Phosphoglucosomerase-catalysed interconversion of hexose phosphates

Kinetic study by ^{13}C n.m.r. of the phosphoglucosomerase reaction in $^2\text{H}_2\text{O}$

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The fate of D-[2- ^{13}C]glucose 6-phosphate exposed to phosphoglucosomerase (glucose-6-phosphate isomerase, EC 5.3.1.9) in $^2\text{H}_2\text{O}$ was monitored by ^{13}C -n.m.r. spectroscopy. The generation of the anomers of both D-[2- ^{13}C]fructose 6-phosphate and D-[2- ^{13}C , 2- ^2H]glucose 6-phosphate followed a single-exponential pattern. The rate constant, which was proportional to the enzyme concentration, was about 14 times higher, however, in the former than in the latter case. The disappearance of D-[2- ^{13}C , 2- ^2H]glucose 6-phosphate occurred in a bi-exponential manner, the rate constants for the fast and the slow processes being in fair agreement with those obtained for the generation of D-[2- ^{13}C]fructose 6-phosphate and D-[2- ^{13}C , 2- ^2H]glucose 6-phosphate respectively. These findings indicate that the process of equilibration of D-[2- ^{13}C]glucose 6-phosphate and D-[2- ^{13}C]fructose 6-phosphate is at least one order of magnitude faster than the intermolecular proton transfer involving the deuterons from the solvent. Such a difference provides strong support to the view that the interconversion of hexose phosphates in the reaction catalysed by phosphoglucosomerase proceeds in two distinct steps, the second of which occurs according to two competing modalities with either an intramolecular or an intermolecular proton transfer.

INTRODUCTION

In a recent study [1] we used the $^1J(^{13}\text{C}-^2\text{H})$ coupling splitting of the $\text{C}_{(1)}$ signal of $^{13}\text{C}_{(1)}$ -enriched D-fructose 6-phosphate in the broadband ^1H -decoupled ^{13}C Fourier-transform n.m.r. spectrum of an equilibrium mixture of D-[1- ^{13}C]glucose 6-phosphate and D-[1- ^{13}C]fructose 6-phosphate, obtained by the phosphoglucosomerase (glucose-6-phosphate isomerase, EC 5.3.1.9) reaction, to demonstrate that deuterium incorporation from $^2\text{H}_2\text{O}$ into the $\text{C}_{(1)}$ atom of the latter is total. In strong contrast, during the phosphoglucosomerase-catalysed 'single-passage' conversion of D-[1- ^{13}C]glucose 6-phosphate into D-[1- ^{13}C]fructose 6-phosphate, obtained by the immediate subsequent irreversible conversion by phosphofructokinase of D-[1- ^{13}C]fructose 6-phosphate into D-[1- ^{13}C]fructose 1,6-bisphosphate, no deuterium incorporation into the $\text{C}_{(1)}$ of D-[1- ^{13}C]fructose 6-phosphate from $^2\text{H}_2\text{O}$ could be detected. This indicated a close-to-100 % intramolecular proton transfer [1,2] from the $\text{C}_{(2)}$ of D-[1- ^{13}C]glucose 6-phosphate to the $\text{C}_{(1)}$ of D-[1- ^{13}C]fructose 6-phosphate during this single-passage conversion. Furthermore, we observed a dramatic isotopic discrimination by the enzyme in the latter conversion, as only 72 % and 51 % of intramolecular transfer were observed when D-[1- ^{13}C , 2- ^2H]glucose 6-phosphate and D-[2- ^2H]glucose 6-phosphate in $^1\text{H}_2\text{O}$ were used as substrates for phosphoglucosomerase rather than D-[1- ^{13}C , 2- ^2H]glucose 6-phosphate in $^2\text{H}_2\text{O}$.

These results and other observations were rationalized with a model [1] in which it is assumed that the substrate–enzyme complex generated during the first step from D-glucose 6-phosphate can liberate D-fructose 6-phosphate through two competing steps, one involving intramolecular proton transfer from the $\text{C}_{(2)}$ of D-glucose 6-phosphate to the $\text{C}_{(1)}$ of D-fructose 6-phosphate, the other one involving intermolecular exchange between the $\text{C}_{(2)}$ hydrogen atom (^1H , ^2H or ^3H) of D-glucose 6-phosphate and the $^1\text{H}^+$, $^2\text{H}^+$ or $^3\text{H}^+$ ions from the solvent before their migration to the $\text{C}_{(1)}$ of D-fructose 6-phosphate. In this way, the isotopic discrimination observed could be explained by an isotope-induced modulation of the relative rates of these two competing steps.

A prerequisite to the validity of this model and our interpretation of the strong contrast in isotopic behaviour between phosphoglucosomerase-catalysed reversible (full deuterium incorporation) and phosphoglucosomerase + phosphofructokinase-catalysed single-passage (no deuterium incorporation) conversion of D-glucose 6-phosphate into D-fructose 6-phosphate is that in $^2\text{H}_2\text{O}$ the intramolecular transfer proceeds sufficiently faster than the intermolecular one as to make the latter unobservable under single-passage conditions. The full incorporation in the reversible phosphoglucosomerase reaction can then straightforwardly be explained by numerous forth-and-back reactions between D-glucose 6-phosphate and D-fructose 6-phosphate, resulting in the

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total replacement of the ^1H atom of the $\text{C}_{(2)}$ of D-glucose 6-phosphate by the ^2H atoms from the $^2\text{H}_2\text{O}$ solvent present in overwhelmingly higher concentration.

The major aim of the present study is to support the proposed model quantitatively by showing that the reversible generation of D-fructose 6-phosphate from D-glucose 6-phosphate through the intramolecular proton transfer is indeed much more rapid than the irreversible deuteration of these hexose 6-phosphates induced by intermolecular transfer from the $^2\text{H}_2\text{O}$. For such a purpose we undertook a kinetic study by ^{13}C -n.m.r. spectroscopy of the reversible phosphoglucosomerase equilibration of D-glucose 6-phosphate and D-fructose 6-phosphate in $^2\text{H}_2\text{O}$. The kinetics was followed by monitoring continuously and simultaneously the $^{13}\text{C}_{(2)}$ n.m.r. signals of the various 2- ^{13}C -enriched hexose 6-phosphates, deuterated or not, involved in the phosphoglucosomerase reaction.

MATERIALS AND METHODS

Sample preparation for kinetic experiments

D-[2- ^{13}C]Glucose (99 % isotopic abundance) and $^2\text{H}_2\text{O}$ (99.8 % isotopic abundance) were purchased from Cambridge Isotope Laboratories (Woburn, MA, U.S.A.) and Merck (Darmstadt, Germany), respectively. Yeast hexokinase (EC 2.7.1.1) and phosphoglucosomerase were obtained from Boehringer (Mannheim, Germany). The 6-phosphate ester of D-[2- ^{13}C]glucose (45 mM) was prepared over 90 min incubation at 37 °C in 20 mM-Tris/HCl buffer, pH 7.0, containing MgCl_2 (6 mM), EDTA (1 mM), ATP (50 mM) and yeast hexokinase (14 units/ml). The reaction was halted by heating for 10 min at 80 °C, and the medium (1.0 ml) was freeze-dried and reconstituted with 0.7 ml of $^2\text{H}_2\text{O}$ in the presence of 5, 25 or 500 munits of phosphoglucosomerase. Under these experimental conditions the residual amount of unphosphorylated D-glucose does not exceed about 3.0 ± 0.3 %.

N.m.r. measurements and acquisitions of the kinetic data

The ^1H -broad-band-decoupled ^{13}C -n.m.r. spectra were recorded fully automatically, under computer control, over periods of 45–60 h. The free induction decays were collected in series files, on a Bruker WM 250 n.m.r. spectrometer operating at 62.90 MHz for ^{13}C , equipped with an Aspect 2000 computer and a 5 mm double-tuned probe for ^1H and ^{13}C acquisition. The signal of $^2\text{H}_2\text{O}$ was taken as internal lock. The spectra were acquired at a temperature of 304 K. The microprogram was written as to start a free-induction-decay acquisition every 40 min for a duration of 500 s, with 100 scans for each acquisition, as to have a relaxation delay of 2.12 s and an acquisition time of 2.88 s, for a pulse duration of 3.0 μs and 32k memory size. The middle of the first acquisition corresponded to times 24 min, 30 min and 20 min respectively for Expts. 1, 2 and 3. The total numbers of free induction decays collected were 100, 96 and 60 for Expts. 1, 2 and 3 respectively. All chemical-shift data are referenced to the chemical shift of the $\text{C}_{(2)}$ resonance of β -[2- ^{13}C]fructose 6-phosphate, taken as 102.4 p.p.m. [3]. The time monitoring of the different species was done with the signal intensities taken as time-dependent variables. No corrections to the intensity saturation, because of probably incomplete T_1 relaxation between two scans,

were performed, as no concentration determinations are needed to characterize the rate profiles of generation or disappearance of the different $^{13}\text{C}_{(2)}$ -enriched hexose 6-phosphates involved.

Statistical treatment of the data

Non-linear-regression analyses were performed with the modules NONLIN and DATA of the statistical package SYSTAT, version 3.0 for HFS (1986), written by L. Wilkinson and M. Bjerknes and published by SYSTAT Inc. All rate constants were fitted with a least-square sum as loss function; the quality of the fit was evaluated with the final value of the loss function and the standard error on each parameter resulting from the fit.

Unless otherwise specified, all rate constants given below are means $\pm$ S.E.M. These means were obtained, as explained in the text, from different fits to the same parameter during the treatment of the time variation of signal intensities from different species. They were calculated with the statistical package STATWORKS, version 1.1 (1985), published by CRICKET SOFTWARE.

All results are given as the means $\pm$ S.E.M. Standard errors on ratios of rate constants were determined by using a method described previously [4].

RESULTS

Methodological considerations

Our basic strategy was to follow the fast equilibration of D-[2- ^{13}C]glucose 6-phosphate and D-[2- ^{13}C]fructose 6-phosphate, associated with the intramolecular proton transfer, by monitoring, as a function of time, the intensity of the $^{13}\text{C}_{(2)}$ singlets, in ^1H -broad-band-decoupled ^{13}C -n.m.r. spectra, of all undeuterated D-[2- ^{13}C]hexose 6-phosphates involved in this process. The slow deuteration of D-[2- ^{13}C]glucose 6-phosphate, resulting from the intermolecular transfer, was followed by monitoring the intensity of the characteristic 1:1:1 triplet patterns [1], resulting from the $^1J(^{13}\text{C}-^2\text{H})$ coupling, of the $\text{C}_{(2)}$ atom of both anomers of D-[2- ^{13}C , 2- ^2H]glucose 6-phosphate. The deuterated D-[2- ^{13}C , 1- ^2H]fructose 6-phosphate anomers are unobservable, as they are deuterated in the ^{13}C -unenriched $\text{C}_{(1)}$ position, so that their $\text{C}_{(2)}$ signals reflect merely the total pools of undeuterated and deuterated species. As explained below, the time decrease of the singlet intensities of the undeuterated β - and α -anomers of [2- ^{13}C]glucose 6-phosphate reflect actually both the fast and the slow processes.

This study was conducted with D-[2- ^{13}C]glucose 6-phosphate rather than D-[1- ^{13}C]glucose 6-phosphate for the two following main reasons. First, the β - and α -anomers of fructose 6-phosphate exhibit two distinguishable well-separated $\text{C}_{(2)}$ signals, instead of the unique broad one resulting from the $\text{C}_{(1)}$ atoms of both β - and α -anomers of D-[1- ^{13}C]fructose 6-phosphate [1]. In this way, time courses can be followed for the two anomers, as well for glucose 6-phosphate as for fructose 6-phosphate (Fig. 1). Secondly, handling of kinetic data would be more inaccurate with D-[1- ^{13}C]hexose 6-phosphates than with D-[2- ^{13}C]hexose 6-phosphates. Indeed, at equilibrium, the signal intensity of each of the 1:1:1 triplet components of [1- ^{13}C , 1- ^2H]fructose 6-phosphate is more than 10-fold smaller than those of the singlet signals of [1- ^{13}C , 2- ^2H]glucose 6-phosphate [1], because the equilibrium concentration of D-fructose 6-

phosphate is 3 times smaller than that of D-glucose 6-phosphate, and because the former when deuterated gives rise to signals split in triplets whereas the latter keeps singlets even when deuterated. Moreover, the triplet components appear to be broader than the singlets. The dynamic range problem created in this way would give triplet signals that are too noisy and that would have too low an amplitude resolution. In contrast, when deuterated D-[2-¹³C]hexose 6-phosphates are used the equilibrium mixture exhibits signal intensities differing by less than 3-fold, because here the signals of the 3-fold more abundant D-[2-¹³C]glucose 6-phosphate are eventually split into triplets as the aldohexose ester becomes deuterated whereas those of the D-[2-¹³C]fructose 6-phosphate remain unsplit even when deuterated.

According to the model proposed [1], the generation of the total pools of β- and α-D-[2-¹³C]fructose 6-phosphate and that of β- and α-D-[2-¹³C,2-²H]glucose 6-phosphate should proceed mono-exponentially, with rate constants depending linearly on the analytical enzyme concentration [E]_T and pairwise identical because of the rapid equilibrium between the β- and α-anomers. The numerical value of the rate constants should be significantly larger for the former generation than for the latter, according to our model hypothesis [1]. We therefore performed a least-square two-parameter fit to the time courses of the signal intensities *I*(*t*) associated with these generations according to the equation:

$$I(t) = I(\infty)(1 - e^{-k_i t}) \quad (1)$$

The rate constants (*k_i*) for equilibration are denoted as *k_{fast}* or *k_{slow}* according to whether the fast process of D-glucose 6-phosphate–D-fructose 6-phosphate equilibration or the slow process of deuteration at the C₍₂₎ atom of D-[2-¹³C]glucose 6-phosphate is concerned. Consistently, the time courses of the signal intensities associated with the decrease of undeuterated β- and α-D-[2-¹³C,2-¹H]glucose 6-phosphate should obey a bi-exponential law, the rate constants of which should actually coincide with *k_{fast}* and *k_{slow}*. We therefore performed a least-square five-parameter fit to the time courses of the signal intensities *I*(*t*) associated with these decreases according to the equation:

$$I(t) = I(\infty) + I_f e^{-k_{fast} t} + I_s e^{-k_{slow} t} \quad (2)$$

The term *I*(∞) was introduced to account for the contribution of unphosphorylated D-glucose (see the

Materials and methods section) and possible incomplete deuteration of the D-[2-¹³C]glucose 6-phosphate pool.

Experimental findings

Three kinetic experiments were performed, with phosphoglucisomerase activities of 5 munits (Expt. 1), 25 munits (Expt. 2) and 500 munits (Expt. 3) per 0.7 ml, over time periods of 60 h for the first two experiments and 45 h for the last one.

At the end of Expt. 1, no triplet-like patterns associated with deuterated D-[2-¹³C]glucose 6-phosphate were observed. This indicates that the enzyme concentration was too low for the deuteration process to be directly observable on the experimental time scale. However, whereas the signal intensities of the D-[2-¹³C]fructose 6-phosphates had reached their stationary equilibrium value, those of the D-[2-¹³C]glucose 6-phosphates had not, exhibiting a value of 70 % of the initial value, lower than the 75 % normally expected for equilibration between D-glucose 6-phosphate and D-fructose 6-phosphate. This apparent discrepancy is explained by the much higher intensity-sensitivity of the narrow singlet signals of the undeuterated D-[2-¹³C]glucose 6-phosphates compared with that of the broad triplet components of the deuterated ones. Thus the failure of the singlet signals of undeuterated D-[2-¹³C,2-¹H]glucose 6-phosphate to reach a stationary value at a time when those of D-fructose 6-phosphate already had is due to the slow deuteration process, which causes the intensity of these signals to decrease further below the equilibrium intensity expected from the sole equilibration of D-[2-¹³C]glucose 6-phosphate and D-[2-¹³C]fructose 6-phosphate in the absence of deuteration.

Despite their inherent limitations, the results of Expt. 1 could be used not only to fit the rate constants of the fast process from the time courses of D-[2-¹³C]fructose 6-phosphate with the mono-exponential law, but also to fit the rate constants of the slow deuteration from the time courses of D-[2-¹³C]glucose 6-phosphate with the bi-exponential law, even if in this experiment the deuteration was too slow to be observed directly. The results are given in Table 1. It should be emphasized that the only relevant bi-exponential fits that were obtained for both Expts. 1 and 2 were those in which the term *I*(∞) in eqn (2) was taken *a priori* to be zero. In the case of Expt. 1 the data relative to β-D-[2-¹³C,2-¹H]glucose 6-phosphate when fitted in terms of two exponentials yielded one rate

Table 1. Rate constants for the phosphoglucisomerase reaction

The data refer to the mono-exponential generation of α- and β-D-[2-¹³C]fructose 6-phosphate (Fru6P) and α- and β-D-[2-¹³C,2-²H]glucose 6-phosphate [Glc6P(²H)] and the bi-exponential disappearance of α- and β-D-[2-¹³C,2-¹H]glucose 6-phosphate [Glc6P(¹H)]. Abbreviation: N.D., not determined.

	Expt. 1 (5 munits of enzyme)		Expt. 2 (25 munits of enzyme)		Expt. 3 (500 munits of enzyme)	
	<i>k_{fast}</i>	<i>k_{slow}</i>	<i>k_{fast}</i>	<i>k_{slow}</i>	<i>k_{fast}</i>	<i>k_{slow}</i>
α-Fru6P	0.045 ± 0.003		0.100 ± 0.005		1.20 ± 0.81	
β-Fru6P	0.045 ± 0.002		0.111 ± 0.005		1.45 ± 0.30	
α-Glc6P(¹ H)	0.031 ± 0.008	0.0022 ± 0.0008	0.114 ± 0.007	0.0068 ± 0.0002	1.59 ± 0.26	0.121 ± 0.002
β-Glc6P(¹ H)	0.017 ± 0.006	−0.0006 ± 0.0025	0.121 ± 0.006	0.0069 ± 0.0001	1.45 ± 0.14	0.119 ± 0.002
α-Glc6P(² H)		N.D.		N.D.		0.112 ± 0.011
β-Glc6P(² H)		N.D.		N.D.		0.102 ± 0.004

constant not significantly different from zero, while the other one was not significantly different from the value obtained by a mono-exponential fit. In both models the least-square loss functions had nearly identical values (results not shown), indicating very flat least-square minima. We interpret this by the fact that the intensity variation due to the deuteration process is too slow within the experiment time scale to be reasonably treatable. Nevertheless, the mono-exponential rate constants found for the D-[2-¹³C,2-¹H]glucose 6-phosphate time courses ($0.019 \pm 0.001 \text{ h}^{-1}$ for either α - or β -D-glucose 6-phosphate) were lower than for the D-[2-¹³C]fructose 6-phosphate ones, suggesting that the mono-exponential decrease due to D-glucose 6-phosphate and D-fructose 6-phosphate equilibration was contaminated by the deuteration process. In this context it is worthwhile to underline that the data relative to α -D-[2-¹³C]glucose 6-phosphate could actually be fitted in terms of two exponentials, even if the two rate constants obtained exhibit a large standard error. Therefore the ratio of rate constants $k_{\text{fast}}/k_{\text{slow}}$ (14.1 ± 6.3) found from the time courses of α -D-[2-¹³C]glucose 6-phosphate in Expt. 1 must be considered as only indicative.

In Expt. 2 the triplets of the deuterated D-[2-¹³C,2-²H]glucose 6-phosphate anomers hardly started to become visible above noise level at the end of the experiment, and the singlet signals for D-[2-¹³C,2-¹H]glucose 6-phosphate had intensities far below the normal equilibrium value, typically 55%.

The data of Expt. 2 gave unambiguously better bi-exponential than mono-exponential fits for the D-[2-¹³C,2-¹H]glucose 6-phosphate intensity time courses (results not shown). The two rate constants k_{fast} obtained from the mono-exponential fits of the β - and α -anomers of D-[2-¹³C]fructose 6-phosphate coincided satisfactorily both mutually and with those obtained from the bi-exponential fits of the β - and α -anomers of D-[2-¹³C]glucose 6-phosphate. The rate constants k_{fast} ($0.112 \pm 0.004 \text{ h}^{-1}$; $n = 4$) and k_{slow} ($0.00682 \pm 0.00006 \text{ h}^{-1}$; $n = 2$) yielded a $k_{\text{fast}}/k_{\text{slow}}$ ratio of 16.4 ± 0.6 , in satis-

factory agreement with the values obtained from Expts. 1 (see above) and 3 (see below).

Fig. 1 gives a stacked plot of seven spectra at increasing experiment times, out of the 45 obtained from the acquisitions during Expt. 3. It clearly reveals that the C₍₂₎ signals of α - and β -D-[2-¹³C]fructose 6-phosphate, at 105.3 and 102.4 p.p.m. respectively, reach a constant intensity in the early stage of the experiment. In contrast, the C₍₂₎ singlets of β - and α -D-[2-¹³C,1-¹H]glucose 6-phosphate, at 75.1 and 72.5 p.p.m. respectively, decrease much more slowly, during the synchronous slow appearance of the C₍₂₎ triplets of β -D-[2-¹³C,2-²H]glucose 6-phosphate [at 75.0, 74.6, 74.2 (± 0.1) p.p.m., $|^1J(^{13}\text{C}-^2\text{H})| = 22 \pm 1 \text{ Hz}$] and of α -D-[2-¹³C,2-²H]glucose 6-phosphate [at 72.4, 72.0, 71.6 (± 0.1) p.p.m., $|^1J(^{13}\text{C}-^2\text{H})| = 22 \pm 1 \text{ Hz}$]. Note, in this respect, the strong overlap between the low-field component of the triplet and the singlet, indicating a ²H isotopic high-field shift of $0.5 \pm 0.1 \text{ p.p.m.}$

The time courses obtained in Expt. 3 for the signal intensities of β - and α -D-[2-¹³C,2-¹H]glucose 6-phosphate, β - and α -D-[2-¹³C,2-²H]glucose 6-phosphate and of the total pools, undeuterated and deuterated together, of β - and α -D-[2-¹³C]fructose 6-phosphate are given in Fig. 2.

Expt. 3 was the only one in which the deuteration was directly monitored through the time-related increase in the intensity of the triplets of D-[2-¹³C,2-²H]glucose 6-phosphate anomers. In this experiment the deuteration process was also indirectly monitored, however, as explained above for Expts. 1 and 2. In Expt. 3 the term $I(\infty)$ amounted to $3.7 \pm 0.2 \%$ of the sum of $I(\infty) + I_t + I_s$. This arises from a small contribution of unphosphorylated D-glucose to the n.m.r. resonance and, possibly, to minor contamination of ²H₂O by ¹H₂O.

The results obtained from the fit of the data according to eqns. (1) and (2) in Expt. 3 are given in Table 1. Two main features need to be underlined. First, the rate constants obtained from the α - and β -anomers, whatever the hexose phosphate under consideration, did not differ significantly from one another. Secondly, the rate con-

Fig. 1. Stacked plot of seven ¹H-broad-band-decoupled ¹³C-N.m.r. spectra out of 45, representing the kinetics of the phosphoglucoisomerase reaction performed on an equilibrium mixture of the β - and α -D-[2-¹³C]glucose 6-phosphate anomers in the presence of an enzyme activity of 500 munits in 0.7 ml of ²H₂O (Expt. 3)

Assignments: α - and β -D-[2-¹³C]fructose 6-phosphate, 105.3 and 102.4 p.p.m. respectively; β - and α -D-[2-¹³C,2-¹H]glucose 6-phosphate, 75.1 and 72.5 p.p.m. respectively; β -D-[2-¹³C,2-²H]glucose 6-phosphate, 75.0, 74.6, 74.2 (± 0.1) p.p.m., $|^1J(^{13}\text{C}-^2\text{H})| = 22 \pm 1 \text{ Hz}$; α -[2-¹³C,2-²H]glucose 6-phosphate, 72.4, 72.0, 71.6 (± 0.1) p.p.m., $|^1J(^{13}\text{C}-^2\text{H})| = 22 \pm 1 \text{ Hz}$.

Fig. 2. Time courses of the signal intensity for the $^{13}\text{C}_{(2)}$ singlets of either D-[2- ^{13}C ,2- ^1H]glucose 6-phosphate (a) or D-[2- ^{13}C]fructose 6-phosphate (b) and for the middle component of the $^{13}\text{C}_{(2)}$ triplets of D-[2- ^{13}C ,2- ^2H]glucose 6-phosphate (c)

White and black symbols refer respectively to the α - and β -anomer of each hexose ester (Expt. 3). Note the 2-fold and 4-fold increase of the ordinate scales in (b) and (c) respectively relative to that in (a).

stants k_{fast} and k_{slow} obtained from the mono-exponential fits of the data associated with [2- ^{13}C ,2- ^2H]glucose 6-phosphate and [2- ^{13}C]fructose 6-phosphate were satisfactorily of comparable value with the rate constants k_{fast} and k_{slow} obtained from the bi-exponential fits of the data associated with [2- ^{13}C ,2- ^1H]glucose 6-phosphate. The high standard error on the fit associated with the time course of α -D-[2- ^{13}C]fructose 6-phosphate is due to the intrinsic low intensity of its signal and, hence, its subsequent noisy character. Averaging out the four values of the rate constants obtained in this way for each process gives $k_{\text{fast}} = 1.41 \pm 0.08 \text{ h}^{-1}$ ($n = 4$) and $k_{\text{slow}} = 0.114 \pm 0.004 \text{ h}^{-1}$ ($n = 4$). The ratio $k_{\text{fast}}/k_{\text{slow}}$ resulting from these values is 12.5 ± 0.9 .

Fig. 3 indicates that the values of both k_{fast} and k_{slow} derived from the experiments performed with increasing amounts of phosphoglucose isomerase were grossly proportional to the enzyme content of the incubation medium, further documenting the internal consistency of our analytical approach. The difference in elevation of

Fig. 3. Relationship in logarithmic co-ordinates between either k_{fast} (upper line) or k_{slow} (lower line) and the amount of phosphoglucose isomerase present in the assay medium (0.7 ml)

The data were derived from the generation of D-[2- ^{13}C]fructose 6-phosphate ($\circ$ and $\bullet$), disappearance of D-[2- ^{13}C ,2- ^1H]glucose 6-phosphate ($\triangle$ and $\blacktriangle$) or appearance of D-[2- ^{13}C ,2- ^2H]glucose 6-phosphate ($\square$ and $\blacksquare$), white and black symbols referring respectively to the α - and β -anomer of each hexose ester. The lines were drawn on the assumption of a rule of proportionality between k_i and enzyme content.

the two lines drawn in Fig. 3 also confirmed that k_{fast} is about 13–14 times higher than k_{slow} . Likewise, linear-regression analyses in linear co-ordinates yielded, for the volume of 0.7 ml used in all experiments, a k_{fast} of $0.0028 \pm 0.0001 \text{ munit}^{-1} \cdot \text{h}^{-1}$ ($r = 0.991$) and a k_{slow} of $0.00023 \pm 0.00001 \text{ munit}^{-1} \cdot \text{h}^{-1}$ ($r = 0.994$), with a $k_{\text{fast}}/k_{\text{slow}}$ ratio of 12.2 ± 0.8 .

DISCUSSION

The present results unambiguously demonstrate that the rate constant for the reversible interconversion of D-glucose 6-phosphate and D-fructose 6-phosphate in $^2\text{H}_2\text{O}$ is much higher than that for the irreversible deuteration of these hexose phosphates. Such a difference may well account for the fact that virtually no deuteration of hexose phosphate could be detected when the mono-directional conversion of D-[1- ^{13}C]glucose 6-phosphate into D-[1- ^{13}C]fructose 6-phosphate in $^2\text{H}_2\text{O}$ was coupled to the irreversible phosphofructokinase-catalysed generation of D-[1- ^{13}C]fructose 1,6-bisphosphate [1]. The

most likely explanation for such a situation consists in the existence of two competing modalities for the generation of D-fructose 6-phosphate (or D-glucose 6-phosphate) from the enzyme-substrate complex, one involving an intramolecular and the other an intermolecular proton transfer [1]. Isotopic preference in the prevalence of each modality is operative, since the relative extent of the intramolecular transfer varies to a large extent, depending on the hydrogen isotope present on the C₍₂₎ (or C₍₁₎) atom of D-glucose 6-phosphate (or D-fructose 6-phosphate) or in the solvent. Such an isotopic preference must be clearly distinguished from the isotopic discrimination in reaction velocity, which itself rather appears to be attributable to differences in the rate of generation of the enzyme-substrate complex [1].

The vast difference in rate constants for the isomerization and deuteration of hexose phosphates, as revealed by the present study, is directly relevant to metabolic studies conducted with labelled D-glucose in cell-free systems, isolated intact cells or the whole organism. Indeed, by analogy with the present results, it can be surmised that the relative extent of D-[2-³H]glucose 6-phosphate detritiation in the reaction catalysed by phosphoglucosomerase will be tightly dependent on the frequency of back-and-forth interconversion of hexose phosphates relative to the net monodirectional flow through the same reaction. Hence, by use of an analytical model comparable with that developed in the present study, it should be possible to obtain information on the respective rates of both back-and-forth interconversion of hexose phosphates and their net production in the reaction catalysed by phosphoglucosomerase, e.g. by monitoring the generation of ³H₂O and tritiated glycolytic end products in cells exposed to D-[2-³H]glucose. It should be realized, however, that even for the monodirectional conversion of each hexose phosphate the relative extent of intramolecular and intermolecular hydrogen transfer depends, not solely on the isotope (¹H, ²H or ³H) present in the hexose phosphate and solvent

and on the identity of the substrate (D-glucose 6-phosphate or D-fructose 6-phosphate), but also on a number of environmental factors such as the ambient temperature or concentration of inorganic phosphate [2,5]. It is also conceivable that the relative extent of hydrogen transfer can be affected by covalent changes of the enzyme in pathological situations, as resulting for instance from its non-enzymic glycation [6].

Taking into consideration this complex regulation of both reaction velocities and hydrogen transfer, it would seem desirable to monitor, in intact cells, both the intracellular content of the hexose phosphates concerned and the generation of further glycolytic intermediates or end products. The ¹³C-n.m.r. technique used in the present study, when suitably adapted to the study of isolated cells, might well represent the most appropriate approach to achieve such a monitoring by a non-invasive procedure.

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