[17] Isotope Exchange

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Introduction

The study of enzyme kinetics by NMR observation of isotope-exchange processes is in many ways very similar to the study of enzyme kinetics with the magnetization and saturation transfer techniques described elsewhere in this volume [15]. In the application of magnetization transfer and saturation transfer techniques, a transient physical label of a chemical group is used as a marker which is transferred between different molecules, namely its high-frequency-induced nonequilibrium magnetization. In isotope-exchange studies an isotopic label is introduced chemically into the molecule or the molecular group under study and the transfer of this label to different molecules is observed. There are distinctive differences between the two methods, in particular as far as the range of kinetic parameters which may be determined is concerned. It should be noted in passing that all these methods may be used at least in principle with *in vitro* as well as *in vivo* systems.

Isotope exchange experiments are basically independent of NMR relaxation times as they depend on the concentration of permanently labeled chemical groups only. This is in contrast to the magnetization and saturation transfer studies as well as to the line-shape analysis method. The inverse of the pseudo-first-order exchange rates for isotope exchange reactions should be roughly in the order of magnitude of the time needed to take a decent NMR spectrum of the sample in order to allow the experimentalist to observe the exchange taking place in, roughly speaking, real time. The time needed to take an NMR spectrum of a biological sample is usually on the order of several minutes. It is also possible to take aliquots of a reaction mixture and quench the ongoing reaction in certain time intervals, thus extending the method to higher and lower exchange rates, respectively. The latter method also makes it possible to determine reaction rates under experimental conditions at which high-resolution NMR spectra may not be obtained satisfactorily, e.g., at temperatures below 273 K or in solutions with very high salt concentrations (several hundred millimolar).

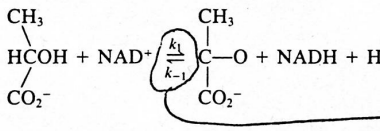
The ways in which the exchange of isotopes may be observed by NMR are manifold. The simplest experimental protocol is the observation of a signal appearing or disappearing in the NMR spectrum of a reaction mixture by direct detection of the substitution of magnetically active nuclei

with inactive isotopes or vice versa during the reaction. In practice, this almost always means observation of deuterium replacement with protons or the reverse reaction. There are also ways to probe exchanges of isotopes of nuclei which are not readily accessible to NMR detection. This is done by use of their influence on nuclei which are easily observed directly, usually protons or phosphorus. Examples for this type of experiment are the oxygen-16 for oxygen-18 exchange processes taking place in labeled inorganic phosphate (P_i) in the presence of ADP, metal ions, and catalytic concentrations of ATPases. These processes may easily be observed via the splitting of the ^{31}P resonance of the phosphate into five different resonances by the differential effect of ^{16}O and ^{18}O on the resonance frequency of P_i. Other indirect methods of isotope detection include the spin-echo technique allowing the observation of the exchange of spin-$\frac{1}{2}$ nuclei of low sensitivity such as carbon-13 or nitrogen-15 via attached protons. A review on isotope exchange studies *in vivo* appeared recently.[1] In the following, the experimental and data evaluation procedures are described with the help of one typical example per experimental category.

Experimental

Proton-Exchange Experiments: Lactate Dehydrogenase (LDH)[2-4]

Direct Detection of Labels: Lactate(CH_3)/Pyruvate(CH_3)/Solvent Proton Exchange.[2,3] As an example for the determination of enzyme-catalyzed isotope exchange by observation of the exchange of deuterium labels with protons, the study of the kinetics of the LDH is described.[2] For the description, it is irrelevant that the original experiment[2] was performed in erythrocytes and not *in vitro*. The reaction catalyzed by LDH is the interconversion between lactate and pyruvate:



(1)

[1] K. M. Brindle and I. D. Campbell, *Q. Rev. Biophys.* **19,** 314, 159 (1987).
[2] K. M. Brindle, F. F. Brown, I. D. Campbell, D. C. Foxall, and R. J. Simpson, *Biochem. Soc. Trans.* **8,** 647.
[3] R. J. Simpson, K. M. Brindle, F. F. Brown, I. D. Campbell, and D. L. Foxall, *Biochem. J.* **202,** 573 (1982).
[4] K. M. Brindle, I. D. Campbell, and R. J. Simpson, *Eur. J. Biochem.* **158,** 299 (1986).

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In addition, protons from pyruvate are exchanged rapidly with solvent deuterons due to protein α-NH_2 catalysis. In the presence of erythrocytes, this is expected to be of considerable magnitude. As the proton content of the solvent during the whole course of the reaction may be neglected in a first approximation, this solvent deuteron exchange reaction is essentially irreversible.

A simplified reaction scheme with respect to the methyl group, neglecting kinetic isotope effects, thus reads

$$\text{Lactate-CH}_3 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{pyruvate-CH}_3 \underset{}{\overset{k_2}{\rightleftharpoons}} \text{pyruvate-CD}_3 \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} \text{lactate-CD}_3 \qquad (2)$$

The parameters k_1, k_{-1}, and k_2 can be determined from the following experiment.

Experimental procedure:

1. Fill packed cells in deuterated buffer in a sample tube.
2. Equilibrate temperature of sample and stock solutions (deuterated solvent) of lactate and pyruvate.
3. Inject desired amount of lactate and pyruvate (e.g., 10 m*M* lactate/10 m*M* pyruvate final concentration as in Brindle *et al.*[5]) in sample tube and mix.
4. Put the sample into the spectrometer, tune probe, and take spectra at appropriate time points (dependent on necessary number of scans per spectrum).

The experiments were performed on a Nicolet NT 470 spectrometer with a proton resonance frequency of 470 MHz.

Data evaluation: The set of differential equations which describes the time dependence of the concentrations of the protonated species according to reaction (2) is

$$\begin{aligned} d/dt[\text{L}] &= -k_1[\text{L}] + k_{-1}[\text{P}] \\ d/dt[\text{P}] &= -(k_{-1} + k_2)[\text{P}] + k_1[\text{L}] \end{aligned} \qquad (3)$$

where L is lactate-CH_3 and P is pyruvate-CH_3. The analytical solution is straightforward[2]:

$$\begin{aligned} [\text{L}](t) &= [\text{L}](0)(a\mathrm{e}^{-bt} - b\mathrm{e}^{-at})/(a - b) \\ [\text{P}](t) &= [\text{P}](0)[b(k_{-1} - a)\mathrm{e}^{-at} - a(k_{-1} - b)\mathrm{e}^{-bt}]/[k_{-1}(b - a)] \end{aligned} \qquad (4)$$

where $a = \frac{1}{2}\{k_1 + k_{-1} + k_2 + [(k_1 + k_{-1} + k_2)^2 - 4k_1k_2]\}^{1/2}$ and $b = k_1 + k_{-1} + k_2 - a$.

A least-squares fit of Eq. (4) to the experimental data by use of a

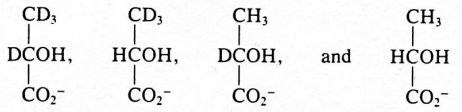
[5] K. M. Brindle, J. Boyd, I. D. Campbell, R. Porteous, and N. Soffe, *Biochem. Biophys. Res. Commun.* **109**(3), 864 (1982).

standard minimization procedure (such as Nelder/Mead "Simplex") yields the rate constants. This can be performed with simple general fit programs fitting the experimental data to the solutions of the Eq. (3) differentials. The (inferior) alternative would be use of a program integrating these sets numerically and fitting the numerical solutions to the experimental data. Both types of program, running on a computer, are used in our laboratory.

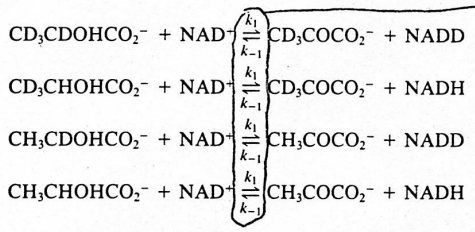
Homonuclear Spin-Echo Experiments: Lactate(2-CH) Exchange[4]

Measurement principle: Use of similar concentrations of reactants and products, as in the experiment described above, is often undesirable. For example, with LDH the product pyruvate is an inhibitor of the enzyme. In addition, pyruvate enables leakage of label by the exchange of methyl protons and solvent deuterons according to reaction (2). For both reasons it is desirable to keep the pyruvate concentration as low as possible. In cases like this, employment of a double-label experiment can be helpful.

In the study of LDH kinetics, the procedure for the double-label experiment is as follows: The experiment is performed in H_2O rather than D_2O. A mixture of completely deuterated and completely protonated lactate results in a mixture of four differently labeled species in the presence of NAD^+ and LDH, namely:



This is the result of the following equilibria (again, kinetic isotope effects are neglected):



(5)

Obviously, in the methyl proton NMR spectrum, only the latter two species may be observed. A spin-echo experiment with the pulse sequence (90°–τ–180°–τ) leads to inversion of the methyl resonances of the completely labeled lactate if τ is chosen so that $\tau = 1/(2J)$. τ = 68 msec, corresponding to J_{HH} = 7.2 Hz, proved to be a good value for lactate. As

[6] O. Lutz, *Z. Naturforsch. A.* **33A**, 380 (1978).

the methyl resonance of the 2-CD lactate is not inverted, the spin-echo sequence gives a signal intensity which is essentially the difference between both species. A spin-echo experiment with a sample containing equal amounts of labeled and unlabeled lactate at isotopic equilibrium, i.e., when $[CH_3—CDOH—CO_2^-]=[CH_3—CHOH—CO_2^-]$, would result in zero intensity for the methyl protein resonance.

Sample preparation: The preparation of completely deuterated lactate is described in ample detail in the original paper.[4] LDH was from Sigma, as were all other chemical except NAD^+, which was obtained from Boehringer in preweighed vials.[4] In the original paper, the experiment was started with equal amounts of $CD_3CDOHCO_2^-$ and $CH_3CHOHCO_2^-$. A concentration of 10 m*M* was used for both.

NMR procedure:

1. Put the sample without enzyme in the spectrometer.
2. Tune the probe by use of an HF-bridge or, preferably, by maximizing the free induction decay signal. In the latter procedure, care must be taken not to saturate the signal, i.e., very small pulse angles should be used. Maximizing the free induction decay proved to be the superior method of probe tuning in all cases in our laboratory.
3. Determine the 90° pulse angle according to one of the fashionable methods, for example by watching the signal phase inversion at a pulse angle of 180° and halving this value: (a) do a single-shot experiment, (b) Fourier transform, (c) phase correct to positive phase, (d) increase pulse width by a factor of two, (e) Fourier transform, (f) use automatic phase correction, (g) if the phase is positive, go to step (d), (h) decrease pulse width to determine the signal zero, (i) halve the value found in step (h).
4. Set up the spin-echo sequence.
5. Readjust the 90° pulse angle to give optimum spin-echo effect.
6. Inject LDH into the sample tube and take spectra at appropriate time points (2 min, 80 scans/time point in the original publication[6]).

The experiments were performed on a Nicolet NT 470 spectrometer with a proton resonance frequency of 470 MHz.

Data evaluation: The differential equations describing the Eq. (5) kinetics are

$$\begin{aligned}
d/dt(\mathrm{DH}) &= -k_1\mathrm{DH}\cdot\mathrm{N} + k_{-1}\mathrm{HO}\cdot\mathrm{ND}\\
d/dt(\mathrm{HH}) &= -k_1\mathrm{HH}\cdot\mathrm{N} + k_{-1}\mathrm{HO}\cdot\mathrm{NH}\\
d/dt(\mathrm{DD}) &= -k_1\mathrm{DD}\cdot\mathrm{N} + k_{-1}\mathrm{DO}\cdot\mathrm{ND}\\
d/dt(\mathrm{HD}) &= -k_1\mathrm{HD}\cdot\mathrm{N} + k_{-1}\mathrm{DO}\cdot\mathrm{NH}\\
d/dt(\mathrm{ND}) &= -k_{-1}\mathrm{CO}\cdot\mathrm{ND} + k_1(\mathrm{DH}\cdot\mathrm{N} + \mathrm{DD}\cdot\mathrm{N})\\
d/dt(\mathrm{NH}) &= -k_{-1}\mathrm{CO}\cdot\mathrm{NH} + k_1(\mathrm{HH}\cdot\mathrm{N} + \mathrm{HD}\cdot\mathrm{N})
\end{aligned} \tag{6}$$

with the abbreviations [$CD_3CHOHCO_2^-$], DH; [$CH_3CHOHCO_2^-$], HH; etc.; [NAD^+], N; NADH, NH; NADD, ND; $CD_3COCO_2^-$, DO; $CH_3COCO_2^-$, HO.

It may be noted that under the given experimental conditions, HH + HD = constant, DH + DD = constant, and ND = NH. The time dependence of the difference DH − HH, which is observed with the spin-echo method, is then:

$$d/dt(\mathrm{DH} - \mathrm{HH}) = -k_1\mathrm{N}(\mathrm{DH} - \mathrm{HH}) + k_{-1}\mathrm{HO}\,\mathrm{ND} \qquad (7)$$

After chemical equilibrium has been obtained, which is a very fast process compared to the establishment of the isotopic equilibrium, ND = NH = constant and OH = constant. Thus, the solution for Eq. (7) is a simple exponential:

$$(\mathrm{DH} - \mathrm{HH}(t) = (1 - e^{-k_1 N t})(k_{-1}\mathrm{HO}\,\mathrm{ND})/(k_1\mathrm{N}) + \mathrm{HH}(0) \qquad (8)$$

This solution can be fitted to the experimental data with k_1 and k_{-1} as free parameters with one of the standard programs.

Indirect Detection of Labels with Spin Echoes[7,8]

Measurement Principle. Indirect detection of nuclear labels with low NMR sensitivity can be performed by observation of spin echoes of attached protons. Although this technique shows its full advantages only when unwanted broad proton signals are to be suppressed, as in cells, it is described here since it proved to be worthwhile to apply this method also *in vitro*. In particular, in cases where the proton resonances cannot be resolved clearly the difference method described below proves to be very attractive. The principle of the measurement consists in the application of a (90°–τ–180°–τ–acquisition) pulse sequence at the proton frequency on the sample exactly as in a standard homonuclear spin-echo experiment. In addition, coincident with the proton 180° pulse, a 180° pulse is applied to the sample at the resonant frequency of the heteronucleus. The pulse sequence is shown in Fig. 1.

Alanine Aminotransferase.[5] Alanine aminotransferase catalyzes the reaction:

[7] R. Freeman, T. M. Mareci, and G. A. Morris, *J. Magn. Reson.* **42,** 341 (1981); R. Freeman and H. D. W. Hill, *J. Chem. Phys.* **54**(1), 301 (1971).
[8] K. M. Brindle, R. Porteous, and I. D. Campbell, *J. Magn. Reson.* **56,** 543 (1984).

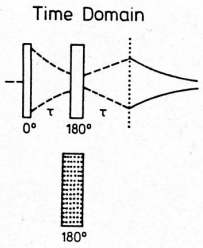
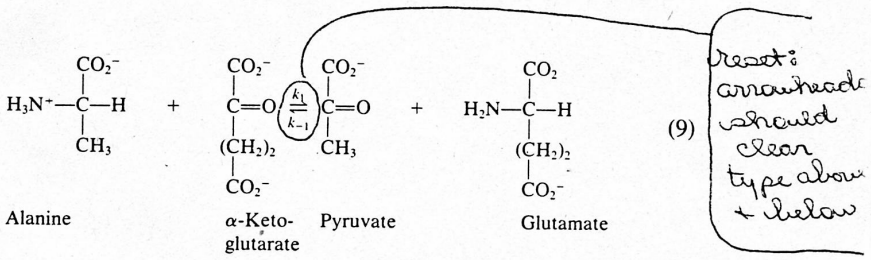


FIG. 1. Pulse sequence for heteronuclear spin-echo experiments. (After Brindle *et al.*[5])



Measurement of the reaction rate of alanine aminotransferase was performed with L-[3-^{13}C]alanine as labeled compound. The methyl spectrum of this compound is a doublet (due to the ^{13}C splitting) of doublets (due to the $C_\alpha H$ splitting). A mixture of labeled and unlabeled alanine then gives a methyl spectrum of a central doublet from the unlabeled compound and two symmetrically located doublets from the labeled compound. A value $\tau = 1/(2J_{HH})$ in the spin-echo sequence, where J_{HH} is the proton–proton coupling constant (in the case of alanine, $J_{HH} = 7.3$ Hz), results in an inversion of all doublet signals in a mixture of labeled and unlabeled alanine. This is illustrated in Fig. 2a. Decoupling of ^{13}C results in a collapse of the outer doublets into the central one giving, in essence, the sum of the signal from the labeled and unlabeled compounds (Fig. 2b). An echo delay of $\tau = n/(2J_c)$ results in an inversion of the signals from protons attached to ^{13}C relative to the signals from protons attached to ^{12}C (Fig. 2c). ^{13}C decoupling during acquisition again leads to the collapse of the ^{13}C splitting and gives, in essence, a signal which corresponds to the difference between the signals from protons attached to the label and the corresponding protons from the unlabeled compound.

Sample preparation: The reaction mixture in the experiment described by Brindle *et al.*[5] contained 0.15 units/ml alanine aminotransferase as obtained from Sigma; 100 m*M* glycylglycine–imidazole buffer, pH 7.4, 0.5 m*M* EDTA, 0.15 m*M* KCl, 10 m*M* pyruvate, and 10 m*M*

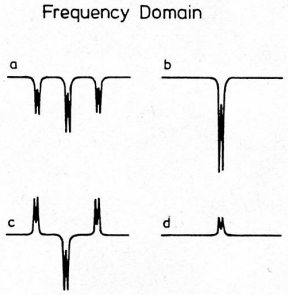


FIG. 2. (a) Effect of the proton spin-echo sequence with $\tau = 1/(2J_{HH})$ on the methyl protons of a mixture of 60% alanine ^{13}C labeled in the methyl group (outer doublets) and 40% unlabeled alanine (central doublet). (b) Application of ^{13}C decoupling during acquisition leads to a collapse of the outer doublets into the central doublet. (c) A 180° pulse ^{13}C at the same time as the proton 180° pulse [$\tau = 1/(2J_0)$] leads to inversion of the unlabeled resonance only. (d) ^{13}C decoupling during acquisition in experiment (c) leads again to a collapse of the outer doublets into the central (inverted) doublet, thus resulting essentially in a difference spectrum. (After Brindle *et al.*[5])

L-[3-^{13}C]alanine. The measurements were performed in H_2O as the alanine aminotranferase catalyzes the exchange of the alanine as well as the pyruvate methyl protons with the solvent.

NMR procedure: Setup of spin-echo sequence: Hardware requirements for the setup of a ^{13}C–proton spin-echo sequence in most spectrometers currently available are as follow (the requirements are analogous for other X nuclei): (1) a probe with a ^{13}C detection coil and a proton decoupling coil; and (2) a computer-controlled, pulsed high-frequency power amplifier tuned to the ^{13}C frequency.

Of course, the preferable equipment available in state-of-the-art spectrometers is a so-called f_1/f_2 switch. This allows interchange of decoupling and observation frequencies, thus making the external power amplifier superfluous. Also, in state-of-the-art instruments, an "inverse" probe with observation and decoupling coils interchanged for superior signal-to-noise ratio may be used.

Procedure with standard equipment for the setup of a proton-detected ^{13}C measurement with the spin-echo method (again, the methods are analogous for other X nuclei):

1. Connect the cable from the external computer-controlled ^{13}C power amplifier to the observation coil plug of the probe.
2. Connect the proton observation channel to the decoupler coil of the probe.

3. Set the spectrometer to the ^{13}C observation mode.

4. Determine the ^{13}C 90° pulse width with the external power amplifier. Use a standard sample where the ^{13}C signal may be detected easily with a single-scan experiment. For the determination of the 90° pulse any of the currently used procedures may be used, for example the method outlined in the section, Homonuclear Spin-Echo Experiments.

5. Insert the sample without initializing the reaction, i.e., without enzyme.

6. Set the spectrometer console to proton observation.

7. Tune the observation coil of the probe to ^{13}C using an HF-bridge with fast external power amplifier pulses. Tune the decoupling coil of the probe to protons as usual, i.e., using an HF-bridge or maximizing the FID with small excitation pulse angles.

8. Determine a value n (odd) for which the equation $1/(2J_{HH}) = n/(2J_{CH})$ is approximately fulfilled. J_{HH} is the proton–proton coupling constant, J_{CH} the carbon–proton coupling constant of the signal under study. For the methyl signal of alanine, $J_{HH} = 7.3$ Hz and $J_{CH} = 130$ Hz; so $n = 15$ yields $t = 58$ msec as a good compromise.

9. Set up the pulse program.

10. Readjust the ^{13}C pulse angle to maximize the spin-echo effect.

11. Add enzyme to the sample.

12. Start the NMR measurement.

Evaluation of data: The differential equations governing the time development of the difference between ^{13}C-labeled pyruvate (P*) and pyruvate (P) and between ^{13}C-labeled alanine (A*) and alanine (A) can be derived from

$$\begin{aligned} d/dt[A^*] &= -k_1[A^*][\alpha] + k_{-1}[P^*][G] \\ d/dt[A] &= -k_1[A][\alpha] + k_{-1}[P][G] \\ d/dt[P^*] &= -k_{-1}[P^*][G] + k_1[A^*][\alpha] \\ d/dt[P] &= -k_{-1}[P][G] + k_1[A][\alpha] \end{aligned} \tag{10}$$

where α is α-ketoglutarate and G is glutamate. The time development for the difference between labeled and unlabeled compounds is (with the convention [a] = [A*] − [A]; [p] = [P*] − [P]:

$$\begin{aligned} d/dt[a] &= -k_1[\alpha][a] + k_{-1}[G][p] \\ d/dt[p] &= -k_1[G][p] + k_1[\alpha][a] \end{aligned} \tag{11}$$

At chemical equilibrium, i.e., $d/dt\ G = 0$ and $d/dt\ \alpha = 0$, an analytical solution of this set of differential equations can be obtained (omitting concentration brackets):

$$a = [a(0) + p(0)]k_{-1}(G/X) + \{a(0) - [a(0) + p(0)]k_{-1}G/X\}e^{-Xt}$$
$$p = [a(0) + p(0)]k_1(\alpha/X) - \{a(0) - [a(0) + p(0)]k_{-1}G/X\}e^{-Xt} \quad (12)$$
$$X = k_{-1}G + k_1\alpha$$

These solutions may be fitted to the experimental data with the rate constants as free parameters to yield k_1 and k_{-1}. In more complicated cases, programs exist which integrate the set of differential equations numerically and fit the constants to the experimental equations in an iterative way.

Creatine Kinase[9]

Creatine kinase catalyzes the reaction:

$$\text{Phosphocreatine} + \text{ADP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{creatine} + \text{ATP}$$

As an alternative to the numerous saturation transfer experiments performed on this system, an isotope-exchange experiment making use of an ^{15}N-labeled creatine ([1-^{15}N]guanidino-1-methylethanoic acid) was used. The ^{15}N label was observed via the ^{31}P resonance.

Sample preparation: An excellent exposition of the synthesis of the labeled compound was given by Greenaway and Whatley.[10] The reaction mixture[9] contained 10 m*M* phosphocreatine, 10 m*M* [^{15}N]creatine, 5 m*M* ATP, 200 m*M* potassium HEPES buffer, pH 7.0, or triethanol-HCl, pH 8.0, 0.01 m*M* EDTA, 2 m*M* dithiothreitol, 120 μM creatine kinase as obtained from Boehringer. $MgCl_2$ was added to result in 1 m*M* free ion concentration as calculated from the known stability constants of the complex.

NMR procedures: The hardware requirements for a ^{31}P-observed, heteronuclear ^{15}N spin-echo experiment are a double-tuned probe (^{31}P and ^{15}N) and an external power amplifier for the ^{15}N frequency with the spectrometer console set for ^{31}P observation (or vice versa). A home-built probe with the center coil tuned to the phosphorus frequency and the outer coil tuned to the nitrogen frequency was used. The experiment was performed with a 10-mm tube on an essentially home-built spectrometer operating at a phosphorus frequency of 73.8 MHz. The instrumental setup for the experiment is analogous to the one described for the proton–^{13}C heteronuclear spin-echo experiment.

Evaluation of data: The differential equations describing the labeled (*) and unlabeled compounds phosphocreatine (PC) and creatine (C) are

[9] K. M. Brindle, R. Porteous, and G. K. Radda, *Biochim. Biophys. Acta* **786,** 18 (1984).
[10] W. Greenaway and F. R. Whatley, *J. Labelled Compd. Radiopharm.* **14,** 611 (1978).

$$d/dt[PC^*] = -k_1[PC^*][ADP] + k_{-1}[C^*][ATP]$$
$$d/dt[PC] = -k_1[PC][ADP] + k_{-1}[C][ATP] \quad \text{etc.} \qquad (13)$$

With pc = [PC*] − [PC] and c = [C*] − [C], integration yields eq. (12) with

$$pc = pc(0) + ck_{-1}/k_1[ATP]/[ADP](1 - e^{-k_1[ADP]t}) \qquad (14)$$

Detection of Oxygen Labels of Inorganic Phosphate: Myosin S_1 ATPase

Measurement Principle. The method of determination of kinetic parameters of nucleotide hydrolases by following the P_i–^{18}O exchange catalyzed by these proteins rests on the fact that all known hydrolases cleave the β-bridge of nucleoside triphosphates at the terminal bond of the bridge oxygen:

$$ADP—O—PO_3 + H_2O \rightleftharpoons ADP + P_i \qquad (15)$$

This means that with every ATP cleavage an oxygen atom from the surrounding water is incorporated into the P_i molecule. The ATP cleavage and resynthesis may go back and forth on the hydrolase several times before product release. Thus, the average number of oxygens exchanged during the overall reaction may obtain any value. Labeling of the ATP terminal phosphoryl group with ^{18}O then yields a specific pattern of isotopic replacements in the product P_i. Of course, as the reaction is reversible, it may also be started with ADP and labeled P_i and either the time course of incorporation of the labels into the terminal phosphoryl group of ATP or the change in the concentrations of the five different P_i species, $P^{18}O_4$, $P^{18}O_3{}^{16}O_1$, ..., $P^{16}O_4$, may be followed. The ^{31}P NMR spectrum shows different signals for the five different species, the signal spacing being about 0.025 ppm.[6,11] In the former case, product ATP must be removed by a secondary reaction to yield reasonable amounts of this compound, e.g., with hexokinase, which transfers the terminal phosphoryl group of ATP to glucose to yield glucose 6-phosphate essentially without disturbing the label content.[12] The concentration of the different P_i species can also be detected by mass spectroscopy.[13] This latter method is somewhat more demanding as far as the chemistry involved is concerned but is much more sensitive than the NMR method, accordingly resulting in lower amounts of enzyme required. The major advantage of the use of NMR as the detection method is the simplicity of the experimental setup and, under certain conditions, the possibility of following the ongoing

[11] M. Cohn and A. Hu, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 200 (1978).
[12] J. J. Sines and D. D. Hackney, *Biochemistry* **25**, 6144 (1986).
[13] D. D. Hackney, K. E. Stempel, and P. D. Boyer, this series, Vol. 64, p. 70.

exchange reaction in virtually real time. One of the systems studied most intensively with the aid of P_i–^{18}O isotope exchange (PIX) is the hydrolase part of myosin, myosin S_1. Here I describe the experimental setup for a simple experiment with observation of the time dependence of the five different P_i species in the presence of myosin S_1 and ADP.

Sample preparation: The preparation of myosin S_1 is essentially standardized. The procedure was described earlier in this series.[14] Procedures for ^{18}O enrichment of P_i, based on isotope exchange, would require a large excess of isotopically enriched water and are thus not economical. An alternative synthesis of ^{18}O-labeled P_i was also described in this series.[13] In our laboratory we applied a somewhat different procedure based on hydrolysis of phosphorus pentachloride with $H_2{}^{18}O$ to phosphoric acid and hydrogen chloride and subsequent isolation of phosphoric acid as the crystalline monopotassium salt:

$H_2{}^{18}O$ (1 g, ^{18}O content approximately 98.8%, Monsanto, OH) is frozen with liquid nitrogen in a two-necked vial which contains a magnetic spin vane and is connected to an adjustable vacuum pump. Phosphorus pentachloride (2.9 g) is transferred quickly to the frozen enriched water. The equipment is protected against humidity while it is slowly warmed to room temperature. A reduced pressure of 66 kPa is applied and the mixture stirred and heated to 80° for a period of 90 min. After evolution of HCl is finished, the reaction mixture is allowed to cool to room temperature. Potassium hydroxide (2 M, in normalized water) is used to adjust the pH to 4.6 and the phosphate is precipitated by adding ethanol (70%, 15–20 ml), collected, and dried: 1.7 g KH_2PO_4, ^{18}O approximately 98%.

The chemical yield usually fluctuates between 75 and 90%. It may contain traces of potassium chloride. As suggested by Risley and van Etten,[15] a reprecipitation to get a virtually KCl-free product may be required, in particular if the product is used for purposes other than NMR observation of ^{18}O exchange. Synthesis of ATP (^{18}O) from this product was described earlier in this series.[13]

The reaction mixture in the experiment described by Rösch *et al.*[16] contained 0.18 m*M* myosin S_1, 100 m*M* labeled P_i, 0.2 m*M* ADP, 0.02 AP_5A (diadenosine pentaphosphate), 1 m*M* $MgCl_2$, 50 m*M* HEPES, pH 7.5, 0.1 m*M* EDTA. The preparation of AP_5A was described earlier. Addition of this compound to the reaction mixture was essential to suppress residual adenylate kinase activity. Traces of sodium azide were added to prevent bacterial growth. In order to obtain the necessary reso-

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[14] S. S. Margossian and S. Lowey, this series, Vol. 85, p. 55.
[15] J. M. Risley and R. L. van Etten, *J. Labelled Compd. Radiopharm.* **15,** 533 (1978).
[16] P. Rösch, R. S. Goody, and H. Zimmermann, *Arch. Biochem. Biophys.* **211**(2), 622 (1981).

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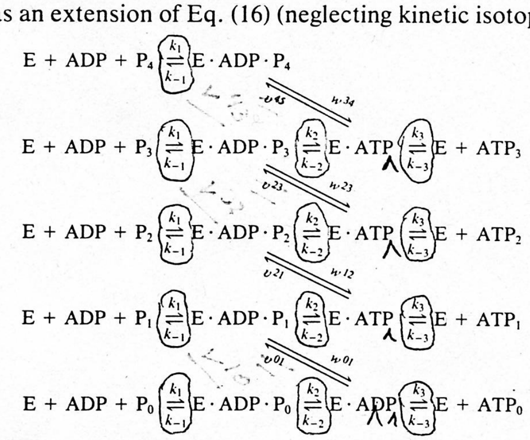
lution, in general two things are essential: First, the pH of the solution must be well above (or below) the pK region of inorganic phosphate, i.e., pH 7.5 or above should be used; second, the temperature must be equilibrated very well before data collection is started.

NMR procedures: The experiments were performed on a Bruker 360 HX NMR instrument with a ^{31}P resonance frequency of 145.78 MHz. In our experiments, the spectral width was 60 Hz with a computer memory of 1K data, resulting in a scan repetition rate of 8.5 sec. The pulse angle was 90° and 200 accumulations/time point with a total of 100 time points required a measurement time of about 2 days. The relative concentration can be determined using the peak height in the spectrum. As the different P_i species have virtually the same T_1 values, it is not necessary to take relaxation into account.

Data evaluation: Analysis becomes more laborious in exchange reactions with multiple isotope labels.[12,13,17] The minimum number of reaction steps for the ^{18}O exchange catalyzed by ATPases is

$$\mathrm{E + ADP + P_i} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E\cdot ADP\cdot P_i} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E\cdot ATP} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \mathrm{E + ATP} \qquad (16)$$

In general, experiments starting with labeled ATP and experiments starting with labeled P_i may be described with the same methods. The description is also the same irrespective of which of the two compounds is the species whose time-dependent label incorporation or loss is followed by the experiment. With the notation $P^{18}O_n{}^{16}O_{4-n} = P_n$ and ADP–O–$P^{18}O_n{}^{16}O_{3-n} = ATP_n$, the kinetic scheme governing the oxygen isotope exchange as an extension of Eq. (16) (neglecting kinetic isotope effects) is



(17)

[17] P. Rösch, *Prog. Nucl. Magn. Reson. Spectrosc.* **18**(2), 123 (1986).

The exchange of a label corresponds to the transition between different lines in the set of equations with reaction rates k_2 and transition probabilities between the differently labeled species w^{mn} and v^{mn}. w^{mn} is the probability of transition from P_n to ATP_m, v^{mn} the probability of transition from ATP_n to P_m. Of course, $\sum_n V^{mn} = \sum_n W^{mn} = 1$.

The differential equations describing the above scheme are

$$
\begin{aligned}
d/dt[P_0] &= -k_1[E][ADP][P_0] + k_{-1}[E\cdot ADP\cdot P_0] \\
&\vdots \\
d/dt[P_4] &= -k_1[E][ADP][P_4] + k_{-1}[E\cdot ADP\cdot P_4] \\
d/dt[ATP_0] &= -k_{-3}[E][ATP_0] + k_3[E\cdot ATP_3] \\
&\vdots \\
d/dt[ATP_3] &= -k_{-3}[E][ATP_3] + k_3[E\cdot ATP_3] \\
d/dt[E\cdot ADP\cdot P_0] &= -(k_{-1} + k_2)[E\cdot ADP\cdot P_0] + k_1[E][ADP][P_0]) \\
&\quad + k_2[E\cdot ATP_0] \\
d/dt[E\cdot ADP\cdot P_1] &= -(k_{-1} + k_2)[E\cdot ADP\cdot P_1] + k_1[E][ADP][P_1] \\
&\quad + k_2(v^{11}[E\cdot ATP_1] + v^{10}[E\cdot ATP_0]) \\
&\vdots \\
d/dt[E\cdot ADP\cdot P_4] &= -(k_{-1} + k_2)[E\cdot ADP\cdot P_4] + k_1[E][ADP][P_4] \\
&\quad + k_2 v^{43}[E\cdot ATP_3] \\
d/dt[E\cdot ATP_0] &= -(k_3 + k_{-2})[E\cdot ATP_0] \\
&\quad + k_2(w^{01}[E\cdot ADP\cdot P_1] + w^{00}[E\cdot ADP\cdot P_0]) \\
&\quad + k_{-3}[E][ATP_0] \\
&\vdots \\
d/dt[E\cdot ATP_3] &= -(k_3 + k_{-2})[E\cdot ATP_3] \\
&\quad + k_2(w^{34}[E\cdot ADP\cdot P_4] + w^{33}[E\cdot ADP\cdot P_3]) \\
&\quad + k_{-3}[E][ATP_3] \\
d/dt[ADP] &= \textstyle\sum_n (d/dt\ P_n)
\end{aligned}
\qquad (18)
$$

In practice, experimental conditions may be found under which one or the other of the rate constants may be neglected. For example, if the experiment is started from labeled P_i, usually k_3 and k_{-3} may be zeroed in a first approximation. Thus, under decent assumptions for w^{mn} and v^{mn}, the other parameters may be obtained directly by a least-squares fit of the numerical solutions of the Eq. (18) differentials to the experimental data. For a P_i molecule with complete rotational freedom in the enzyme-bound

[18] A. G. Weeds and R. S. Taylor, *Nature (London)* **257,** 54 (1975).

state, the values for v^{mn} and w^{mn} are given by

$$\begin{aligned} v^{00} &= v^{11} = v^{22} = v^{33} = 1 - z \\ v^{10} &= v^{21} = v^{32} = v^{43} = z \\ w^{00} &= 1;\ w^{11} = 3/4;\ w^{22} = 1/2;\ w^{33} = 1/4 \\ w^{01} &= 1/4;\ w^{12} = 1/2;\ w^{23} = 3/4;\ w^{34} = 1 \end{aligned} \tag{19}$$

z is the fractional $H_2{}^{18}O$ content of the solvent, i.e.,

$$z = H_2{}^{18}O/(H_2{}^{16}O + H_2{}^{18}O) \tag{20}$$

All other v^{mn} and w^{mn} are zero. The fractional content of $H_2{}^{18}O$ may either be assumed to be invariant during the experiment (usually $z =$) or may be varied according to

$$\begin{aligned} z(t) &= (H_2{}^{18}O + K)/(H_2{}^{16}O + H_2{}^{18}O) \\ K &= [\Sigma n P_n(0) + \Sigma n ATP_n(0) - \Sigma n P_n(t) - \Sigma n ATP_n(t)] \end{aligned} \tag{21}$$

Of course, the values for v^{mn} and w^{mn} can be adapted to situations where the prerequisite of complete rotational freedom is not fulfilled. Extensive calculations along this line were done by Sines and Hackney.[12] The computer calculations may be performed with one of the mentioned programs being able to numerically integrate and least-squares fit the differential equations to the experimental kinetic parameters. In the simplest experiment, the one starting from P_i and the E · ADP complex, the number of reversals of the ATP-synthesis step may be calculated as $N = k_2/k_{-1}$, and k_1 can be determined easily. This can be done to a very good approximation even with numerical procedures which are not based on the solution of the complete set of Eq. (18) differentials.

An example of the results obtained with this method is given in Fig. 3. It shows in the upper part the calculated time dependence of the five different P_i species in the presence of myosin S_1, ADP, and $MgCl_2$ under the conditions outlined above. The lower part of Fig. 3 shows the final spectrum of the experiment as it was obtained after 23.5 hr (upper trace) and the computer simulation according to the fitted rate constants.

Outlook

Observation of isotope exchange opened the possibility to measure kinetic parameters which could not be determined by any other method. NMR is a simple way to follow the time course of the isotopic exchange procedure. In most NMR experiments, not much chemistry is involved. Even in experiments where competitive methods such as mass spectrometry for the ^{18}O exchange experiments are available, NMR proves to be by far the simplest way to get the desired information. On the other hand,

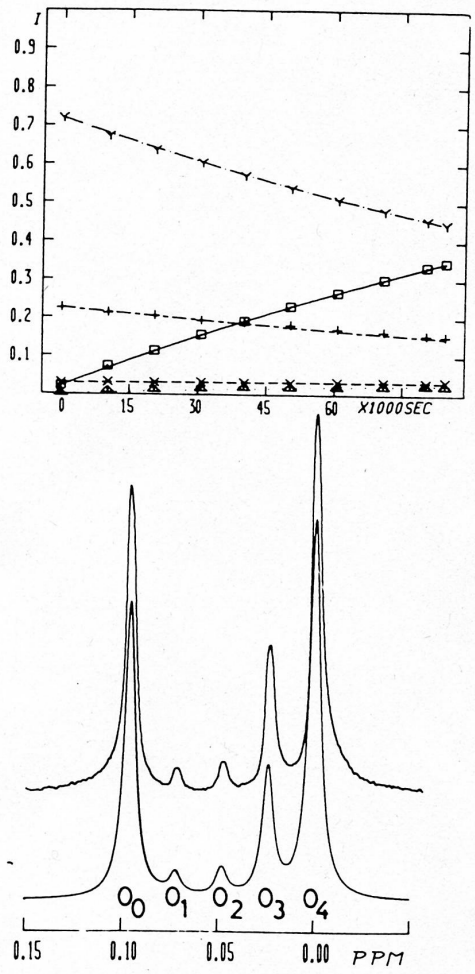


FIG. 3. *Upper:* Time development of the concentration of the five different $P^{18}O_n{}^{16}O_{4-n}$ species under the experimental conditions described in the text. ■: $n = 4$; ■: $n = 3$; ■: $n = 2$; ■: $n = 1$; ■: $n = 0$. *Lower:* Last measured spectrum from the reaction mixture (upper trace) and spectrum simulated according to the fitted parameters. (From Rösch *et al.*[16])

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NMR requires sample amounts which worry many biochemists. In these cases, if alternative methods exist, researchers tend to employ these alternatives. A good example is again the ^{18}O exchange experiment. The groups most active in the field recently seem to employ the additional chemistry involved in the mass spectrometric approach in order to economize on the biological sample. Another point is that NMR studies at high field are still very expensive and it may be necessary to resort to methods which are not as simple as NMR, but are less costly.

Acknowledgments

I would like to thank Dr. Roger S. Goody for years of fruitful collaboration. Dr. David D. Hackney (Pittsburgh, PA) sent me manuscripts prior to publication. Mr. Herbert Zimmermann (Heidelberg, West Germany) worked out the preparation procedure for labeled inorganic phosphate.

for reading

[18] Interpreting Protein Dynamics with Nuclear Magnetic Resonance Relaxation Measurements

By ROBERT E. LONDON

Introduction

The extraction of a dynamic description of protein structure from NMR relaxation measurements constitutes a complex theoretical problem which has resisted reduction to the direct application of a set of simple methodological rules. This situation reflects the fact that the problem is not merely one of determining rates of motion within the framework of a particular dynamic model, but of formulating a description of the motion. Given the rather theoretical nature of this problem, it might seem inappropriate to treat this topic in a series devoted to methodology. However, there are two important reasons for the discussion of the effects of protein dynamics in this volume. First, one of the principle motivations for magnetic resonance studies of proteins has been the desire to characterize the "solution structure" of proteins, and to compare this structure with the more static picture provided by crystallographic analysis. Thus, the dynamic aspects of protein structure which strongly modulate the observed NMR spectra constitute one of the most unique contributions of the method. Second, since dynamic aspects of protein structure determine the NMR relaxation parameters, an appreciation of how these will affect the data becomes important at the design, execution, and interpretation