Statistical Description of Isotope Exchange Processes: A Multisite Model for the ^{18}O Exchange

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An analytical procedure has been developed for the determination of isotope exchange processes as exemplified by the ^{18}O exchange catalysed by enzyme-nucleotide complexes. The model is able to handle more than one type of active site per reaction solution and is also able to distinguish between different types of inequivalence of the oxygens of enzyme bound P_i. Use of transition matrix formalism and basic statistical considerations lead directly to the simple model. A data refinement procedure is introduced and model calculations are shown.

Kinetic experiments involving ^{18}O exchange processes have been very helpful in elucidating certain features of nucleoside triphosphate cleavage and similar processes. (For a recent review, see Hackney *et al.*, [1].) The fact on which the experiments rest is that during synthesis of a nucleoside triphosphate from the nucleoside diphosphate and P_i an oxygen atom is released which stems from P_i (Fig. 1). This cleavage and synthesis process may be described in the case of a nucleoside triphosphate NTP by the simple equation

$$\begin{aligned} \mathrm{NTP} + \mathrm{E}\cdot\mathrm{NDP} &\underset{k_{\mathrm{diss}}}{\overset{k_{\mathrm{ass}}}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{NTP} + \mathrm{NDP} \\ &\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{NDP}\cdot P_i + \mathrm{NDP} \\ &\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{NDP} + P_i + \mathrm{NDP}\,. \quad (1) \end{aligned}$$

For n different types of mutually independent reactive sites a set of n different equations of the type of Eqn. (1) has to be considered.

Two different types of ^{18}O exchange experiments yielding similar types of information may be performed, one starting from labelled NTP, *i.e.* NTP ($\gamma^{18}O_n{}^{16}O_{4-n}$), $0 \leqq n \leqq 4$, the other starting from labelled inorganic phosphate, *i.e.* $P^{18}O_n{}^{16}O_{4-n}$, $0 \leqq n \leqq 4$. For the NTP ($\gamma^{18}O$) experiment n is usually less than or equal to three. In principle, at least four pieces of information may be extracted from appropriately designed experiments: The rate constant with which the labelled compound enters the exchange cycle (either step 2 or the NTP association step in Eqn. [1]), the number of cycles the labelled compound undergoes before P_i is released to the bulk solution, the number of different active sites involved, and the number of oxygens which are accessible to the exchange process.

The analysis of the time dependence of the concentration of the various P_i species in the reaction solution can be performed with at least two techniques, namely mass spectroscopy and ^{31}P NMR spectroscopy.

The ^{31}P NMR spectroscopic approach has the advantage that it is more easily executed because P_i can be monitored directly in the solution. In con-

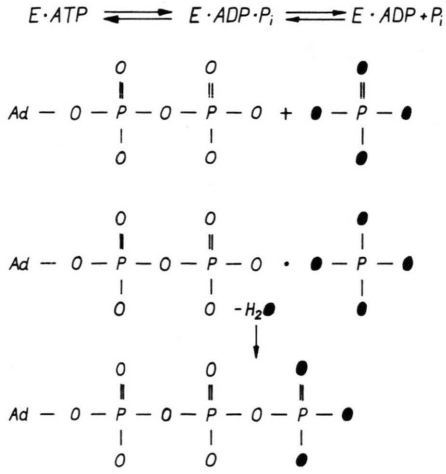

Fig. 1. Nucleoside triphosphate synthesis on nucleotides from nucleoside triphosphate and inorganic phosphate results in the loss of one oxygen atom from P_i. Cleavage of nucleoside triphosphate into diphosphate and P_i incorporates an oxygen from the surrounding H_2O into P_i.

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trast, mass spectrometry requires quantitative conversion of the P_i species into a volatile compound, but has the advantage that smaller quantities of sample are needed and that it is, therefore, more accurate.

The methods used for the analysis of the experimental results are manifold. They range from serious calculations of the exchange process (Hackney *et al.* [1]; Hackney [2]; Rösch *et al.* [3]; Rösch [4]) to very speculative interpretations. So far, all calculations have been restricted to models with only one type of active site in the reaction solution. One purpose of this contribution is to introduce a general model for ^{18}O exchange processes with ^{18}O fed into the exchange cycle from either side of Eqn. (1) for an in principle unlimited number of types of reactive sites in the reaction mixture; we also introduce a data fitting procedure with the purpose of extracting the kind of information mentioned above from the experimental data. Experimental results on myosin/ADP interaction evaluated with this model will be discussed in a subsequent paper.

Results

Many time dependent processes in nature on a molecular and submolecular level which involve the interconversion of different substates of a system can be described by a differential equation of the form

$$\frac{\partial}{\partial t}\boldsymbol{A}(t) = \sum_{j=1}^{k}\sum_{i=1}^{n} {}_j^i\mathscr{T}(t, x_1 \ldots)\, {}_j\boldsymbol{B}(t) - \sum_{i=1}^{n^*} {}^i\mathscr{D}(t, y_1 \ldots)\, \boldsymbol{A}(t)\,. \qquad (2)$$

Here, the vector $\boldsymbol{A}(t)$ describes the subsystem under observation, ${}_j\boldsymbol{B}(t)$ are subsystems which interconvert with $\boldsymbol{A}(t)$, and ${}_j^i\mathscr{T}(t, x_1 \ldots)$ are second rank tensors which describe the transition from ${}_j\boldsymbol{B}(t)$ to $\boldsymbol{A}(t)$. The tensors ${}_j^i\mathscr{T}(t, x_1 \ldots)$ are called transition matrices and have the dimension of probability per unit of time (usually sec^{-1}). They may be dependent on time and other parameters $x_1 \ldots$. n is the number of independent pathways from $\boldsymbol{B}(t)$ to $\boldsymbol{A}(t)$. ${}^i\mathscr{D}$ describes the losses of $\boldsymbol{A}(t)$, n^* being the number of different processes by which $\boldsymbol{A}(t)$ is lost. From a reaction chemical point of view, Eqn. (2) can be interpreted as a general first order rate equation for the vectors $\boldsymbol{A}(t)$ and ${}_j\boldsymbol{B}(t)$. Now, in order to proceed immediately to the actual case of ^{18}O exchange, the conditions of experiments following Eqn. (1) can be chosen in such a way that Eqn. (1) is unidirectioned for the NTP ($\gamma^{18}O$) experiment, *i.e.* P_i, once released from the E · NDP complex, is not bound to this complex again. This can be accomplished for example by using a high concentration of NTP. For experiments starting from the P_i side the assumption is that there is no NTP set free from the E · NTP state into the bulk solution.

These conditions suggest the treatment of the isotope exchange as a simple first order process with respect to the product P_i.

Eqn. (2) is simplified with these conditions, because now we have the special case that only one species, either NTP or P_i itself, is converted to a new vector $\boldsymbol{A}(t)$, *i.e.* $k = 1$. If it is further assumed that the system is not changing during the experiment, *i.e.* the temperature is constant, the enzyme is stable etc., we can substitute the partial derivative by a total one. If, in addition the vector elements of $\boldsymbol{A}(t)$ can only be decreased by the same processes which cause the transition described by $\mathscr{T}$ (*i.e.* $n = n^*$) we can rewrite Eqn. (2):

$$\frac{d}{dt}\boldsymbol{A}(t) = \sum_{i=1}^{n}(-{}^i\mathscr{D}\,\boldsymbol{A}(t) + {}^i\mathscr{T}\,\boldsymbol{B}(t)) = \mathscr{T}\,\boldsymbol{B}(t) - \mathscr{D}\,\boldsymbol{A}(t)\,. \qquad (3)$$

The elements of $\boldsymbol{A}$ are the concentrations of $P^{18}O_n{}^{16}O_{4-n}$, $0 \leqq n \leqq 4$, the elements of $\boldsymbol{B}$ either the concentrations of $NTP(\gamma^{18}O_n{}^{16}O_{4-n})$, or $P^{18}O_n{}^{16}O_{4-n}$ for the experiment starting out from $NTP(\gamma^{18}O)$ and $P_i(^{18}O)$, respectively. The basic scheme of the experiments is given in Fig. 2. In the following we use a more compact notation:

$O_n = P^{18}O_n{}^{16}O_{4-n}$, $\boldsymbol{O} = (O_0, O_1, O_2, O_3, O_4)^\top$;
$\gamma_n = NTP(\gamma^{18}O_n{}^{16}O_{4-n})$, $\gamma = (\gamma_0, \gamma_1, \gamma_2, \gamma_3, \gamma_4)^\top$.

In most experiments γ_4 will be zero due to the preparation procedures for NTP.

All that remains to be done is to calculate the 5×5 transition probability matrices $\mathscr{T}$ and $\mathscr{D}$ and solve Eqn. (3). The elements of the matrix $\mathscr{T}$, T_{ij}, give the probability that the j^{th} element of the vector γ or the vector $\boldsymbol{O}$ is transformed to the i^{th} element of $\boldsymbol{O}$. If we make the assumption that the enzyme cannot distinguish between the various oxygen isotopes we may extract a rate constant ${}^ik$ out of ${}^i\mathscr{T}$ and ${}^i\mathscr{D}$, so that ${}^i\mathscr{T} = {}^ik\,{}^i\mathscr{T}^*$ and ${}^i\mathscr{D} = {}^ik\,{}^i\mathscr{D}^*$.

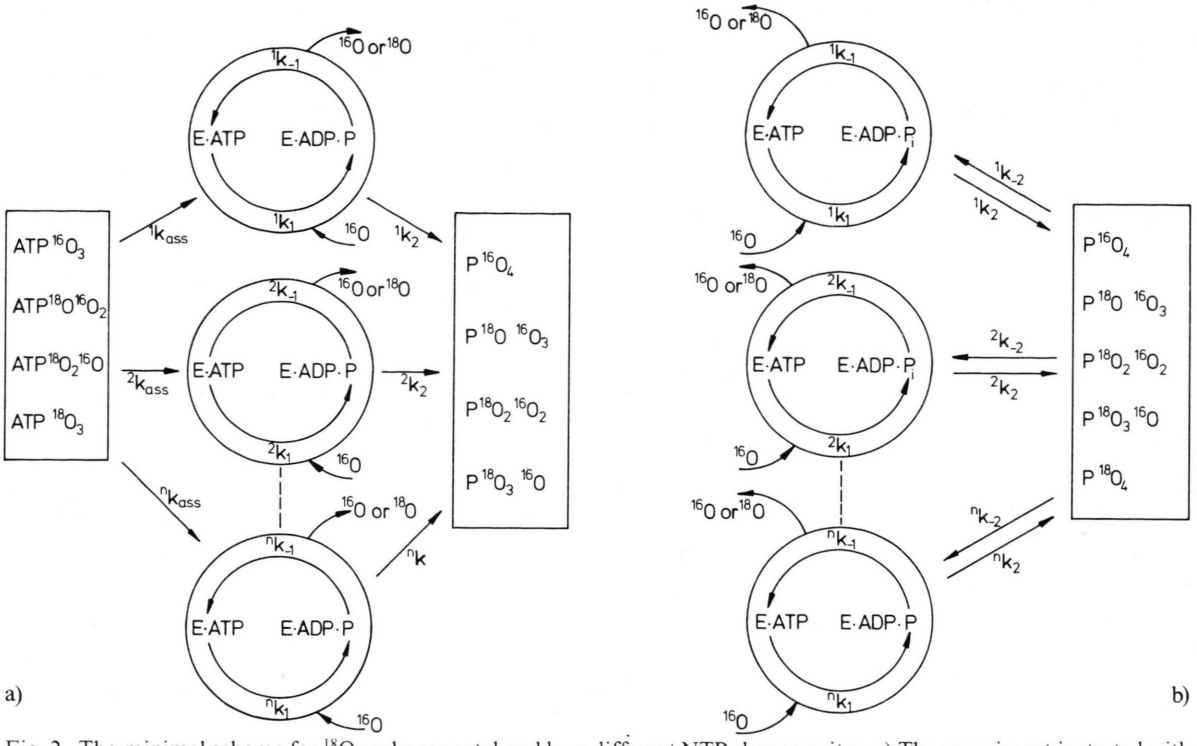

Fig. 2. The minimal scheme for ^{18}O exchange catalysed by n different NTP cleavage sites. a) The experiment is started with NTP (γ-^{18}O). Either ^{16}O or ^{18}O is released during the exchange cycle by NTP cleavage. ^{16}O is fed into the cycle from the surrounding water for the synthesis of NTP. After an average of ${}^{n}N = {}^{n}k_{-1}/{}^{n}k_2$ cycles P_i is released containing the same number of labels or fewer labels than the original NTP (γ-^{18}O). b) Same as a), but this time the ^{18}O label is entering the exchange cycle from the P_i site. For both experiments $[H_2{}^{18}O] \ll [H_2{}^{16}O]$ is assumed.

${}^{i}\mathcal{T}^*$ has the dimension of probability, ${}^{i}k$ has the dimension of inverse time (usually $\sec^{-1}$); ${}^{i}k$ is readily seen to be the rate at which NTP(γ-^{18}O) or P_i (^{18}O) enters the exchange cycle, *i.e.* ${}^{i}k = k_{ass}[\mathrm{E}\cdot\mathrm{NDP}]\cdot[P_i]$ or ${}^{i}k = k_{-2}[\mathrm{E}\cdot\mathrm{NDP}]\cdot[\mathrm{NTP}]$. In both cases, the enzyme is assumed to be saturated with nucleotides at all times.

The important point to notice here is that the matrix $\mathcal{T}$ can be calculated in just the same way for both types of experiment and depends only on the probabilitites $T^*_{mn}(\bar{N})$ that a P_i molecule in the $\mathrm{E}\cdot P_i$ state – which it may enter from either direction – loses (or gains) $n-m$ of originally n labels during an average of $\bar{N}$ repetitions of the exchange cycle $\mathrm{E}\cdot\mathrm{ATP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E}\cdot\mathrm{ADP}\cdot P_i\cdot\mathrm{A}$ short thought shows that $\bar{N} = k_{-1}/k_2$.

The diagonal of the matrix $\mathcal{T}^*(\bar{N})$ corresponds to the probability of no change in the system, the upper right triangle corresponds to probabilities that P_i looses labels, the lower left triangle corresponds to probabilities that P_i gains labels. It may be noted that ${}^{i}\mathcal{T}^*$ is normalized to 1 columnwise.

Further decomposition of ${}^{i}\mathcal{T}^*$ proceeds as follows: ${}^{i}\mathcal{T}^*$ is a function of the average number of repetitions of the exchange cycle $\bar{N} = k_{-1}/k_2$. With the aid of a probability density ${}^{i}P(X=N)$,

$$\sum_{N=0}^{\infty} N\,{}^{i}P(X=N) = \bar{N}\,, \qquad \sum_{N=0}^{\infty} {}^{i}P(X=N) = 1\,,$$

we therefore have

$${}^{i}\mathcal{T}^*(\bar{N}) = \sum_{N=0}^{\infty} {}^{i}\mathcal{T}^*(N)\,{}^{i}P(X=N)\,.$$

The matrix elements of ${}^{i}\mathcal{T}^*(N)$ are dependent on a specific number N of repetitions of the exchange cycle. Accordingly, ${}^{i}\mathcal{T}^*(N)$ is still normalized to 1 columnwise.

Eqn. (4) now has its final form:

$$\frac{d}{dt}\boldsymbol{O}(t) = \sum_{i=1}^{n} {}^{i}k \left(\sum_{N=0}^{\infty} {}^{i}\mathcal{T}^*(N)\,{}^{i}P(X=N)\,\boldsymbol{B}(t) - {}^{i}\mathcal{D}\boldsymbol{A}(t) \right). \tag{5}$$

The solution of this equation may be obtained by standard methods.

The $P_i(^{18}O)$ experiment

For the experiment starting from the right hand side of Eqn. (1), $\boldsymbol{A}(t) = \boldsymbol{B}(t) = \boldsymbol{O}(t)$. Also, $\mathscr{T}$ is the matrix **1**; this can most easily be seen by realizing that every interaction of a P_i molecule causes a transition of one molecule from this species to a new one (which, of course, is identical to the old one with a probability $T^*_{ii}(\bar{N})$).

$$\boldsymbol{O}(t) = \exp \sum_{i=1}^{n} {}^{i}k \left(\sum_{N=0}^{\infty} {}^{i}\mathscr{T}^*(N)\, {}^{i}P(X=N) - \mathbf{1} \right) t\, \boldsymbol{O}(0)\,. \tag{6}$$

The NTP($\gamma^{18}O$) experiment

For the experiment starting out from the left hand side of Eqn. (1) $\boldsymbol{B}(t) = \boldsymbol{\gamma}(t)$. $\boldsymbol{\gamma}(t)$ itself follows a differential equation:

$$\frac{d}{dt}\boldsymbol{\gamma}(t) = -\sum_{i=1}^{n} {}^{i}k\, \boldsymbol{\gamma}(t) \tag{7}$$

with the interpretation of ${}^{i}k$ as above. From the solution of (7) and the condition that P_i, once released, does never again enter the exchange cycle ($\mathscr{T} = \mathbf{0}$):

$$\boldsymbol{O}(t) = \boldsymbol{O}(0) + \sum_{i=1}^{n} {}^{i}k \sum_{N=0}^{\infty} {}^{i}\mathscr{T}^*(N)\, {}^{i}P(X=N) \Big/ \sum_{i=1}^{n} {}^{i}k$$
$$\boldsymbol{\gamma}(0) \left(1 - \exp\left(t \sum_{i=1}^{n} {}^{i}k\, {}^{i}\mathscr{T}^*(N)\, {}^{i}P(X=N) \right) \right). \tag{8}$$

The Matrix ${}^{i}\mathscr{T}^*(N)$ for $[H_2{}^{18}O] = 0$.

The probabilities ${}^{i}\mathscr{T}^*_{mn}(N)$ can readily be calculated from scratch for different types of inaccessibility of the P_i oxygens to the exchange process in the bound state. This was done earlier for the exchange process starting from the P_i side and the results for $n - m \geqq 0$ are repeated here for the sake of completeness (and the corrections of a few printing errors) (Table I) (Rösch *et al.*, [3]; Rösch, [4]). The ${}^{i}\mathscr{T}^*_{mn}(N)$ simplify appreciably for the experiment starting out from the NTP side, because NTP($\gamma^{18}O$) enters the state E · NDP · P_i with three labels at most. Also, the calculation for the different cases of inaccessibility of oxygens during the exchange process is simplified: A minute's thought shows that only the three nonbridge oxygens of the γ-group can be protected from the exchange process, so that the number of inaccessible oxygens which still allows an exchange is two at most (the case $\gamma_4 \neq 0$ and three inaccessible oxygens is trivial). The arguments leading to the calculation of the probabilities ${}^{i}\mathscr{T}^*_{mn}$ are similar to the ones given for the exchange process starting from the $P_i(^{18}O)$ site and are not repeated here. The analytical results are given in Table II.

Table I. The expressions for ${}^{i}T^*_{mn}(N)$ for the NTP($\gamma^{18}O$) experiment; l is the number of inaccessible oxygens.

m	n	$l=1$	$l=2$
0	0	1	1
1	1	$\frac{1}{3}\left(1+2\left(\frac{2}{3}\right)^N\right)$	$\frac{1}{3}\left(2+\left(\frac{1}{2}\right)^N\right)$
2	2	$\frac{1}{3}\left(\left(\frac{1}{3}\right)^N+2\left(\frac{2}{3}\right)^N\right)$	$\frac{1}{3}\left(1+2\left(\frac{1}{2}\right)^N\right)$
3	3	$\left(\frac{1}{3}\right)^N$	$\left(\frac{1}{2}\right)^N$
0	1	$\frac{2}{3}\left(1-\left(\frac{2}{3}\right)^N\right)$	$\frac{1}{3}\left(1-\left(\frac{1}{2}\right)^N\right)$
1	2	$\frac{2}{3}\left(1-\left(\frac{1}{3}\right)^N\right)$	$\frac{2}{3}\left(1-\left(\frac{1}{2}\right)^N\right)$
2	3	$2\left(\left(\frac{2}{3}\right)^N-\left(\frac{1}{3}\right)^N\right)$	$1-\left(\frac{1}{2}\right)^N$
0	2	$\frac{1}{3}\left(1-2\left(\frac{2}{3}\right)^N+\left(\frac{1}{3}\right)^N\right)$	0
1	3	$1-2\left(\frac{2}{3}\right)^N+\left(\frac{1}{3}\right)^N$	0

In all calculations of ${}^{i}\mathscr{T}^*_{mn}(N)$ it has been assumed that $[H_2{}^{18}O] = 0$ throughout the experiment. Taking a non zero concentration of ^{18}O in the solution into account is trivial and has to proceed along the line of argument shown below. In the actual experimental case, NTP will contain three labels at most, so that $\gamma_4 = 0$, $\boldsymbol{O}_4 = 0$, and $\mathscr{T}^*_{m4}$ is meaningless. This again is a point when the condition $[H_2{}^{18}O] = 0$ must be stressed, because otherwise $\mathscr{T}^*_{mn} \neq 0$, $m > n$, and $O_4 \neq 0$ although $\gamma_4 = 0$.

Computational Results

Although the solution of Eqn. (5) for the NTP($\gamma^{18}O$) experiment, Eqn. (8), is useful for data refinement and model calculations even with small computers this is not the case for the solution for the $P_i(^{18}O)$ experiment, Eqn. (6). Of course, the

Table II. The expressions for ${}^{i}T^{*}_{mn}(N)$ for the $P_i(^{18}O)$ experiment; l is the number of inaccessible oxygens.

m	n	$l=0$	$l=1$	$l=2$	$l=3$
0	0	1	1	1	1
1	1	$\left(\frac{3}{4}\right)^N$	$\frac{1}{4}+\frac{3}{4}\left(\frac{2}{3}\right)^N$	$\frac{1}{2}\left(1+\left(\frac{1}{2}\right)^N\right)$	$\frac{1}{4}(3+\delta_{N0})$
2	2	$\left(\frac{1}{2}\right)^N$	$\frac{1}{2}\left(\left(\frac{2}{3}\right)^N+\left(\frac{1}{3}\right)^N\right)$	$\frac{1}{3}\left(\frac{1}{2}+2\left(\frac{1}{2}\right)^N+\frac{1}{2}\delta_{N0}\right)$	$\frac{1}{2}(1+\delta_{N0})$
3	3	$\left(\frac{1}{4}\right)^N$	$\frac{3}{4}\left(\frac{1}{3}\right)^N+\frac{1}{4}\delta_{N0}$	$\frac{1}{2}\left(\left(\frac{1}{2}\right)^N+\delta_{N0}\right)$	$\frac{1}{4}(1+3\delta_{N0})$
4	4	δ_{N0}	δ_{N0}	δ_{N0}	δ_{N0}
0	1	$1-\left(\frac{3}{4}\right)^N$	$\frac{3}{4}\left(1-\left(\frac{2}{3}\right)^N\right)$	$\frac{1}{2}\left(1-\left(\frac{1}{2}\right)^N\right)$	$\frac{1}{4}(1-\delta_{N0})$
1	2	$2\left(\left(\frac{3}{4}\right)^N-\left(\frac{1}{2}\right)^N\right)$	$\frac{1}{2}\left(1+\left(\frac{2}{3}\right)^N\right)-\left(\frac{1}{3}\right)^N$	$\frac{1}{3}\left(2-\left(\frac{1}{2}\right)^N-\delta_{N0}\right)$	$\frac{1}{2}(1-\delta_{N0})$
2	3	$3\left(1-\left(\frac{1}{2}\right)^N\right)\left(\frac{1}{2}\right)^N$	$\frac{3}{4}\left(2\left(\frac{2}{3}\right)^N-\left(\frac{1}{3}\right)^N-\delta_{N0}\right)$	$\frac{1}{2}\left(1+\left(\frac{1}{2}\right)^N\right)-\delta_{N0}$	$\frac{3}{4}(1-\delta_{N0})$
3	4	$4\left(\left(\frac{1}{4}\right)^N-\delta_{N0}\right)$	$3\left(\left(\frac{1}{3}\right)^N-\delta_{N0}\right)$	$2\left(\left(\frac{1}{2}\right)^N-\delta_{N0}\right)$	$1-\delta_{N0}$
0	2	$1-2\left(\frac{3}{4}\right)^N+\left(\frac{1}{2}\right)^N$	$\frac{1}{2}-\left(\frac{2}{3}\right)^N+\frac{1}{2}\left(\frac{1}{3}\right)^N$	$\frac{1}{6}\left(1-2\left(\frac{1}{2}\right)^N+\delta_{N0}\right)$	0
1	3	$3\left(\left(\frac{3}{4}\right)^N-2\left(\frac{1}{2}\right)^N+\left(\frac{1}{4}\right)^N\right)$	$\frac{3}{4}\left(1-\left(\frac{2}{3}\right)^N-\left(\frac{1}{3}\right)^N+\delta_{N0}\right)$	$\frac{1}{4}\left(1-2\left(\frac{1}{2}\right)^N+\delta_{N0}\right)$	0
2	4	$6\left(\left(\frac{1}{2}\right)^N-2\left(\frac{1}{4}\right)^N+\delta_{N0}\right)$	$3\left(\left(\frac{2}{3}\right)^N-2\left(\frac{1}{3}\right)^N+\delta_{N0}\right)$	$1-2\left(\frac{1}{2}\right)^N+\delta_{N0}$	0
0	3	$1-3\left(\left(\frac{3}{4}\right)^N-\left(\frac{1}{2}\right)^N\right)-\left(\frac{1}{4}\right)^N$	$\frac{1}{4}\left(1-3\left(\left(\frac{2}{3}\right)^N-\left(\frac{1}{3}\right)^N\right)-\delta_{N0}\right)$	0	0
1	4	$4\left(\left(\frac{3}{4}\right)^N-3\left(\left(\frac{1}{2}\right)^N-\left(\frac{1}{4}\right)^N\right)-\delta_{N0}\right)$	$1-3\left(\left(\frac{2}{3}\right)^N-\left(\frac{1}{3}\right)^N\right)-\delta_{N0}$	0	0
0	4	$1-4\left(\frac{3}{4}\right)^N+6\left(\frac{1}{2}\right)^N-4\left(\frac{1}{4}\right)^N+\delta_{N0}$	0	0	0

general solution may also be arrived at by setting up the characteristic equation and determination of eigenvalues and eigenvectors. But this also would not exactly speed up the data refinement procedure. Again, the assumption $[H_2{}^{18}O]=0$ for all times t simplifies matters: the lower left triangle of the matrix expression in Eqn. (5) vanishes and the vector equation can be decomposed in a set of one homogeneous and four inhomogeneous differential equations which are readily solved analytically by the standard Lagrangian method. The solution vector is given in Table III.

Computer programs simulating the time dependence of the vector $\boldsymbol{O}(t)$ and programs calculating the expected ^{31}P-NMR spectrum at any one time have been set up for experiments starting from the $P_i(^{18}O)$ side. In addition, a program for fitting the solution of Eqn. (5) as given in Table III to the experimental results with ${}^{i}k_{-1}/{}^{i}k_2$ and ${}^{i}k_{-2}$ as free parameters for the different exchange models has

Table III. The solution vector for Eqn. (5) as described in the main text.

$$O_4(t) = O_4(0)\, E(5, t)$$
$$O_3(t) = T_8\, E(4, t) + T_9\, E(5, t)$$
$$O_2(t) = T_5\, E(3, t) + T_6\, E(4, t) + T_7\, E(5, t)$$
$$O_1(t) = T_1\, E(2, t) + T_2\, E(3, t) + T_3\, E(4, t) + T_4\, E(5, t)$$
$$O_0(t) = \sum_{i=0}^{4} O_i(0) - \sum_{i=1}^{4} O_i(t)$$
$$T_1 = O_1(0) - O_2(0)\, G(2, 3, 1) - O_3(0)\, (G(3, 4, 1) - G(2, 4, 1)\, G(2, 3, 1) + G(2, 4, 1)\, G(2, 3, 2)) - O_4(0)\, (G(4, 5, 1) - G(2, 5, 1)\, G(3, 4, 1) + G(2, 5, 1)\, G(3, 4, 2) - G(3, 5, 1)\, G(2, 3, 1) + G(2, 5, 1)\, G(2, 4, 1)\, G(2, 3, 1) - G(2, 5, 1)\, G(2, 4, 2)\, G(2, 3, 1) - G(2, 5, 1)\, G(2, 4, 1)\, G(2, 3, 2) + (G(2, 5, 1)\, G(2, 4, 2) + G(3, 5, 1))\, G(2, 3, 3))$$
$$T_2 = (O_2(0) - O_3(0)\, G(2, 4, 1) - O_4(0)\, (G(3, 5, 1) - G(2, 5, 1)\, G(2, 4, 1) + G(2, 5, 1)\, G(2, 4, 2)))\, G(2, 3, 1)$$
$$T_3 = O_3(0)\, (G(3, 4, 1) + G(2, 4, 1)\, G(2, 3, 2)) - O_4(0)\, (G(2, 5, 1)\, G(3, 4, 1) + G(2, 5, 1)\, G(2, 4, 1)\, G(2, 3, 2))$$
$$T_4 = O_4(0)\, (G(4, 5, 1) + G(2, 5, 1)\, G(3, 4, 2) + (G(2, 5, 1)\, G(2, 4, 2) + G(3, 5, 1))\, G(2, 3, 3))$$
$$T_5 = (O_2(0) - O_3(0)\, G(2, 4, 1) - O_4(0)\, (G(3, 5, 1) - G(2, 5, 1)\, G(2, 4, 1) + G(2, 5, 1)\, G(2, 4, 2))$$
$$T_6 = (O_3(0) - O_4(0)\, G(2, 5, 1))\, G(2, 4, 1)$$
$$T_7 = O_4(0)\, (G(2, 5, 1)\, G(2, 4, 2) + G(3, 5, 1))$$
$$T_8 = O_3(0) - O_4(0)\, G(2, 5, 1)$$
$$T_9 = O_4(0)\, G(2, 5, 1)$$
$$G(K, L, M) = \sum_{i=1}^{n} {}^{i}k\, {}^{i}T_{L-K\,L-1} / \sum_{i=1}^{n} {}^{i}k\, ({}^{i}T_{M+L-2M+L-2} - {}^{i}T_{L-K\,L-K})$$
$$E(M, t) = \exp\left(-\sum_{i=1}^{n} {}^{i}k\, (1 - {}^{i}T_{M-1M-1})\, t\right)$$

n = Number of active sites.

been established. The latter is a least squares routine relying heavily on the SIMPLEX algorithm and a primitive hill climbing procedure. More complicated methods, like the Fletcher algorithms, have proved to be of not much use in our particular case. The program always leads to a unique solution, *i.e.* a global minimum, for a single reactive site in the model experiments we tested if the starting value for the rate constant is not too different from the true value. This can easily be achieved by taking $k = \frac{1}{1 - T_{44}} \frac{1}{t} \log \frac{O_4(0)}{O_4(t)}$, which follows from the expression for $O_4(t)$ in Table III. By taking the experimental values for $O_4(0)$ and $O_4(t)$ one ends up with a sufficiently accurate estimate for k. For k_{-1}/k_2 starting values are chosen by a random number generator in a predetermined range.

The four parameter fit which is necessary for models with two reactive sites demands some caution in the interpretation of the results. As model calculations show, the global minimum is only hit if the starting values of the free parameters are rather close to the real values, because the four dimensional hypersurface is crowded with local minima. It is rather hopeless to discuss more than the basic model for this case, *i.e.* no non exchanging oxygens and the geometric distribution for ${}^{i}P(X = N)$.

It should be noted that the calculation uses only relative concentrations of $P_i(^{18}O)$ species. Fig. 3a shows the time dependence of $P^{18}O_n{}^{16}O_{4-n}$ species of a hypothetical experiment with a sample containing two different reactive sites. Fig. 3b shows a few of the ^{31}P spectra of the same hypothetical experiment expected at the times indicated. This particular example has been chosen because it is one of the cases which show a pattern with two maxima, one of them an internal one, for a certain time point, making the contribution of two active sites obvious. Fig. 4 shows various examples of theoretical spectra with different values for k_{-1}/k_2 and k_{-2} at a specific time point.

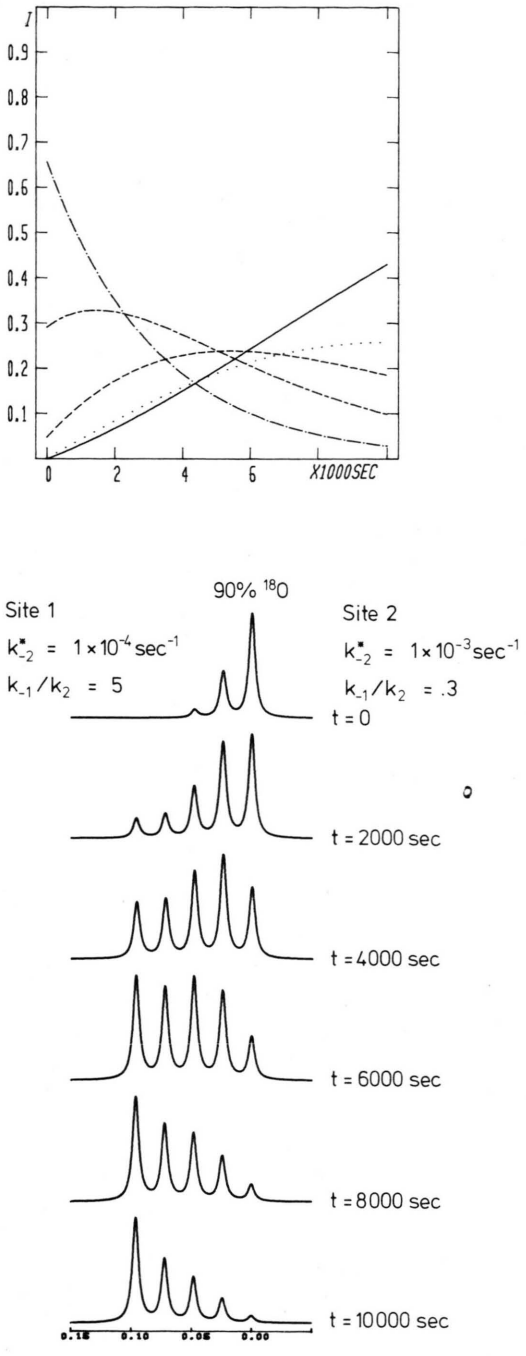

Fig. 3. The time dependence of the species $P^{18}O_n{}^{16}O_{4-n}$, $0 \leqq n \leqq 4$, for a reaction mixture with two types of active site. Parameters: ${}^1k_{-2} = 10^{-4}$ sec^{-1}, ${}^2k_{-2} = 10^{-3}$ sec^{-1}, ${}^1k_{-1}/{}^2k_2 = 5$, ${}^2k_{-2}/{}^2k_2 = .3$. Initial ^{18}O enrichment: 90%. O_4 –·–·–; O_3 –··–··–; O_2 - - - - - - -; O_1 ·····; O_0 ——. b) Hypothetical ^{31}P-NMR sample spectra as expected at various times under the conditions of a). The unusual pattern with two maxima, one internal, is indicative for the presence of more than one type of active site under the condition that all P_i-oxygens are exchanging ($l = 0$).

The Matrix $\mathscr{T}^*(N)$ for $[H_2{}^{18}O] \neq 0$

So far we neglected the finit concentration of ^{18}O in the bulk water. Although this is justified in general, the desire to complete the formalism and the possibility of experiments where the label in the bulk water is added by purpose (*e.g.* for the study of isotope effects in the exchange process) tempts us to give the procedure for calculating the theoretical concentration of $P^{18}O_n{}^{16}O_{4-n}$ under the assumption of ^{18}O presence in the bulk water.

The formal way to do this is actually quite trivial: The complete matrix ${}^i\mathscr{T}^*(N)$ must be calculated and used in the solution of Eqn. (5), *i.e.* Eqn. (6) for the $P_i(^{18}O)$ experiment and Eqn. (8) for the NTP($\gamma^{18}O$) experiment. This can be done easily by realizing that ^{18}O exchange cycles form a Markov chain of events. Without going too much into the details of Markov's theory – which is described, *e.g.*, in [6, 7] – the basic idea is as follows: For N independent and successiv events described by the transition matrix $\mathscr{y}(N)$ the equation holds: $\mathscr{y}(N) = \mathscr{y}^N(1)$. Talking about the $P_i(^{18}O)$ experiment this means for our purpose that for four equally accessible P_i oxygens in the bound state ($l = 0$) ${}^i\mathscr{T}^*(N) = {}^i\mathscr{T}^{*N}(1)$. The transition matrix ${}^i\mathscr{T}^*(1)$ is given by:

$$ {}^i\mathscr{T}^*(1) = \begin{pmatrix} 1-x & (1-x)/4 & 0 & 0 & 0 \\ x & (3-2x)/4 & (1-x)/2 & 0 & 0 \\ 0 & 3/4\,x & 1/2 & 3/4\,(1-x) & 0 \\ 0 & 0 & x/2 & (1+2x)/4 & 1-x \\ 0 & 0 & 0 & x/4 & x \end{pmatrix}. $$

$$ x = [H_2{}^{18}O]/([H_2{}^{18}O] + [H_2{}^{16}O]) $$

For one non exchanging oxygen ($l = 1$) we are evidently left with a 4×4 transition matrix $\mathscr{A}(N)$ describing the exchange of the three oxygens free to participate. Now, every composition of the three oxygens as far as the label/non-label contents is concerned can be arrived at in two different ways: either the non exchanging oxygen is a label or not. This means that $\mathscr{A}(N)$ has to be multiplied column-wise by the elements of the $l \times (5-l)$ matrix $\mathscr{y}$ giving the probability that a certain composition (*i.e.* columns of the matrix $\mathscr{A}(N)$) is arrived at. The resulting 5×5 matrix ${}^i\mathscr{T}^*$ is constructed according to

$$ {}^iT^*(N)_{m+k,\,m+n} = \sum_{\substack{m+k=c \\ m+n=c'}} A^N(1)_{kn}\, Y_{mn}; \qquad m = 0, l;\quad n, k = 0, 4-l $$

where the sum is taken over constant values of $m+n$ and $m+k$, *i.e.* terms with values m, n, k yielding a fixed value of $m+n$ and $m+k$ are summed up.

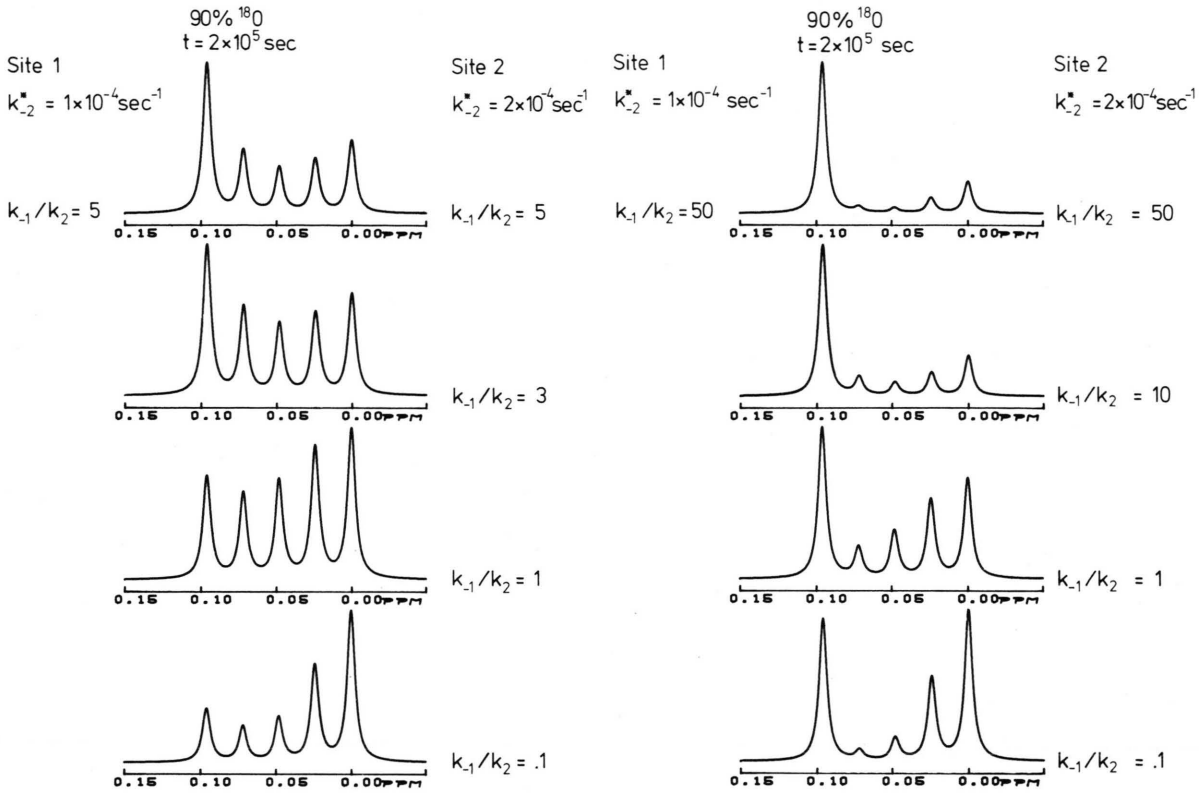

Fig. 4. The dependence of the appearance of the ^{31}P-NMR spectra of P_i $^{18}O_n{}^{16}O_{4-n}$ solutions in the presence of two different types of active site are shown for constant incubation time, *i.e.* $t = 2 \times 10^5$ sec, and constant initial ^{18}O enrichment, *i.e.* 90%.

The matrices $\mathscr{A}(1)$ and $\mathscr{Y}$ are given by:

$$\mathscr{A}(1) = \begin{pmatrix} 1-x & (1-x)/3 & 0 & 0 \\ x & (2-x)/3 & 2/3(1-x) & 0 \\ 0 & 2/3x & (1+x)/3 & 1-x \\ 0 & 0 & x/3 & x \end{pmatrix}$$

$$\mathscr{Y} = \begin{pmatrix} 1 & 1/4 \\ 3/4 & 1/2 \\ 1/2 & 3/4 \\ 1/4 & 1 \end{pmatrix}.$$

For two non exchanging oxygens the same procedure holds with $\mathscr{A}(1)$ and $\mathscr{Y}$ given by ($l = 2$):

$$\mathscr{A}(1) = \begin{pmatrix} 1-x & (1-x)/2 & 0 \\ x & 1/2 & 1-x \\ 0 & x/2 & x \end{pmatrix}$$

$$\mathscr{Y} = \begin{pmatrix} 1 & 1/2 & 1/6 \\ 1/2 & 2/3 & 1/2 \\ 1/6 & 1/2 & 1 \end{pmatrix}.$$

For $l = 3$

$$\mathscr{A}(1) = \begin{pmatrix} 1-x & 1-x \\ x & x \end{pmatrix}$$

$$\mathscr{Y} = \begin{pmatrix} 1 & 3/4 & 1/2 & 1/4 \\ 1/4 & 1/2 & 3/4 & 1 \end{pmatrix}.$$

The procedure for calculating the matrices ${}^{i}\mathscr{T}^{*}(N)$ for the NTP($\gamma^{18}O$) experiment is basicly the same: First the concentration of the different species in the stage after the first cleavage has to be calculated (because we assume the start of the exchange cycle with P_i entering E · ADP · P_i the first time). This can be done by:

$$\boldsymbol{O}(0) = \begin{pmatrix} \gamma_0 & 0 \\ \gamma_1 & \gamma_0 \\ \gamma_2 & \gamma_1 \\ \gamma_3 & \gamma_2 \\ 0 & \gamma_3 \end{pmatrix} \cdot \begin{pmatrix} 1-x \\ x \end{pmatrix}.$$

The matrices $\mathscr{A}(1)$ are still the same as above; the $\mathscr{Y}$ matrices change for $l \neq 0$ due to the fact that we postulate that the non exchanging oxygen is a former nonbridge oxygen of NTP. They are given by:

$$l = 1$$

$$\mathscr{Y} = \begin{pmatrix} 1 & 1/3(1-x) \\ 1/3(2+x) & 1/3(2-x) \\ 1/3(1+x) & 1/3(3-x) \\ 1/3x & 1 \end{pmatrix}$$

$$l = 2$$

$$\mathscr{Y} = \begin{pmatrix} 1 & 2/3(1-x) & 1/3(1-x) \\ 1/3(1+2x) & 2/3 & 1/3(3-2x) \\ 1/3x & 2/3x & 1 \end{pmatrix}$$

$$l = 3$$

$$\mathscr{Y} = \begin{pmatrix} 1 & 1-x & 1-x & 1-x \\ x & x & x & 1 \end{pmatrix}.$$

It should be emphasized at this point that the treatment of the ^{18}O exchange as a Markov chain demonstrates clearly the similarity of the information which can be obtained by both types of ^{18}O exchange experiments. At this point one should also add that data refinement or model calculations based on the Markov formalism, *i.e.* with $[H_2{}^{18}O] \neq 0$, are quite time consuming due to the many matrix multiplications involved in getting $\mathscr{T}(N)$ and the matrix exponential in Eqn. (6).

Discussion

The theoretical treatment given here was divided into two parts: One part giving the complete solution of the rate equation governing the isotope exchange process under no prerequisites as far as the label content of the medium is concerned, the other giving the solution under the assumption that no label is contained in the medium. The latter method has been described because it gives a roughly 10 fold time advantage over the former one as far as typical computer calculations are concerned. For experiments along the usual line the concentration of the label in the medium is always less than about .5%. The error introduced by disregarding this label concentration would yield results slightly too low for k_{-1}/k_2 and k_{-2}, the deviation from the true value being of the order of the label concentration in the medium. This is definitely less than errors introduced by other experimental imperfections, so that one may use this data refinement procedure for the usual experiments. It may be mentioned in passing that the incorporation of $H_2{}^{18}O$ in the bulk water in the probability calculations has also been tried earlier [2].

Another noteworthy point is the form of the solutions of Eqn. (5) as given above: For the NTP($\gamma{}^{18}O$) case the solutions for different active sites in the sample is a linear superposition of the solutions for single active sites, whereas this is not the case for the $P_i(^{18}O)$ experiments.

A word of caution should be said about the assumption of a really random choice of a specific P_i out of the bulk solution by the enzyme-nucleotide complex. As a matter of fact, it is by no means self evident that a P_i molecule, once released from the E · ADP complex, immediately leaves into the bulk solution. This is probably the major drawback of the $P_i(^{18}O)$ experiment (as for many other kinetic considerations). At present, this assumption can only be justified by the success of the model based on it.

In conclusion it may be said that the model presented is able to take some of the guesswork out of the interpretation of experimental isotope exchange data. It should be mentioned that the above model is of course also applicable to other isotope exchange processes, as described for example by Rose [5].

Acknowledgements

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