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Evaluation of the partitioning of bound inorganic phosphate during medium and intermediate phosphate ⇌ water oxygen exchange reactions of yeast inorganic pyrophosphatase

(mass spectrometry/^{18}O/enzyme mechanism)

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ABSTRACT **During the rapid exchange of oxygens of P_i with water catalyzed by yeast inorganic pyrophosphatase, P_i at the catalytic site may either dissociate or undergo reversible loss of an oxygen to water. The effective partitioning of bound P_i during exchange starting with medium P_i containing ^{18}O in all four oxygens has been evaluated by mass spectral analysis of the change in the distribution of P_i species containing zero to four ^{18}O oxygens per P_i. This analysis indicates that the rate of P_i release from the enzyme is only 1.4 times faster than the rate of reformation of the anhydrous intermediate. A similar partitioning of bound P_i is observed during PP_i hydrolysis, indicating that hydrolysis and medium exchange have common intermediates. The approach should be applicable to study of related phosphate oxygen exchanges.**

Enzymes that catalyze hydrolysis of phosphate compounds frequently will also catalyze a facile exchange of oxygens of P_i with water—that is, a medium $P_i \rightleftharpoons HOH$ exchange. Examples are inorganic pyrophosphatase (1), alkaline phosphatase (2, 3), and the ATPase of sarcoplasmic reticulum (4). A general scheme for such exchanges is given by

$$E + P_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot P_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (EP) + H_2O$$

in which (EP) represents some intermediate that has lost an oxygen atom from P_i to water. For sarcoplasmic reticulum ATPase (5) and *Escherichia coli* alkaline phosphatase (6), this intermediate is a covalent phosphorylated enzyme, whereas for pyrophosphatase an intermediate-bound PP_i participates (C. A. Janson, C. Degani, and P. D. Boyer, unpublished data).

An understanding of the catalytic process for an enzyme can be considerably aided if the relative rates of intermediate steps can be ascertained. Previous work has indicated that P_i release may be partially rate-limiting in the exchange reactions of the CaATPase of sarcoplasmic reticulum (5), but a more direct approach is needed. For enzymes catalyzing a medium $P_i \rightleftharpoons$ HOH exchange, an equivalent participation of the oxygen atoms of an enzyme-bound P_i in the exchange reaction allows a unique procedure for the quantitative evaluation of the partitioning of the bound P_i. The procedure is based on the use of P_i that is highly labeled with ^{18}O and measurement of the change in the distribution of the five isotopic $[^{18}O]P_i$ species† as the exchange reaction proceeds in unenriched water. If the partition coefficient, P_c [$P_c = k_2/(k_2 + k_{-1})$],‡ is close to 1 (i.e., $k_{-1} \ll k_2$) then each time a P_i binds to the enzyme it will undergo many reversals of step 2 before it is released and lose all four of its ^{18}O oxygens by exchange with water. This would result in the $P^{18}O_4$ species of the medium being replaced directly by the $P^{18}O_0$ species with no accumulation of intermediate species. If P_c is close to zero (i.e., $k_{-1} \gg k_2$, the Michaelis–Menten assumption) then only one oxygen atom can be exchanged per productive encounter of P_i with the enzyme. This would result in a complex cascade in which $P^{18}O_4$ goes initially to $P^{18}O_3$ with subsequent formation of other intermediate species.

The distribution of $[^{18}O]P_i$ species can be quantitated by conversion of the P_i to the volatile tris(trimethylsilyl)phosphate derivative and direct analysis by mass spectrometry. A similar method for the derivatization and analysis of ^{18}O-enriched P_i has been reported (8).

The evaluation of the partition coefficient of bound P_i during pyrophosphatase-catalyzed medium $P_i \rightleftharpoons HOH$ exchange is reported here. The value is considerably greater than zero and is in harmony with the extent of intermediate $P_i \rightleftharpoons HOH$ oxygen exchange observed during PP_i hydrolysis.

MATERIALS AND METHODS

Enriched P_i (≈84% ^{18}O) was obtained from Miles Laboratories. Yeast inorganic pyrophosphatase (EC 3.6.1.1) was obtained from Sigma Chemical Co. and *N,O*-bis(trimethylsilyl)acetamide and *N*-(trimethylsilyl)diethylamine were from Pierce Chemical Co.

P_i samples were purified and prepared for derivatization by anion exchange chromatography. In order to minimize contamination by extraneous P_i, all glassware was given a final rinsing in 1 M HCl and then in deionized water. The sample was adjusted to pH ≥ 8.5 with 1 M Tris, diluted to an ionic strength of ≤0.01, and applied to a column (0.5 × 1 cm) of anion exchange resin (Bio-Rad, AGl-X4, 200–400 mesh, Cl^- form). The column was washed with water and then with 10 mM HCl until the eluent was at pH 2. The P_i was eluted with 1.5 ml of 30 mM HCl.

Initially the samples were converted to the triethylammonium salt and derivatized with *N,O*-bis(trimethylsilyl)acetamide in acetonitrile. However, *N*-(trimethylsilyl)diethylamine was found to be a better reagent because it and its by-products

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† These are $P^{18}O_4$, $P^{18}O_3$, $P^{18}O_2$, $P^{18}O_1$, and $P^{18}O_0$.

‡ This treatment assumes that the bound P_i undergoes rapid rotation which completely scrambles all four oxygens between reversals of step 2 and that exchange is not limited by access of water to the site. Although it is reasonable that P_i rotation should be fast, it is possible that scrambling may be incomplete and that all four oxygens may not participate equally. For pyrophosphatase, significant scrambling must occur in order to explain the incorporation of more than one water oxygen during medium and intermediate exchange as reported here. Also, bound P_i has been shown to undergo extensive scrambling between reversals during the analogous intermediate exchange of myosin (ref. 7 and unpublished data).

are more volatile. In the standard procedure, 10–100 nmol of P_i as H_3PO_4 from lyophilization of the sample in 30 mM HCl were treated with 50 μl of dry CH_2Cl_2 and 5 μl of *N*-(trimethylsilyl)diethylamine for 2–3 hr at room temperature in a tube sealed with a Teflon-lined screw cap. This solution could be applied to a probe and directly introduced into the mass spectrometer. A brief pump-down of the probe removed solvent and excess reagent and by-products. Tris(trimethylsilyl)phosphate could then be volatilized in essentially pure form by moving the probe into the heated area of the mass spectrometer. It is possible to work with smaller P_i samples, but contamination becomes an increasing problem.

Mass spectra were obtained on an Associated Electronic Industries MS-9 spectrometer using electron impact ionization. Either the cluster at *m/e* 314 (parent ion) or the cluster at *m/e* 299 (loss of a methyl group) was used, and no systematic differences were detected in their isotopic ratios. Several scans were taken and the results were averaged.

The reactions with pyrophosphatase were stopped by vigorous vortex mixing in the presence of liquid chloroform. This rapidly and irreversibly denatures the enzyme.

In the calculation of the ratios of the P_i species, the distribution of intensity versus mass number must be corrected for the spillover due to ^{29}Si, ^{30}Si, and the other isotopes besides ^{18}O. For tris(trimethylsilyl)phosphate containing no ^{18}O, this spillover can be calculated from known natural abundance ratios (9) and is 0.25804 for P + 1, 0.14172 for P + 2, 0.02422 for P + 3, and 0.00687 for P + 4 expressed as a fraction of the observed parent peak, P. These expected ratios are observed within experimental error in nonenriched P_i samples. For enriched P_i, the correction procedure involves starting with the parent peak and subtracting out its contribution to the peaks at higher mass. The corrected peak at P + 2 then represents the contribution of the species with one $^{18}O/P_i$ and the spillover from this corrected peak is used to correct the peaks at even higher mass. This procedure is repeated until all of the P_i species have been determined. Highly ^{18}O-enriched samples are usually further complicated by the presence of ^{17}O in significant amounts. Any deviation of P + 1, P + 3, P + 5, and P + 7 from the expected amount due to spillover has been assumed in the calculation to be due to ^{17}O and has been added to the contribution of the peak at one lower mass (i.e., ^{17}O is treated as if it were ^{16}O).

RESULTS

Tests for Random Distribution and Scrambling. It was deemed essential to test if the procedures used gave distribution patterns in accord with theoretical predictions. Fig. 1*A* gives the distribution of the ^{18}O-containing species that was obtained with the enriched P_i used in these experiments. The major component is the $P^{18}O_4$ species with essentially no $P^{18}O_0$ present. The values for a random distribution[§] at the observed average enrichment of 84.3% are included and the agreement is good. Analysis of CO_2 produced from the P_i by pyrolysis with guanidine·HCl (10) indicated an enrichment of 83.5% in ^{18}O

[§] For a random distribution with an average ^{18}O-enrichment of *X* mol fraction, the amounts of the five species are: $P^{18}O_0 = (1 - X)^4$; $P^{18}O_1 = 4(1 - X)^3(X)$; $P^{18}O_2 = 6(1 - X)^2(X)^2$; $P^{18}O_3 = 4(1 - X)(X)^3$; and $P^{18}O_4 = (X)^4$.

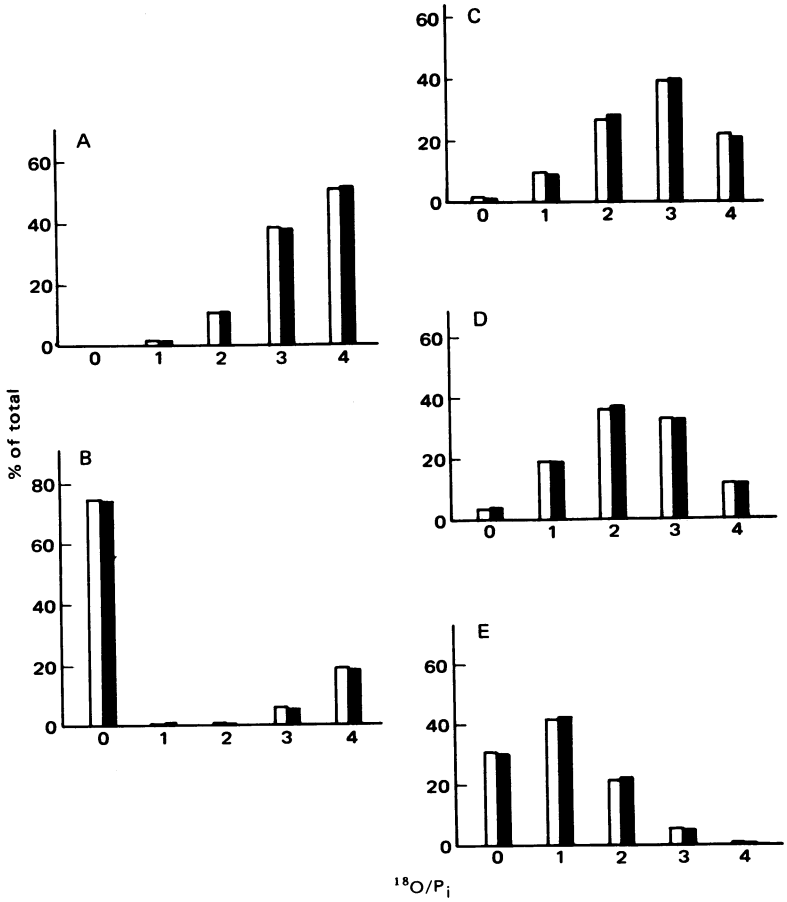

FIG. 1. Distribution of P_i species in control samples. (*A*) Original [^{18}O]P_i (84.3% ^{18}O). (*B*) Nonrandom mixture (23.7% ^{18}O). (*C–E*) Samples equilibrated with ^{18}O-enriched water: *C*, 68.0% ^{18}O; *D*, 56.9% ^{18}O; *E*, 25.8% ^{18}O. □, Observed; ■, theoretical (as described in text).

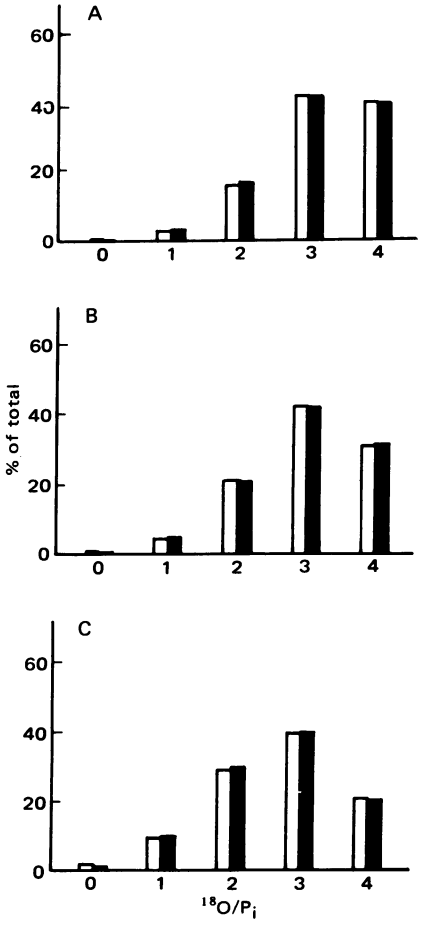

FIG. 2. Distribution of P_i species during nonenzymic exchange. Reaction conditions were 5 mM 84.3% [^{18}O]KH_2PO_4 and 15 mM KCl at 100° (reflux) in unenriched water. Samples were removed at increasing time intervals as indicated and the distributions of ^{18}O-containing species were determined. (*A*) At 1532 min; 79.2% ^{18}O. (*B*) At 2959 min; 75.0% ^{18}O. (*C*) At 4661 min; 67.0% ^{18}O. □, Observed; ■, theoretical random distribution.

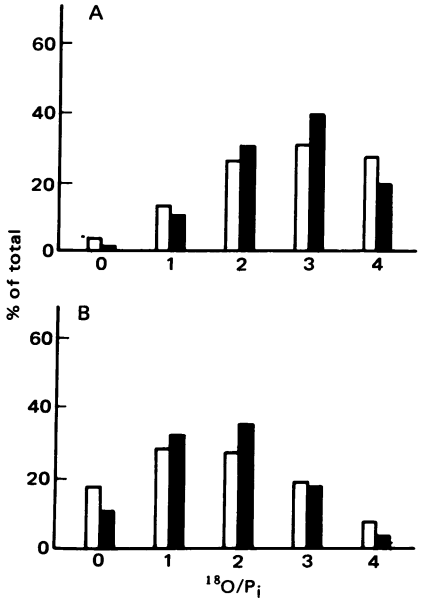

FIG. 3. Distribution of P_i species during pyrophosphatase-catalyzed exchange. Reaction conditions were 6 mM 84.3% [^{18}O]KH_2PO_4, 5 mM $MgCl_2$, and 15 mM KCl (pH 7.6 with KOH) at 25° in unenriched water with 18 μg of pyrophosphatase per ml and incubation times as indicated. (*A*) Incubation for 10 min; 66.1% ^{18}O. (*B*) Incubation for 30 min; 42.5% ^{18}O. □, Observed; ■, theoretical random distribution.

and 0.4% in ^{17}O. An ^{17}O-contribution of this magnitude was observed in the original volatile P_i spectrum.

When enriched P_i with a random distribution undergoes exchange in unenriched water by a process with an effective P_c of zero, the distribution will remain random throughout the exchange process. If $P_c > 0$, however, the distribution at intermediate times will become skewed with the $P^{18}O_4$ and $P^{18}O_0$ species present in relatively higher amounts at the expense of the mixed species.

A critical test of the suggested approach is that it must be able to distinguish random from nonrandom distributions. P_i samples at several average ^{18}O enrichments were prepared by extensive pyrophosphatase-catalyzed equilibration of P_i with ^{18}O-enriched water. At isotopic equilibrium between the P_i and HOH, the distribution of P_i species should be random. Fig. 1 *C–E* shows the distribution patterns obtained. The agreement with the theoretical random distributions is good.

A second requirement is the absence of oxygen interchange (scrambling) between species during volatile P_i preparation. To test for any possible scrambling, a nonrandom sample was generated by mixing 1.3 μmol of 92% [^{18}O]P_i with 4 μmol of unenriched P_i. This mixture was derivatized by the standard method, and its distribution is shown in Fig. 1*B*. The distribution of the 92% sample was independently determined, and the theoretical distribution is a linear combination of the two components in a ratio of 1.3:4. Scrambling would have resulted in an increase in the intermediate peaks at the expense of the extreme ones, and thus no scrambling had occurred. Also, a duplicate lyophilized H_3PO_4 sample was left for 3 days at room temperature before derivatization, without any indication of scrambling. These results demonstrate that the observed distribution corresponds closely to the actual distribution in the starting P_i preparation.

Nonenzymic Exchange. P_i as KH_2PO_4 is known to undergo slow exchange in HOH at elevated temperature. This reaction would be expected to have an effective P_c of zero with the oxygen atoms being replaced one at a time. Distribution patterns during exchange are shown in Fig. 2. They show that the P_i retains a random distribution pattern throughout the exchange reaction. The observed rate of 4.5×10^{-5} min^{-1} for approach to isotopic equilibrium is in good agreement with an earlier value of 6×10^{-5} min^{-1} under similar but not identical conditions (11).

Pyrophosphatase-Catalyzed Medium Exchange. The distribution patterns accompanying medium $P_i \rightleftharpoons$ HOH exchange catalyzed by pyrophosphatase are shown in Fig. 3. These patterns are markedly different from the nonenzymic exchange, with the distribution becoming significantly nonrandom during the exchange process. The deviations correspond to the kind expected for $P_c > 0$ as the accumulation of intermediate species is depressed, but a more quantitative evaluation of P_c is desired. The ratio, R_4, of the rate of loss of the $P^{18}O_4$ species to the rate of loss of the average ^{18}O enrichment can be related to P_c by

$$R_4 = \frac{P_c(E \cdot P_i)k_{-1}/P_i \text{ pool size}}{\bar{O}(E \cdot P_i)k_{-1}/\text{oxygen pool size}}$$

in which $\bar{O}$ is the average number of oxygen atoms that are exchanged per encounter with the enzyme and has previously been shown to be equal to $4P_c/(4 - 3P_c)$ (5). The oxygen pool size is 4 times that of the P_i pool size and thus

$$R_4 = 4 - 3P_c \quad \text{or} \quad P_c = \frac{4 - R_4}{3}.$$

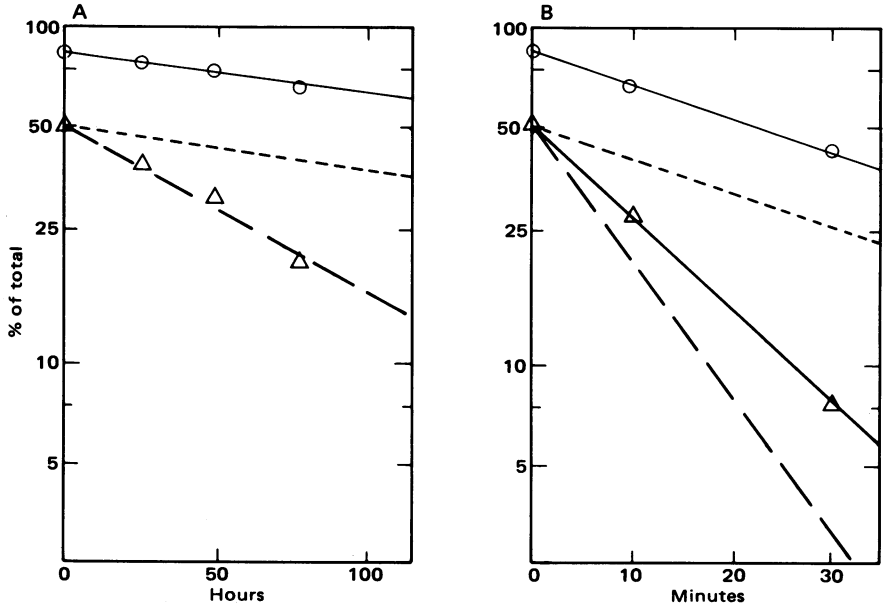

FIG. 4. Time course for average ^{18}O enrichment and $P^{18}O_4$ species during medium $P_i \rightleftharpoons HOH$ exchange. (*A*) Nonenzymic exchange (data from Fig. 2); (*B*) pyrophosphatase-catalyzed exchange (data from Fig. 3). O, Average ^{18}O enrichment. Δ, $P^{18}O_4$ species: — — theory for $R_4 = 4$, - - - - - theory for $R_4 = 1$.

Fig. 4 shows that R_4 for the nonenzymic exchange reaction was 4, as expected for $P_c = 0$. With pyrophosphatase, R_4 is equal to 2.73, which corresponds to $P_c = 0.41$; for this value, k_{-1} is 1.4 times greater than k_2.

Pyrophosphatase-Catalyzed Intermediate Exchange. During hydrolysis of free PP_i, if the same (EP) intermediate is formed then more than one water oxygen will be incorporated into each PP_i hydrolyzed because the partitioning of this intermediate should be the same as that observed during medium exchange. Table 1 gives the results of an experiment which evaluates $\overline{O}'$, the average number of water oxygens present in P_i for each PP_i hydrolyzed. The observed value for $\overline{O}'$ is 1.32 at short times; the slightly higher values at longer times probably represent a small contribution from the medium exchange of the P_i that is released. A control experiment in which the PP_i was subjected to acid hydrolysis in $H^{18}OH$ yielded an $\overline{O}'$ value of 1.02.

The $\overline{O}'$ value can be related to P_c by

$$\overline{O}' = \overline{O}/P_c = \frac{4}{4 - 3P_c}$$

Table 1. Intermediate $P_i \rightleftharpoons HOH$ oxygen exchange during PP_i hydrolysis catalyzed by pyrophosphatase

Fraction of PP_i hydrolyzed	$\overline{O}'$*
0.23	1.32
0.73	1.35
0.98	1.40

Reaction conditions were 7.2 mM $MgCl_2$, 20 mM KCl, and 32 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), adjusted to pH 7.5 with KOH, at 25° with 19 μg of pyrophosphatase per ml and an initial $[PP_i]$ of 2.85 mM. Samples were analyzed for ^{18}O content by standard methods (10) and samples were also taken for P_i analysis to determine the rate and extent of hydrolysis. The initial rate was 212 μmol of PP_i hydrolyzed per min per mg. The PP_i was purified by anion exchange chromatography and contained no detectable P_i.

* Number of water oxygens present in P_i for each PP_i hydrolyzed.

$$\text{or } P_c = \frac{4\overline{O}' - 4}{3\overline{O}'}.$$

The P_c value calculated from the observed $\overline{O}'$ is 0.32. The bound P_i thus partitions similarly during both medium and intermediate exchange reactions.

DISCUSSION

These results clearly demonstrate that more than one oxygen atom is exchanged per productive encounter of P_i with pyrophosphatase during medium $P_i \rightleftharpoons HOH$ exchange and that P_i release is not much more rapid than reformation of the anhydrous intermediate. This conclusion is supported by the observation of an intermediate exchange reaction during hydrolysis that exhibits a similar P_c for the bound P_i, as would be expected if both exchange reactions have the common intermediate (EP).

The evaluation procedure described in this communication should be quite general for the analysis of medium $P_i \rightleftharpoons HOH$ exchange reactions and the way in which the P_c for this exchange varies in response to changes in pH, metal ions, and other factors can give important insight into the catalytic mechanism. Previous work with *E. coli* alkaline phosphatase (8) can be interpreted as indicating an effective P_c of near zero for the $P_i \rightleftharpoons HOH$ exchange of this enzyme. The medium $P_i \rightleftharpoons HOH$ exchange of myosin S1 has recently been shown (7) to have a P_c of 0.99 by these methods. It is also possible to obtain information about the distribution of P_i species from ^{31}P NMR (12), although this method requires relatively concentrated P_i samples and use of high magnetic field strengths in order to obtain a comparable quantitative description of the distribution.

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