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^{31}P Magnetization transfer in the phosphoglyceromutase-enolase coupled enzyme system

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Abstract. The rate of exchange of phosphoryl groups between 2-phosphoglycerate, 3-phosphoglycerate and phosphoenolpyruvate by the coupled phosphoglyceromutase-enolase enzyme system using one- and two-dimensional ^{31}P NMR spectroscopy was measured. Magnetization exchange in one-dimensional experiments was achieved by saturation transfer with selective irradiation at both one and two sites in this three-site exchange system using the DANTE pulse sequence. The two-dimensional magnetization exchange experiment avoids the need to selectively saturate at one or more frequencies which may be difficult in complex exchange systems. Analysis of the two-dimensional exchange experiment by the back transformation method yielded exchange rate constants in good agreement with the saturation transfer method.

Key words: ^{31}P NMR, phosphoglyceromutase-enolase, magnetization transfer, coupled enzyme system

Introduction

Nuclear magnetic resonance magnetization transfer methods (Forsén and Hoffman 1963) have been widely used in the measurement of equilibrium-reaction rate constants in biological systems. The techniques have been applied to both in vitro enzyme catalysed reactions such as adenylate kinase (Brown and Ogawa 1977) and phosphoglyceromutase (Robinson et al. 1984) and in vivo in the study of creatine kinase in rat heart (Matthews et al. 1982) and sodium transport in rat kidney (Freeman et al. 1983).

The techniques of saturation and inversion transfer are strictly only applicable to two-site-exchange systems. If one of the reactants is undergoing other chemical exchange reactions then it is impossible to determine unambiguously all rate constants in the exchange system. Ugurbil (1985) has proposed a method of effectively reducing a multi-site exchange system to a two-site system. This involves a conventional saturation transfer experiment on the two species of interest while maintaining constant saturation of all other species involved in the exchange network. This method, of course, requires the NMR spectrometer to generate a number of saturation frequencies. Most conventional spectrometers do not possess this capability.

The two-dimensional magnetization exchange experiment (Jeener et al. 1979) is capable of yielding estimates of exchange rate-constants in multiple-exchange-site systems and does not require any selective saturation frequencies. A numerical method of analyzing the two-dimensional exchange spectrum (2D EXSY) has been proposed (Perrin and Gipe 1984; Bremer et al. 1984). This technique has been used to analyse the kinetics of two enzyme systems, adenylate kinase and phosphoglyceromutase (Mendz et al. 1986) and the non-enzymic hydration/dehydration of acetaldehyde (Kuchel et al. 1988).

In this study the two-dimensional exchange experiment is extended to a two enzyme three-exchange-site system, phosphoglyceromutase and enolase catalysing exchange of a phosphate group between 3-phosphoglycerate, 2-phosphoglycerate and phosphoenolpyruvate. Where applicable, single- and multiple-site saturation transfer measurements using DANTE saturation pulse sequences were also carried out. Good agreement between the one-dimensional and two-dimensional methods of measuring rate constants was observed.

Experimental

Rabbit muscle phosphoglyceromutase (E.C. 2.7.5.3) and enolase (E.C. 4.2.1.11) were obtained from Boehringer Mannheim, North Ryde, NSW, Australia.

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3-Phosphoglycerate was obtained from Sigma, St. Louis, MO, USA.

Enzyme solutions were prepared by washing with buffer through an Amicon ultrafiltration cell using a PM-10 membrane. The buffer used was 50 m*M* *N-Tris* (Hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES), 2 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* dithiothreitol, 1 m*M* 2,3-bisphosphoglycerate, pH 6.8. Substrate was introduced to the reaction by addition of 3-phosphoglycerate (66 m*M*) which had been passed through a column of Chelex 100 resin to remove paramagnetic impurities.

The phosphoglyceromutase and enolase activities of the experimental samples were measured by the assays described in Beutler (1975) and were found to be 3480 ± 300 and 1263 ± 23 IU ml^{-1}, respectively, at 37 °C.

^{31}P-NMR spectra were acquired at 162 MHz using a Varian XL-400 spectrometer at 37 °C. Saturation transfer measurements were carried out using a DANTE pulse sequence (Morris and Freeman 1978). Typically 16 transients were averaged into 8 K memory locations using a spectral width of 1000 Hz. For single-site saturation the DANTE pulse width was 1.3 μs and the delay between pulses was 333 μs giving excitation sidebands spaced 3000 Hz apart. For two-site saturation the delay was adjusted so that the frequency sidebands fell on the peaks of interest and the pulse width was increased to 2 μs to give sufficient power to saturate the spin populations.

Two-dimensional phase sensitive exchange spectra (2D EXSY) were obtained using the hypercomplex method (States et al. 1982) and the Varian supplied 2D-phase sensitive nuclear Overhauser enhancement pulse program. Eight transients were added into 1 K memory points using 256 t_1 increments and zero filling to 1 K in the t_1 domain. A field/frequency lock was obtained using a ^2H_2O capillary. Chemical shifts were referenced to inorganic phosphate which was arbitrarily set at 0 ppm.

Results and discussion

The reaction catalysed by the phosphoglyceromutase-enolase enzyme system is

$$3\,PG \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} 2\,PG \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} PEP$$

Phosphoglyceromutase catalyses the reaction between 3-phosphoglycerate (3-PG) and 2-phosphoglycerate (2-PG). Enolase catalyses the reaction between 2-PG and phosphoenolpyruvate (PEP). The *k*'s are the first-order rate constants characterizing the equilibrium reactions and are the experimental variables to be determined.

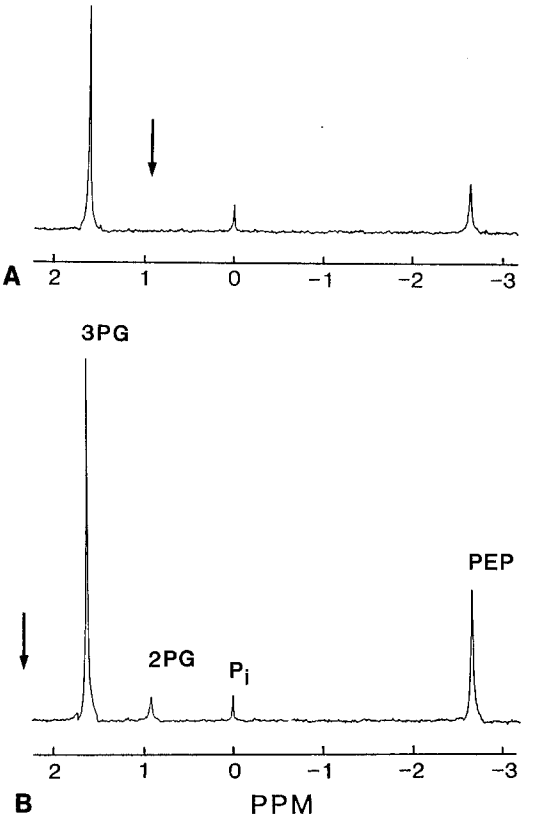

Fig. 1 A and B. Saturation transfer catalysed by phosphoglyceromutase-enolase in a mixture of 3-phosphoglycerate (3-PG), 2-phosphoglycerate (2-PG) and phosphoenolpyruvate (PEP). The saturation frequency is at the position indicated by the arrow. **A** Saturation at the 2-PG frequency. **B** Control experiment. The experimental conditions are given in the text

In this three-site-exchange system only k_1 and k_{-2} can be determined from a conventional one-site saturation transfer experiment (Ugurbil 1985) by saturating the 2-PG phosphorus nuclei. 3-PG and PEP only exchange with 2-PG, the centre member of the system. 2-PG exchanges with both 3-PG and PEP and saturating one of these does not give the true exchange rate constants k_{-1} and k_2. These latter rate constants may be calculated from k_1 and k_{-2} and the relevant equilibrium constants which may be determined from the integrated intensity ratios of the 'fully relaxed' NMR spectrum (Fig. 1 B).

Figure 1 shows the result of such a saturation transfer experiment. Saturation of the 2-PG peak (spectrum *A*) results in a decreased intensity of the 3-PG and PEP peaks compared with the control (spectrum *B*). The relationship between *k* and the reduction in intensity ΔM of the peak not undergoing saturation is given by (Brown and Ogawa 1977; Robinson et al. 1984)

$$\Delta M = k T_1^{sat} M_E, \qquad (1)$$

where M_E is the equilibrium intensity of the peak and T_1^{sat} is the T_1 measured during saturation of the exchange partner.

The T_1^{sat} values for 3-PG and PEP during saturation of 2-PG were 5.74 ± 0.08 and 3.26 ± 0.09 s.

From Eq. (1) using these values and including peak intensity error estimates (see e.g., Kuchel et al. (1988) for the general error formula), k_1 and k_{-2} and their standard deviations were determined to be 0.063 ± 0.010 and $0.172 \pm 0.027\ s^{-1}$. The mole ratio 3-PG: 2-PG: PEP was measured as 10.20:1.00:5.24 from the spectrum of Fig. 1 B. Using the relevant equilibrium constants determined from the mole ratios, and the two measured rate constants, the calculated values of k_{-1} and k_2 were 0.64 ± 0.10 and $0.90 \pm 0.14\ s^{-1}$, taking into account errors in the estimation of the equilibrium constants.

Direct measurement of k_{-1}, the 2-PG to 3-PG rate constant is less precise as 2-PG is also involved in an exchange reaction with PEP. However, as has been shown by Ugurbil (1985) a three-site exchange system can be converted to an "effective" two-site system (in this example 3-PG $\rightleftarrows$ 2-PG) by saturating the third member of the exchange system (here PEP). The rate constant k_{-1} was measured by simultaneously saturating both 3-PG and PEP in the saturation transfer experiment and only PEP in the control experiment. Analysis of the data was as given by Ugurbil (1985).

Most spectrometers are not equipped with multiple saturation channels making this type of experiment difficult if not impossible. The frequency spectrum of a DANTE pulse sequence consists of a series of sidebands separated in frequency by the inverse of the delay time between the DANTE pulses (Morris and Freeman 1978). In favourable circumstances it is thus possible to saturate at more than one site with this pulse sequence. In this example by placing the transmitter frequency on the PEP resonance and adjusting the delay time between DANTE pulses it was possible to saturate simultaneously both PEP and 3-PG or PEP and 2-PG and also saturate only PEP to obtain the relevant control spectra. Use of this double saturation method on a *different* sample from that used in the one site saturation method discussed above gave values for k_1 and k_{-1} of 0.058 and $0.593\ s^{-1}$. The one-site saturation method (saturating 2-PG) on this sample gave k_1 as 0.056 and a calculated value for k_{-1} of $0.577\ s^{-1}$. Attempts to measure k_2 and k_{-2} by the double saturation method were not successful. In one of these experiments both 3-PG and 2-PG had to be saturated. As these resonances differ in chemical shift by only 121.5 Hz, the time interval between DANTE pulses, 8.2 ms, was too long to maintain efficient saturation of the 2- and 3-PG signals.

Two-dimensional exchange spectroscopy (2D EXSY) avoids the need to saturate selectively a signal in a crowded region of a spectrum and eliminates the need for multiple-site saturation in exchange reactions consisting of three or more sites. It has been successfully applied to analyze the exchange reactions catalysed by the enzymes phosphoglyceromutase and adenylate kinase (Mendz et al. 1986).

The 2D EXSY spectrum can be viewed as the solution of the Bloch-McConnell equations that describe relaxation and magnetization exchange in a system. Thus,

$$M = \exp(R\,t_m)\,M_0, \tag{2}$$

where M is the matrix comprising the peak volumes of the 2D EXSY spectrum, R is the matrix of exchange and relaxation rate constants relevant to the system under study and t_m is the mixing time. M_0 is a diagonal matrix representing the 2D EXSY spectrum acquired with a mixing time of zero s. To avoid the need for another time-consuming 2D EXSY experiment it is usually replaced with αS_0 where S_0 is the diagonal matrix of mole ratios obtained from a fully relaxed 1D spectrum (e.g., Fig. 1 B) and α is an unknown scaling factor (Kuchel et al. 1988). Use of the mole ratio method gives rise to false values of diagonal elements in the R matrix but leaves the off-diagonal elements, which specify the exchange rate constants, unaffected. In this system the symbolic R matrix is

$$\begin{bmatrix} \frac{1}{T_1^{3PG}} + k_1 & -k_{-1} & 0 \\ -k_1 & \frac{1}{T_1^{2PG}} + k_{-1} + k_2 & -k_{-2} \\ 0 & -k_2 & \frac{1}{T_1^{PEP}} + k_{-2} \end{bmatrix}$$

The 2D EXSY spectrum of this system, using the same enzyme sample as for the one-site saturation transfer measurements, with a mixing time of 1.9 s, is shown in Fig. 2. The matrix of peak volumes corresponding to Fig. 2 was

$$\begin{bmatrix} 7.16 & 9.03 & 56.60 \\ 6.31 & 2.74 & 7.18 \\ 129.00 & 9.77 & 10.50 \end{bmatrix}$$

M is this peak-volume matrix rotated 90° so that the diagonal of the 2D EXSY spectrum runs from top-left to bottom-right as in the standard mathematical representation of a matrix. Symmetrically related cross peaks in the 2D EXSY spectrum should theoretically have equal intensities (Macura and Ernst 1980). However the matrix M was not symmetrized (by replacing the cross peak volumes by their means) as this gives a false impression of the accuracy of the data and the resulting estimate of the rate constants (Kuchel et al. 1988).

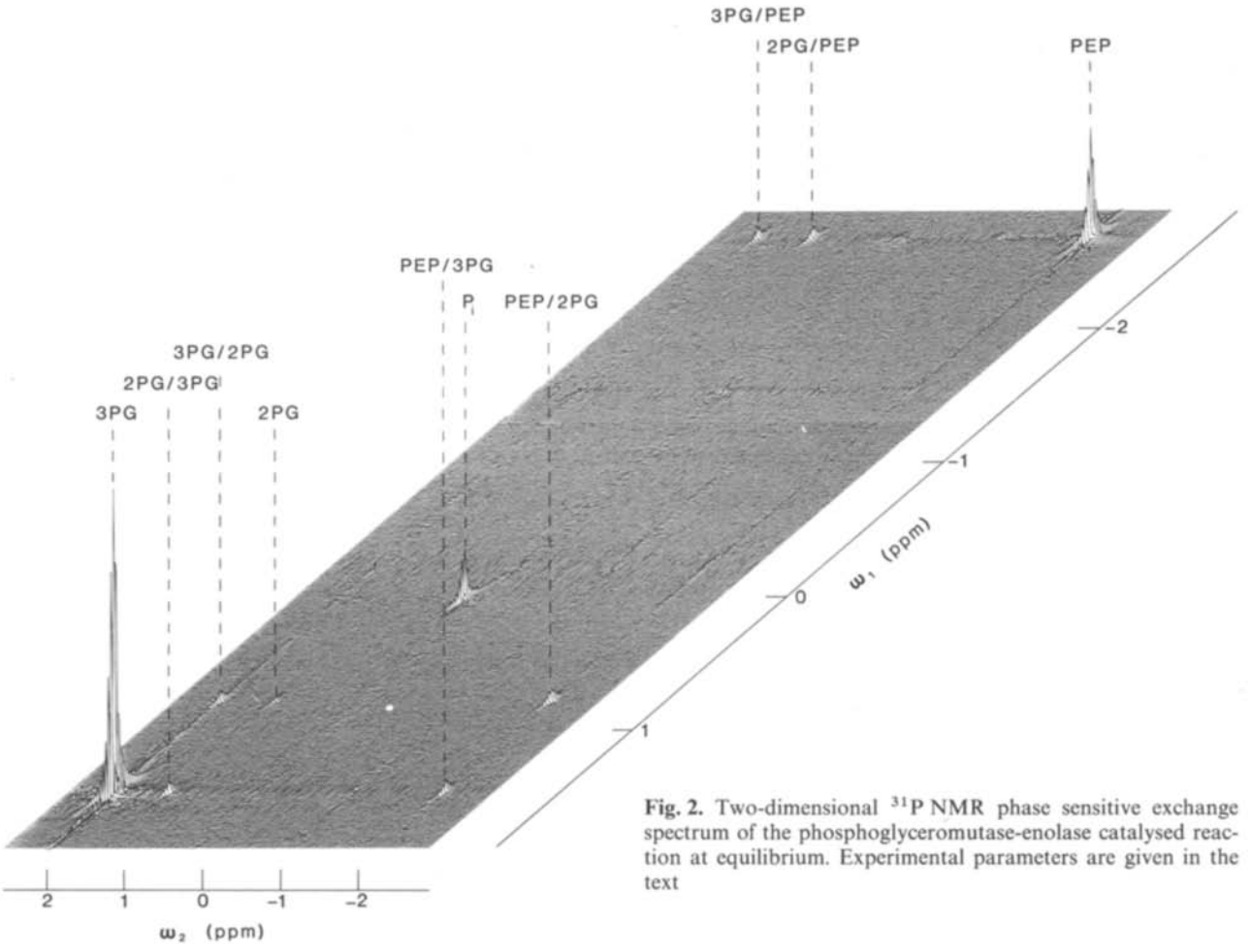

Fig. 2. Two-dimensional ^{31}P NMR phase sensitive exchange spectrum of the phosphoglyceromutase-enolase catalysed reaction at equilibrium. Experimental parameters are given in the text

From M, t_m, and S_0, obtained from the one-dimensional spectrum of Fig. 1 B the calculated rate constant matrix was,

$$\begin{bmatrix} -2.519 & -0.895 & -0.017 \\ -0.057 & -1.434 & -0.149 \\ -0.002 & -0.988 & -2.383 \end{bmatrix}$$

yielding the values $k_1 = 0.057 \pm 0.007$, $k_{-1} = 0.895 \pm 0.096$, $k_2 = 0.988 \pm 0.087$ and $k_{-2} = 0.149 \pm 0.023$ s^{-1}. Error estimates were determined by the method of Kuchel et al. (1988).

The two-dimensional exchange method of measuring equilibrium rate constants yields values comparable with those obtained by one-dimensional magnetization transfer. Although the 2D EXSY experiment takes approximately 10 times as long as the saturation transfer experiment to achieve a comparable signal to noise ratio, it provides a viable alternative to saturation transfer in enzyme catalysed multiple-exchange-site systems eliminating the need to saturate simultaneously at a number of sites.

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References

Beutler E (1975) Red cell metabolism, 2nd edn. Grune and Stratton, New York

Bremer J, Mendz GL, Moore WJ (1984) Skewed exchange spectroscopy. Two-dimensional method for the measurement of cross relaxation in ^{1}H NMR spectroscopy. J Am Chem Soc 106:4691–4696

Brown JR, Ogawa S (1977) ^{31}P-nuclear magnetic resonance kinetic measurements on adenylate kinase. Proc Natl Acad Sci USA 74:3627–3631

Forsén S, Hoffman RA (1963) Study of moderately rapid chemical exchange reactions by means of nuclear magnetic double resonances. J Chem Phys 39:2892–2901

Freeman D, Bartlett S, Radda G, Ross B (1983) Energetics of sodium transport in the kindey saturation transfer ^{31}P-NMR. Biochim Biophys Acta 762:325–336

Jeener J, Meier BA, Bachmann P, Ernst RR (1979) Investigation of exchange processes by two-dimensional NMR spectroscopy. J Chem Phys 71:4546–4553

Kuchel PW, Bulliman BT, Chapman BE, Mendz GL (1988) Variances of rate constants estimated from 2D NMR exchange spectra. J Magn Reson 76:136–142

Macura S, Ernst RR (1980) Elucidation of cross relaxation in liquids by two-dimensional NMR spectroscopy. Mol Phys 41:95–117

Matthews PM, Bland JL, Gadian DG, Radda GH (1982) A ^{31}P-NMR saturation transfer study of the regulation of creatine kinase in the rat heart. Biochim Biophys Acta 721: 312–320

Mendz GL, Robinson G, Kuchel PW (1986) Direct quantitative analysis of enzyme-catalyzed reactions by two-dimensional nuclear magnetic resonance spectroscopy: Adenylate kinase and phosphoglyceromutase. J Am Chem Soc 108:169–173

Morris GA, Freeman R (1978) Selective excitation in Fourier transform nuclear magnetic resonance. J Magn Reson 29: 433–462

Perrin CL, Gipe RK (1984) Multisite kinetics by quantitative two-dimensional NMR. J Am Chem Soc 106:4036–4038

Robinson G, Chapman BE, Kuchel PW (1984) ^{31}P NMR spin-transfer in the phosphoglyceromutase reaction. Eur J Biochem 143:643–649

States DJ, Haberkorn RA, Ruben DJ (1982) A two-dimensional nuclear Overhauser experiment with pure absorption phase in four quadrants. J Magn Reson 48:286–292

Ugurbil K (1985) Magnetization-transfer measurements of individual rate constants in the presence of multiple reactions. J Magn Reson 64:207–219