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

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Z. Naturforsch. **36 c**, 534–538 (1981); received October 21, 1980/March 24, 1981

^{18}O-Exchange, Enzyme Kinetics, ^{31}P-NMR, Myosin S1, Nucleotidases

Enzymes which hydrolyse ATP cause an exchange of ^{18}O of labelled P_i 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 P_i species is presented. Application to the ^{18}O exchange catalysed by myosin S1 as observed by ^{31}P-NMR shows excellent agreement with values of the rate constants determined earlier.

I. Introduction

Observation of the exchange of isotopically labelled inorganic phosphate against ^{16}O of the surrounding H_2O 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 ^{31}P-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 S 1, which is probably the hydrolyzing protein best characterised by ^{18}O exchange methods, was chosen to prove the validity and usefullness 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 S 1 was prepared according to [4]. The solution used for the experiment was 10 mg/ml S 1 (A 1), 50 mM KCl, 50 mM Hepes, 0.1 mM EDTA, 0.12 mM AP_5A (di-adenosine-pentaphosphate), 100 mM K_2HPO_4 with ^{16}O atoms partially replaced ($\sim$ 80%) by ^{18}O. 10% D_2O was added to provide a lock signal for the spectrometer.

NMR spectra were obtained with a Bruker HX 360 spectrometer working at a ^{31}P 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:

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

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 ($m, n \leqq 4$) are exchanged after N_i reversals of step 1 ($N_i = k_{-1}/k_2$) by $P^0_{mn}(N_i)$ it can be calculated by the first principles of probability theory under the assumption $[H_2{}^{16}O] \gg [H_2{}^{18}O]$:

$$P^0_{00}(N_i) = 1$$

$$P^0_{01}(N_i) = \left(\frac{3}{4}\right)^{N_i}$$

$$P^0_{11}(N_i) = \sum_{I=1}^{N_i} \left(\frac{3}{4}\right)^{I-1}\left(\frac{1}{4}\right)$$

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$$P^0_{02}(N_i) = \left(\frac{1}{2}\right)^{N_i}$$

$$P^0_{12}(N_i) = \sum_{I=1}^{N_i} \left(\frac{1}{2}\right)^{I-1} \left(\frac{1}{2}\right) \left(\frac{3}{4}\right)^{N_i-I}$$

$$P^0_{22}(N_i) = \sum_{J=2}^{N_i} \sum_{I=1}^{J-1} \left(\frac{1}{2}\right)^{I-1} \left(\frac{1}{2}\right) \left(\frac{3}{4}\right)^{J-I-1} \left(\frac{1}{4}\right)$$

$$P^0_{03}(N_i) = \left(\frac{1}{4}\right)^{N_i}$$

$$P^0_{13}(N_i) = \sum_{I=1}^{N_i} \left(\frac{1}{4}\right)^{I-1} \left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{N_i-I}$$

$$P^0_{23}(N_i) = \sum_{J=2}^{N_i} \sum_{I=1}^{J-1} \left(\frac{1}{4}\right)^{I-1} \left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{J-I-1} \cdot \left(\frac{1}{2}\right) \left(\frac{3}{4}\right)^{N_i-J}$$

$$P^0_{33}(N_i) = \sum_{K=3}^{N_i} \sum_{J=2}^{K-1} \sum_{I=1}^{J-1} \left(\frac{1}{4}\right)^{I-1} \cdot \left(\frac{3}{4}\right) \left(\frac{1}{2}\right) \left(\frac{3}{4}\right)^{K-J-1} \left(\frac{1}{4}\right) \left(\frac{1}{2}\right)^{J-I-1}$$

$$P^0_{04}(N_i) = 0 \quad \text{if} \quad N_i \neq 0, \quad = 1 \quad \text{if} \quad N_i = 0$$

$$P^0_{14}(N_i) = \left(\frac{1}{4}\right)^{N_i-1}$$

$$P^0_{24}(N_i) = \sum_{I=2}^{N_i} \left(\frac{1}{4}\right)^{I-2} \left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{N_i-I}$$

$$P^0_{34}(N_i) = \sum_{K=3}^{N_i} \sum_{I=2}^{K-1} \left(\frac{1}{4}\right)^{I-2} \left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{K-I-1} \cdot \left(\frac{1}{2}\right) \left(\frac{3}{4}\right)^{N_i-K}$$

$$P^0_{44}(N_i) = \sum_{K=4}^{N_i} \sum_{J=3}^{K-1} \sum_{I=2}^{J-1} \left(\frac{1}{4}\right)^{I-2} \left(\frac{3}{4}\right) \left(\frac{1}{2}\right)^{J-I-1} \cdot \left(\frac{1}{2}\right) \left(\frac{3}{4}\right)^{K-J-1} \left(\frac{1}{4}\right)$$

$$P^0_{mn}(N_i) = 0 \quad \text{for} \quad m > n\,. \tag{2}$$

For a distribution of values N_i around a mean value N with a distribution function

$$P(X = N_i), \quad \sum_{N_i=0} P(X = N_i) = 1\,,$$

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

$$P_{mn}(N) = \sum_{N_i} P(X = N_i)\, P^0_{mn}(N_i) \tag{3}$$

can now be used to establish and solve the rate equations which govern the ^{18}O-exchange process (and similar processes):

$$\frac{\mathrm{d}}{\mathrm{d}t} O_n(t) = k \left\{ \sum_{m>n} O_m(t)\, P_{m-nm}(N) - \sum_{m \leq n} O_n(t)\, P_{mn}(N) \right\}. \tag{4}$$

Here, O_n, $0 \leqq n \leqq 4$, designates the number of P_i molecules with n labelled oxygen atoms $P^{18}O_n{}^{16}O_{4-n}$ and k is a rate constant corresponding to $k_{-2} \cdot [\mathrm{ADP} \cdot \mathrm{SI}] \cdot [\mathrm{PO_4}]$ 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} replacing $P_{mn}(N_i)$:

$$\begin{aligned} O_1(t) = {} & C_1 \exp(-k\, P_{11}\, t) \\ & + C_2 \exp(-k\,(P_{12} + P_{22})\, t) \\ & + C_3 \exp(-k\,(1 - P_{03})\, t) \\ & + C_4 \exp(-k\,(1 - P_{04})\, t) \\ & + C_5 \exp(-k\,(1 - P_{03})\, t) \\ & + C_6 \exp(-k\,(1 - P_{04})\, t) \\ & + C_7 \exp(-k\,(1 - P_{04})\, t) \end{aligned}$$

$$\begin{aligned} O_2(t) = {} & C_8 \exp(-k\,(P_{12} + P_{22})\, t) \\ & + C_9 \exp(-k\,(1 - P_{03})\, t) \\ & + C_{10} \exp(-k\,(1 - P_{04})\, t) \end{aligned}$$

$$O_3(t) = C_{11} \exp(-k(1-P_{03})t) + C_{12} \exp(-k(1-P_{04})t)$$

$$O_4(t) = O_4(0) \exp(-k(1-P_{04})t)$$

$$O_0(t) = \sum_{i=0}^{4} O_i(0) - \sum_{i=1}^{4} O_i(t)$$

$$C_1 = O_1(0) - C_2 - C_3 - C_4 - C_5 - C_6 - C_7$$

$$C_2 = -O_2(0)\,P_{12} - C_3(1-P_{03}-P_{11}) - C_4(1-P_{04}-P_{11})/(1-P_{02}-P_{11})$$

$$C_3 = P_{13}\,P_{12}(O_3(0) - O_4(0) - P_{14}/(P_{04}-P_{03}))/(P_{02}-P_{03})/(1-P_{03}\,P_{11})$$

$$C_4 = P_{12}\,(P_{14}\,P_{13}\,O_4(0)/(P_{04}-P_{03}) + P_{24}\,O_4(0))/(P_{02}-P_{04})/(1-P_{04}-P_{11})$$

$$C_5 = -P_{23}(O_3(0) - O_4(0)\,P_{14}/(P_{04}-P_{03}))/(1-P_{03}-P_{11})$$

$$C_6 = -P_{14}\,P_{23}\,O_4(0)/(P_{04}-P_{03})/(1-P_{04}-P_{11})$$

$$C_7 = -P_{34}\,O_4(0)/(1-P_{04}-P_{11})$$

$$C_8 = -C_2(P_{12}+P_{22}-P_{11})/P_{12}$$

$$C_9 = -C_3(1-P_{03}-P_{11})/P_{12}$$

$$C_{10} = -C_4(1-P_{04}-P_{11})/P_{12}$$

$$C_{11} = O_3(0) - O_4(0)\,P_{14}/(P_{04}-P_{03})$$

$$C_{12} = O_4(0)\,P_{14}/(P_{04}-P_{03})\,.$$

The average number of labelled oxygens in PO_4 exchanged between the time when a $P^{18}O_4$ molecule enters the stage $ADP \cdot P_i$ and the time when it is released into the surrounding H_2O as $P^{18}O_n{}^{16}O_{4-n}$ is given by

$$\overline{^{18}O} = \sum_{l=1}^{4} P_{l4}(N)\,l$$

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 $P(X=N_i) \geqq P(X=N_i)/100$, where $P(X=K)$ is the maximum of the probability function. The P_{mn} are calculated and normalized to 1, then the experimental data, also normalized to $\sum_{n=0}^{4} O_n = 1$, 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^0_{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 for $m=n$, = 0 otherwise) for $N_i \geqq 100$, introducing an error which is certainly negligible.

c) ^{18}O-exchange catalysed by S 1

Fig. 1 shows the ^{31}P NMR spectrum of $K_2HP^{18}O_n{}^{16}O_{4-n}$, $n = 0, 4$, 1.5 h after incubation with S 1. We used two different distribution functions $P(X=N_i)$ for the evaluation of our data, the Poisson distribution $\left(P(X=N_i) = \frac{N^{N_i}}{N_i!}\exp(-N)\right)$ and the geometric distribution $\left(P(X=N_i) = \left(\frac{N}{N+1}\right)^{N_i}\frac{1}{N+1}\right)$. 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 $k_{-1}/k_2 = 65$, $k_2 = .123\ \mathrm{M}^{-1}\ \mathrm{sec}^{-1}$. Our experiment was performed at

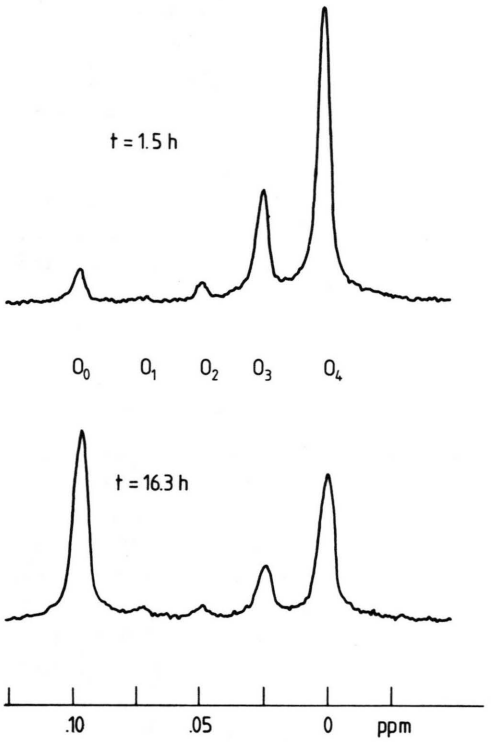

Fig. 1. The spectrum of labelled P_i 1.5 hrs and 16.3 hrs after incubation with S 1. O_n designates the resonance of the species $P^{18}O_n{}^{16}O_{4-n}$, $n = 1, 4$. The spectra are drawn to scale with chemical shift values referenced to $P^{18}O_4$.

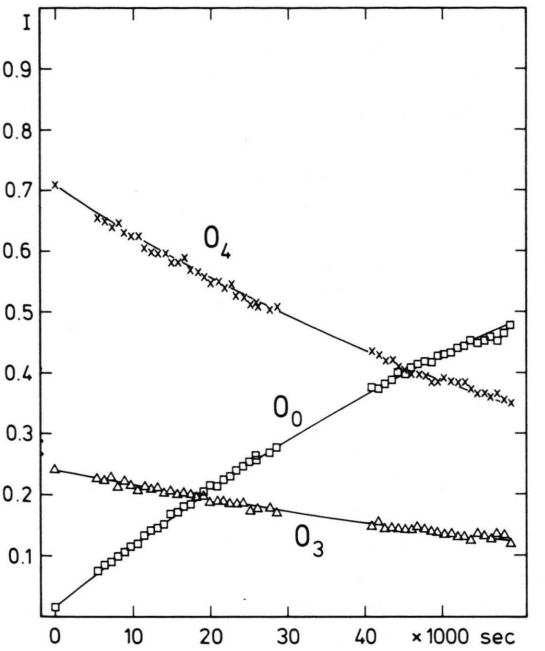

Fig. 3. Time dependence of the concentration of the three most abundant P_i species; designation as in Fig. 1.

15 °C and pH 7.5. The resulting values are completely in line with earlier experiments which yielded $k_{-2} = .23$ M^{-1} 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}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: $k_{-1}/k_2 = 17$, $k_2 = .117$ M^{-1} sec^{-1}. For the Poisson distribu-

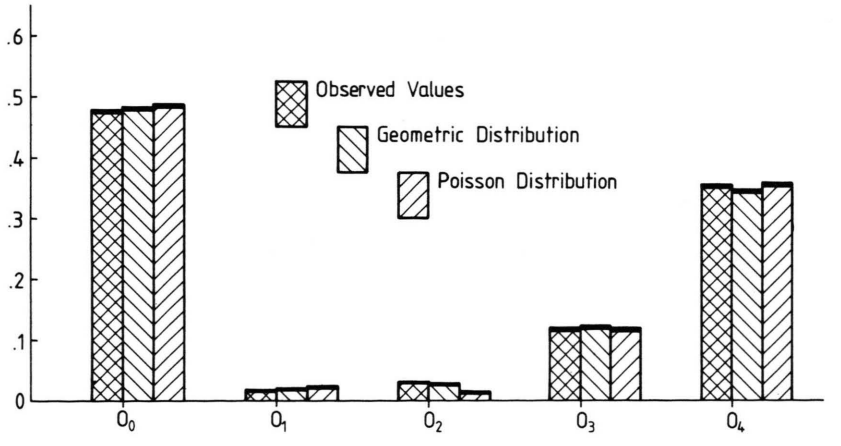

Fig. 2. Distribution of P_i species at the end of our experiment. Comparison with the calculated values for the Poisson and the geometric distribution shows the better fit of the latter one.

tion $k_{-1}/k_2 = 17$ corresponds to $\overline{^{18}O} = 3.93$, $k_{-1}/k_2 = 65$ for the geometric distribution to $\overline{^{18}O} = 3.77$.

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 $P^{18}O_4$ 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 $P^0_{mn}(N_i)$. The same is true if one drops the assumption $[H_2{}^{16}O] \gg [H_2{}^{18}O]$.

Acknowledgements

P.R. would like to thank Prof. Mildred Cohn cordially; he had the privilege to spend part of the years 1978 and 1979 in her laboratory. All theoretical considerations in this paper were done during this period. He also thanks the Max-Planck-Gesellschaft for a fellowship during this time.

$K_2HP^{18}O_4$ was a gift from H. Zimmermann. Discussions with Dr. Edler from the Deutsches Krebsforschungszentrum were helpful.

This work was supported by the Deutsche Forschungsgemeinschaft.

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