The Formation of Enzyme-bound and Medium Pyrophosphate and the Molecular Basis of the Oxygen Exchange Reaction of Yeast Inorganic Pyrophosphatase*

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Yeast inorganic pyrophosphatase, with 10 mM $^{32}P$ and 10 mM Mg$^{2+}$ present at pH 7.3 to 7.8, rapidly forms enzyme-bound pyrophosphate equivalent to about 5% of the total catalytic sites on the two enzyme subunits. The enzyme thus appears to bind PPi, so as to favor thermodynamically its formation from Pi. The enzyme catalyzes a measurable equilibrium formation of free PPi, at a much slower rate. Under similar conditions, the enzyme catalyzes a rapid exchange of oxygen atoms between Pi and water with the relative activation by metals being Mg$^{2+} >$ Zn$^{2+} >$ Co$^{2+} >$ Mn$^{2+}$. Millisecond mixing and quenching experiments demonstrate that the rate of formation and cleavage of the enzyme-bound PPi is rapid enough to explain most or all of the oxygen exchange reaction.

In 1958, Cohn reported that inorganic pyrophosphatase catalyzes a rapid exchange of oxygens of inorganic orthophosphate with water oxygens, that is, a Pi + HOH exchange (1). A satisfactory explanation for this exchange reaction has not been available. Cohn established that this exchange did not result from the overall reversal of the hydrolysis of medium PPi (1). Other possibilities suggested are that the exchange may result from reversible formation of a phosphoryl enzyme from P, and enzyme (1,2) or a reversible formation of a pentacovalent intermediate from enzyme-bound PPi and water (1,3). The present paper gives evidence for an exchange mechanism not previously presented.

An explanation for ATP synthesis by oxidative phosphorylation currently being studied in our laboratory is based in part on a molecular explanation for the rapid Pi = HOH exchange reactions catalyzed by mitochondria (see Ref. 4). This explanation proposes that the oxygen exchanges associated with oxidative phosphorylation accompany the reversible cleavage of ATP at the catalytic site. If such a proposal for the exchanges of oxidative phosphorylation were valid, it seemed quite possible that the rapid Pi = HOH exchange reaction catalyzed by yeast inorganic pyrophosphatase might result from a correspondingly rapid and reversible formation of enzyme-bound PPi from P, without release of the bound PPi to the medium.

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The purpose of the present paper is to present evidence demonstrating that yeast inorganic pyrophosphatase will indeed catalyze the rapid and reversible formation of enzyme-bound PPi from P, with Mg$^{2+}$ or Mn$^{2+}$ present. The techniques used for these measurements were sufficiently sensitive to allow direct measurement of the equilibrium amounts of pyrophosphate formed. Amounts observed were somewhat less than those predicted by the data of Flodgaard and Fleron (5). In agreement with an earlier measurement of Cohn (1), the rate of formation of medium PPi is too slow to account for the $^{18}$O exchange, However, enzyme-bound PPi is formed from and cleaved to P, sufficiently rapidly to account for most, or all, of the observed oxygen exchange.

**EXPERIMENTAL PROCEDURES**

Materials—For most studies, inorganic pyrophosphatase from baker's yeast was purchased from Sigma Chemical Co. The enzyme was prepared as described by Cooperman et al. (7) and had a specific activity, when used, of 700. Enzyme activity was assayed by measuring the P released from PP, essentially as described elsewhere (6,7). A unit of pyrophosphatase activity is defined as that amount of enzyme which hydrolyzes 1 pmol of PPi/min at pH 7.2 and 30°C. The molarity of the enzyme preparation was estimated by absorbance at 280 nm using the value of A$_{280}$ = 1.45 (8) and a molecular weight of 64,000 (9). The molarity of active sites taken as twice the enzyme molarity was based on the evidence that the enzyme contains two subunits and two catalytic sites/molecule (10,11).

$^{32}P$, from ICN Corp., was purified before use as described by Cross and Boyer (12), with use soon after the acid-heating step of the purification, or by separation on an anion exchange column, as described below, to free it from polyphosphates.

Separation of P, from PP, and Determination of $^{32}PP$—A typical procedure was as follows. Reactions were quenched by making 0.3 to 0.4 M perchloric acid at 0°C. If necessary, denatured protein was removed by 10-min centrifugation at 10,000 g near 0°C. The sample was adjusted to pH 1.8 to 2.2 with KOH and the potassium perchlorate formed was removed by centrifugation. The supernatant solution was transferred to a column of Dowex 1 (Cl-, 0.5 × 5.0 cm, X4, 100 to 200 mesh) which previously had been washed with 0.01 M HCl. The P, was eluted with 10 ml of 0.01 M HCl, 0.05 M KCl and the PPi was eluted with 10 ml of 0.05 M HCl, 0.05 M KCl. One milliliter of 60 mM ammonium molybdate in 4 M sulfuric acid and 2 ml of 6 M HCl were added to each sample and the P, and any $^{32}P$ present were removed by four extractions with isobutyl alcohol:benzene (1:1, v:v), with 1 µmol of carrier P, added before the first and third extractions. The $^{32}PP$, remaining in the lower layer was determined by scintillation counting or counting Cerenkov radiation. Concentrations of $^{32}PP$ were calculated on the basis of two $^{32}P$, incorporated into each PPi. This obviously holds for net PPi formation from $^{32}P$, in measurement of enzyme-bound $^{32}PP$ formation after $^{32}P$, addition in rapid mixing.

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experiments, the assumption is made that both enzyme-bound P, molecules that form enzyme-bound PP, are derived from the medium. This assumption, although plausible, may not be justified, and this introduces some uncertainty in the rate estimations.

**O Exchange Measurements**—The P, in the supernatant from perchloric acid precipitation was isolated as KH2PO4 (13) and 31O incorporated from H2O into P, was determined by the guanidinium pyrosylation procedure (14).

The extent of oxygen exchange was calculated as described by Boyer and Bryan (13) from the relationship, 4 X P, ln(1/F) = mm P, = HOH (mm of H2O exchanged with P,) = pmol of O exchange/ml. Where P, = mm P, in the reaction medium and F, the fraction of isotope exchange, is the ratio between atom per cent excess 31O in P, of the reaction medium before addition of any carrier and atom per cent excess of 31O in HOH.

Identification and Characterization of the 32P-labeled Product as Pyrophosphate—For these assessments, migration on anion exchange resin and enzymic and acid hydrolyses in the presence of authentic carrier PP, were measured. For enzymic hydrolysis of a 100-µl aliquot of the lower layer (after isobutyl alcohol-benzene extraction), the following were added: 25 µl of 8 mM MgCl2, 10 µl of 2 mM EGTA, 100 µl of 1 M Tris-Cl-0.8 ml of H2O, and 50 µl of enzyme (containing about 0.1 µmol enzyme). The reaction mixture was incubated at 30°C for 15 min, sufficient for hydrolysis of nearly all the PP, present. The 32P released due to the enzymic reaction was extracted by isobutyl alcohol-benzene as the phosphomolybdate complex.

Two conditions were used for acid hydrolysis. In one, a 100-µl aliquot of a lower layer, after molybdate addition and solvent extraction, was added to 3 ml of 1 M HCl, and the reaction mixture was heated at 100°C for 10 min. This treatment hydrolyzes almost all of the PP, present. 32P released was determined as described above.

For a second assessment, the relative rates of total PP, cleavage and 32P release at lower temperature were measured. To 1.8 ml of a lower layer, 0.2 ml of 9 M HCl was added (final HCl concentration = 1.49 M) and the reaction mixture was incubated at 60°C. Samples were withdrawn at appropriate times for determination of total P, and of 32P.

**Gel Filtration of Enzyme-Pyrophosphate Complex (E-PPi)**—A reaction mixture in a final volume of 75 µl contained 0.1 M Tris-Cl at pH 7.3, 0.1 mM EGTA, 35 mM MgCl2, 5 mM 32P, (4.97 x 1010 cpm), and 30 µg enzyme. After 30 s, the reaction mixture was loaded on a column of Sephadex G-25 (0.6 x 3 cm) which previously had been equilibrated with 1 ml of 0.1 M Tris-Cl buffer at 7.3, having the same composition of reaction mixture, including 31P, except for the absence of enzyme.

The column was eluted with 50 mM Tris-Cl buffer at pH 7.3. Fractions of four drops each were collected in test tubes containing 0.5 ml of 0.44 M perchloric acid. The presence of protein was detected by turbidity and, after addition of carrier P, and PP, the presence of 32PP, was detected after separation from 31P, as described above.

**RESULTS**

Relative Oxygen Exchange Rates in Presence of Various Metal Ions—Cohn demonstrated that the pyrophosphatase-catalyzed oxygen exchange was stimulated by Mg2+ or Co2+ ions (1). Activation by several metal ions under conditions of our experiments was assessed. Results are reported in Table I. The observed oxygen exchange rates were linear with time (not shown). Values are given in Table I for the apparent first order velocity constants for exchange. The order of effective- 

| Metal Ion | pH | Oxygen Exchange Rate | Activity (pmol/min/pmol enzyme) |
|-----------|----|---------------------|---------------------------------|
| Mg2+      | 5.9 | 100 ± 5             | 1.1                             |
| Mg2+      | 6.9 | 110 ± 10            | 1.0                             |
| Mg2+      | 7.6 | 140 ± 10            | 1.0                             |
| Mn2+      | 5.9 | 300 ± 20            | 1.1                             |
| Mn2+      | 6.7 | 1500 ± 200          | 1.1                             |
| Mn2+      | 7.6 | 3000 ± 600          | 1.1                             |

The values give the capacity to form bound pyrophosphate from P, were a relatively high concentration of enzyme, use of 32P containing little or no 32PP, and an adequate method for separation of any small amount of 32PP, formed from the relatively large amount of 32P, present. Although our approaches, as will be shown, sufficed for the demonstration of the formation of enzyme-bound PP, from P, the quantitation of the amount of formation was not as precise as desired. The amounts measured were equivalent to only a fraction of the total concentration of catalytic sites, which is necessarily much less than the 32P, molarity. Zero-time values often approached experimental values. Data were quite sufficient, however, to establish major conclusions.

In preliminary experiments not reported in detail here, an apparent formation of 31P, from pyrophosphatase and 32P, was observed, and the amount of 32PP, formed was roughly proportional to the enzyme concentration. On the basis of these results, conditions were chosen for subsequent experimentation. For measurements of the formation of the apparent 31P, rapid mixing experiments, a trace amount of 32P, was added to enzyme in the presence of nonlabeled P, and Mg2+. This helped avoid any possible changes in enzyme properties that might follow the initial dissolving of the lyophilized enzyme or the initial exposure of dissolved enzyme to Mg2+ and P,. If formation of enzyme-bound 31P, from P, and its subsequent hydrolysis were the basis for the P, = HOH exchange, enzyme-bound PP, would already be present at a steady state concentration and would be rapidly labeled by addition of a trace of 32P.
In other experiments, much longer incubations were used and the total 32PP, formed was measured. Results of such experiments are reported in Table III. With Mg" at pH 7.6, and in other similar experiments, the amount of PP, formed appeared to exceed that expected if the enzyme were only catalyzing attainment of equilibrium for the reaction

\[
2P, \rightleftharpoons PP, + HOH
\]

based on the reported value for the equilibrium of MgPP, hydrolysis (5). Such results were reported in preliminary form (16) and justified more extensive experimentation on the rate and extent of 32PP, formation.

Prior to making the more extensive studies given later in this paper, it was deemed necessary to test for the authenticity of the 32P-labeled product as PP, and to check on the indicated binding to protein by an independent method. Such tests are given in the following sections. Also, as mentioned under "Experimental Procedures," a commercial enzyme preparation was used for most of the present experiments. It was thus desirable to check on bound PP, formation with an enzyme of known preparative procedure. With a highly purified pyrophosphatase prepared as described by Cooperman et al. (7), PP, formation per unit of enzyme activity was identical within experimental error with that noted with the commercial enzyme.

Identification of the Labeled Product as 32PP, — For these tests, a sample of the 32P-labeled product prepared under conditions similar to those given in Table III was mixed with authentic nonlabeled PP. The 32P-labeled product and authentic PP, were found to be completely converted to 32Pi by inorganic pyrophosphatase action in the presence of Mg" or by heating in 1 N HCl at 100°C for 10 min. In addition, in a

**Table III**

| Mg" and P° concentration | Temperature °C | Enzyme concentration mM | PPi concentration mM | Oxygen exchange rate μatoms/mmol enzyme/min |
|---------------------------|----------------|--------------------------|---------------------|----------------------------------------|
| 5                         | 22             | 5.0                      | 0.29                |                                        |
|                           |                | 3.75                     | 0.27                |                                        |
|                           |                | 2.5                      | 0.18 ± 0.01         |                                        |
|                           |                | 1.87                     | 0.14                |                                        |
|                           |                | 1.25                     | 0.11 ± 0.01         | 3100 ± 600                             |
|                           |                | 0.125                    | 0.091               | 2100 ± 400                             |
|                           |                | 0.0125                   | 0.044               | 2700 ± 400                             |
|                           |                | 0.00125                  | 0.040               |                                        |
| 4                         | 2.50           | 1.25                     | 0.06 ± 0.01         | 220                                    |
|                           |                | 0.125                    | 0.018               | 210 ± 50                               |
|                           |                | 0.0125                   | 0.014               |                                        |
|                           |                | 0.00125                  | 0.012               |                                        |
| 10                        | 22             | 1.25                     | 0.28 ± 0.004        |                                        |
|                           |                | 0.125                    | 0.19 ± 0.01         |                                        |
|                           |                | 0.0125                   | 0.18 ± 0.01         | 4600 ± 600                             |
|                           |                | 0.00125                  | 0.16 ± 0.004        | 4000                                   |
|                           |                | 0.00125                  | 0.20                | 4200 ± 150                             |

**Fig. 1.** Rate of acid hydrolysis of the 32P-labeled reaction product and inorganic pyrophosphate. A 32P-labeled fraction with carrier PP, was prepared by exposure of pyrophosphatase to 32P, in the presence of Mg", with ion exchange column separation, exposure to acid molybdate and isobutyl alcohol-benzene extraction, followed by measurement of 32P, (cpm) and total P, released during acid hydrolysis at 40°C in 1.49 M HCl as described under "Experimental Procedures." a, hydrolysis of pyrophosphatase. Plot of (D - D) (log scale) versus time (O-O). b, hydrolysis of reaction product. Plot of (cpm, - cpm,/) (log scale) versus time (A-A-A).

Gradient elution on Dowex-1 (0.01 M HCl → 0.2 M HCl and 0.05 M KCl), the radioactive 32P product and authentic PP, appeared in maximum concentration in the same fraction (tube no. 114) with comparable specific activity in preceding and following fractions.

As a further test, the relative rate of acid hydrolysis of the 32P-labeled product and authentic pyrophosphate at 40°C was measured (see "Experimental Procedures"). Results of these measurements are given in Fig. 1. The data of Fig. 1 show that the rate of liberation of 32P, from the labeled product and of P, from authentic PP, were identical. The first order kinetic plots show strict linearity over two half-lives. These acid and enzymic hydrolysis tests thus allow identification of the 32P-labeled product as 32PP,.

Direct Demonstration of Enzyme-bound Pyrophosphate — Although the preceding experiments gave evidence that most of the 32P, formed was enzyme-bound, confirmation of this important conclusion by independent means appeared desirable. Very small traces of 32PP, would be expected in the medium, but if all or nearly all of the 32PP, present were that bound to the enzyme, appropriate gel filtration should show that most of the 32PP, migrates with the protein peak and not where free PP, migrates.

Results of a gel filtration experiment are given in Fig. 2. Enzyme was exposed to 32P, under conditions where 32PP, is formed. The enzyme solution was then placed on a Sephadex column to which sufficient 32P-containing medium had been added to assure that, as pyrophosphatase moved ahead of low molecular weight substances, it would always be equilibrated with 32P. Any 32PP, that was not protein-bound would lag behind the pyrophosphatase in the column and appear in the eluate after the protein. Shown in Fig. 2 are the fractions where protein and PP, would be expected to appear on the basis of independent trials with the columns used. Nearly all the 32PP, in the sample where pyrophosphatase was equilibrated with 32P, appears with the protein peak. These results demonstrate that most of the 32PP, formed is indeed protein-bound.
Pyrophosphatase Oxygen Exchange

With conditions as used for Fig. 2, a small amount of the total $^{32}$PP, formed would be expected to represent medium PP, present at equilibrium concentration with the $P_i$ (5). This may account for the apparent presence of some $^{32}$PP, migrating after the protein (Fig. 2).

Measurement of the Total Pyrophosphate Formed under Equilibrium Conditions—These measurements were undertaken in part because the sensitivity of the methods appeared adequate to measure directly both the amount of PP, present at equilibrium and its rate of formation. Also, a direct measurement of the rate of medium PP, formation from medium $P_i$ would give another method of checking the possibility that reversal of the overall reaction contributed to the $P_i = HOH$ exchange.

As a means of differentiating between enzyme-bound and medium PP, at equilibrium, measurements were made of total $^{32}$PP, formation from $^{32}$P, at various enzyme concentrations. Results are presented in Table III. With 5 mM $P_i$ and 5 mM $Mg^{2+}$, total PP, formation drops to a constant value of 0.035 to 0.040 $\mu$M at 22°C and 0.01 to 0.015 $\mu$M at 4°C as enzyme concentration is decreased, indicating that these amounts represent equilibrium levels. Data presented later in this paper give assurance that the incubation times used were sufficient for >98% of enzyme PP, concentration to be reached even at the lowest enzyme concentration used. At the highest enzyme concentration (5 $\mu$M) the total PP, formed is 0.29 $\mu$M, of which only about 0.04 $\mu$M represents an equilibrium concentration and thus about 0.25 $\mu$M, equivalent to about $30\%$ of the enzyme molarity, is enzyme-bound.

With 10 mM $P_i$ and $Mg^{2+}$, the amount of total PP, present in all measurements at 0.0125 $\mu$M or less enzyme (conditions where enzyme-bound PP, would be negligible) was 0.175 ± 0.03 $\mu$M. This may be compared with a value of 0.46 $\mu$M calculated from measurements of Flodgaard and Fleron under similar conditions (5).

Also shown in Table III are some measurements of oxygen exchange rates under the same conditions. The exchange rate per mol of enzyme shows little or no change as enzyme concentration is decreased. Thus, the enzyme is not changing properties as it is diluted.

Rate of $^{32}$P, Labeling of Medium PP, at Equilibrium—As a means of checking on the possible contribution of reversal of the overall hydrolytic reaction to oxygen exchange, the rate of attainment of an equilibrium concentration of medium PP, at low enzyme concentration was measured. Results of one series of measurements with 10 mM $^{32}$P, and 10 mM $Mg^{2+}$ are given in Fig. 3. From a semilog plot of the data, a $t_{1/2}$ for approach to isotopic equilibrium was calculated to be 4.8 min and the initial rate of PP, labeling was 75 $\mu$mol/mmol of enzyme/min. This may be compared to a rate of 20,000 $\mu$mol of PP, cleaved/mmol of enzyme/min under comparable conditions but with 5 mM PP, instead of P, present.

Under the same conditions (Table III), the rate of oxygen exchange is $4,300$ $\mu$atoms of oxygen exchanged/mmol of enzyme/min. Clearly, and in confirmation of Cohn (1), the rate of formation and cleavage of medium PP, does not account for the exchange. However, the reversal of overall hydrolysis is considerably faster than the lower estimate given by Cohn.

The Kinetic Competency of Bound Pyrophosphate as an Intermediate in the $P_i = HOH$ Exchange—If the formation and hydrolysis of enzyme-bound pyrophosphate is responsible for the $P_i = HOH$ exchange, it should be theoretically possible to demonstrate that the rate of formation and cleavage of the bound pyrophosphate is sufficiently rapid to account for the exchange. The difficulty of obtaining precise measurements of $E \cdot PP_i$ formation, particularly with millisecond mixing and quenching, did not favor a critical test of kinetic competency. However, it seemed possible to assess if the rates of formation and cleavage of $E \cdot PP_i$ fall within the range required to explain the oxygen exchange.

For this purpose, use was made of a simple millisecond mixing and quenching apparatus described elsewhere (17). Results are shown in Figs. 4 and 5. At 22°C (Fig. 4), the labeling of $E \cdot PP_i$ appears to be completed within 20 ms indicating a $t_{1/2}$ of 10 ms or less. At 4°C, the formation of enzyme-bound PP, is much slower with a $t_{1/2}$ of roughly 50 ms. As noted from Table III, some of the PP, formed at 1 min is due to equilibrium formation.

The experiments required high amounts of $^{32}$P and this, together with the time required for the individual separations necessary for each experimental point, the variability encountered, and the limitation of the apparatus used to cover a wider mixing time and temperature range, made attempts to get more complete and precise data unwarranted at this stage.

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**Fig. 2.** Demonstration of $^{32}$PP, binding to pyrophosphatase by gel filtration. Pyrophosphatase was equilibrated with $^{32}$P, and $Mg^{2+}$ and the mixture was separated by gel filtration as described under "Experimental Procedures." For a control, $Mg^{2+}$ was omitted. Positions where protein and free PP, would appear, indicated in the figure, were determined in separate experiments.

**Fig. 3.** Approach to isotopic equilibrium of $^{32}$PP, and $^{32}$P. A 5.0-ml reaction mixture of 50 mM Hepes, 10 mM $Mg^{2+}$, 10 mM $P_i$, 135 mM KCl, and 0.25 mM enzyme at pH 7.5 and 25°C was incubated for 2 h. Purified, pH-adjusted $^{32}$P, (10$^6$ cpm) was added and 0.5-ml aliquots were withdrawn at times indicated, quenched, and assayed as in Table III. $^{32}$O measurements under identical conditions gave a value of 4800 $\mu$atom/min/mmol of enzyme. Values are averages of two experiments and the line is theoretical assuming an infinity value of 0.165 $\mu$m.
It is apparent that pyrophosphatase has the capacity to favor formation of PP, from P, at the catalytic site considerably above that expected from the known $\Delta G^\circ$ for hydrolysis in solution. Factors that might favor such formation of PP, are a preferential tight binding of PP, a low water activity, a high effective local concentration of P, and an increased proton availability.

In the presence of the higher concentrations of enzyme used in our experiments, an equilibrium concentration of free PP, in solution is soon attained. As enzyme concentration is reduced, the amount of enzyme-bound PP, is correspondingly reduced and the remaining PP, at the lowest enzyme concentrations greatly exceeds the total enzyme concentration (Table III). Such PP, is in equilibrium with P, under the experimental conditions. Estimates of the equilibrium concentration of PP, from data in Table III give values somewhat lower than the more extensive measurements of Flodgaard and Fleron (5). They used an indirect coupled assay for measurement of PP, present. Our results, by direct assay, appear to us to be valid but the measurements are not extensive. Further experimental assessment is indicated if more certain, precise values are needed.

For discussion of the millisecond mixing experiments (Figs. 4 and 5), factors governing the labeling of PP, by $^{32}$P, must be related to the rate of oxygen exchange. For the formation of enzyme-bound PP, from medium P, and the exchange of water oxygens with medium P, at least two reaction steps must be involved, as indicated in Equation 1.

$$E \cdot P_i + P_i \xrightarrow{k_1} E \cdot 2P_i \xrightarrow{k_2} E \cdot PP_i + HOH (1)$$

Theoretical relationships governing oxygen exchange in such a system but without the complications of equilibrium formation have been developed more fully in another paper from this laboratory on the Pi + HOH exchange catalyzed by the sarcoplasmic reticulum ATPase (18). For the present discussion, some quite simple considerations will suffice.

Two limiting possibilities may be envisaged for the oxygen exchange, with either the first step of Equation 1 rapid compared to the second, or vice versa. The rate of $E \cdot 2P_i$, once formed, is conveniently expressed by the partition coefficient, $P_i$, where $P_i = k_{2}/(k_{1} + k_{2})$. As $P_i$ approaches 0, then each medium $P_i$ that forms $E \cdot PP_i$ will return to the medium $P_i$ pool with one oxygen atom acquired from water. The oxygen exchange rate would equal the rate of bound $PP_i$ formation. As $P_i$ approaches 1, a medium $P_i$ upon forming the enzyme-bound $P_i$ will acquire nearly four oxygens from water before returning to the medium.7

An estimate of the value for the partition coefficient has been made from measurements of the distribution of the $^{18}$O, species during exchange starting with fully $^{18}$O-labeled P, (19). The results show that the partition coefficient does not approach either extreme and would be expected to be in the range of 0.2 to 0.4 for our experimental conditions. From the data of Table III at 22°C, with 5 mM Mg$^{2+}$ and 1.25 mM enzyme, $E \cdot PP_i$ is 0.105 $\mu$m minus 0.04 or 0.065 $\mu$m, and the constant relating $E \cdot PP_i$ concentration to the exchange rate is thus 800 s$^{-1}$.

If $P_i$ were close to 0, the $t_{1/2}$ for labeling of $E \cdot PP_i$ would be 0.69/k or 0.9 ms. As $P_i$ values increase, labeling of enzyme-bound $P_i$ would not need to be as rapid to account for the oxygen exchange because more than 1 atom of oxygen would

7 All four oxygens of $E \cdot P_i$ are assumed to have the same probability of exchange. This assumption is in harmony with unpublished findings of D. D. Hackney in this laboratory on patterns of "O loss from fully $^{18}$O-labeled P, during oxygen exchange catalyzed by pyrophosphatase.
be replaced for each P bound. Thus, if $P_i$ were close to 1, the $t_{1/2}$ would be close to 4 times greater, or $\sim 3.6$ ms. At intermediate values, the relation between $E\cdot PP_i$ and time would deviate slightly from hyperbolic (18), and the apparent $t_{1/2}$ would be between 1 and 4 ms. As mentioned above, $P_i$ is somewhat greater than 0 and labeling of half of the $E\cdot PP_i$ would be expected in no more than 2 to 3 ms. Data of Fig. 4 at $22^\circ C$ are consistent with this. Results of oxygen exchange measurements and $E\cdot PP_i$ formation at $4^\circ C$ by similar approaches lead to a lower limit of 10 ms for $P_i$ approaching 0 and an upper limit of 40 ms for $P_i$ approaching 1. An estimate of the apparent half-time of labeling from data of Fig. 5 is 30 to 40 ms.

These results show that the formation and cleavage of enzyme-bound pyrophosphate from medium $P_i$ accounts for most, if not all, of the $P_i = HOH$ exchange. Such a conclusion is in harmony with other related considerations. The reasonable assumption may be made that $E\cdot PP_i$ has similar rates of cleavage whether formed from $P_i$ or $PP_i$. Thus, the appreciable fraction of $E\cdot PP_i$ present when only $P_i$ and the enzyme are mixed would be cleaving at a rapid rate and account for the rapid net hydrolysis observed when $E$ and $PP_i$ are mixed and form $E\cdot PP_i$. The rate of formation of $E\cdot PP_i$ from $E$ and $P_i$ obviously must be rapid in order to maintain an appreciable fraction of the total enzyme in the form of $E\cdot PP_i$. Thus, a rapid $P_i = HOH$ exchange would be expected to result from the rapidly reversible formation of $E\cdot PP_i$ from $E$ and $P_i$.

Our data establish that enzyme-bound pyrophosphate is rapidly and preferentially converted to medium $P_i$. With the promise that the same type of bound $PP_i$ participates in the net hydrolysis reaction, the results show that once the product $E\cdot PP_i$ complex is formed, it is about 30 times more likely to be cleaved to $P_i$ than to return to medium $PP_i$.

The high rate of $P_i = HOH$ exchange noted in Table III occurs with less than 10% of the enzyme as $E\cdot PP_i$. When only $P_i$ is present, more of the enzyme would be expected to be present as $E\cdot PP_i$; and thus, oxygen exchange would be greater. This means that some intermediate $P_i = HOH$ exchange might be observed accompanying net $PP_i$ hydrolysis. Other experiments in progress in this laboratory in cooperation with Dr. David Hackney show that this is indeed the case. A full analysis of present published and unpublished data gives promise of assigning reasonable values for all rate constants in enzymatic hydrolysis of pyrophosphate.

For the $P_i = HOH$ exchange, a possibility previously considered was that the exchange results from the reversible formation of a phosphorylated enzyme from $P_i$. This would be akin to the exchange catalyzed by alkaline phosphatase (20) and by transport ATPases (21, 22). Although experiments of Nazarova and Avakova (23) have indicated a binding of $^{32}P_i$ interpreted as representing a phosphorylated intermediate of the hydrolysis reaction, conditions for the detection of the binding were unusual and rapid interchange with medium $P_i$ or $PP_i$ was not established. In the careful experiments of Sperow et al. (2) and of Rapoport et al. (24), no evidence for a phosphoryl enzyme intermediate has been obtained. It is difficult to prove that something does not exist, however, and it remains conceivable that in our experiments a phosphorylated enzyme precedes the formation of enzyme-bound $PP_i$ from $P_i$. Also, Raykov et al. (25–27) have reported evidence interpreted as showing a covalently bound $PP_i$, formed in the presence of enzyme, $F_2$, $PP_i$, and $Mg^{2+}$. We thus checked the effect of $F_2$ on enzyme-bound $PP_i$ formation. At 10 mM $Mg^{2+}$ and 10 mM $^{32}P_i$, 75 mM $F_2$ decreased the amount of $^{32}PP_i$ formed from 0.15 to 0.01 $\mu$M and the oxygen exchange to 0. With 100 mM $Mg^{2+}$ present, 75 mM $F_2$ did not change the amount of $^{32}P$ formation.

Other suggestions have been that the oxygen exchange results from the reversible formation of a pentacovalent derivative from bound $PP_i$ and water (1, 3). Our data give no support to such suggestions. If a relatively rapid and reversible formation of any such derivative occurred, the oxygen exchange rate should have considerably exceeded the rate of formation of bound $PP_i$ from medium $P_i$. Also, formation of considerable $E\cdot PP_i$ would not be expected.

As noted in the introduction, these studies with pyrophosphatase were prompted by suggested mechanisms for oxygen exchange accompanying oxidative phosphorylation. Related studies from this laboratory with myosin have demonstrated formation of bound ATP from $^{32}P_i$ (28). These results, the findings of Bagshaw and Trentham (29), and the demonstration of water-oxygen incorporation into myosin-bound ATP (30) are consistent with the reversible cleavage of bound ATP being responsible for the oxygen exchanges observed with muscle. Evidence favoring this explanation comes from data showing a capacity of myosin to cause scrambling of the $\beta,\gamma$-bridge oxygens of ATP (31).

With other enzymes, unlike with pyrophosphatase, a $P_i = HOH$ exchange could result from dynamic reversal of formation of a phosphorylated enzyme. With alkaline phosphatase, oxygen exchange do appear to result from such reversible enzyme phosphorylation (20). Also, recent studies with sarcoplasmic reticulum ATPase show that the rate of formation and cleavage of the phosphoryl enzyme from $P_i$ can account for the rapid $P_i = HOH$ exchange observed (18). Present evidence makes it tenable to suggest that all exchanges of phosphate oxygen with water catalyzed by enzymes are associated with dynamic reversal of cleavage of covalent phosphorylated substances.

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