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KINETIC STUDIES OF ^{18}O EXCHANGE FROM INORGANIC PHOSPHATE CATALYZED BY Mg^{2+}-ACTIVATED UNADENYLYLATED GLUTAMINE SYNTHETASE (E. coli w)*

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SUMMARY: Oxygen-18 exchange out of $[^{18}O]P_i$ catalyzed by Mg^{2+}-activated unadenylylated glutamine synthetase from E. coli was followed by ^{31}P-NMR in the presence of the other substrates, ADP and L-glutamine. The pattern of the $^{16}O/^{18}O$ in the species $P^{18}O_4$, $P^{18}O_3{}^{16}O_1$, $P^{18}O_2{}^{16}O_2$, $P^{18}O_1{}^{16}O_3$, $P^{16}O_4$ during the exchange followed a binomial distribution consistent with indiscriminate removal of any of the four oxygens of P_i. The rate constant for $^{16}O/^{18}O$ exchange was 410±40 min^{-1} while the rate constant for net reaction (ATP formation) was 62±4 min^{-1}. Thus exchange proceeds ~7 times faster than net reaction, a finding in accord with that of Stokes and Boyer (J. Biol. Chem. (1976) 251, 5558) for the Mn^{2+}-activated adenylylated glutamine synthetase. A model for the overall catalytic events first derived from rapid kinetic fluorescence experiments (Rhee and Chock, Proc. Natl. Acad. Sci. USA, (1976) 73, 476) was successfully used to fit the oxygen exchange data in this paper.

An important aspect of the metabolic regulation of Escherichia coli glutamine synthetase is the covalent adenylylation of each of the 12 subunits. As a consequence of this reaction, the enzyme changes from a catalytic form that requires Mg^{2+} for activity (unadenylylated) to one that is maximally active with Mn^{2+} but inactive with Mg^{2+} (adenylylated) (1). In addition, the change in metal ion specificity is accompanied by different V_{max} values and pH optima (2). To understand what rate constant or constants are altered in the overall catalytic mechanism with different metal ions and enzyme of different states of adenylylation, it is important to study one rate or set of rates in the overall mechanism so that quantitative changes can be measured and

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assessed. We have recently studied the kinetics of oxygen exchange out of inorganic phosphate catalyzed by glutamine synthetase in the following reaction, which is the reverse of the overall biosynthetic reaction.

$$ADP + P_i + \text{L-glutamine} \xrightleftharpoons{M^{2+}} ATP + \text{L-glutamate} + NH_4^+$$

Recently several methods of analysis of the $^{18}O/^{16}O$ content among the five species of inorganic phosphate, i.e., $P^{16}O_4$, $P^{16}O_3{}^{18}O_1$, $P^{16}O_2{}^{18}O_2$, $P^{16}O_1{}^{18}O_3$, $P^{18}O_4$ have been reported. These include 1) mass spectral analysis of both the tris-(trimethylsilyl) phosphate ester (3) and the trimethylphosphate esters (4) and 2) a direct ^{31}P-NMR analysis that relies on the isotopic shift differences exerted by ^{18}O and ^{16}O in the five species listed above (5). We have applied the $^{31}P(^{18}O/^{16}O)$-NMR method to our investigation. Our studies were designed to complement those of Stokes and Boyer (6) who demonstrated that oxygen exchange from $[^{18}O]P_i$ to L-glutamine (adenylylated enzyme, Mn^{2+}-activated) occurred at a rate five times faster than net formation of L-glutamate. Also our experiments could supply information on the rotational freedom or restriction of P_i on the enzyme and the relative rates of P_i release compared to enzymatic interconversion steps involving $^{18}O/^{16}O$ exchange.

The results of our studies indicate that there is rapid exchange of ^{18}O out of $[^{18}O]P_i$ compared to net formation of ATP with the Mg^{2+}-activated unadenylylated enzyme. Thus, under similar reaction conditions both Mn^{2+}-activated adenylylated subunits and Mg^{2+}-activated unadenylylated subunits catalyze exchange reactions with nearly the same relative rate ratios.

MATERIALS AND METHODS

Glutamine synthetase was isolated from Escherichia coli in a state of low adenylylation ($E_{\overline{1-7}}$) as described previously (7). The subscript refers to the average number of adenylyl groups per twelve subunits. All chemicals were products of Sigma. The enzyme activity was determined by the procedure as described earlier (8). Protein concentration was measured by the method of Lowry et al. (9) and by spectral measurements as described by Ginsburg et al. (2). Potassium phosphate (KH_2PO_4) was enriched with ^{18}O by the procedure of Boyer and Bryan (10) using 99.4% $H_2{}^{18}O$ (Miles Laboratories).

For the enzymatic exchange reactions, a typical reaction mixture (12 ml) contained: L-glutamine, 30 mM; ADP, 10 mM; $[^{18}O]P_i$, 25 mM; $MgCl_2$, 10 mM; glucose, 40 mM; hexokinase, 0.25 mg/ml; KCl, 100 mM; HEPES, 10 mM and glutamine synthetase, 0.49 mg/ml. The reaction was conducted at 37°C and at pH 7.0. Aliquots of 1.0 ml were withdrawn at 0, 5, 12, 20, 30, 45, 60, 120, 240, 540 and 1080 min, pipetted into test tubes containing acidified charcoal (pH 2), and vortexed to remove the enzyme as well as nucleotides. The solution was centrifuged. To the supernatant, D_2O (20% final volume) and EDTA (0.1 mM) were added; the pH was adjusted to 7.5 and the solution (2.5 ml) transferred to a 10-mm NMR tube. A Teflon vortex supressor was placed in the tube. ^{31}P-NMR spectra were recorded on a Brüker WH 360/180 spectrometer at 145.7 MHz. Spectra were recorded in the Fourier Transform Mode using a 22 μsec (90° flip angle) with 8K data points and 500 Hz spectral width. The % distribution of ^{18}O in P_i was obtained from the individual peaks by a curve digitizer or by

measuring the intensities of the individual peaks. The estimated error was <2% for measurements of the larger peaks and <15% for the smaller peaks (Fig 1). The exchange of ^{18}O out of P_i was analyzed by a computer program which fits the ^{18}O distribution with time to the model (3):

$$P^{18}O_4 \xrightarrow{k} P^{18}O_3{}^{16}O_1 \xrightarrow{3/4k} P^{18}O_2{}^{16}O_2 \xrightarrow{1/2k} P^{18}O_1{}^{16}O_3 \xrightarrow{1/4k} P^{16}O_4$$

An interactive chemical reaction simulator program was written in FORTRAN by Dr. William E. Brugger of our department for a MODCOMP II computer. The algorithm used involves calculation of the reaction time course (11) by means of Taylor series expansions of the differential equations describing the changes in concentration of the chemical species in the system (3).

RESULTS

Oxygen exchange experiments were conducted with E. coli glutamine synthetase under initial rate conditions for the back reaction. Thus, enzyme of low adenylylation state was used (1.7) at pH 7.0 with Mg^{2+}, ADP, $[^{18}O]P_i$ (77.6 atom % ^{18}O) and L-glutamine as described in Materials and Methods. The ATP produced by the above reaction reacted with glucose via the hexokinase reaction resulting in no net change of ADP but P_i and L-glutamine were consumed during the reaction.

Figure 1A shows the ^{31}P NMR spectrum of 77.6 atom % $[^{18}O]P_i$. Cohn and Hu (5) first reported that inorganic phosphate with different numbers of ^{18}O atoms could be resolved by ^{31}P NMR. The percent of total of the five species agrees with that predicted by a binomial distribution of the given atom % ^{18}O. Glutamine synthetase was then added to the reaction mixture and aliquots were withdrawn at various time intervals as described in Methods. Figure 1B shows the ^{31}P NMR spectrum of a sample after 2 hrs of the reaction at 37°C. The ^{18}O content of the sample dropped to 58.9 atom % and the $^{18}O/^{16}O$ distribution is still binomial. Figure 1C shows the spectrum after 18 hrs reaction. The distribution is binomial and ^{18}O content is 47.5 atom %.

The percentage distribution of $^{18}O/^{16}O$ species was calculated for samples withdrawn at various time intervals and the data are plotted in Fig 2. The curves drawn in the Figure are from a model (see Methods) that assumes random removal of ^{18}O from any of the P_i species and that the bound P_i has rotational freedom. The rate constant used to generate the curves for loss or production of an individual species is 410±40 min^{-1}.

In a separate experiment, the initial velocity of ATP production was measured under identical reaction conditions by assaying the glucose-6-phosphate formed during the reaction with NADP and glucose-6-phosphate dehydrogenase, and was found to be 62±4 min^{-1}. Thus, ^{18}O exchange out of P_i is ∿7 times faster than the overall back reaction.

DISCUSSION

The data in this report demonstrate that oxygen atoms can be exchanged out of P_i under initial velocity conditions with Mg^{2+}-activated unadenylylated

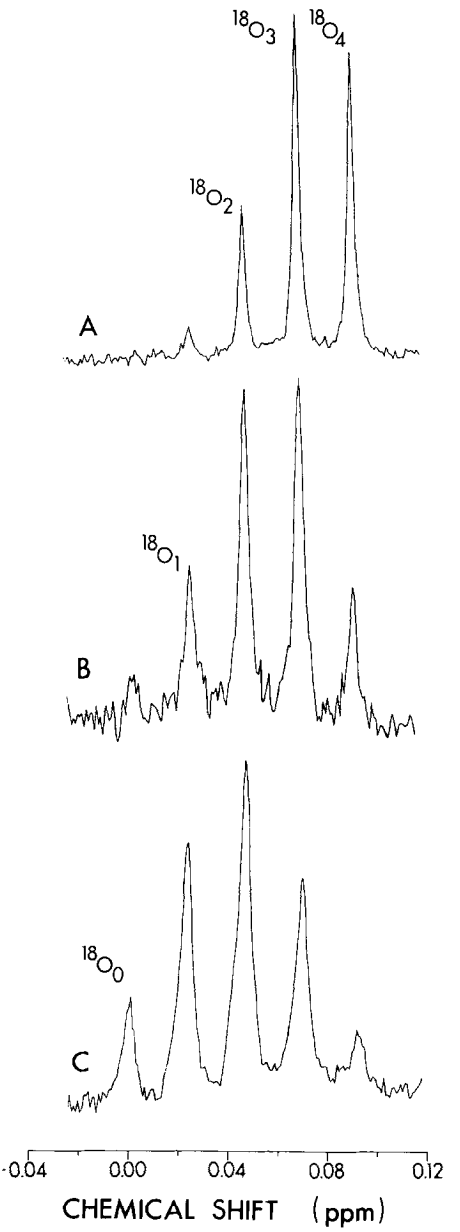

Fig. 1: ^{31}P nuclear magnetic resonance spectra of ^{18}O-enriched inorganic phosphate at 145.7 MHZ. The species $^{18}O_o$ is used as a reference point and assigned a chemical shift of 0 ppm in each spectrum. A, initial $KH_2P^{18}O_4$, 77.6% ^{18}O, 25 mM; 10 scans with 4.1 sec aquisition time. B. After reaction with 9.2 μM glutamine synthetase for 2 h at 37°C in 10 mM HEPES buffer, pH 7.0 (58.9% ^{18}O in phosphate), 10 scans with 4.1 s acquisition time. C. After 18 h of reaction with glutamine synthetase (47.5% ^{18}O in phosphate); 10 scans with 4.1 s acquisition time. $^{18}O_4$, $^{18}O_3$, $^{18}O_2$, $^{18}O_1$ and $^{18}O_o$ refer to $P^{18}O_4$, $P^{18}O_3\,^{16}O_1$, $P^{18}O_2\,^{16}O_2$, $P^{18}O_1{}^{16}O_3$, and $P^{16}O_4$ respectively.

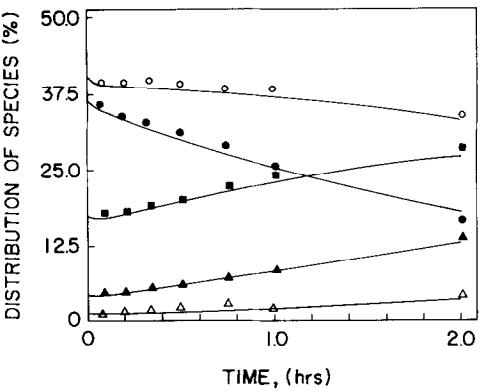

Fig. 2: Time-course of phosphate and oxygen exchange reaction catalyzed by Mg^{2+}-activated glutamine synthetase. Fractional concentration of the species $P^{18}O_4$(●), $P^{18}O_3{}^{16}O_1$(○), $P^{18}O_2{}^{16}O_2$(■), $P^{18}O_1{}^{16}O_3$(▲), $P^{16}O_4$(△) respectively, is plotted versus time (see results) during the course of the reaction. The solid lines are the computer fit while the symbols are the experimental values.

glutamine synthetase from E. coli. The exchange is ∿7 times faster than net formation of ATP from P_i, ADP and L-glutamine. Stokes and Boyer (6) were the first to demonstrate this type of exchange using the Mn^{2+}-activated fully adenylylated enzyme. In order to explain their findings Stokes and Boyer deduced that the γ-carboxyl of bound glutamate was free to rotate and thus either oxygen was available for reaction.

We can discuss our experimental results (and those of Stokes and Boyer) in terms of the kinetic scheme given below.

$$\begin{array}{ccc} Mg{\cdot}E_{1.7} & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & Mg{\cdot}E_{1.7}{\cdot}ATP{\cdot}Glu{\cdot}NH_3 \quad (I) \\ k_5 \uparrow\downarrow k_{-5} & & k_{-2} \uparrow\downarrow k_2 \\ Mg{\cdot}E_{1.7}{\cdot}ADP{\cdot}P_i{\cdot}Gln & \underset{k_4}{\overset{k_{-4}}{\rightleftharpoons}} I_2 & \underset{k_3}{\overset{k_{-3}}{\rightleftharpoons}} I_1 \end{array}$$

This scheme is the one determined by Rhee and Chock (12) by rapid kinetic methods for the Mg^{2+}-activated unadenylylated enzyme under conditions nearly identical to those that we have employed. The major difference is that our data were taken at 37°C while those of Rhee and Chock were at 9-15°C. Also, it is assumed that ADP, P_i and glutamine are in rapid equilibrium with the enzyme, a point made by Stokes and Boyer (6), and that the relative order of release of substrates follows that of Wedler and Boyer (13).

Our experimental results show that at some stage in the above scheme there is a partition of an enzyme complex such that net return of P_i is ∿7 times faster than net formation of ATP. The P_i released from the enzyme also could have undergone exchange of ^{16}O for ^{18}O requiring that the following series of chemical changes have taken place: P_i must have reacted with glutamine to form γ-glutamyl phosphate followed by reaction with ADP to form ATP and [^{18}O] glutamate; the chemical composition of the enzyme complex at this stage is $Mg{\cdot}E_{1.7}{\cdot}ATP{\cdot}Glu{\cdot}NH_3$ (I) or an enzyme conformer of this complex, i. e., I_1 ($Mg{\cdot}E^*_{1.7}{\cdot}ATP{\cdot}Glu{\cdot}NH_3$). This enzyme complex can go on to release ATP which is trapped by the hexokinase coupling system; alternatively the carboxyl of the [^{18}O] glutamate in the enzyme complex could rotate resulting in either ^{16}O or ^{18}O participating in the reaction to form P_i from ATP, glutamate and ammonia. Since the ^{18}O content of P_i was 47.5% after 18 hr, this must mean that some of the P_i bound to the enzyme reacts directly to form ATP and is trapped without oxygen exchange. This conclusion is based on the initial concentration of L-glutamine and the initial ^{18}O content of P_i.

The partition analysis method of Cleland (14) was used to derive the rate expressions for isotope exchange out of P_i (k_{ex}) and for net formation of ATP (k_r) from two different complexes. The first complex was I ($Mg{\cdot}E_{1.7}{\cdot}ATP{\cdot}Glu{\cdot}NH_3$) and the second was I_1 ($Mg{\cdot}E^*_{1.7}$ $ATP{\cdot}Glu{\cdot}NH_3$). The rate expressions are given below:

Case 1: Partition from I ($Mg{\cdot}E_{1.7}$ $ATP{\cdot}Glu{\cdot}NH_3$)

$$k_r = [I]\, k_{-1}; \qquad k_{ex} = [I]\left[\frac{k_2k_3k_4k_5}{k_3k_4k_5 + k_{-2}k_4k_5 + k_{-2}k_{-3}k_5 + k_{-2}k_{-3}k_{-4}}\right]$$

Case 2: Partition from I_1 ($Mg{\cdot}E^*_{1.7}{\cdot}ATP{\cdot}Glu{\cdot}NH_3$)

$$k'_r = [I_1]\left[\frac{k_{-1}k_{-2}}{k_2 + k_{-1}}\right]; \qquad k'_{ex} = [I_1]\left[\frac{k_3k_4k_5}{k_4k_5 + k_{-3}k_5 + k_{-3}k_{-4}}\right]$$

Using the values of the rate constants given by Rhee and Chock (12) and a value of $k_{-1} = 6\ s^{-1}$ calculated from V_{max} (forward)/V_{max}(reverse) = 50 (15), the ratios k_{ex}/k_r and k'_{ex}/k'_r were calculated to be ∿6 and ∿10. A statistical correction of 0.5 to 0.8 should be applied for ^{18}O exchange since rapid rotation of a carboxyl with ^{18}O and ^{16}O would show exchange only half of the time but there are four oxygen atoms in P_i to exchange if P_i is rapidly rotating in the complex. The exchange rates are then k_{ex}/k_r = 3-5 and k'_{ex}/k'_r = 5-8. It seems that partition from complex I_1 best fits our experimental data of ∿7 oxygen exchanges out of [^{18}O]P_i per net formation of ATP.

Since it is known that the values of the individual rate constants are temperature dependent (12), it may be fortuitous that the analysis used above agrees with our experimental data. However, while it is uncertain whether I_1

is really an enzyme conformer of I, it is clear that an enzyme complex containing ATP, glutamate and NH_3 is required for oxygen exchange out of P_i. Calculation of k_{ex}/k_r for the I_2 complex gave a partition value of ∿110, clearly too high to fit our data. Further exchange experiments at lower temperatures should clarify the above discussion.

While the above analysis represents only one model for the catalytic events carried out by glutamine synthetase, we are encouraged by the fit to the experimental data from both the fluorescence methods and the ^{18}O exchange approach and are pursuing these studies in the presence of other metal ions, allosteric effectors and inhibitors. The ^{18}O exchange should be a very sensitive indicator of changes of rate constants (16) resulting from enzyme-induced perturbations by the above effectors of the catalytic activity of glutamine synthetase.

Our data can be placed in context with recently published data on $P_i \rightleftharpoons H_2O$ exchange catalyzed by myosin (17), inorganic pyrophosphatase (18), and alkaline phosphatase (3). Hackney and Boyer (18) presented a lucid description of the analysis of data for changes in $^{16}O/^{18}O$ distribution in P_i. This method is particularly useful for distinguishing random from nonrandom distributions throughout the exchange processes. From their analysis we conclude that, since the distribution of isotopic species of P_i in this work remained random throughout the time course of the reaction, that P_i is rapidly rotating on the enzyme and only one oxygen of P_i can be exchanged per enzymatic encounter.

These conclusions necessitate that $P_{i\ on\text{-}off} >> L\text{-glutamine}_{on\text{-}off}$, since L-glutamine is the source for oxygen exchange. Additionally some P_i reacts without exchange or reversal to form ATP that is trapped by the glucose-hexokinase reaction.

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