following assignment of the carbamates of the four diastereomers of 3-methylpentan-2-ol (in order of increasing retention time): 2*S*, 3*S*; 2*R*, 3*R*; 2*R*, 3*S*, and 2*S*, 3*R*.

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Registry No. 1 (ketone), 78-93-3; **1** (alcohol), 14898-79-4; **2** (ketone), 563-80-4; **2** (alcohol), 1572-93-6; **3** (ketone), 765-43-5; **3** (alcohol), 6516-09-2; **4** (ketone), 107-87-9; **4** (alcohol), 26184-62-3; **5** (ketone), 591-78-6; **5** (alcohol), 26549-24-6; **6** (ketone), 108-10-1; **6** (alcohol), 14898-80-7; **7** (ketone), 565-61-7; **7** (alcohol), 99212-18-7; **8** (ketone), 589-38-8; **8** (alcohol), 6210-51-1; **9** (ketone), 110-43-0; **9** (alcohol), 6033-23-4; **10** (ketone), 106-35-4; **10** (alcohol), 26549-25-7; **11**, 123-19-3; **12** (ketone), 111-13-7; **12** (alcohol), 6169-06-8; **13** (ketone), 110-93-0; **13** (alcohol), 58917-26-3; **14** (ketone), 821-55-6; **14** (alcohol), 70419-06-6; **15**, 502-56-7; **16** (ketone), 693-54-9; **16** (alcohol), 33758-16-6; **17**, 112-12-9; **18**, 5166-53-0; **19** (ketone), 5891-21-4; **19** (alcohol), 99212-19-8; alcohol dehydrogenase, 9031-72-5.

Direct Quantitative Analysis of Enzyme-Catalyzed Reactions by Two-Dimensional Nuclear Magnetic Resonance Spectroscopy: Adenylate Kinase and Phosphoglyceromutase

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Abstract: The back-transformation method for the analysis of two-dimensional nuclear magnetic resonance cross-relaxation and exchange data has been employed to obtain the rate constants of the phosphoglyceromutase and adenylate kinase catalyzed reactions in vitro. The results are in excellent agreement with those obtained by other methods. It is shown that a single two-dimensional experiment for each catalyzed reaction is sufficient to calculate the rate constants. The study of the adenylate kinase reaction demonstrates the potential of the method for direct analysis of higher order reactions without having to simplify them in terms of pseudo-first-order steps.

I. Introduction

Complete characterization of metabolic pathways requires the determination of the rate constants of enzyme-catalyzed reactions. Nuclear magnetic resonance (NMR) spectroscopy is a particularly important method for the study of steady-state enzyme kinetics because of its ability to follow noninvasively in vivo intracellular processes and because of its potential to monitor individual metabolites. One-dimensional (1D) and two-dimensional (2D) Fourier transform NMR techniques have been applied to the measurements of the rates of reactions; saturation and inversion transfer of magnetization are the most widely used 1D methods. They are employed to measure "intermediate-to-slow" rate constants by following the direct transfer of magnetization between exchanging chemical species. ^{31}P NMR has been used to study the adenylate kinase[1,2] and phosphoglyceromutase-catalyzed[3] reactions involving high-energy phosphate esters. However, the application of these 1D methods presents difficulties; the most important one is the limit in the selectivity that can be achieved by irradiating a set of resonances in a crowded or complex spectrum.

Jeener et al.[4] designed a 2D technique, the exchange experiment, to measure chemical exchange and cross-relaxation rates. Ferretti and co-workers have demonstrated that it can be applied to the study of in vitro[5,6] and in vivo[1] enzyme-catalyzed reactions. A fundamental advantage of this technique is that under suitable conditions species which have undergone chemical or magnetic exchange are connected by off-diagonal cross-peaks on a two-dimensional spectral map whose diagonal represents the one-dimensional spectrum.

Thus, in principle, the limitation imposed by crowded spectral regions on selective irradiation can be overcome and all exchange pathways are observed simultaneously in a single experiment. However, the lack of a suitable method of analysis of the exchange experiment has limited the obtaining of quantitative information to very simple cases[4,8] and has made necessary a large number of experiments.

Kumar et al.[9] examining the variation of NOESY (Nuclear Overhauser enhancement spectroscopy) cross-peaks as a function of mixing time demonstrated a qualitative correlation between the time rate of increase of cross-peak intensity and the distance between atoms found from X-ray data. Bodenhausen and Ernst[10,11] devised a new type of exchange experiment, accordion spectroscopy, capable of measuring cross-relaxation rates. In *J*-coupled systems this technique requires the elimination of zero-quantum effects by means other than the standard random variation of the mixing time, using, for instance, a field-gradient pulse.

Rate constants for first-order reactions can be obtained by carrying out a number of experiments at different mixing times and fitting the relevant mathematical expression to the cross-peak volumes vs. mixing time data. The parameters thus fitted are employed to calculate the rate constants from the analytically solved rate equations. This method was used for the yeast phosphoglucose isomerase reaction with excellent results,[5] but *it required 12 experiments*. The analyses of more complex reactions

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have required assumptions about the reaction mechanisms. If a reaction can be simplified to pseudo-first-order, the solutions of the resultant set of coupled differential equations describing it depend on the relaxation rates and the longitudinal relaxation times. In the limit of zero mixing time the solutions depend only on the exchange rate constants. Initial rates are calculated by fitting sums of exponential to cross-peak volume time-courses measured from experimental data at different mixing times. The rates of the creatine phosphokinase reaction were obtained in this way from *the data of 11 experiments*.[6] The adenylate kinase exchange reaction was similarly analyzed by assuming the formation of an ATP-enzyme complex and assuming that the reaction occurs in two steps, one comprising the exchange between ADP and AMP, which was analyzed with use of the initial rate approximation, and a second step involving the dissociation of ATP from the enzyme complex; the latter rate constants were determined by curve fitting. The whole analysis involved *ten 2D NMR experiments*.[6]

A common feature of these analyses is that the data obtained from cross-peaks corresponding to exchanging species are used individually in either initial rate determination or curve fitting. Recently we have devised a method to calculate rate constants using a back-transformation of the matrix of exchange-mixing coefficients.[12] The method makes use of all the peaks measured in a 2D experiment, and in principle, a single experiment is sufficient to calculate the flux rates of reactants and rate constants.

In the present study the method was applied to the phosphoglyceromutase and adenylate kinase reactions. The reaction rates obtained are in good agreement with those measured by conventional biochemical methods. In addition, we obtained spin–lattice and spin–spin relaxation times for the latter reaction and for its products. The data show that the behavior of individual cross peaks in the two-dimensional map can be explained in terms of their relaxation rates without the need to postulate more than a single rate-limiting step.

II. Materials and Methods

A. Experimental. Rabbit muscle phosphoglyceromutase (PGM; EC 2.7.5.3) and adenylate kinase (AK; EC 2.7.4.3) were obtained as crystalline suspensions in concentrated ammonium sulfate from Sigma, St. Louis, MO. Each enzyme was prepared by dialysis against the experimental buffer (100 volumes, three changes in 6h, 4 °C); the buffers used were 0.2 M Tris/0.2 M tetramethylammonium chloride/4 mM dithiothreitol pH 7.0 for experiments involving phosphoglyceromutase and 0.2 M Tris/0.2 M tetramethylammonium chloride/4 mM dithiothreitol/1 mM EDTA pH 8.0 for the adenylate kinase experiments.

Each NMR sample consisted of an equilibrium mixture of the enzyme under study and its reactants in the appropriate buffer; samples also contained 10% ^2H_2O for field/frequency locking and were contained in 10-mm precision tubes.

In the experiments on PGM, substrate was introduced to the reaction by the addition of 3-phosphoglycerate dissolved in the experimental buffer. 3PG concentrations in these solutions were measured enzymatically by using the method described by Beutler.[13] In the case of AK the substrate solutions (in the experimental buffer) contained ADP and sufficient magnesium sulfate to ensure that the free Mg^{2+} concentration in the final sample was equal to half the total substrate concentration. Concentrations of the ADP solutions were estimated by measuring the absorbance of 260 nm ($E = 1.54 \times 10^4$ M^{-1} cm^{-1}).[14] All substrate solutions were passed through columns of Chelex 100 ion-exchange resin (Bio-Rad, Epping, NSW, Australia) to remove paramagnetic ions.

Enzymatic activities of the samples used were estimated by using the assays described by Beutler.[13] An enzyme-free equilibrium mixture of the substrates of adenylate kinase was prepared by ultrafiltration of a preequilibrated sample (UM10 membrane; Amicon, Danvers, MA). The filtrate, after the addition of 10% ^2H_2O, was used in the NMR experiment.

The enzyme-kinetic rate constants determined by the 2D-exchange method were compared with estimates of the rate constants obtained independently. In the case of adenylate kinase, a saturation transfer experiment[3] was performed by using the same NMR sample. For phosphoglyceromutase, the unidirectional fluxes at equilibrium in the NMR sample were estimated from the enzymatic activity by appalication of eq 31 of ref 3.

B. NMR Methods. ^{31}P spectra were collected with a Bruker WM-400 spectrometer operating at 162 MHz in the Fourier transform mode, with quadrature detection, at 310 K. The 1D spectra of both reactions were obtained with a 90° radio frequency pulse (17–18 μs). For the PGM reaction the spectral width was 200 Hz over 8K data points acquired with a recycling time of 30 s for typically 16 transients. The spectral width for the AK reaction was 4400 Hz and the acquisition time 1.3 s, with a total recycling time of 15 s for typically 40 transients. Longitudinal relaxation times, T_1, were measured by the inversion-recovery method.[15] The 180° pulse was 35 μs and 256 experiments were run with delay times in the range of 0.0001 to 15 s. Transverse relaxation times, T_2^* and T_2, were measured by using the spin–echo[16] and Carr–Purcell–Meiboom–Gill (CPMG)[17,18] pulse sequences. Each run consisted of an experiment with delay times between 0.0006 and 0.5 s. Relaxation times were calculated by using a nonlinear regression computer program.[19]

Two-dimensional spectra were obtained with standard Bruker software for the NOESY sequence $(t_w-\pi/2-t_1-\pi/2-t_m-\pi/2-t_2)_n$. The acquisition parameters for the PGM experiments were $t_w = 28.5$ s, $t_1 = 0.005$ to 0.32 s, $t_m = 2.12$ s with a random variation of up to ±15%, $t_2 = 1.28$ s, and $n = 16$. A spectral width of 200 Hz over 512 data points was used. To determine peak volumes 64 experiments were transformed to 128 × 128 spectra with no window functions. The frequency limits for measuring peak volumes were obtained from a contour map after transforming the spectra with sine-bell multiplication. For the AK reaction and for the enzyme-free equilibrium mixture 256 experiments were carried out with $t_w = 11$ s, $t_1 = 0.00023$ to 0.0589 s, $t_m = 0.5$ s with a random variation of ±15%, $t_2 = 0.2$ s, and $n = 16$. A spectral width of 4400 Hz over 1K data points was used. Peak volumes were determined from a matrix of 1K × 1K spectra transformed with no window functions.

C. Background Theory. The exchange matrix **R** is constructed from the system of coupled differential equations describing the chemical exchange and dipolar relaxation processes taking place with each of the different species in the system during the mixing time. Its elements contain rate constants and cross-relaxation and spin–lattice relaxation rate constants.[4]

In the back-transformation method[12] it is shown that the matrix of exchange mixing coefficients, **A**, can be expressed as

$$\mathbf{A} = \mathbf{S} \exp(-\Lambda t_m)\mathbf{S}^{-1}$$

where t_m is the mixing time and **S** is a matrix of eigenvectors; **R** is such that

$$\Lambda = \mathbf{S}^{-1}\mathbf{R}\mathbf{S}$$

where λ_i are the diagonal elements, eigenvalues of **R**, of the diagonal matrix Λ, the diagonalization process was carried out numerically.[12]

The exchange matrix is then calculated from the expression

$$\mathbf{R} = -(1/t_m)\mathbf{S}(\ln \Gamma_i)\mathbf{S}^{-1}$$

where the diagonal matrix Γ has elements $\gamma_i = \exp(-\lambda_i t_m)$.

III. Results

Phosphoglyceromutase. The reaction catalyzed by phosphoglyceromutase is

$$\text{3-PG} \underset{k_{23}}{\overset{k_{32}}{\rightleftharpoons}} \text{2-PG}$$

The PGM reaction is of methodological interest because the forward and reverse reactions have very different flux rates as indicated by the 1D ^{31}P NMR spectrum of the equilibrium mixture shown in Figure 1. Moreover, calculation of the NMR peak volumes corresponding to the different species is difficult owing to the very different concentrations of reactants and the presence of large "wings" in the transformed 2D spectrum (Figure 2). An effective way to calculate the volumes of each resonance was to obtain the limits from a spectrum transformed with sine-bell window functions on both frequency axes. These limits were used

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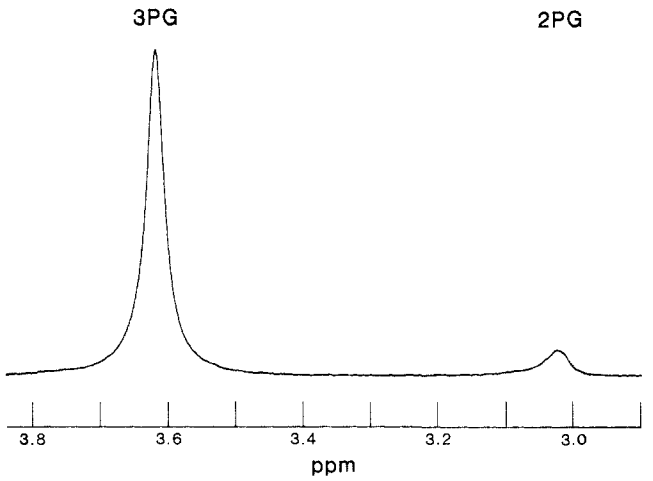

Figure 1. One-dimensional ^{31}P NMR spectrum of the phosphoglyceromutase-catalyzed reaction at equilibrium. 3PG (initial concentration 121 mM) was incubated at 310 K with PGM (1609 IU mL^{-1}). The spectrum was obtained by Fourier transformation without window functions of 16 transients accumulated with the acquisition parameters described in the text.

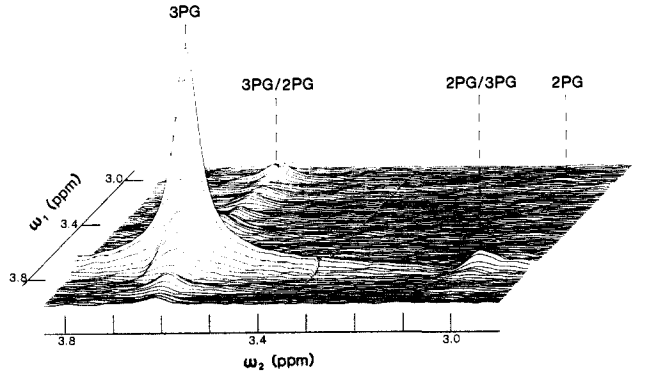

Figure 2. Two-dimensional ^{31}P NMR spectrum of the PGM-catalyzed reaction at equilibrium. Experimental conditions are the same as for Figure 1. Acquisition and transformation parameters are described in the text.

Table I. Calculated Rate Constants of the PGM and AK Reactions

	PGM		AK	
	k_{32} (s^{-1})	k_{23} (s^{-1})	k_1 (s^{-1})	k_{-1} (s^{-1})
2D exchange	0.19 ± 0.03	1.58 ± 0.20	0.72 ± 0.05	0.92 ± 0.06
biochemical assay	0.18 ± 0.03	1.56 ± 0.20		
saturation transfer			0.75 ± 0.10	0.97 ± 0.13

to measure the volumes from a spectrum with no window functions. Dipolar contour maps of the reaction species are shown in Figure 3, and the method of choosing peak limits is outlined in the caption. Molar fractions of both phosphate moieties were calculated from the 1D spectrum in Figure 1. The elements of the matrix of mixing coefficients were calculated to be 30.0, 1.94, 1.94, and 0.25. The exchange matrix corresponding to this first-order monomolecular reaction has the same form as that described by Jeener et al.[4] The calculated rate constants using the theory mentioned in the Methods are given in Table I.

Adenylate Kinase. The 1D ^{31}P NMR spectrum of the adenylate kinase reaction

$$2ADP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AMP + ATP$$

is shown in Figure 4. The 2D ^{31}P contour map and stackplot of the spectra of reactants are shown in Figure 5. It is possible to measure in this spectrum the exchange cross-peaks between ADP and ATP or AMP species as well as a cross-peak between

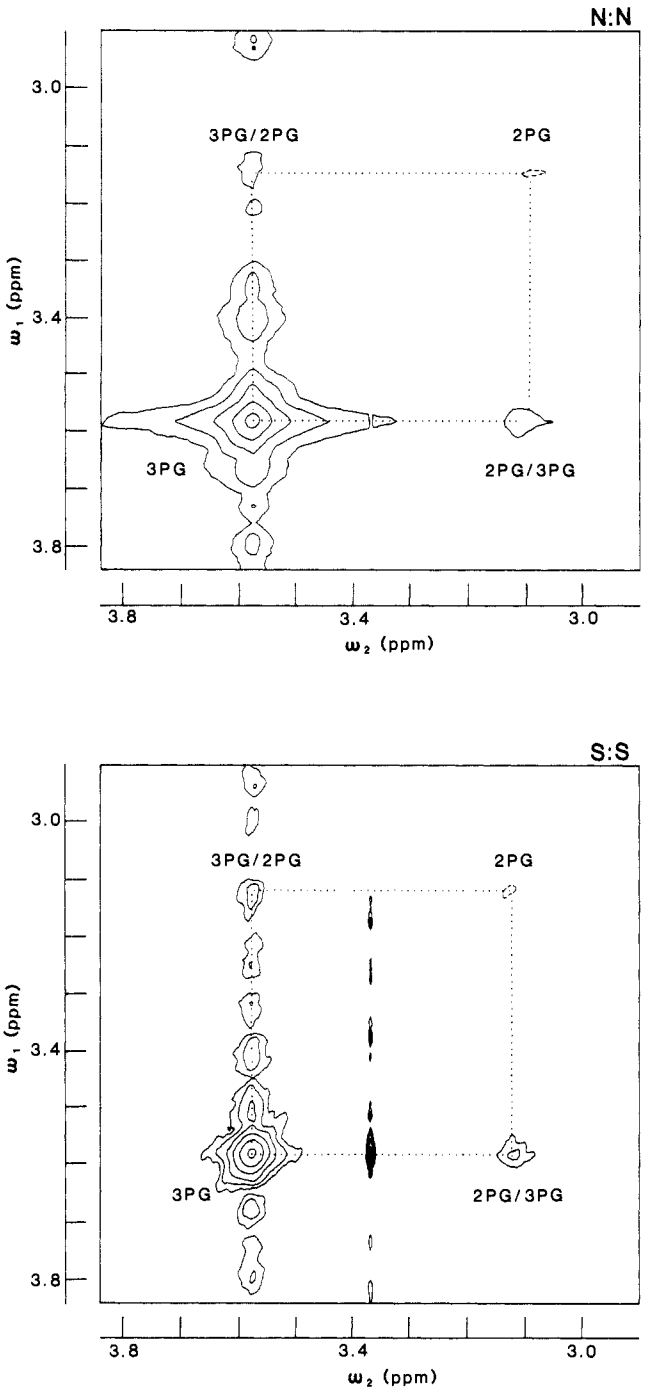

Figure 3. Dipolar contour maps of the PGM reaction at equilibrium. The top plot was obtained with no window functions in the double Fourier transformation. The bottom plot is the result of transforming with sine-bell window functions in both frequency domains. The figure illustrates the method for choosing the limits of peak volumes.

AMP and ATP corresponding to a "second pass" of adenosine phosphate through the enzyme. A phase-sensitive transformation of this 2D experiment shows auto-peaks and cross-peaks of equal sign; no negative cross-peaks exist thus indicating that no cross-correlation occurs between phosphorus nuclei with short correlation times. The 2D contour map and stackpot of the equilibrium mixture of the three adenosine phosphates without the enzyme are also shown in Figure 5; as expected, cross-peaks corresponding to cross-correlation or exchange between moieties are not present in this spectrum.

The matrix of exchange coefficients formed with volumes of the peaks in the 2D plot of the spectrum of the reaction is shown in Figure 6. The theoretical exchange matrix is also shown in Figure 6. The volume of the βATP auto-peak is smaller than the volume of the α- and γATP auto-peaks. On a molar basis the

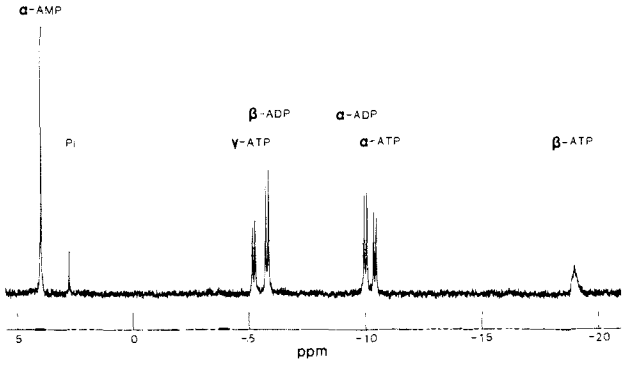

Figure 4. One-dimensional ^{31}P NMR spectrum of the steady-state adenylate kinase-catalyzed reaction. ADP (initial concentration 37.7 mM) was incubated at 310 K with AK (727 IU mL^{-1}). The spectrum was obtained by Fourier transformation without window functions of 40 transients accumulated with the acquisition parameters described in the text.

Table II. Relaxation Times of Phosphorus Nuclei in the Adenylate Kinase Reaction (+AK) and in an Equilibrium Mixture without the Enzyme (-AK)

	T_1 (ms)		T_2 (ms)		T_2^* (ms)	
	+AK	-AK	+AK	-AK	+AK	-AK
αAMP	2900±100	4500±60	291.0±4.0	563.0±9.0	268±10	449±23
αADP	1390±40	2038±28	96.4±2.0	170.0±6.0	144±9	209±30
βADP	2260±40	3000±50	157.1±3.2	192.0±5.0	210±10	351±30
αATP	1390±40	1410±23	144.0±4.0	97.0±5.0	223±14	305±40
βATP	1620±60	1550±100	11.4±1.1	9.8±0.8	14±1	12±1
γATP	2181±30	2359±28	24.9±3.0	33.0±4.0	191±11	162±29

volumes of the latter two peaks compare well with the volumes of the peaks of the ADP nuclei. Correspondingly, cross-peaks that result from exchange of the βATP are smaller than cross-peaks arising from the α- and γATP and α- and βADP. Very similar ratios of volumes were obtained for the equilibrium mixture without the enzyme. Ferretti and co-workers[4] observed that the AMP/αADP cross-peaks were larger than the βATP/βADP ones, and inferred that the former reaction had a greater rate. This conclusion was used to suggest a two-step mechanism in which the rate-limiting step involved the release of ATP from the product–enzyme complex. The model allowed them to simplify their scheme for the reaction and to calculate pseudo-first-order rate constants for both steps. Our results also indicate this difference in cross-peak volumes (Figure 6), but they can be explained without having to postulate a second step in the reaction. Table II shows the calculated T_1, T_2, and T_2^* relaxation times for the

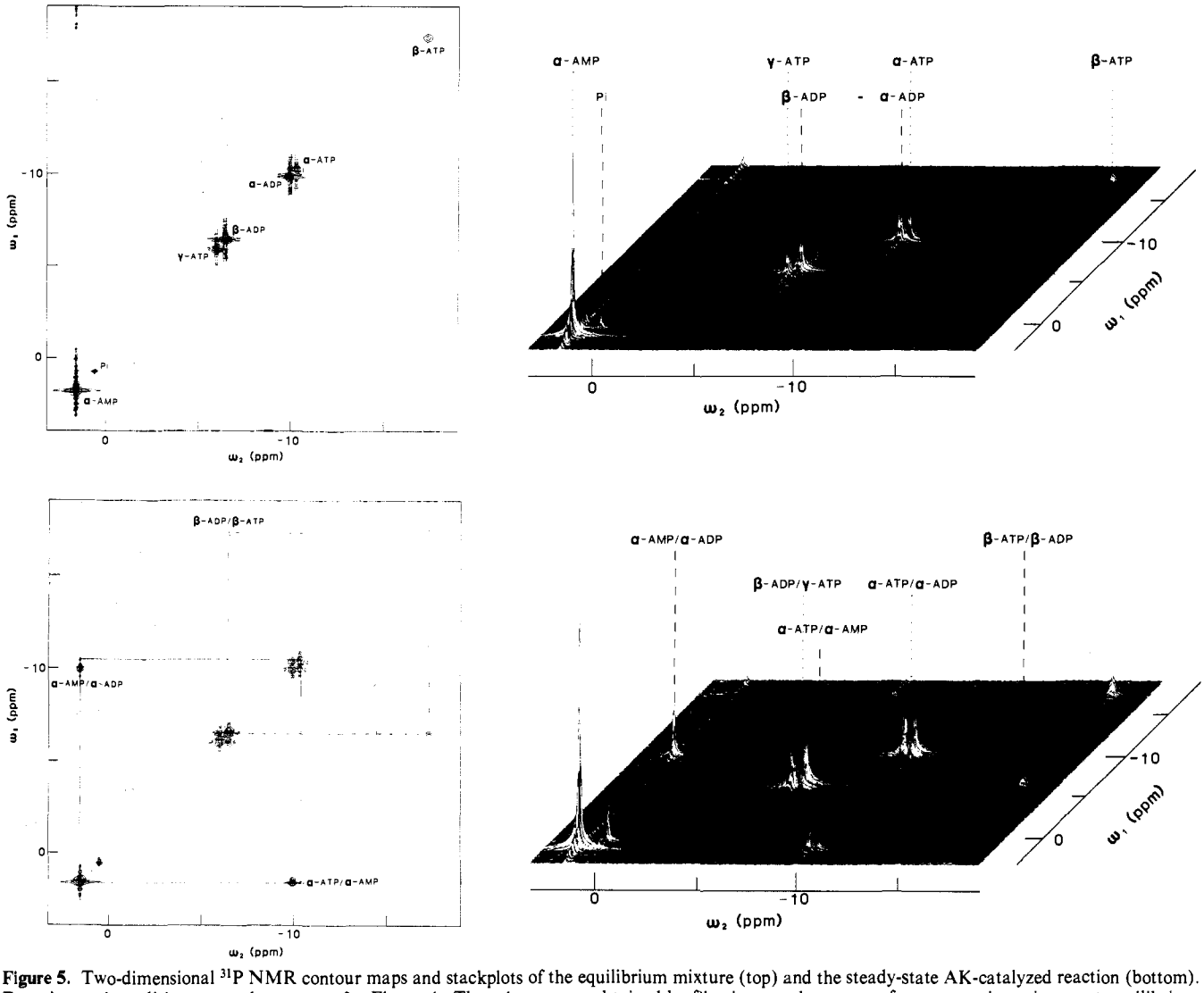

Figure 5. Two-dimensional ^{31}P NMR contour maps and stackplots of the equilibrium mixture (top) and the steady-state AK-catalyzed reaction (bottom). Experimental conditions were the same as for Figure 4. The mixture was obtained by filtering out the enzyme from a reaction mixture at equilibrium under similar experimental conditions. Acquisition and transformation parameters are described in the text.

	α-AMP	γ-ATP	β-ADP	α-ADP	α-ATP	β-ATP
α-AMP	30.91	0.0	0.0	7.38	1.28	0.0
γ-ATP	0.0	12.45	6.09	0.0	0.0	0.0
β-ADP	0.0	6.09	14.27	0.0	0.0	2.60
α-ADP	7.38	0.0	0.0	9.20	3.92	0.0
α-ATP	1.28	0.0	0.0	3.92	8.85	0.0
β-ATP	0.0	0.0	2.60	0.0	0.0	7.24

$$\begin{bmatrix} -R_\alpha^M - \frac{k_{-1}}{2}[T] & 0 & 0 & k_1[D] & \frac{k_{-1}}{2}[M] & 0 \\ 0 & -R_\gamma^1 - 2R_{\gamma\beta}^1 - \frac{k_{-1}}{2}[M] & k_1[D] & 0 & 0 & -R_{\gamma\beta}^1 + \frac{k_{-1}}{2}[M] \\ 0 & R_{-1}[M] & -R_\beta^0 - 2k_1[D] & 0 & 0 & \\ k_{-1}[T] & 0 & 0 & -R_\alpha^0 - 2k_1[D] & k_{-1}[M] & 0 \\ \frac{k_{-1}}{2}[T] & 0 & 0 & k_1[D] & -R_\alpha^1 - \frac{k_{-1}}{2}[M] & 0 \\ 0 & -R_{\beta\gamma}^1 + \frac{k_{-1}}{2}[M] & k_1[D] & 0 & 0 & -R_\beta^T - 2R_{\beta\gamma}^T - \frac{k_{-1}}{2}[M] \end{bmatrix}$$

Figure 6. Top: Matrix of exchange coefficients measured from the data in Figure 5. Bottom: Exchange matrix for the AK reaction. The R^i_p denote spin–lattice relaxation rates and the R^i_{pq} cross-relaxation rates. [M], [D], and [T] represent concentrations of the mono-, di-, and triphosphates of adenosine.

six phosphorus nuclei and for the same nuclei in an equilibrium mixture obtained by filtering out the enzyme (see Methods). The spin–spin lattice relaxation times of βATP are much shorter than the rest, in both the presence and absence of the enzyme. The short transverse relaxation times would account for the smaller peak volumes.

The rate constants calculated from the 2D data and from the saturation transfer in the AK reaction are given in Table I; because it is a higher-order reaction, the nonzero off-diagonal elements of the exchange matrix contain both rate constants and the equilibrium chemical concentrations of different phosphates. Consequently, it is necessary to calibrate the 2D spectrum taking into consideration also the molar fractions of each species. The values in Table I were derived from a calibration carried out with use of the AMP auto- and cross-peaks and the molar fractions measured on the 1D spectrum.

IV. Discussion and Conclusions

The back-transformation method of analysis has yielded rate constants which compare well with those obtained by biochemical assays[13] for the PGM reaction, in which the ratio of rate constants for the forward and reverse reactions is approximately 1:10. The potential of the method becomes more apparent in the analysis of the AK reaction. One experiment is, in principle, sufficient to obtain the kinetic parameters of this second-order bimolecular reaction without having to introduce simplifying assumptions. We have also shown that the smaller volumes of the β auto- and cross-peaks can be explained in terms of the dynamics of the ATP molecule independently of it being involved in the AK reaction. Consequently there is no need to assume the existence of a second step in the reaction for the dissociation of the ATP–enzyme complex.

The method makes possible the monitoring of steady-state in vitro and in vivo enzyme-catalyzed reactions within a reasonable time, since it only requires a single experiment. It should be noted that a judicious choice of the mixing time, t_m, is necessary in order to obtain good results. The simplicity of the analysis makes the back-transformation the most direct method at present available for obtaining rate constants of complex exchange reactions. And the simple application of the Jeener exchange experiment makes it a feasible technique within the hardware limitations of any modern spectrometer.

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Registry No. 3-PG, 820-11-1; 2-PG, 2553-59-5; ADP, 58-64-0; AMP, 61-19-8; ATP, 56-65-5; PGM, 9032-62-6; AK, 9013-02-9.