The Active Chemical State of D-Glyceraldehyde 3-Phosphate in its Reactions with D-Glyceraldehyde 3-Phosphate Dehydrogenase, Aldolase and Triose Phosphate Isomerase

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Glyceraldehyde 3-phosphate exists as the geminal diol and the free aldehyde in the molar ratio 29:1 in aqueous solution. The rate constant of the conversion of diol into aldehyde is $8.7 \times 10^{-2} \text{ sec.}^{-1}$ in the pH range 7.3–8.6 at 20°. The free aldehyde is the substrate for D-glyceraldehyde 3-phosphate dehydrogenase. Over a wide concentration range of enzyme the rate of conversion of diol into aldehyde is the rate-limiting process in the catalytic oxidation of D-glyceraldehyde 3-phosphate by NAD⁺. Aldolase and triose phosphate isomerase both liberate D-glyceraldehyde 3-phosphate as the aldehyde. This suggests that the relatively slow diol–aldehyde interconversion does not restrict the rate of glycolysis.

The state of a substrate when it binds to an enzyme is an important factor in elucidating the elementary processes of an enzyme-catalysed reaction. If the states of the substrate are only slowly interconvertible, then in certain concentration ranges of substrate and enzyme this rate of interconversion may become the rate-limiting process in the enzyme-catalysed reaction of the substrate.

The state of aldehydes and carbohydrates that are found in biological systems has been frequently studied (Rutter, 1961; Müller-Hill & Wallenfels, 1964; Bailey, Fishman & Pentchev, 1968; Dolin, 1968; Naylor & Fridovich, 1968). We have examined the states of G3P* and investigated the influence of these states on its reactions with GPDH, TIM and aldolase (EC 4.1.2.13).

MATERIALS AND METHODS

Preparation, purity and assay of enzymes. The activity of the enzymes was determined in either direct or linked assays by measuring the rate of NADH production or oxidation spectrophotometrically. The molar extinction coefficient of NADH at 340 nm. was taken as 6220 (Horecker & Kornberg, 1948).

GPDH from lobster tail muscle was prepared and assayed as described by Trentham (1968). The enzyme (0.1 µg./ml.)

was also assayed at 25° in a medium that contained G3P (1.67 mm), NAD⁺ (0.48 mm or 1.80 mm), Na₂HAsO₄ (30 mm), EDTA (1 mm) and triethanolamine (200 mm) adjusted to pH 8.6 with HCl. This is a typical medium of the stopped-flow experiments. The specific activity of the enzyme was 180 µmoles of NADH produced/min./mg. of enzyme.

Aldolase was extracted from rabbit white skeletal muscle and fractionated on CM-cellulose (Whatman C-22). The enzyme was crystallized to constant specific activity by repeated (NH₄)₂SO₄ fractionation. The enzyme activity was determined at 25° by using a linked assay system (Racker, 1947). The assay medium contained TIM (0.2 unit/ml.), L-glycerol 3-phosphate dehydrogenase (0.2 unit/ml.), F1,6P (20 mm), NADH (1.5 mm) and triethanolamine (200 mm) adjusted to pH 7.5 with HCl. $E_{1\text{cm}}^{1\%}$ at 280 nm. was taken as 9.1 (Baranowski & Neiderland, 1949). The specific activity was 12 µmoles of F1,6P cleaved/min./mg. of enzyme. The enzyme (1.0 µg./ml.) was also assayed at 25° in a medium that contained GPDH (0.2 unit/ml.), F1,6P (2.0 mm), NAD⁺ (1.67 mm), Na₂HAsO₄ (30 mm) and triethanolamine (200 mm) adjusted to pH 8.5 with HCl. The specific activity was 10 µmoles of F1,6P cleaved/min./mg. of enzyme. This assay medium is similar to the medium of the aldolase stopped-flow experiment.

TIM was extracted from chicken muscle and precipitated with acetone at –5°. The enzyme was fractionated on CM-Sephadex (Pharmacia, Uppsala, Sweden) and crystallized from (NH₄)₂SO₄. The enzyme was homogeneous when analysed by isoelectric fractionation (Vesterberg & Svensson, 1966) and analytical ultracentrifugation. Protein concentration was estimated by the method of Warburg & Christian (1941) as described by Layne (1957). This procedure gives a working value of $E_{1\text{cm}}^{1\%}$ at 280 nm. as 9.0. Enzyme activities after suitable dilutions in 0.5% bovine serum albumin were determined at 25° by using linked assay systems. The rates of isomerization of both G3P and DHAP were determined. For the former the assay medium

* Abbreviations: G3P, D-glyceraldehyde 3-phosphate; DLG3P, DL-glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; F1,6P, fructose 1,6-diphosphate; 3PG, 3-phosphoglycerate; GPDH, D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12); TIM, triose phosphate isomerase (EC 5.3.1.1).

contained L-glycerol 3-phosphate dehydrogenase (1 unit/ml.), G3P (3 mM), NADH (0.15 mM) and triethanolamine (100 mM) adjusted to pH 7.0 with HCl. The specific activity was 10800 μ moles of G3P isomerized/min./mg. of enzyme. For the latter the assay medium contained GPDH (3.6 units/ml.), DHAP, NAD^+ (1.0 mM), Na_2HAsO_4 (6 mM) and triethanolamine (100 mM) adjusted to pH 8.5 with HCl. The specific activities with DHAP at 1.0 mM and 3.0 mM were 136 and 249 μ moles of DHAP isomerized/min./mg. of enzyme respectively. This assay medium is similar to the medium of the TIM stopped-flow experiment.

The purity of each of the above enzymes was checked with respect to contamination by the other two. The degree of contamination was too small to be of significance in the experiments described.

Reagents. L-Glycerol 3-phosphate dehydrogenase, DLG3P (as the barium salt of the diethyl acetal), DHAP (as the cyclohexylammonium salt of the dimethyl ketal), F1,6P (as the trisodium salt), NADH and NAD^+ were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany). DHAP was prepared from the ketal by the method of Ballou & Fischer (1956). Bovine serum albumin (fraction V) was obtained from the Armour Pharmaceutical Co. Ltd. (Eastbourne, Sussex). G3P was prepared and assayed as described by Trentham (1968). For proton-magnetic-resonance (p.m.r.) studies D_2O replaced water in the acetal hydrolysis and Ba^{2+} ion-exchange step. Triethanolamine was redistilled under reduced pressure and stored at -18° . HCl was prepared by isopiestic distillation (Irving & Cox, 1958). Water was glass-distilled. All other reagents were A.R. grade where possible and were used without further purification.

Spectroscopic and kinetic measurements. The u.v.-absorption measurements were made in the 1 cm. thermostatically controlled cuvette of a Uvispek H700 spectrophotometer (Hilger and Watts Ltd.) provided with a Gilford recording attachment and a Sunvic recorder (type 10S).

The stopped-flow apparatus used was similar to that described by Gutfreund (1965). The interchangeable mixer and observation cell consisted of eight mixing jets and a 1 cm. light-path. The solution in the observation chamber was 3 msec. old when flow stopped. Monochromatic light was obtained from a quartz-tungsten-iodine lamp and a Bausch and Lomb grating monochromator. Transmission of light was recorded on a Tektronix storage oscilloscope, which amplified the output of an EMI 9592B photomultiplier. Reactions were studied at room temperature ($21 \pm 2^\circ$).

The temperature-jump apparatus was designed by Dr L. C. de Maeyer and built by Messanlagen G.m.b.H. (Göttingen, Germany). This equipment has been described and the theoretical concepts defined by Eigen (1968).

The p.m.r. spectra were recorded with a Varian HA-100 spectrophotometer locked on the HOD signal. τ values are quoted with reference to the internal standard, sodium 3-(trimethylsilyl)propane-1-sulphonate (τ 10.00). A neutral deuterated phosphate buffer reagent was prepared by freeze-drying equimolar concentrations of Na_2HPO_4 and NaH_2PO_4 in D_2O .

RESULTS AND DISCUSSION

As part of a continuing programme of analysis of the elementary processes involved in the GPDH-catalysed reaction, the dependence of the rate of

Fig. 1. Dependence of the rate of NADH production on GPDH concentration. Stopped-flow traces were recorded at 340 nm. The reaction mixtures contained G3P (0.53 mM), NAD^+ (0.48 mM), Na_2HAsO_4 (30 mM), EDTA (1 mM) and triethanolamine (200 mM) adjusted to pH 8.6 with HCl. In addition the solutions contained GPDH [0.44 mg./ml., 12 μ M in sites (A), and 0.147 mg./ml., 4 μ M in sites (B)] as indicated. Each trace shows duplicate runs. Associated with the upper traces are base-line traces which were coincident. The base-line traces were obtained with reaction mixtures containing all reagents except G3P. In each experiment one syringe contained GPDH and NAD^+ and the other G3P. The other reagents were at equal concentrations in each syringe.

NADH production on GPDH concentration was studied. It was apparent that over a wide range of conditions, when GPDH was present at concentrations greater than 0.1 mg./ml., the final steady-state rate of NADH production was not proportional to GPDH concentration. In general, NADH production followed triphasic kinetics. When the second and third phases were clearly resolved, the final steady-state rate and the amplitude of the second phase were independent of enzyme concentration (Fig. 1). The amplitude of the first phase, whose rate was in general too fast to measure, and the rate of the second phase were functions of enzyme concentration.

The dependence of the rate of NADH production on G3P concentration was studied (Figs. 2a and 2b). When G3P concentration was increased threefold from 0.53 mM to 1.60 mM, the final steady-state rate of NADH production increased 2.97-fold from $4.35 \times 10^{-5} \text{ Msec.}^{-1}$ to $1.29 \times 10^{-4} \text{ Msec.}^{-1}$. The NADH produced in the pre-steady-state process was increased 3.05-fold from 17.4 μ M to 53 μ M. The NADH yield was determined by extrapolating the line representing the steady-state rate back to the zero-time axis and measuring the extinction difference between this intercept and the solutions before mixing.

These results indicate that G3P exists in two

Fig. 2. (a) Dependence of the rate of NADH production on G3P concentration. Stopped-flow traces were recorded at 340nm. The reaction mixtures contained GPDH (0.44 mg/ml.), NAD^+ (0.48 mM), Na_2HAsO_4 (30 mM), EDTA (1 mM) and triethanolamine (200 mM) adjusted to pH 8.6 with HCl. In addition the solutions contained G3P [1.60 mM (A) and 0.53 mM (B)] as indicated. Associated with the upper traces are base-line traces which were coincident. The base-line traces were obtained with reaction mixtures containing all reagents except G3P. In each experiment one syringe contained GPDH and NAD^+ and the other G3P. The other reagents were at equal concentration in each syringe. (b) Description of the rate of change of $E_{340}^{1 \text{ cm.}}$ of the upper traces in (a). The base-line traces correspond to zero extinction. The 50 cyc./sec. a.c. mains supply has been averaged out.

forms, only one of which is active as a substrate for GPDH. A 1.60 mM solution of G3P contains $53 \mu\text{M}$ active form, since this is the yield of NADH in the pre-steady-state phase. The ratio of active to inactive G3P is then 1:29. Under certain conditions the interconversion of these two forms becomes rate-limiting in the GPDH-catalysed oxidation of G3P by NAD^+ .

The hydration of aldehydes is a well-characterized reaction (Bell, 1966). The two forms of G3P that are proposed are the aldehyde, G3P_{ald} , and the diol, G3P_{diol} . First-order rate constants k_{+1} and k_{-1} are assigned to the forward and back reactions:

The p.m.r. spectrum should indicate the proportion of G3P present as G3P_{ald} , since the proton α to the carbonyl group has a characteristic τ value. The spectrum of DLG3P (90 mM) in 0.75 M-phosphate buffer in D_2O under neutral conditions had a signal at 0.43 τ . The intensity of the signal was about 1% of that of those protons of DLG3P that are not exchangeable with water. Allowance was made for the signals from the ethanol that is liberated in the acetal hydrolysis. Since each molecule of DLG3P contains three non-exchangeable

protons in addition to the α -proton, about 4% of DLG3P exists as the aldehyde. G3P_{ald} must be the active form.

The steady-state rate of NADH production (Figs. 2a and 2b) is equal to $k_{+1} [\text{G3P}_{\text{diol}}]$, so that $k_{+1} = 8.7 \times 10^{-2} \text{ sec}^{-1}$. Since $k_{-1}/k_{+1} = 29$, $k_{-1} = 2.5 \text{ sec}^{-1}$.

The rapid first phase of NADH production is associated with the first turnover of GPDH. The extinction change/mole of NADH produced in this step will probably be less than 6220 on account of the GPDH- NAD^+ absorption band (Racker & Krimsky, 1952). The NADH yield in the pre-steady-state phase was not corrected for this, since a precise value of the correction was not known. Its maximum effect would be to decrease k_{-1} by 10%.

The diol-aldehyde interconversion was studied in the absence of enzyme to test whether or not GPDH catalyses the reaction. The rate constant, $\bar{k}$, associated with the relaxation process after a rapid temperature perturbation of the diol-aldehyde equilibrium was measured (Fig. 3). The maximum signal was observed at 280 nm. and so the extinction may be assigned to the carbonyl absorption band. The mean value of $\bar{k}$ from 11 traces was 2.33 sec^{-1} (S.E.M. 0.09 sec^{-1}). The agreement between $\bar{k}$ and the sum of the rate constants, $k_{+1} + k_{-1}$, determined from the flow experiments suggests that GPDH does not catalyse the diol-aldehyde interconversion.

No change in the relaxation time was observed

and the amplitude of the signal was proportional to DLG3P concentration as the concentration was varied from 10mM to 50mM. At 10mM- and 20mM-DLG3P the signal/noise ratio was low. However, the flow and relaxation experiments covered a 50-fold range of G3P concentration. These results suggest that polymerization of the aldehyde was not contributing significantly to the inactive form of G3P.

By using stopped-flow methods, k_{+1} and k_{-1} were determined at pH 7.4 and 8.1. Triethanolamine, which may possibly form an adduct with G3P_{ald} or catalyse the hydration, was not present in these studies. GPDH and the substrates were present at concentrations already described (Fig. 2a). At pH 7.3 the reaction mixture in potassium chloride (0.3M) was buffered by arsenate (30mM). At pH 8.1 the reaction mixture was buffered by pyrophosphate (15mM). At both pH values there was no detectable difference in k_{+1} and k_{-1} from the values at pH 8.6. However, the sensitivity of the analyses was less since the rates of the second and third phases of NADH production were not so well resolved because of diminished GPDH activity.

The relatively low rate of conversion of G3P_{d10l} into G3P_{ald} is probably responsible in part for the complex kinetic pattern of NADH production in

the GPDH-catalysed reaction that has been observed at high GPDH/G3P_{ald} concentration ratios (Chance & Park, 1967).

Linked systems. In a coupled reaction catalysed by TIM and GPDH, DHAP may be isomerized to G3P, which may in turn be oxidized by NAD⁺. The rate of NADH production depends on whether TIM liberates G3P as the diol or the aldehyde. This is shown by considering the two alternatives outlined in Scheme 1.

If TIM is present at sufficiently high concentration then the equilibrium between DHAP and the form of G3P liberated from the enzyme may be maintained. In Scheme 1(a):

$$d(\text{NADH})/dt = k_0[\text{GPDH}]$$

where k_0 is the catalytic-centre activity of GPDH appropriate to the concentration of G3P_{ald} that is maintained in equilibrium with DHAP. However, if G3P_{d10l} is liberated from TIM, then the diol-dehydration step will be rate-limiting at sufficiently high concentration of GPDH. In this case (Scheme 1b):

$$[d(\text{NADH})/dt]_{\text{max.}} = k_{+1}[\text{G3P}_{\text{d10l}}]$$

in the steady state, where [G3P_{d10l}] is the concentration of G3P_{d10l} that is maintained in equilibrium with DHAP.

The DHAP/G3P concentration ratio at equilibrium is 22:1 (Burton & Waley, 1968). An equilibrated solution of TIM and its substrates therefore contains DHAP, G3P_{d10l} and G3P_{ald} in the proportions 660:29:1. G3P (2.92mM) was allowed to equilibrate in the presence of TIM. The concentrations of DHAP, G3P_{d10l} and G3P_{ald} free in solution were then 2.79mM, 123μM and 4.2μM respectively. When G3P_{ald} is 4.2μM, the catalytic-centre activity of GPDH may be estimated as 131sec.⁻¹ (Fig. 2b). This value is deduced from the measured rate of NADH production when the concentration of G3P_{ald} is estimated to be 4.2μM.

The rate of NADH production was 3.5 × 10⁻⁴msec.⁻¹ when the equilibrated triose phosphates were mixed with GPDH (0.44mg./ml.), NAD⁺ and arsenate in a stopped-flow apparatus (Fig. 4). If TIM liberates G3P_{ald} (Scheme 1a) the predicted rate of NADH production would be 3.95 × 10⁻⁴msec.⁻¹, whereas if TIM liberates G3P_{d10l}

Fig. 3. Spectrophotometric record at 280nm. and 23° of the relaxation of DLG3P after a rapid temperature perturbation. The solution contained DLG3P (50mM), Na₂HAsO₄ (30mM), EDTA (0.1mM) and triethanolamine (200mM) adjusted to pH 8.6 with HCl.

Scheme 1.

(Scheme 1b) the maximum predicted rate of NADH production would be $1.1 \times 10^{-5} \text{ Msec.}^{-1}$. When the concentration of GPDH is decreased ninefold or the concentration of triose phosphates is decreased threefold, the rates of NADH production were those predicted by Scheme 1(a). We conclude, therefore, that TIM liberates G3P_{ald} .

In a coupled reaction catalysed by aldolase and GPDH, F1,6P may be cleaved to yield G3P, which may in turn be oxidized by NAD^+ . The rate of NADH production depends on whether aldolase liberates G3P as the diol or the aldehyde. This is shown by considering the two alternatives outlined in Scheme 2.

If GPDH is present at sufficiently high concentration then all the G3P_{ald} will be removed as it is synthesized. In Scheme 2(a):

$$d(\text{NADH})/dt = \bar{k}_0[\text{aldolase}]$$

where $\bar{k}_0$ is the catalytic-centre activity of aldolase. However, if G3P_{diol} is liberated from aldolase (Scheme 2b) then:

$$[d(\text{NADH})/dt]_{\text{max.}} = k_{+1}[\text{G3P}_{\text{diol}}]$$

and

$$d[\text{G3P}_{\text{diol}}]/dt = -k_{+1}[\text{G3P}_{\text{diol}}] + \bar{k}_0[\text{aldolase}]$$

Provided that $\bar{k}_0[\text{aldolase}]$ is constant the second equation becomes a simple first-order differential equation whose solution is:

$$[\text{G3P}_{\text{diol}}] = (\bar{k}_0[\text{aldolase}]/k_{+1})(1 - e^{-k_{+1}t})$$

When t is less than 1 sec., since k_{+1} equals $8.7 \times 10^{-2} \text{ sec.}^{-1}$, this reduces to:

$$[\text{G3P}_{\text{diol}}] = \bar{k}_0[\text{aldolase}]t$$

In Scheme 2(b), therefore, for $t < 1 \text{ sec.}$:

$$[d(\text{NADH})/dt]_{\text{max.}} = k_0 k_{+1}[\text{aldolase}]t$$

There is a qualitative difference in the rate of NADH production in the two schemes in that in the former the rate is constant whereas in the latter the rate is proportional to time.

The rate of NADH production was independent of time and equalled $8.2 \times 10^{-5} \text{ Msec.}^{-1}$ when F1,6P was mixed with aldolase (0.50 mg./ml.), GPDH, NAD^+ and arsenate in a stopped-flow apparatus (Fig. 5). If aldolase liberates G3P_{ald} (Scheme 2a), the predicted rate of NADH production would be $8.3 \times 10^{-5} \text{ Msec.}^{-1}$. The constant rate of NADH production is inconsistent with Scheme 2(b). Moreover, after 100 msec., the maximum rate of NADH production predicted by Scheme 2(b) is more than 100-fold less than that actually observed. If the aldolase concentration is decreased 16-fold and fourfold the rates of NADH production were $6.1 \times 10^{-6} \text{ Msec.}^{-1}$ and $2.1 \times 10^{-5} \text{ Msec.}^{-1}$. These are the rates predicted by Scheme 2(a). In the last experiment the same rate of NADH production was observed when F1,6P and aldolase were switched from one syringe to the other. This experiment minimizes the possibility that any association of aldolase and GPDH might influence the rate of NADH production. We conclude that aldolase liberates G3P as the aldehyde.

Fig. 4. Spectrophotometric record of the rate of NADH production in a coupled reaction catalysed by GPDH and TIM. Stopped-flow traces were recorded at 340 nm. The reaction mixtures contained GPDH (0.44 mg./ml.), TIM (1.71 mg./ml.), G3P (0.13 mM), DHAP (2.79 mM), NAD^+ (0.48 mM), Na_2HAsO_4 (30 mM), EDTA (0.1 mM) and triethanolamine (200 mM) adjusted to pH 8.6 with HCl. The trace shows duplicate runs. One syringe contained GPDH, NAD^+ and EDTA and the other TIM, G3P and DHAP. The other reagents were at equal concentrations in each syringe. G3P (2.92 mM) was equilibrated to a mixture of DHAP and G3P before the reaction by preincubation with TIM.

Scheme 2.

Fig. 5. Spectrophotometric record of the rate of NADH production in a coupled reaction catalysed by GPDH and aldolase. Stopped-flow traces were recorded at 340 nm. The reaction mixtures contained GPDH (0.44 mg./ml.), aldolase (0.55 mg./ml.), F1,6P (16 mM), NAD^+ (0.48 mM), Na_2HAsO_4 (30 mM), EDTA (0.1 mM) and triethanolamine (200 mM) adjusted to pH 8.6 with HCl. The trace shows duplicate runs. One syringe contained GPDH, aldolase, NAD^+ and EDTA and the other F1,6P. The other reagents were at equal concentrations in each syringe.

Since no burst of NADH was observed we also conclude that the rate-limiting step of the aldolase-catalysed reaction occurs before or synchronous with G3P_{ald} release.

It is likely that there are both active and inactive states of DHAP and F1,6P. However, in the above experiments the rate-limiting processes do not depend on the rate of conversion from inactive into active form of these substrates.

From a more physiological viewpoint, the $\text{G3P}_{\text{diol}}\text{--G3P}_{\text{ald}}$ equilibrium *in vivo* is expected to be displaced markedly in favour of G3P_{ald} owing to the specific binding of this form to the three enzymes, GPDH, TIM and aldolase, which are probably present in at least as great a molar concentration as G3P (Williamson, 1966; D. R. Trentham, C. H. McMurray & C. I. Pogson, unpublished work). This reinforces the view that extrapolations from results obtained with dilute enzyme solutions *in vitro* to conditions *in vivo* should be treated cautiously (Srere, 1967). The existence of different chemical states of G3P (and probably of DHAP) that are only slowly interconvertible may in part explain the apparent disequilibrium of the TIM reaction *in vivo* (Garfinkel, 1963).

Since GPDH, TIM and aldolase all react with G3P_{ald} , the slow $\text{G3P}_{\text{diol}}\text{--G3P}_{\text{ald}}$ interconversion is unlikely to influence the flux of glycolysis.

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