**18O-Exchange by Hydrolyzing Enzymes: An ab initio Calculation**

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Enzymes which hydrolyse ATP cause an exchange of 18O of labelled Pi in the presence of ADP. A theory for the evaluation of rate constants from an observation of the time dependence of the concentration of the various Pi species is presented. Application to the 18O exchange catalysed by myosin S1 as observed by 31P-NMR shows excellent agreement with values of the rate constants determined earlier.

I. Introduction

Observation of the exchange of isotopically labelled inorganic phosphate against 16O of the surrounding H2O by ATP hydrolyzing enzymes in the presence of ADP has proved to be a very successful method for the evaluation of rate constants involved in the hydrolysis process. The basic experiment is simple to perform with the aid of a mass spectrometer [1], or, even simpler, by direct observation of the various labelled species by 31P-NMR [2]. A recent review summarizes experiments and methods involved [3]. Although an attempt to simulate the experimental results with extensive use of the partition coefficient seemed to be quite successful [3] we thought it worth while to develop a method for fitting the experimental data which starts out from the very first principles of probability theory. This ab initio method, presented below, has several advantages as compared to the alternative methods. A particular case, myosin S1, which is probably the hydrolyzing protein best characterised by 18O exchange methods, was chosen to prove the validity and usefulness of our approach.

II. Materials and Methods

The computer program used to fit the theoretical curves to the experimental data was written in FORTRAN and run on a Norsk Data computer.

Myosin S1 was prepared according to [4]. The solution used for the experiment was 10 mg/ml S1 (A 1), 50 mM KCl, 50 mM HEPES, 0.1 mM EDTA, 0.12 mM ATP (di-adenosine-pentaphosphate), 100 mM K2HPO4 with 18O atoms partially replaced (~ 80%) by 16O. 10% D2O was added to provide a lock signal for the spectrometer.

NMR spectra were obtained with a Bruker HX 360 spectrometer working at a 31P resonance frequency of 145.7 MHz at a sample temperature of 15°C. The spectral width was 60 Hertz, and 1 K computer memory was used. The pulse angle was 90 degrees, the repetition rate 0.12 sec⁻¹. 50 spectra were recorded, 100 scans each, as a function of time.

III. Results and Discussion

a) Theory

The basic assumption of all considerations concerning oxygen exchange of inorganic phosphate catalysed by a hydrolyzing enzyme is the equation:

\[
\text{ATP} \xrightarrow{k_1} \text{ADP} \cdot \text{P}_i \xrightarrow{k_2} \text{ADP} + \text{P}_i. \quad (1)
\]

For the following calculation it is essential to assume that in the ADP · P_i complex on the enzyme a free rotation of P_i is possible, rendering four equivalent oxygen atoms. Denoting the probability that m oxygens out of n labelled ones (m, n \equiv 4) are exchanged after N_i reversals of step 1 (N_i = k_-i/k_2) by \( P_{m,n}^0(N_i) \) it can be calculated by the first principles of probability theory under the assumption [H_2^{16}O] \gg [H_2^{18}O]:

\[
P_{00}(N_i) = 1
\]

\[
P_{01}(N_i) = \left( \frac{3}{4} \right)^{N_i}
\]

\[
P_{11}(N_i) = \sum_{i=1}^{N_i} \left( \frac{3}{4} \right)^{i-1} \left( \frac{1}{4} \right)
\]

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it applies that
\[
\sum_{N_i=0}^{\infty} P(X = N_i) N_i = N = \frac{k_1}{k_2}.
\]
This gives a more realistic view of the processes involved in (1) by allowing a freely chosen probability function \(P(X = N_i)\) and the resulting distribution for \(N_i\) rather than assuming a single value \(N\) for the \(N_i\)'s. In addition, the choice of \(P(X = N_i)\) always implies a specific model of the reaction (1). Thus, the function \(P(X = N_i)\) which results in the best fit for the experimental data gives further insight into the mechanism of the enzyme.

The functions
\[
P_{mn}(N) = \sum_{N_i} P(X = N_i) P_{mn}(N_i)
\]
can now be used to establish and solve the rate equations which govern the \(^4\)O-exchange process (and similar processes):
\[
\frac{d}{dt} O_n(t) = k \left\{ \sum_{m > n} O_m(t) P_{m-n}\ln(N) - \sum_{m = n} O_m(t) P_{m-n}(N) \right\}. \tag{4}
\]
Here, \(O_n\), \(0 \leq n \leq 4\), designates the number of \(P_i\) molecules with \(n\) labelled oxygen atoms \(P_{180}^{16}O_{4-n}\) and \(k\) is a rate constant corresponding to \(k_{-2}\cdot[ADP \cdot SI] \cdot [PO_4] \) in (1).

Of course, the conservation equation holds:
\[
\sum_{n=0}^{4} O_n(t) = \sum_{n=0}^{4} O_n(0) \quad \text{for all } t.
\]

The solution vector for this set of 5 differential equations is easily obtained by the standard Lagrangian method for the solution of a set of inhomogeneous linear differential equations with constant coefficients and yields, with \(P_{mn}(N_i)\) replacing \(P_{mn}(N)\):

\[
O_1(t) = C_1 \exp(-kP_{11} t) \\
+ C_2 \exp(-k(P_{12} + P_{22}) t) \\
+ C_3 \exp(-k(1 - P_{03}) t) \\
+ C_4 \exp(-k(1 - P_{04}) t) \\
+ C_5 \exp(-kP_{05} t) \\
+ C_6 \exp(-k(1 - P_{04}) t) \\
+ C_7 \exp(-k(1 - P_{04}) t)
\]
\[
O_2(t) = C_8 \exp(-k(P_{12} + P_{22}) t) \\
+ C_9 \exp(-k(1 - P_{03}) t) \\
+ C_{10} \exp(-k(1 - P_{04}) t)
\]

For a distribution of values \(N_i\) around a mean value \(N\) with a distribution function
\[
P(X = N_i), \quad \sum_{N_i=0}^{\infty} P(X = N_i) = 1,
\]

\[
P_{02}(N_i) = \left(\frac{1}{2}\right)^{N_i} \\
P_{12}(N_i) = \sum_{j=1}^{N_i} \left(\frac{1}{2}\right)^{j-1} \left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{N_i-j} \\
P_{22}(N_i) = \sum_{j=2}^{N_i} \sum_{i=1}^{j-1} \left(\frac{1}{2}\right)^{j-1} \left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{j-i} \left(\frac{1}{4}\right) \\
P_{03}(N_i) = \left(\frac{1}{4}\right)^{N_i} \\
P_{13}(N_i) = \sum_{j=1}^{N_i} \left(\frac{1}{4}\right)^{j-1} \left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{N_i-j} \\
P_{23}(N_i) = \sum_{j=2}^{N_i} \sum_{i=1}^{j-1} \left(\frac{1}{4}\right)^{j-i} \left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{N_i-j} \\
P_{33}(N_i) = \sum_{k=3}^{N_i} \sum_{j=2}^{k-1} \sum_{i=1}^{j-1} \left(\frac{1}{4}\right)^{j-1} \\
\left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{k-j} \left(\frac{1}{4}\right)^{N_i-j} \\
P_{04}(N_i) = \sum_{k=4}^{N_i} \sum_{j=3}^{k-1} \sum_{i=2}^{j-2} \left(\frac{1}{4}\right)^{j-2} \left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{k-j} \\
\left(\frac{1}{2}\right)^{N_i-k} \\
P_{14}(N_i) = \left(\frac{1}{4}\right)^{N_i-1} \\
P_{24}(N_i) = \sum_{j=2}^{N_i} \sum_{i=1}^{j-1} \left(\frac{1}{4}\right)^{j-2} \left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{N_i-j} \\
P_{34}(N_i) = \sum_{k=3}^{N_i} \sum_{j=2}^{k-1} \sum_{i=1}^{j-1} \left(\frac{1}{4}\right)^{j-1} \\
\left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{k-j} \left(\frac{1}{4}\right)^{N_i-j} \\
P_{44}(N_i) = \sum_{k=4}^{N_i} \sum_{j=3}^{k-1} \sum_{i=2}^{j-2} \left(\frac{1}{4}\right)^{j-2} \left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{k-j} \\
\left(\frac{1}{2}\right)^{N_i-k} \\
P_{mn}(N_i) = 0 \text{ for } m > n. \tag{2}
\]
\[ O_3(t) = C_{11} \exp \left( -k (1 - P_{03}) t \right) + C_{12} \exp \left( -k (1 - P_{04}) t \right) \]
\[ O_4(t) = O_4(0) \exp \left( -k (1 - P_{04}) t \right) \]
\[ O_6(t) = \sum_{i=0}^{4} O_i(0) - \sum_{i=1}^{4} O_i(t) \]
\[ C_1 = O_1(0) - C_2 - C_3 - C_4 - C_6 - C_7 \]
\[ C_2 = O_2(0) P_{12} - C_3 (1 - P_{03} - P_{11}) - C_4 (1 - P_{04} - P_{12}) \]
\[ C_3 = P_{13} O_3(0)/P_{04} - O_4(0)/P_{04} \]
\[ C_4 = P_{10} (P_{14} P_{13} O_3(0) - O_4(0)/(P_{04} - P_{03})\]
\[ C_5 = -P_{23} (O_3(0)/P_{04} - O_4(0)/P_{04}) \]
\[ C_6 = -P_{14} P_{25} O_4(0)/P_{04} \]
\[ C_7 = -P_{34} O_4(0)/(1 - P_{04} - P_{12}) \]
\[ C_8 = -C_2(P_{12} + P_{22} - P_{11})/P_{12} \]
\[ C_9 = -C_3 (1 - P_{03} - P_{12}) \]
\[ C_{10} = -C_4 (1 - P_{04} - P_{12})/P_{12} \]
\[ C_{11} = O_3(0) - O_4(0) P_{14}/P_{04} \]
\[ C_{12} = O_4(0) P_{14}/P_{04} \]

The average number of labelled oxygens in PO₄ exchanged between the time when a P⁰⁰O₄ molecule enters the stage ADP • P* and the time when it is released into the surrounding H₂O as P¹⁸O₄⁻¹⁶O₄⁻₈⁻₉ is given by
\[ ^{18}O = \sum_{i=1}^{4} P_{14}(N) \]

which establishes the connection between the present work and work done earlier with different methods of data evaluation.

b) Computer program

The working principal of the computer program we use to fit our experimental data to the theory just presented is as follows:

Within a range of values \( N \) defined by the operator the first value is chosen. This value is assumed to be the mean value of a probability distribution described by \( P(X=N_i) \). The program calculates the \( N_i \)'s and the probability for specific values \( N_i \) with \( P(X=N_i) \) being the maximum of the probability function. The \( P_{mn} \) are calculated and normalized to 1, then the experimental data, also normalized to \( \sum_n O_n = 1 \), are fitted with \( k \) as a free parameter applying the regula falsi. The root mean square is calculated and the value of \( N \) with the smallest root mean square is assumed to be the correct result for \( k_{-1}/k_2 \).

In our program we used forms of the probabilities \( P_{mn} \) which are somewhat more concise than the ones in (1). In addition, to avoid excessive computing times we assumed \( P_{mn}(N_i) = \delta_{mn} (= 1 \text{ for } m = n, = 0 \text{ otherwise}) \) for \( N_i \geq 100 \), introducing an error which is certainly negligible.

c) ^{18}O-exchange catalysed by S1

Fig. 1 shows the ^{31}P NMR spectrum of K₂HP¹⁸O₄⁻¹⁶O₄⁻₉, \( n = 0, 4, 1.5 \) h after incubation with S1. We used two different distribution functions \( P(X=N_i) \) for the evaluation of our data, the Poisson distribution \( (P(X=N_i) = \frac{N^N_i}{N_i!} \exp(-N)) \) and the geometric distribution \( (P(X=N_i) = \frac{(N-1)}{N} \frac{1}{N+1}) \). The geometric distribution resulted in a root mean square three times less than the Poisson distribution, thus indicating that the former one is more appropriate. The parameters obtained with this distribution are \( k_{-1}/k_2 = 65 \), \( k_2 = 0.123 \text{ m}^{-1} \text{ sec}^{-1} \). Our experiment was performed at...
15 °C and pH 7.5. The resulting values are completely in line with earlier experiments which yielded $k_{-2} = .23 \, \text{M}^{-1} \text{sec}^{-1}$ and $(k - 1)/k_2 = 50$ at 22 °C and pH 8.0 [5] and experiments which yielded $k_{-1}/k_2 = 17$ at 22 °C and pH 7.0 and $k_{-1}/k_2 = 150$ at 25 °C and pH 7.0 [6]. (These data were derived with the implicit assumption of a geometric distribution of the $N_i$'s.)

Fig. 2 shows a comparison of the $^{18}\text{O}$ distribution as determined experimentally and as fitted with the Poisson distribution and the geometric distribution 16.3 h after incubation with S 1. This again shows that the geometric distribution is closer to reality. For the sake of completeness the values derived with the Poisson distribution are given: $k_{-1}/k_2 = 17$, $k_2 = .117 \, \text{M}^{-1} \text{sec}^{-1}$. For the Poisson distribu-
tion \( k_{-1}/k_2 = 17 \) corresponds to \( ^{18}O = 3.93 \), \( k_{-1}/k_2 = 65 \) for the geometric distribution to \( ^{18}O = 3.77 \).

Fig. 3 finally shows the time dependence of the concentration of the \( PO_4 \) species as calculated with the geometric distribution.

It should be emphasised that the geometric distribution assumes a specific model for the action of the enzyme, namely that all reversals of step 2 are equivalent, i.e. the enzyme does not "memorize" exchanges done prior to a specific one.

IV. Conclusions

The ab initio calculations for the evaluation of \( ^{18}O \)-exchange data seems to be more accurate than the model resting on the simple determination of the partition coefficient as described in [3] for two reasons: Firstly, in its simplest form the latter makes use only of the time dependence of \( PO_4 \) and the sum over all species, whereas our method uses all the information contained in the distribution of the various species. Secondly, the partition coefficient \( P_c \) gets close to 1 at large values of \( N \). This means that even small errors in \( P_c \) may result in large

errors in \( N \), as may easily be seen by the relation 
\[
1/N = (1/P_c) - 1.
\]

Unfortunately, large values of \( N \), i.e. values of \( P_c \) close to 1, are not uncommon. In contrast, the accuracy of the ab initio calculations is virtually independent of the value of \( N = k_{-1}/k_2 \).

Another advantage of the ab initio calculation is the flexibility of the model. To simulate different cases of \( ^{18}O \)-exchange one has simply to choose different probabilities \( P_{mn}(N_i) \). The same is true if one drops the assumption \([H_2^{16}O] > [H_2^{18}O]\).

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