Does phosphoglucosomerase display anomeric specificity or selectivity towards α -D-glucose 6-phosphate?

An assessment by two-dimensional phase-sensitive ^{31}P exchange spectroscopy (EXSY) n.m.r.

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The study by two-dimensional phase-sensitive ^{31}P exchange spectroscopy (EXSY) n.m.r. of hexose 6-phosphates inter-conversion in the reaction catalysed by yeast phosphoglucosomerase reveals that the enzyme displays anomeric sel-

ectivity, rather than specificity, towards α -D-glucose 6-phosphate. Indeed, β -D-glucose 6-phosphate participates for about 20 % to the total and direct conversion of the aldohexose into oxohexose ester.

INTRODUCTION

The specific recognition of the α -anomer of D-glucose 6-phosphate by phosphoglucosomerase in the reversible conversion between D-fructose 6-phosphate and D-glucose 6-phosphate is well supported by numerous reports [1–3], including n.m.r. studies [4,5]. More recently the strong anomeric preference of phosphoglucosomerase towards β -D-fructose 6-phosphate was evidenced [4] from phase-sensitive two-dimensional (2D) ^{13}C - $\{^1\text{H}\}$ exchange spectroscopy (EXSY) n.m.r. experiments [6–11] using ^{13}C -enriched substrates in the C-2 position of the D-hexose 6-phosphates. This unambiguous conclusion from the latter study [4] was at variance with earlier proposals [1–2] that favoured the less abundant α -anomer of D-fructose 6-phosphate.

Although the same ^{13}C study [4] leaves no doubt about the well-documented anomeric preference of phosphoglucosomerase towards α -D-glucose 6-phosphate, the possibility of restricted recognition of β -D-glucose 6-phosphate by phosphoglucosomerase was briefly discussed. The present paper presents an accurate assessment of the question of the anomeric *selectivity* rather than *specificity* of phosphoglucosomerase towards α -D-glucose 6-phosphate. Our study uses phase-sensitive 2D ^{31}P - $\{^1\text{H}\}$ EXSY n.m.r. spectroscopy [6–10], which provides the necessary high signal-to-noise ratio as compared with ^{13}C n.m.r. [4] and phase-insensitive ^{31}P EXSY n.m.r. [5]. The rate constants, on the other hand, were obtained by a so-called 'normal mode analysis' [12], proposed earlier by Gipe and Perrin [8,10], and used as 'method C' in the previous ^{13}C -n.m.r. study [4].

MATERIALS AND METHODS

Chemicals and sample preparation

D-Fructose 6-phosphate (disodium salt; Boehringer-Mannheim) was dissolved at a concentration of 40 mM in a Tris/HCl buffer (pH 7.6, 16 mM) prepared in 20 % $^2\text{H}_2\text{O}$ (v/v) and containing MgCl_2 (1.6 mM), EDTA (0.8 mM) and NaN_3 (0.8 mM). Yeast phosphoglucosomerase (EC 5.3.1.9), also obtained from Boehringer-Mannheim, was added to achieve a concentration of 1500 units/ml.

N.m.r. measurements

The phase-sensitive 2D ^{31}P - $\{^1\text{H}\}$ EXSY [6–11] spectra were recorded at 27 °C at 202.46 MHz on a Bruker AMX 500 spectrometer equipped with a X32 computer, using a 5 mm ^{31}P - $\{^1\text{H}\}$ probehead. The ^{31}P resonances were referenced to external 85 % H_3PO_4 , to which ~ 5 % of $^2\text{H}_2\text{O}$ was added. The 2D ^{31}P - $\{^1\text{H}\}$ EXSY maps were obtained from the basic nuclear-Overhauser-enhancement-spectroscopic [6] three 90° pulse sequence with time proportional phase incrementing [11] and 16-order phase cycling; 16 scans were accumulated with gated Waltz decoupling for each of the 128 t_1 increments, zero-filled to 512 W in the F_1 dimension, using 512 W in the F_2 dimension without zero-filling, 2 s relaxation delay and a spectral window of 567 Hz (acquisition time 452 ms). Preliminary experiments with longer relaxation delays did not reveal any significant change in the relative integrals of the resonances, indicating essentially full T_1 relaxation within the recycling delay. Eight experiments were performed under such conditions with mixing times set at 400, 500, 600, 700, 800, 900, 1100, 1300 ms. The free induction decays were multiplied by a shifted sine-bell:

$$F(t) = \sin\{[(\pi - \phi)(t/AT)] + \phi\}$$

with $\phi = \pi/2$ prior to Fourier transformation. The 2D spectra were phase- and baseline-corrected along both dimensions. The auto- and cross-peak volumes were determined using the standard Bruker UXNMR software. The rate constants of interest were evaluated by the approach of Perrin and Gipe [8,10], using method C described previously [4]. The resulting rate constants, expressed in s^{-1} , are presented as means $\pm$ S.D. over the eight experiments performed.

RESULTS

The standard one-dimensional (1D) ^{31}P - $\{^1\text{H}\}$ n.m.r. spectrum revealed the same pattern as was observed previously [5]. Integration of the three ^{31}P resonances at 7.18, 7.08 and 6.62 p.p.m. provided equilibrium molar fractions of 0.499, 0.284 and 0.217 for respectively β -D-glucose 6-phosphate, α -D-glucose 6-phosphate and the pool of α -D-fructose 6-phosphate and β -D-

Figure 1 Contour plot of the 2D EXSY $^{31}\text{P}\{-^1\text{H}\}$ n.m.r. spectrum obtained from an equilibrium mixture of β -D-glucose 6-phosphate, α -D-glucose 6-phosphate and D-fructose 6-phosphate in the presence of phosphoglucosomerase

The mixing time was 1100 ms; ^{31}P resonances from low to high field on the diagonal and the 1D spectrum on top of the 2D EXSY map: β -D-glucose 6-phosphate, α -D-glucose 6-phosphate and D-fructose 6-phosphate. Cross-peaks represent direct one-step and/or relayed two-step chemical transfers from a chemical species with a resonance frequency on the vertical F_1 -axis to another one with another resonance frequency on the horizontal F_2 -axis.

fructose 6-phosphate, in good agreement with previously obtained data [4,5].

As an example, Figure 1 presents the contour plot of the 2D phase-sensitive $^{31}\text{P}\{-^1\text{H}\}$ EXSY n.m.r. spectrum obtained with a mixing time of 1100 ms. The general principles underlying the interpretation of such spectra were reviewed [7,8]. The eight 2D $^{31}\text{P}\{-^1\text{H}\}$ EXSY spectra, obtained from acquisitions with mixing times of 400, 500, 600, 700, 800, 900, 1100 and 1300 ms, revealed cross-peaks the volumes of which were identical within experimental error (typically 5%) for pairs symmetrical with respect to the diagonal. As a consequence, the normal mode analysis [12] of Perrin and Gipe [8,10] was applied on the mean of the cross-peak pairs symmetric with respect to the diagonal, so as to smooth out errors due to the numerical normal mode analysis. A recent study [4] showed this method ('method C') to provide the most reliable rate constants.

Table 1 gives an overview of the rate constants obtained for each of the possible isomer conversions. The pairs of rate constants of mutually reverse conversions obey the Law of Mass Action, as they have to, since the cross-peak volumes of resonances symmetric with respect to the diagonal have been symmetrized to their average, according to method C, and in agreement with theoretical expectation. This results in the *relative* equilibrium fluxes ($=k_{ij}x_j$, with k_{ij} the rate constant for the conversion of j into i and x_j the equilibrium molar fraction of j), to be 0.050 s^{-1} for the interconversion between the D-fructose 6-phosphate pool and β -D-glucose 6-phosphate, 0.194 s^{-1} for the interconversion between the D-fructose 6-phosphate pool and α -D-glucose 6-phosphate, and 0.144 s^{-1} for the interconversion between the anomers of D-glucose 6-phosphate. Incidentally, these values correspond to an equilibrium rate of D-glucose 6-phosphate to D-fructose 6-phosphate interconversion close to $0.6\text{ mmol}\cdot\text{min}^{-1}\cdot\text{ml}^{-1}$, in fair agreement with the nominal activity of yeast phosphoglucosomerase in these experiments.

These results show a slight participation of $\sim 20\%$ of the β -D-glucose 6-phosphate anomer in the direct conversion of the D-glucose 6-phosphate pool into the D-fructose 6-phosphate pool by phosphoglucosomerase. This conclusion suggests phosphoglucosomerase to be anomeric-selective rather than anomeric-specific, since about one from five conversions appears to involve directly the β -anomer of D-glucose 6-phosphate rather than the α -one. These observations confirm the trend suggested by our ^{13}C study [4], but are at first glance at variance with the observations of Balaban and Ferretti in their early ^{31}P study [5].

Table 1 Rate constants of interconversions at chemical equilibrium of the hexose 6-phosphates of the phosphoglucosomerase reaction

Abbreviations: F6P, pool of the spectrally indistinguishable α -D-fructose 6-phosphate and β -D-fructose 6-phosphate; α -G6P, α -D-glucose 6-phosphate; β -G6P, β -D-glucose 6-phosphate.

Mixing time (ms)	Isomer conversion ...	Rate constant (s^{-1})					
		F6P to β -G6P	β -G6P to F6P	F6P to α -G6P	α -G6P to F6P	β -G6P to α -G6P	α -G6P to β -G6P
1300		0.248	0.108	0.881	0.673	0.275	0.485
1100		0.228	0.099	0.883	0.675	0.282	0.497
900		0.230	0.100	0.874	0.668	0.281	0.494
800		0.234	0.101	0.901	0.689	0.297	0.523
700		0.202	0.088	0.896	0.685	0.290	0.511
600		0.213	0.092	0.944	0.722	0.293	0.516
500		0.280	0.121	0.885	0.676	0.292	0.514
400		0.223	0.097	0.883	0.675	0.297	0.523
Averages ...		0.232	0.101	0.893	0.683	0.288	0.508
S.D. ...		0.024	0.010	0.022	0.017	0.008	0.014
Variance coefficient (%) ...		10.2	10.2	2.5	2.5	2.8	2.8

As in the ^{13}C study [4], the variance coefficient of the rate constants for the D-fructose pool and β -D-glucose 6-phosphate is higher than for the other ones. This is due to the lower value of the rate constant and the consecutive hybrid character of the build-up curves associated with these conversions, being a superposition of a 'weak' one-step transfer between the D-fructose 6-phosphate pool and β -D-glucose 6-phosphate and a strong relayed two-step transfer via α -D-glucose 6-phosphate.

DISCUSSION

The present paper presents a more accurate assessment of the question of the anomeric *selectivity* rather than *specificity* of phosphoglucisomerase towards α -D-glucose 6-phosphate. Our study uses phase-sensitive 2D ^{31}P - $\{^1\text{H}\}$ EXSY n.m.r. spectroscopy [6–10], which provides the necessary high signal-to-noise ratio as compared with ^{13}C - $\{^1\text{H}\}$ n.m.r., albeit at the expense of spectral discrimination between the α - and β -anomers of D-fructose 6-phosphate [4,5], which is lost in ^{31}P - $\{^1\text{H}\}$ EXSY n.m.r. The rate constants, on the other hand, were obtained by a so-called 'normal mode analysis' [12], proposed previously by Gipe and Perrin [8,10] and used as method C in the previous ^{13}C n.m.r. study [4]. The favourable conditions of cross-peak volume determination related to both the high sensitivity of the ^{31}P nucleus and the absence of overlapping in the resonances of interest to this question, as well as applying this method C to a set of data acquired with different mixing times, resulting in a sharp average value of all rate constants of interest, exclude the source of errors usually invoked [12] against this approach [8,10]. The possibility that β -D-glucose 6-phosphate might be recognized as well by phosphoglucisomerase, even to a limited extent, was briefly discussed on the basis of build-up curves with ill-defined profiles and conversion-rate-constant averages with high variation coefficients, despite their indisputable statistically significant non-zero value [4]. An early study [5] using ^{31}P EXSY n.m.r. did not favour such a possibility. However, the availability at that time of essentially only phase-insensitive EXSY spectroscopy as well as the poorly accurate [12] initial-rate approximation [7–12] left little chance to the potentially weaker one-step build-up associated with the conversion from β -D-glucose 6-phosphate into β -D-fructose 6-phosphate to be really observed in addition to the well-marked two-step build-up associated with the relayed conversion β -D-glucose 6-phosphate $\rightarrow$ α -D-glucose 6-phosphate $\rightarrow$ β -D-fructose 6-

phosphate. This holds also to a lesser extent for our own ^{13}C study [4], where the necessary trade-off between reasonable data acquisition times and the sensitivity due to the ^{13}C nucleus limited somewhat the signal-to-noise ratio, and therefore hampered weak short mixing-time cross-peaks of the one-step build-up for the conversion from β -D-glucose 6-phosphate into β -D-fructose 6-phosphate from being observed with high accuracy [4,5,12].

The finding that β -D-glucose 6-phosphate may act to a limited extent as a substrate in the reaction catalysed by phosphoglucisomerase is not incompatible with the results of previous experiments in which the anomeric preference of phosphoglucisomerase was explored by generating extemporaneously the anomers of D-glucose 6-phosphate from pure α - or β -D-glucose 6-phosphate [13,14]. It may also account, at least in part, for the obvious occurrence of a sizeable glycolytic flux in intact cells exposed to pure β -D-glucose [13,15,16].

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