^{18}O-Exchange by Hydrolyzing Enzymes: Extension of the Model to P_i Molecules with Inequivalent Oxygen Atoms in the Bound State

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Enzymes causing an exchange of oxygens from P_i with the surrounding water oxygens are very common. A statistical model for the data evaluation for an observation of this oxygen exchange by isotope methods is presented. It is shown how different cases of inequivalence of the four P_i oxygens may be uncovered. The number of reversals of the oxygen exchange step on the enzyme and the apparent second order rate constant for the binding of P_i to the enzyme are obtained as a result of the data fitting procedure. Cobalt phosphatase, zinc phosphatase, and myosin subfragment 1 are treated as examples.

Introduction

Magnetic resonance spectroscopy provides various methods to determine rate constants of chemical processes. To measure these constants for different interconverting chemical species one has always to introduce a label on at least one of them and observe the change of the concentration of this label in the course of time.

The label may be, in the simplest types of experiment, the different chemical shift of the interconverting species itself (measurement of rate constants either by determination of the linebroadening caused by the exchange or by 2*D* methods); other labels are non-Boltzmann distribution of the spins belonging to a certain species (saturation transfer experiments) or chemical markers, *e.g.* different isotopes of the same element.

The chemical labels may be subdivided into two categories: labels which can be observed directly by an NMR experiment, *e.g.* protons in ^{1}H-^{2}H exchange processes, or labels which can only be observed indirectly via their influence on atoms which are accessible to NMR experiments. An experiment of the latter type is the ^{18}O-^{16}O exchange catalysed by a complex of a hydrolyzing enzyme with the respective substrates, *e.g.* Myosin · ADP · Mg^{2+}. The first experiments of this type were analysed with the aid of a mass spectrometer [1] and only recently it has been shown that ^{18}O may be used as an indirect label in NMR experiments due to the different influence of ^{18}O and ^{16}O on the chemical shift of adjacent phosphorus atoms [2].

The equation describing an ^{18}O-^{16}O exchange caused by hydrolyzing enzymes is described under the simplest assumptions by an equation of the form:

$$\mathrm{ATP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ATP}\cdot P_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{ADP} + P_i\,. \qquad (1)$$

For the evaluation of data two similar mathematical methods have been described, one relying on the partition coefficient for the reaction (1) [3], the other one based on direct probability calculations for the exchange ^{18}O-^{16}O to occur and a least squares fit to the experimental data [4].

Both approaches have so far only been presented for the approximation of P_i rotating freely in the enzyme ATP · P_i complex, rendering four equivalent oxygens. In this paper we demonstrate the extension of the formalism of the second method to deal with inequivalent oxygen atoms of bound P_i. Thus we have to calculate exchange probabilities for these cases which we can then use in the solution of the differential equations

$$\frac{\mathrm{d}}{\mathrm{d}t}\mathrm{O}_n(t) = k \sum_{m>n} \mathrm{O}_m(t)\, P_{m-n,m}(N) - k \sum_{m \leqq n} \mathrm{O}_n(t)\, P_{mn}(N) \qquad (2)$$

as is given in [4].

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Results and Discussion

In agreement with the notation introduced in [4] we designate the probability that m oxygens out of n labelled ones of the species $P^{18}O_n{}^{16}O_{4-n}$, $0 \leqq n \leqq 4$, are exchanged during an average of N reversals of step 1 in Eqn. (1) by P_{mn}. For example, P_{22} is the probability that 2 labels of the compound $P^{18}O_2{}^{16}O_2$ exchange during an average of N reversals; in other words, P_{22} is the probability that $P^{18}O_2{}^{16}O_2$ entering the intermediate step of Eqn. (1) is released as $P^{16}O_4$.

As described in [4] this probability may be considered as a product of a distribution function $P(X = N_i)$ and probabilities $P_{mn}(N_i)$, *i.e.* $P_{mn} = \sum_{N_i} P(X = N_i)\, P_{mn}(N_i)$, where $P_{mn}(N_i)$ are exchange probabilities for exactly N_i reversals of step (1) of Equation (1). A procedure rendering the values $P_{mn}(N_i)$ for a model allowing for free rotation of the enzyme bound P_i molecule is described in [4]. The main objective of this paper is to present the values for $P_{mn}(N_i)$ for three different cases of inequivalence oxygen atoms of the enzyme bound P_i molecule.

A simple model for the simulation of an enzyme bound P_i molecule with inequivalent oxygen atoms, as far as its behaviour in reaction (1) is concerned, has been suggested in [15]: If P_i on the enzyme is somehow locked in a specific position, then not all the oxygens may be accessible for the exchange procedure. This yields probabilities, designated $P^l_{mn}(N_i)$ below, for models with one, two or three non-exchanging oxygens. l is indicating the number of non-exchanging oxygens.

$l = 1$

The case where only three oxygens are accessible to the exchange process may be dealt with as shown here for the example $P^l_{22}(N_i)$:

The probability $P^l_{22}(N_i)$ is the product of the probability that both ^{18}O atoms are accessible to the exchange process ($p = \frac{1}{2}$) and the probability that they really get exchanged. The probability that one ^{18}O gets exchanged in the first reversal of step 1 under the condition that both ^{18}O atoms are accessible is $p = \frac{2}{3}$. The probability that the second ^{18}O gets exchanged in the second reversal is $p = \frac{1}{3}$. Therefore $P^l_{22}(2) = \frac{1}{2}\frac{2}{3}\frac{1}{3} = \frac{1}{9}$. The procedure is similar for $N_i > 2$: The probability that none of the ^{18}O atoms is exchanged during the first $J-1$ reversals under the condition that both ^{18}O atoms are accessible is simply $p = \frac{1}{3}\cdot\frac{1}{3} = \ldots = (\frac{1}{3})^{J-1}$. The probability that one of the ^{18}O atoms is then exchanged in the J-th reversal is – as above – $p = \frac{2}{3}$, leaving us with one ^{18}O. The probability that this ^{18}O atom is not exchanged during the next $(I-J-1)$ reversals is $p = \frac{2}{3}\frac{2}{3} = \ldots = (\frac{2}{3})^{I-J-1}$. The probability that the ^{18}O is exchanged in the I-th reversal is trivially $P = \frac{1}{3}$. Summation over all possible values of I and J then yields the desired values for $P^l_{22}(N_i)$:

$$P^l_{22}(N_i) = \frac{1}{2}\sum_{I=2}^{N_i}\sum_{J=1}^{I-1}\left(\frac{1}{3}\right)^{J-1}\frac{2}{3}\left(\frac{2}{3}\right)^{I-J-1}\frac{1}{3}.$$

Of course, $P^l_{22}(N_i) = 0$ for $N_i < 2$.

The complete set of $P^l_{mn}(N_i)$ is given in Table I.

$l = 2$

The considerations yielding the probabilities $P^2_{mn}(N_i)$, *i.e.* for the model of an enzyme bound P_i molecule with two non-exchanging oxygens, are very similar to the ones which led to the probabilities $P^1_{mn}(N_i)$. Again $P^2_{22}(N_i)$, $N_i \geqq 2$, will be considered as an example:

The probability that both labels are accessible to the exchange process is $p = \frac{1}{6}$. Evidently, the probability that one of these is exchanged in the first reversal is unity, *i.e.* $p = 1$. The probability that up to the J-th reversal no exchange of the remaining ^{18}O will happen is $p = (\frac{1}{2})^{J-2}$. A summation over the possible values for J yields then:

$$P^2_{22}(N_i) = \frac{1}{6}\sum_{J=2}^{N_i}\left(\frac{1}{2}\right)^{J-2}\frac{1}{2}.$$

The complete set of $P^2_{mn}(N_i)$ is shown in Table I.

$l = 3$

This case is trivial because those probabilities are directly given by the probability that the only accessible oxygen is a labelled one. The solutions are given in Table I.

Intermediate cases

The most general model would be a combination of the case $l = 0, 1, 2, 3$ according to

$$P_{mn}(N_i) = \sum_{l=0}^{3} a_l\, P^l_{mn}(N_i)\,, \tag{3}$$

normalized to $\sum_{m=0}^{n} P_{mn}(N_i) = 1$.

Table I. Values for $P_{mn}(N_i)$ for $N_i > 0$; values with $m > n$ are identical to zero for all $N_i > 0$. For $N_i = 0$, $P_{0n}(N_i) = 1$, all other $P_{mn} = 0$.

m	n	$l=1$	$l=2$	$l=3$
0	0	1	1	1
0	1	$\frac{1}{4}+\frac{3}{4}\left(\frac{2}{3}\right)^{N_i}$	$\frac{1}{2}+\left(\frac{1}{2}\right)^{N_i+1}$	$\frac{3}{4}$
0	2	$\frac{1}{2}\left(\frac{2}{3}\right)^{N_i}+\frac{1}{2}\left(\frac{1}{3}\right)^{N_i}$	$\frac{1}{6}+\frac{2}{3}\left(\frac{1}{2}\right)^{N_i}$	$\frac{1}{2}$
0	3	$\frac{3}{4}\left(\frac{1}{3}\right)^{N_i}$	$\left(\frac{1}{2}\right)^{N_i+1}$	$\frac{1}{4}$
0	4	$\frac{1}{4}\sum_{J=1}^{N_i}\left(\frac{2}{3}\right)^{J-1}$	0	0
1	2	$\frac{1}{6}\sum_{J=1}^{N_i}\left(\frac{2}{3}\right)^{J-1}+\frac{1}{3}\sum_{J=1}^{N_i}\left(\frac{1}{3}\right)^{J-1}\left(\frac{2}{3}\right)^{N_i-J}$	$\sum_{J=2}^{N_i+1}\left(\frac{1}{2}\right)^{J}$	$\frac{1}{4}$
1	3	$\frac{1}{2}\sum_{J=1}^{N_i}\left(\frac{1}{3}\right)^{J-1}\left(\frac{2}{3}\right)^{N_i-J}+\frac{1}{4}\left(\frac{1}{3}\right)^{N_i-1}$	$\frac{2}{3}\sum_{J=1}^{N_i}\left(\frac{1}{2}\right)^{J}$	$\frac{1}{2}$
1	4	$\left(\frac{1}{3}\right)^{N_i-1}$	$\left(\frac{1}{2}\right)^{N_i}+\frac{1}{2}\sum_{J=1}^{N_i}\left(\frac{1}{2}\right)^{J}$	$\frac{3}{4}$
2	2	$\frac{1}{9}\sum_{I=2}^{N_i}\sum_{J=1}^{I-1}\left(\frac{1}{3}\right)^{J-1}\left(\frac{2}{3}\right)^{I-J-1}$	$\left(\frac{1}{2}\right)^{N_i}$	1
2	3	$\frac{1}{6}\sum_{I=2}^{N_i}\sum_{J=1}^{I-1}\left(\frac{1}{3}\right)^{J-1}\left(\frac{2}{3}\right)^{I-J-1}+\frac{1}{6}\sum_{J=2}^{N_i}\left(\frac{1}{3}\right)^{J-2}\left(\frac{2}{3}\right)^{N_i-J}$	$\frac{1}{6}\sum_{J=2}^{N_i}\left(\frac{1}{2}\right)^{J-1}$	0
2	4	$\frac{2}{3}\sum_{J=2}^{N_i}\left(\frac{1}{3}\right)^{J-2}\left(\frac{2}{3}\right)^{N_i-J}$	$\sum_{J=2}^{N_i}\left(\frac{1}{2}\right)^{J}$	0
3	3	$\frac{1}{18}\sum_{I=3}^{N_i}\sum_{J=2}^{I-1}\left(\frac{1}{3}\right)^{J-2}\left(\frac{2}{3}\right)^{I-J-1}$	$\sum_{J=2}^{N_i}\left(\frac{1}{2}\right)^{J-1}$	0
3	4	$\frac{2}{9}\sum_{I=3}^{N_i}\sum_{J=2}^{I-1}\left(\frac{1}{3}\right)^{J-2}\left(\frac{2}{3}\right)^{I-J-1}$	0	0
4	4	0	0	0

Computer program

The computer program we use is basically the one introduced in [4], extended to deal with the additional models by using either of the cases $l = 0, 1, 2, 3$. In addition, we set up simulation programs which simulate the time dependence of the concentration of the various P_i species and the NMR spectrum expected at any one time for a given $N = k_{-1}/k_2$ and k_{-2}.

For N_i larger than 100 we use approximations as shown in Table II.

The combined model, Eqn. (3), is simulated by generating values of a_l, $0 \leqq l \leqq 3$, with a random number generator, generating evenly distributed numbers in the interval (0, 1).

The minimum value of the standard deviation of the model with specific parameters N, k, l (or a_l) is looked for and assumed to be the correct description of the exchange process. To this end N is varied

Table II. Approximations for P_{mn} as used in our Computer program for $N_i > 100$.

m	n	$l=1$	$l=2$	$l=3$
0	0	1	1	1
0	1	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{3}{4}$
0	2	0	$\frac{1}{6}$	$\frac{1}{2}$
0	3	0	0	$\frac{1}{4}$
1	1	$\frac{3}{4}$	$\frac{1}{2}$	$\frac{1}{4}$
1	2	$\frac{1}{2}$	$\frac{2}{3}$	$\frac{1}{2}$
1	3	0	$\frac{1}{2}$	$\frac{3}{4}$
1	4	0	0	1
2	2	$\frac{1}{2}$	$\frac{1}{6}$	0
2	3	$\frac{3}{4}$	$\frac{1}{2}$	0
2	4	0	1	0
3	3	$\frac{1}{4}$	0	0
3	4	1	0	0

in a certain interval as described in [4] and the solution of Eqn. (2) corresponding to the specific model is fitted to the experimental data by a least squares fit according to the regula falsi with k as a free parameter.

Computer simulations

Fig. 1 shows the expected distribution of the $P^{18}O_n{}^{16}O_{4-n}$, $0 \leqq n \leqq 4$, species for a fairly typical case, *i.e.* $N = 20$, $k_{-2} = 10^{-4}$ sec^{-1}, in the time range from 0 to 2×10^4 sec with the assumption of a geometric distribution of values N_i around the mean value N. For $t = 0$ we assumed a binomial distribution for all species with a total ^{18}O enrichment of 90%. The four cases $l = 0, 1, 2, 3$ can be distinguished easily.

Fig. 2 shows the spectra for the four different cases calculated with the parameters given above

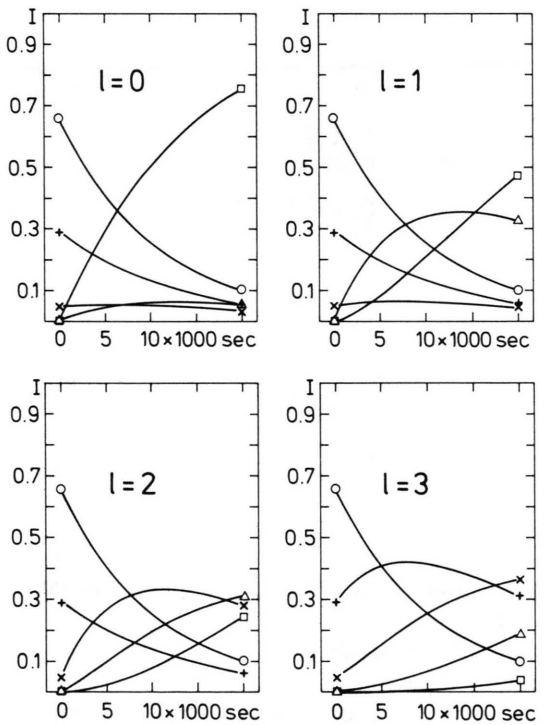

Fig. 1. Simulated time dependence of the concentration of $P^{18}O_n{}^{16}O_{4-n}$ species for the models $l = 0, 1, 2, 3$ for a reaction with $N = 20$, $k_{-2} = 10^{-4}$ sec^{-1} from 0 to 2×10^4 sec. First and final points are marked as follows:
□ $n = 0$; + $n = 3$;
△ $n = 1$; ○ $n = 4$.
× $n = 2$;

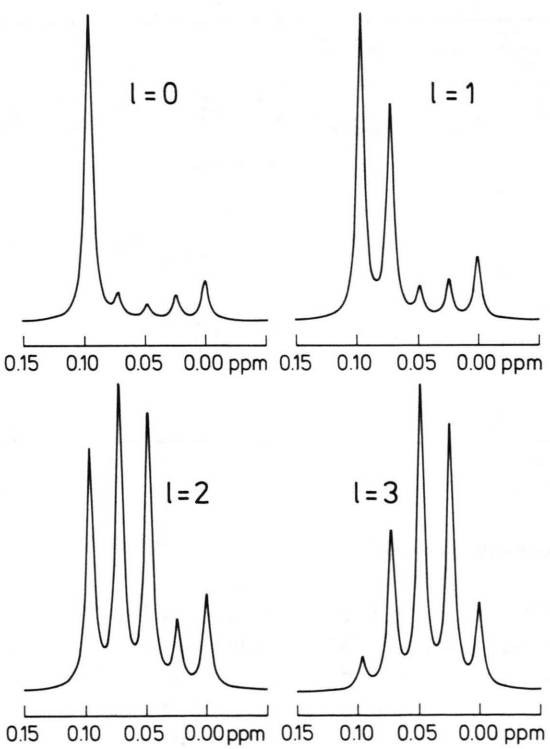

Fig. 2. Simulated ^{31}P spectra which are to be expected under the conditions of Fig. 1 after 2×10^4 sec of reaction time. The resonances represent the species $P^{18}O_n{}^{16}O_{4-n}$ with $n = 4$ at 0 ppm and n decreasing with decreasing field.

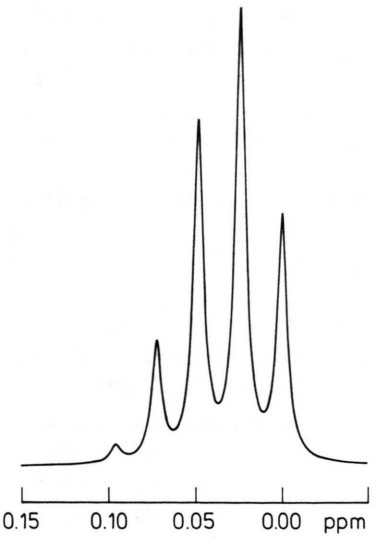

Fig. 3. ^{31}P spectrum simulated for the reaction of zinc phosphatase with $P^{18}O_n{}^{16}O_{4-n}$ as described in the main text for $l = 0$.

after 2×10^4 sec. Again, a clear cut difference is to be seen for the different cases.

Data evaluation of a few experiments

^{18}O exchange catalysed by alkaline phosphatases was one of the first exchange reactions of this type monitored by ^{31}P NMR. Although these enzymes catalyse a reaction of the type

$$E - P_i \underset{k_{-1}}{\overset{k_1}{\leftrightarrows}} E \cdot P_i \underset{k_{-2}}{\overset{k_2}{\leftrightarrows}} E + P_i \qquad (4)$$

rather than the reaction described in (1) it is evident that the formalism just developed can be applied.

Table III. Results of the data evaluation for three experiments. The experiments with alkaline phosphatases are published in [6], the one with myosin S1 in [7]. (SD = standard deviation.)

Enzyme	l	N	k [sec^{-1}]	SD	^{18}O exchanged [%]
Zinc phosphatase	0	.07	1.6×10^{-3}	.0083	1.7
	1	.08	1.5×10^{-3}	.0083	1.9
	2	.11	1.1×10^{-3}	.0083	2.6
Cobalt phosphatase	0	2.8	4.2×10^{-4}	.0073	40.6
	1	3.5	4.2×10^{-4}	.12	39.9
	2	4.3	4.8×10^{-4}	.21	33.8
Myosin S1	0	38	5.9×10^{-6}	.0081	89.5
	1	6	5.7×10^{-6}	.2047	49.4
	2	3	5.1×10^{-6}	.2256	30.1

The method employed for data evaluation in [6] has been a Monte Carlo type calculation. The result of the Monte Carlo type calculations has only been an estimate for the number of reversals, with no information on k_{-2} and the inequivalence of P_i oxygens in the $E \cdot P_i$ complex. In contrast, our method of data fitting proved to be accurate and convenient even

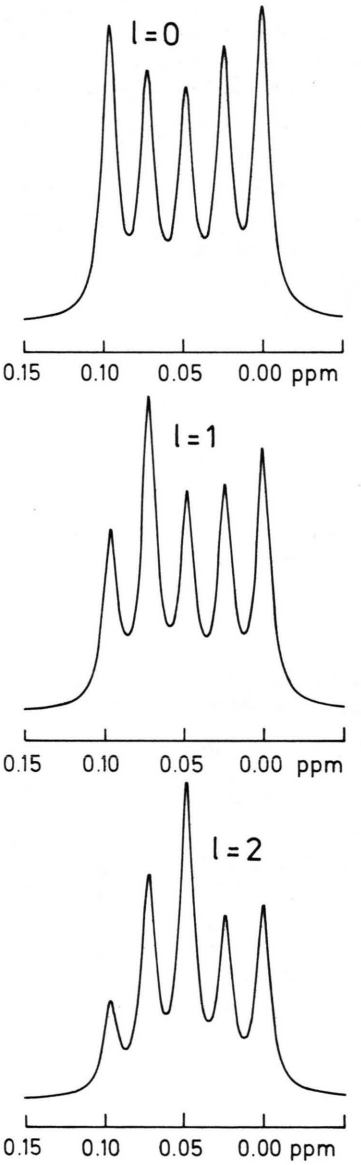

Fig. 4. ^{31}P spectra simulated for the reaction of cobalt phosphatase with $P^{18}O_n{}^{16}O_{4-n}$ as described in the text for the parameters yielding the best fit to either of the models $l = 0, 1, 2$.

with an extremely low input of information, yielding k_{-2} and information on the inequivalence of P_i oxygens in the $E \cdot P_i$ state in addition to a reliable value for the number of reversals k_{-1}/k_2.

The values obtained for data already published [6] and for own measurements [7] are shown in Table III.

For the zinc phosphatase a clear decision as to which model applies could not be made, because the minimum standard deviation was the same for all models. This is due to the fact that with $N = .07$ the probability of an exchange of more than one atom per interaction is only .004. In addition, the distributions at only two timepoints, *i.e.* $t = 0$ and $t = 3$ h, have been published. More data would have clearly helped in deciding which model applies. Fig. 3 shows the NMR spectrum simulated for the $l = 0$ case after three hours of reaction, so that it may be compared with Fig. 1 B in [6]. The spectra for the models $l \neq 0$ cannot be distinguished from this one by eye.

For the cobalt enzyme the number of data points published was the same as for the zinc enzyme, but the higher value of N made a decision for the model $l = 0$ very clear cut (see Table III). The minimum standard deviation for this case was about 17 times less than for the $l = 1$ model and 30 times less than for the $l = 2$ model. Again, this difference can be seen directly from the simulated NMR spectra after 55 min of reaction and comparison with Fig. 1 C in [6]. The simulated spectra are shown in Fig. 4.

The correct model for myosin S1 is clearly also the model $l = 0$. Simulated spectra for this experiment and the time course for the reaction will be given elsewhere [7].

It should be noted that no minimum value of the standard deviation for either of the three sets of data could be obtained for the case $l = 3$.

The column "0 exchanged" in the table gives the percentage of the average number of oxygens exchanged during each contact of the P_i molecules with the enzymes, corresponding to $P_{11} \times 100$.

A general rule follows for large values of N as to which model may apply for a specific system: Under the condition $O_n(0) > O_{n-1}(0)$, $1 \leqq n \leqq 4$, which holds under normal labelling conditions (*i.e.* ^{18}O enriched to more than 80%), it can be shown from (2) and from the approximation for the P^l_{mn} for large values of N as given in this paper and in (4) that

$$\frac{d}{dt} O_n(0) < 0 \quad \text{for} \quad n > l .$$

This means that the appropriate model may be chosen without resorting to extensive calculation procedures for sufficiently high values of N by just comparing the slopes of the plots of the $P^{18}O_n{}^{16}O_{4-n}$ concentrations versus time.

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