is induced upon acylation of the enzyme (Kelemen et al., 1975). In these conditions, the reaction appears to be monophasic (i.e., pseudo-first-order kinetics) as illustrated in Figure 2. This can be taken as an indication that the four active sites are equivalent in this particular reaction.

The apparent pseudo-first-order rate constant of acylation shows, as depicted in Figure 3, a hyperbolic dependence with respect to the 1,3-$P_2$Glyc concentration. The observed amplitude of the reaction decreases, however, with increasing substrate concentrations. This indicates that, before the irreversible acylation of the enzyme, a fast bimolecular change in absorbance occurs within the dead time of the stopped-flow apparatus. This fast reaction may correspond to the formation of a noncovalent complex (ES) between the apoenzyme (E) and 1,3-$P_2$Glyc (S) as illustrated by the reaction

$$E + S \overset{K_S}{\rightleftharpoons} ES \xrightarrow{k_{ac}} E\text{–}Ac + P_i \qquad (3)$$

Accordingly, the apparent rate constant for acylation is given by

$$k_{app} = \frac{k_{ac}(S)}{K_S + (S)} \qquad (4)$$

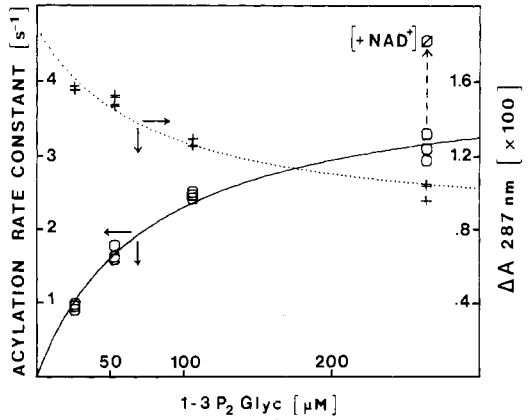



FIGURE 3: Kinetics of the acylation of apo-Glyc-3-P-dehydrogenase by 1,3-$P_2$Glyc. (0) Apparent rate constant of acylation as a function of 1,3-$P_2$Glyc concentration. The arrow shows the effect of preincubation of the enzyme with 0.6 $\mu$M $NAD^+$. The solid curve is calculated according to eq 4 (see text). (+) Amplitude of the observed change in absorbance as a function of 1,3-$P_2$Glyc concentration. The solid curve is calculated according to eq 5 as discussed in the text. Experimental conditions are as given in Figure 2.

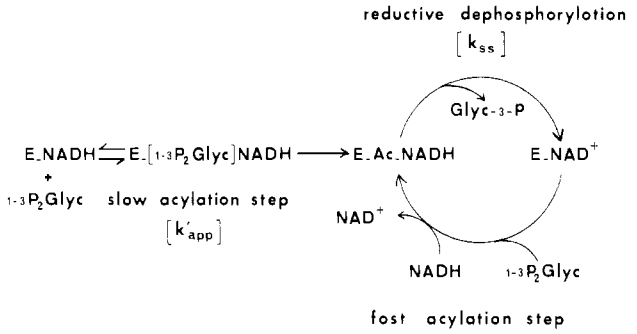
and the amplitude associated with the slow acylation step ($\Delta A_{ac}$) can be expressed as

$$\Delta A_{ac} = \left[ \Delta\epsilon_{EAc} - \Delta\epsilon_{ES}\frac{(S)}{(S) + K_S} \right] E_t \qquad (5)$$

where $\Delta\epsilon_{ES}$ and $\Delta\epsilon_{EAc}$ are the total changes in molar absorbance associated with the formation of the noncovalent ES and the covalent EAc complexes, respectively. This last equation predicts the decrease of the observed amplitude with increasing substrate concentrations if $\Delta\epsilon_{ES} > 0$. From the data of Figure 3, the $K_S$ and $k_{ac}$ parameters can be estimated to be 78 $\mu$M and 4.1 $s^{-1}$, respectively. This behavior contrasts with that reported for holo($NAD^+$)Glyc-3-P-dehydrogenase where no saturation by 1,3-$P_2$Glyc was observed (Harrigan & Trentham, 1973; Seydoux and Bernhard, unpublished experiments). The total change in molar extinction upon acylation of the enzyme can be estimated to be 2750 $M^{-1}$ $cm^{-1}$, in good agreement with previous estimations (Kelemen et al., 1975). The change in molar extinction associated with the formation of the noncovalent enzyme–substrate complex is only about half of this value. These results support the conclusion of a previous investigation (Kelemen et al., 1975) where similar qualitative difference spectra were observed upon acylation by 1,3-$P_2$Glyc and noncovalent binding of Glyc-3-P and orthophosphate. As shown in Figure 3, addition of very small amounts of $NAD^+$ (i.e., about 2% of the total active sites concentration) to the apoenzyme does increase significantly the rate of acylation. Since apo-Glyc-3-P-dehydrogenase as prepared herein was found previously to contain less than 0.3% of $NAD^+$ (Seydoux et al., 1973), the present effect of added $NAD^+$ makes it very unlikely that the observed acylation of apoenzyme is due to trace amounts of $NAD^+$ initially present as contaminant. Assuming that activation of the acylation reaction is proportional to the extent of $NAD^+$ binding to the enzyme, we calculate that the rate of the holo($NAD^+$) enzyme acylation should be at least 2 orders of magnitude faster than with apoenzyme. Hence, although $NAD^+$-free apo-Glyc-3-P-dehydrogenase can be acylated by 1,3-$P_2$Glyc, these results confirm the dramatic activation of the acylation reaction by $NAD^+$ which has been reported previously (Trentham, 1971).

*Positive Lag Phase as a Consequence of the Slow Acylation of Apo-Glyc-3-P-dehydrogenase by 1,3-$P_2$Glyc.* From steady-state measurements (not shown here), the turnover

Scheme I: Kinetic Scheme Describing the Reductive Dephosphorylation of 1,3-$P_2$Glyc Catalyzed by Glyc-3-P-dehydrogenase When the Reaction Is Initiated with Holo(NADH) Enzyme




number and the apparent $K_m$ for 1,3-$P_2$Glyc at nearly saturating (50 $\mu$M) concentration of NADH have been estimated, under similar experimental conditions, to be 30 $s^{-1}$ per active site and 23.5 $\mu$M, respectively. Thus, the observed rate of acylation of the apoenzyme (i.e., maximum rate constant of 4.1 $s^{-1}$) is much too slow to account for this steady-state turnover rate. Furthermore, a simple kinetic interpretation of the lag phase can be given on the basis of the two-step mechanism (eq 1) for the reductive dephosphorylation of 1,3-$P_2$Glyc as described in Scheme I. The acylation of the initially present holo(NADH) enzyme proceeds slowly in the first step. After acylation, the reductive deacylation of the acyl enzyme produces $NAD^+$ in situ. Hence, the oxidized coenzyme facilitates further reacylation of the enzyme by 1,3-$P_2$Glyc, and the acylation step is no longer rate limiting. Under zero-order conditions for the steady-state cycle, Scheme I leads to an equation similar to eq 1. The rate constant $k'_{app}$ (initiation step) is related to the empirical parameters of eq 2 as follows

$$k'_{app} = k = a/b \qquad (6)$$

and the steady-state turnover $k'_{ss}$ is proportional to the $a$ parameter. Thus, eq 6 predicts that the inverse of the lag time ($a/b$) should be equal to the rate constant ($k$) of the transient process. This relationship is accurately verified when the progress curves obtained with sufficient excess of NADH and 1,3-$P_2$Glyc are fitted to eq 2 (see, for example, progress curves a as given in Figures 4 and 5). It should be noted, however, that the rate constant (18 $s^{-1}$) and the apparent Michaelis constant (130 $\mu$M) associated with the transient, as obtained in Figure 1, are significantly larger than those associated with the apoenzyme acylation (i.e., 4.1 $s^{-1}$ and 78 $\mu$M, respectively). This may indicate that NADH slightly activates the acylation of the enzyme. Alternatively, traces of $NAD^+$ may contaminate the NADH solution.

*Effect of the Preincubation of the Apoenzyme with 1,3-$P_2$Glyc or $NAD^+$.* If our interpretation of the lag phase is correct, the lag should be eliminated either by preacylation of the enzyme with 1,3-$P_2$Glyc or by preformation of the holo($NAD^+$) enzyme which is likely to be very rapidly acylated by the substrate. This has been tested in the stopped-flow apparatus by initiating the reaction with the enzyme preincubated with either 1,3-$P_2$Glyc or $NAD^+$. The initial part of the progress curves (about 3–25% of the total reaction, depending on the final 1,3-$P_2$Glyc concentrations) was fitted to eq 2. As shown in Figure 4, the lag phase decreases progressively upon preincubation of the apoenzyme with substoichiometric amounts of 1,3-$P_2$Glyc. The acylation of the enzyme is stoichiometric in these conditions (Seydoux et al., 1973). Strikingly, the positive lag phase disappears completely

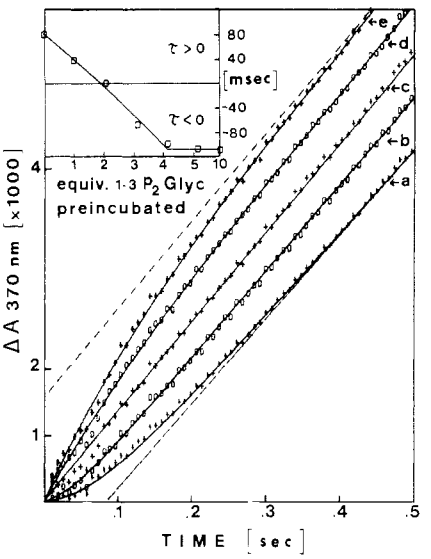



FIGURE 4: Influence of the preincubation of Glyc-3-P-dehydrogenase with 1,3-$P_2$Glyc on the pre-steady-state kinetics of the enzyme. One syringe contained the enzyme (0.035 $\mu$M final concentration) preincubated with stoichiometric amounts of 1,3-$P_2$Glyc: (a) 0; (b) 1; (c) 2.0; (d) 3.1; and (e) 5.1 mol of 1,3-$P_2$Glyc per mol of enzyme tetramer. The other syringe contained 1,3-$P_2$Glyc and NADH (final concentration 97 and 53 $\mu$M, respectively) in standard Tris–sulfonate buffer. The solid lines are calculated from eq 2 with the following numerical values of the parameters: final slope, $a = 0.0128 \pm 0.0002$ $A$/s (average value for all curves); $b = 9.84 \times 10^{-4}$ $A$, $k = 11.1$ $s^{-1}$ (curve a); $b = 4.7 \times 10^{-4}$ $A$, $k = 22.7$ $s^{-1}$ (curve b); $b = 0$ (curve c); $b = -8.76 \times 10^{-4}$ $A$, $k = 7.2$ $s^{-1}$ (curve d); $b = -1.42 \times 10^{-3}$ $A$, $k = 7.67$ $s^{-1}$ (curve e). The insert shows the variation of the lag time ($\tau_{obsd}$) with respect to the extent of preacylation of the enzyme. The solid line is calculated from eq 7.

when half of the four available sites of the enzyme tetramer have been acylated (see insert of Figure 4). Further preacylation of the enzyme induces a new transient phase which, at first sight, appears as a pseudoburst preceding the steady-state production of $NAD^+$. The amplitude of this pseudoburst which reaches a limiting value when the enzyme is fully acylated is, however, about 3 times larger than the total sites concentration. Thus, the complete preacylation of the enzyme seems rather to induce a transient suractivation of the enzymic reaction which disappears after several turnovers. This has been confirmed by numerical analysis of three nearly complete progress curves obtained with apoenzyme, diacylated, and fully acylated enzyme, respectively. These progress curves (not shown here) were differentiated numerically with respect to time, and the resulting rate vs. substrate curves appear to converge, after the transient phase, to the same rate value. Thus, this numerical analysis of complete progress curves demonstrates that the final steady state of the reaction is not dependent on the initial preincubation of the enzyme with 1,3-$P_2$Glyc.

As shown in Figure 5, preincubation experiments with $NAD^+$ lead to similar results. The binding of $NAD^+$ to the enzyme is not stoichiometric, and the amount of bound $NAD^+$ was calculated according to the values of the respective dissociation constants for the two classes of $NAD^+$ binding sites as previously determined under similar conditions (Kelemen et al., 1975).

As illustrated in Figure 6, the positive lag phase disappears when the two tight binding sites are nearly saturated by $NAD^+$. Upon saturation of the two remaining loose sites, the negative lag phase appears and reaches a constant value when the enzyme is saturated by $NAD^+$. As found with 1,3-$P_2$Glyc, the limiting steady-rate values are invariant with respect to

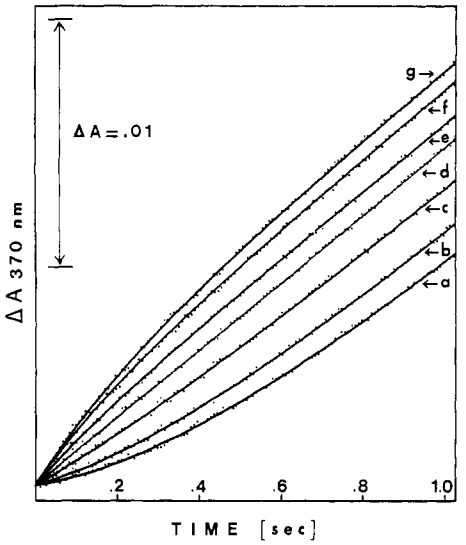



FIGURE 5: Influence of the preincubation of Glyc-3-P-dehydrogenase with $NAD^+$ on the pre-steady-state kinetics of the enzyme (final concentration 0.047 $\mu$M) preincubated with 0 (a); 0.5 (b); 2.0 (c); 3.6 (d); 5.1 (e); and 10.2 (f) mol of $NAD^+$ per mol of enzyme tetramer. Curve g, used as reference curve, was obtained by preincubation of the enzyme with 5.1 mol of 1,3-$P_2$Glyc per mol of enzyme tetramer. The other syringe contained NADH and 1,3-$P_2$Glyc in standard Tris–sulfonate buffer (final concentration 50 and 24.6 $\mu$M, respectively). Solid curves are calculated from eq 2 with the following numerical values of the parameters: final slope, $a = 0.0135 \pm 0.0002$ $A/s$ (average value for all curves); $b = 5.85 \times 10^{-3}$ $A$, $k = 1.98$ $s^{-1}$ (curve a); $b = 3.90 \times 10^{-3}$ $A$, $k = 2.37$ $s^{-1}$ (curve b); $b = 1.65 \times 10^{-3}$ $A$, $k = 2.55$ $s^{-1}$ (curve c); $b = 0$ (curve d); $b = -1.04 \times 10^{-3}$ $A$, $k = 4.75$ $s^{-1}$ (curve e); $b = -2.05 \times 10^{-3}$ $A$, $k = 4.53$ $s^{-1}$ (curve f); $b = -3.1 \times 10^{-3}$ $A$, $k = 3.71$ $s^{-1}$ (curve g).

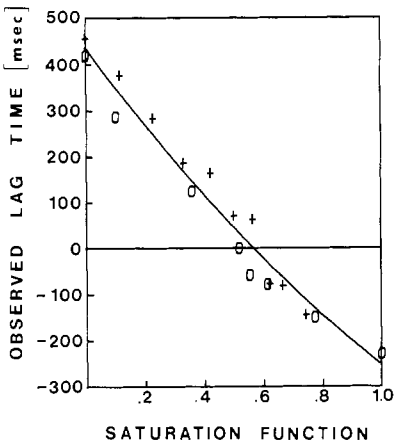



FIGURE 6: Influence of the preincubation of Glyc-3-P-dehydrogenase with $NAD^+$ on the observed lag time in the reductive dephosphorylation of 1,3-$P_2$Glyc. Experimental conditions are similar to those of Figure 5. Two independent data sets are shown: enzyme concentrations (0) 0.047 and (+) 0.118 $\mu$M. The solid curve is calculated according to eq 7 and numerical values of the parameters are as given in Table I. The saturation function is equivalent to $m/2$ in eq 7.

the preincubation conditions. The transient behavior of the enzyme nearly saturated by $NAD^+$ is similar to that observed with fully preacylated enzyme (see Figure 5). The respective values of the kinetic parameters corresponding to the positive and negative lag phases at two distinct concentrations of 1,3-$P_2$Glyc are given in Table I. It should be noted that the decrease of the positive lag time upon increasing the substrate concentration is much larger than that of the negative lag time. This may be taken as an indication that these two phenomena do not arise from complementary kinetic processes. On the other hand, the ratio of the initial and final slopes of the negative lag phase is invariant with respect to a fourfold increase of the 1,3-$P_2$Glyc concentration.

Table I: Kinetic Parameters Associated with the Positive and Negative Lag Phase Phenomena in the Reductive Dephosphorylation of 1,3-$P_2$Glyc Catalyzed by Sturgeon Glyc-3-P-dehydrogenase[a]

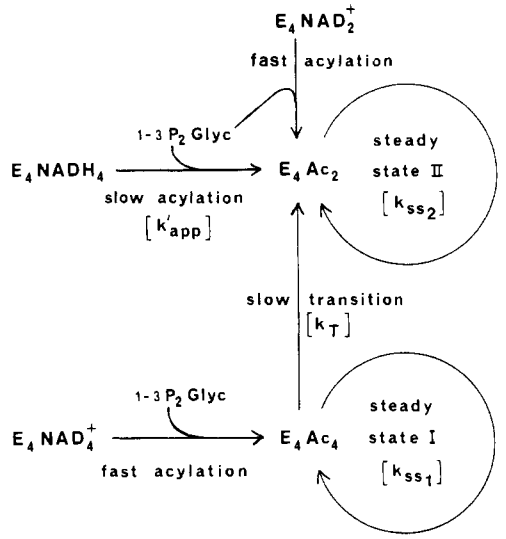
| 1,3-$P_2$Glyc ($\mu$M) | $\tau_+^{max}$ [b] (ms) | $\tau_-^{max}$ [b] (ms) | $k_T^+$ [c] ($s^{-1}$) | $k_T^-$ [c] ($s^{-1}$) | $r$ [d] |
|---|---|---|---|---|---|
| 24.6 | 418 | −230 | 2.30 | 3.95 | 1.86 |
| 97 | 81 | −109 | 11.1 | 7.16 | 1.84 |

[a] Experimental conditions: standard Tris–sulfonate buffer, pH 7.0; 10 °C; 50 $\mu$M NADH. [b] Maximum values of the positive ($\tau_+^{max}$) and negative ($\tau_-^{max}$) lag time are defined from eq 2 and are observed upon initiation of the reaction with apoenzyme and fully acylated enzyme, respectively. [c] $k_T^+$ ($= k'_{app}$) and $k_T^-$ are the transient rate constants as defined in eq 2 for the positive and negative lag phase, respectively. [d] Ratio of the initial and final slopes of the transient when the reaction is initiated with fully acylated enzyme.

## Discussion

The present kinetic results indicate a clear distinction between two pairs of sites of tetrameric Glyc-3-P-dehydrogenase in the transient kinetics of the reductive dephosphorylation of 1,3-$P_2$Glyc. The preacylation of the first pair of sites does allow immediate attainment of the steady-state rate. Further preacylation of the second pair of sites leads to a suractivation phase (i.e., negative lag phase) which is replaced by the normal steady state after a few turnovers. This pseudoburst kinetics which is observed upon either binding of $NAD^+$ or acylation by 1,3-$P_2$Glyc of the second pair of sites is a reproducible process which cannot be interpreted mechanistically in terms of a "burst" kinetics. Such an interpretation would require that the excess of preacylated sites (i.e., half of the total number of acylated sites) undergoes deacylation during the first turnover of the enzyme. Accordingly, the burst size should not exceed in any case the total active sites concentration. This is clearly not consistent with the present results. Thus, a plausible interpretation of the negative lag phase is that the initially fully acylated (and fully active) enzyme is slowly converted into a diacylated species which predominates largely in the final steady state. A schematic illustration of this interpretation is given in Scheme II which involves a slow transition (with a first-order rate constant $k_T$) between two steady states (I and II). Steady states I and II correspond respectively to the reaction of a fully acylated species and to the reaction of the diacylated species which accumulates irreversibly. According to the data of Table I, the turnover rate constant ($k_{ss1}$) of steady state I is about twice as fast as that of steady state II ($k_{ss2}$). In addition, Scheme II includes the slow acylation of the holo(NADH) enzyme by 1,3-$P_2$Glyc which occurs when the reaction is initiated with the unacylated apoenzyme (pseudo-first-order rate constant $k'_{app}$). This initiation step which depends on the substrate concentration (see Scheme I) does provide a simple explanation of the positive lag phase since further reacylation of the enzyme is facilitated by $NAD^+$ which is a product of the reaction. This interpretation is supported by the present data and is consistent with previous results (Trentham, 1971). Furthermore, the invariance of the positive lag phase with respect to the enzyme concentration rules out the occurrence of an association process of the supposedly dissociated and inactive enzyme upon mixing with the substrates. It can be also shown that this behavior is not consistent with an autocatalytic process where significant amounts of the product $NAD^+$ are released in the reaction mixture (Carlier & Pantaloni, 1976).

Scheme II: Kinetic Interpretation of the Effect of Preincubation of Glyc-3-P-dehydrogenase with 1,3-$P_2$Glyc or $NAD^+$ in the Reductive Dephosphorylation of the Substrate[a]




[a] Input and output of substrate and coenzymes are omitted in steady-state cycles for simplicity. Initiation of the reaction with $E_4(NADH)_4$ (or $E_4$) gives rise to a positive lag phase. A negative lag phase occurs when the reaction is initiated with $E_4(NAD_4^+)$ or $E_4(Ac_4)$. No lag phase is observed when the reaction is initiated with $E_4(NAD_2^+)$ or $E_4(Ac_2)$.

By assumption that positive and negative lag phases are independent processes which occur when the first pair of sites is unacylated and when the second pair of sites is fully acylated, respectively, the observed lag time ($\tau_{obsd}$) is given by

$$\tau_{obsd} = \left(1 - \frac{m}{2}\right)^2 \frac{1}{k'_{app}} - \frac{m^2}{4}\left(\frac{k_{ss2}}{k_{ss1}} - 1\right)\frac{1}{k_T} \qquad (7)$$

where $m$ denotes the acylated (or $NAD^+$-saturated) fraction of each class of sites. As shown in Figures 4 and 6, eq 7 does account adequately for the dependence of the observed lag time with respect to the extent of acylation or $NAD^+$ binding to the enzyme tetramer. As a consequence of the fast acylation of the holo($NAD^+$) enzyme, the effector role of bound $NAD^+$ is strictly equivalent to that of the covalently bound 3-phosphoglyceroyl group. It is not possible, however, to correlate the disappearance of the positive lag phase with the saturation of the first pair of tight $NAD^+$ binding sites (Kelemen et al., 1975) since very similar theoretical curves can be calculated by assuming that the positive lag phase disappears upon formation of a randomly half-saturated species with no distinction between tightly and loosely bound $NAD^+$ molecules. This interpretation of the negative lag phase phenomenon has some formal analogy with previous kinetic treatment of mnemonic enzyme systems (Ricard et al., 1977). It is important to point out here that the nonequivalence among sites which is demonstrated in the present study does not seem to provide a significant increase of the catalytic efficiency of tetrameric Glyc-3-P-dehydrogenase. In fact, the fully acylated enzyme appears to be about twice as active as the diacylated enzyme. This is clearly not in agreement with the previous hypothesis of a "kinetic cooperativity" which postulates that oligomeric enzymes gain catalytic efficiency from the phasing out of half of the coupled active sites (Lazdunski, 1972). We rather believe that the present "negative lag phase" phenomenon results from the strong quaternary constraints which favor the accumulation of an asymmetrical, diacylated species of the enzyme during the catalytic process. It is difficult at the present time to propose a detailed molecular mechanism for the slow transition between the two steady states. Our kinetic interpretation, as given in Scheme II, implies that some kinetic branching occurs in steady-state cycle I, leading either to the final steady state (II) or to further cycling of the fully acylated species. It is not clear which intermediate of the reaction pathway (Trentham, 1971; Duggleby & Dennis, 1974; Seydoux et al., 1976) is involved in this branching of the overall reaction. However, the present results are consistent with the accumulation at the steady state of an enzyme species in which only half of the four active sites are acylated by the physiological substrate 1,3-$P_2$Glyc. In this sense, the anticooperative phenomena (i.e., half-site reactivity and negative cooperativity), which have been extensively reported with sturgeon Glyc-3-P-dehydrogenase and other muscle enzymes, appear to be also involved in the physiological reaction of the enzyme.

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