Reversible Binding of P\textsubscript{i} by Beef Heart Mitochondrial Adenosine Triphosphatase*  

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Beef heart mitochondrial ATPase (F\textsubscript{1}) exhibited a single binding site for P\textsubscript{i}. The interaction with P\textsubscript{i} was reversible, partially dependent on the presence of divalent metal ions, and characterized by a dissociation constant at pH 7.5 of 80 \( \mu \text{M} \). A variety of substances known to influence oxidative phosphorylation or the activity of the soluble ATPase (F\textsubscript{0}) also influenced P\textsubscript{i} binding by the enzyme. Thus aurovertin, an inhibitor of oxidative phosphorylation, which was bound tightly by F\textsubscript{1}, and inhibited ATPase activity, enhanced P\textsubscript{i} binding via a 4-fold increase in the affinity of the enzyme for P\textsubscript{i} (K\textsubscript{D} = 20 \( \mu \text{M} \)) but did not alter binding stoichiometry. Anions such as SO\textsubscript{4}\textsuperscript{2-}, SO\textsubscript{3}\textsuperscript{2-}, chromate, and 2,4-dinitrophenolate, which stimulated ATPase activity of F\textsubscript{1}, also enhanced P\textsubscript{i} binding. Inhibitors of ATPase activity such as nickel/hathophenanthroline and the protein ATPase inhibitor of Pullman and Monroy (Pullman, M. E., and Monroy, G. C. (1963) J. Biol. Chem. 238, 3762-3769) inhibited P\textsubscript{i} binding. The adenine nucleotides ADP, ATP, and the ATP analog adenylyl imidodiphosphate as well as the P\textsubscript{i} analog arsenate, also inhibited P\textsubscript{i} binding. The observations suggest that the P\textsubscript{i} binding site was located in or near an adenine nucleotide binding site on the molecule.

The cold-labile ATPase of oxidative phosphorylation was first described about 15 years ago (1). It was proposed at that time that the ATPase activity of the soluble beef heart enzyme was an artifact of isolation and that when the enzyme was bound to the mitochondrial membrane, it served as a phosphate transfer catalyzer in the terminal transphosphorylation step of oxidative phosphorylation (2). In the intervening years, it also has been suggested that F\textsubscript{1} functioned as a component part of a system which synthesized ATP via reversal of the hydrolytic reaction (3, 4) and that energy-dependent conformational changes which altered the affinity of adenine nucleotide binding sites on F\textsubscript{1} were critical features of ATP synthesis in oxidative phosphorylation (5, 6).

It has become clear that a detailed understanding, at the molecular level, of the mechanism of action of F\textsubscript{1}, in oxidative phosphorylation will constitute an important element in the final elucidation of the overall mechanism of the reaction. Investigations thus far have revealed that the soluble beef heart enzyme, as well as other energy transducing ATPases, is characterized by considerable complexity, consisting of five chemically distinct subunits and as many as 10 subunit/oligomer (7). In addition, the enzyme could be isolated with 5 mol of nucleotide tightly bound/mol of protein (6) and five adenine nucleotide binding sites/oligomer of molecular weight 347,000 (7, 8) were demonstrated. As isolated in this laboratory, three of the five sites on beef heart F\textsubscript{1} were occupied by very tightly bound adenine nucleotides (8) and two sites were engaged in readily reversible binding of adenine nucleotides (8). The function of all of the sites has yet to be elucidated. However, one or more may be control sites (8-10) and at least one site is the hydrolytic site (11, 12).

Although the groundwork has thus been prepared for an approach to the role of adenine nucleotide binding sites in the mechanism of action of F\textsubscript{1}, little if any attention has been given to the second major chemical component in the forward reaction of oxidative phosphorylation, namely P\textsubscript{i}. If indeed F\textsubscript{1} serves as the P\textsubscript{i} transfer enzyme in the synthesis of ATP during oxidative phosphorylation, as has been proposed (2), it is reasonable to expect that the enzyme will exhibit binding sites for P\textsubscript{i}. The purpose of this paper is to describe specific, reversible interactions of F\textsubscript{1} with P\textsubscript{i} and to show that the enzyme exhibits a single binding site for this ligand.

**EXPERIMENTAL PROCEDURES**

**Materials**

AMP-P(NH)P, [\(^{3}H\)AMP-P(NH)P, \(^{35}S\)EDTA, and \(^{35}P\), enzyme grade, were purchased from ICN Corp., Isotope and Nuclear Division. \(^{35}P\) was used without further purification. \([\(^{3}H\)ADP was obtained from Schwarz/Mann. Filtration membranes (XM-50) for the pressure dialysis cell were obtained from Amicon. Sephadex G-50 fine was purchased from Pharmacia, Piscataway, N. J. ATP and ADP were obtained from Sigma. All other chemicals were of reagent grade quality. Solutions of cobalt and manganese were prepared fresh from the dry salt for each experiment. F\textsubscript{0} (13), nucleotide-depleted F\textsubscript{1} (8), aurovertin (14), and the protein inhibitor of Pullman and Monroy (15, 16) were prepared as described.

**Methods**

F\textsubscript{1} was prepared for P\textsubscript{i} binding studies in the following way. An aliquot of the ammonium sulfate suspension of the enzyme (10), containing up to 5 mg of protein, was centrifuged for 5 min at 5,000 rpm in a glass tube of the SS-34 rotor of the Sorvall centrifuge (10,000 rpm). The supernatant fluid was poured off and the walls of the tube were carefully dried with filter paper before the protein sediment was dissolved by adding 80 to 100 \( \mu \text{L} \) of buffer. The buffer consisted of

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1 The abbreviations used are: F\textsubscript{1}, beef heart mitochondrial ATPase; AMP-P(NH)P, adenylyl-5'-yl imidodiphosphate.
either 50 mM Tris/acetate, pH 7.5, or 50 mM Tris/acetate, pH 7.5, and 0.25 M sucrose. The dissolved sample was applied to a centrifuge column (Step 2), as described below, for separation of residual ammonium sulfate. The centrifuge column was prepared with Sephadex G-50 membranes which had been equilibrated with the same buffer used to dissolve the protein. It should be emphasized that no loss in ATPase activity of Fo, these conditions, the centrifuge column collected was used to desalt samples of the enzyme in the absence of ATP. These results may be contrasted with ordinary gel permeation chromatography in which losses of 50% or more occurred (17). The ratio of the absorbance at 260 nm to that at 260 nm of the enzyme samples which emerged from the column averaged 1.27. This value corresponded to approximately 3 mol of adenine nucleotide/mol of enzyme and suggested that the two sites which engaged in readily reversible binding were unoccupied in these preparations (8).

Protein concentration was measured by the method of Lowry et al. (18) using bovine serum albumin as standard. The values obtained were divided by 1.18 to convert to dry weight of F0 (19). The molecular weight of F0, used in all calculations, 347,000 (20), was based on the dry weight of protein determined in this way. Radioactivity was determined by adding 3.0 ml of Triton/ouleic scintillant to an aliquot sample volume of 0.3 ml and counting in a scintillation counter. The Triton/ouleic scintillant was prepared by dissolving 0.1 g of 1,4-bis-(2-methyl-5-phenylazoxy)benzene, 5.5 g of 2,5-diphenylouleic, and 333 ml of Triton X-100 in 300 ml of ouleic. Additional ouleic was added to a final volume of 1 liter. Counting was continued to an error of 0.5% for those experiments in which dissociation constants were calculated.

The binding of P0 to F0, was measured by one of two procedures. The first made use of the Paulus pressure dialysis cell and Amicon XM-50 membranes (21) as described previously (5, 11). The second method made use of a small Sephadex column for separation of free and protein-bound ligand. A disposable 1-ml plastic tuberculin syringe (Plastipak No. 6002, Becton-Dickenson & Co., Rutherford, N. J.) was fitted with a porous polyethylene disc (1.6 mm thick, 70 µ pore size, Bel-Products, Pekannock, N. J.) and filled to the 1-ml calibration mark with Sephadex G-50 fine, previously swollen in 50 mM Tris/acetate, pH 7.5.

Step 1 - The column was placed in a test tube (15 x 125 mm), allowed to stand until no further liquid drained from it (3 to 5 min) and then was transferred to a six-place swinging bucket rotor (No. 221) of the model CL centrifuge (International Equipment Company, Needham Heights, Mass.). The centrifuge rotor was preset to one-half maximum speed (900 rpm, 100 x g at the tip of the syringe) and centrifugation was initiated by activating a controlling clock preset to 3 min. After centrifugation, the length of the gel column was decreased 30 to 40% and the diameter of the gel also was somewhat reduced.

Step 2 - A sample of reaction mixture (50 to 100 µl) containing F0, radioactive ligand, buffer, and other ions as described in the text was transferred to the top of the Sephadex column and centrifugation was repeated as before, using a clean test tube to collect the effluent. Under these conditions, the volume of the collected fraction varied from 90 to 110 µl and the recovery of proteins such as F0, bovine serum albumin, and hemoglobin was 98 to 100%. Because the recovery of protein was thus reproducibly high, it sufficed for many purposes to collect the effluent, containing protein and bound radioactive ligand, directly in a scintillation vial and to measure the radioactivity of the entire sample. Alternatively, the volume of the effluent could be determined by weighing and appropriate aliquots were taken for measurement of radioactivity and protein.

The ability of the centrifuge column procedure to separate free from protein-bound ligand is illustrated in Table I. In Experiment 1, 100 µl of reaction mixture applied to the column in Step 2 contained 384,000 cpm of 32P. When the reaction mixture was complete (Line 1), 8228 cpm of 32P, were associated with the protein in the column effluent. If EDTA was substituted for Mg2+ or if F0, was omitted, the observed radioactivity was at or slightly above background (Lines 2 and 3). In Experiment 2, 3.5 x 106 cpm were applied to the column in Step 2. Under these conditions, 174,450 cpm of 32P were associated with the enzyme in the column effluent (Line 1). In Lines 2 and 3 it may be seen that the radioactivity in the effluents of the control samples was suitably low. These residual low values of radioactivity were subtracted from experimental values before calculating P0, binding data which is presented in the figures and tables. Separations of free from protein-bound ligand of the kind illustrated in Table I were always observed when 50- to 100-µl aliquots of reaction mixture were applied to the centrifuge columns in Step 2 and similar separations were usually observed when the volume of reaction mixture was as large as 200 µl. Above 200 µl, however, considerable amounts of apparently unbound 32P, appeared in the protein fraction.

Fig. 1 shows a comparison of ligand binding to F0, as measured by the Paulus pressure dialysis cell (21) and the centrifuge technique. It may be seen that essentially the same results were obtained when binding of AMP-P(NH)2 by nucleotide-depleted F0, (8) was examined by the two methods.

Three observations regarding the properties of the centrifuge column are relevant to a discussion of the mode of separation of free ligand from protein. Direct measurement of the time of transit of F0, through the column, carried out with the aid of a device for automatic labeling of 100 µl of sample on the column while the rotor was revolving, indicated that the bulk of the protein emerged from the column in a volume of 10 to 20 µl about 30 s after application of the sample. An additional 80 to 90 µl of buffer followed during the next 60 to 90 s of centrifugation. Experiments in which the reaction mixture applied to the column in Step 2 contained a colored ion such as chromate also indicated that unbound ions were sequestered in the upper one-third of the column. The third observation was obtained from experiments in which the Sephadex used to fill the centrifuge column was pre-equilibrated with 32P, of known concentration. Application in Step 2 of 100 µl of water to such a column resulted, after centrifugation, in the appearance of 32P, in the column effluent of the same concentration as that originally equilibrated with the Sephadex.

These observations indicate that a considerable dehydration of the protein sample, accomplished by loss of most if not all unbound ions probably occurred in the upper one-third of the column. A concentrated protein sample emerged from the column followed by a volume of buffer which may have been held within the gel beads in the lower portion of the column. It would thus appear that the centrifuge column procedure differs in important ways from gel permeation chromatography as normally used.

In addition to its sensitivity and rapidity in ligand binding studies, the centrifuge column procedure thus exhibits two further features of interest. First, it may be used to exchange buffers in small protein samples with virtually no loss of protein. Second, it may be used to achieve a 5- to 10-fold concentration of protein in small samples and is particularly useful for purposes of gel electrophoresis. The concentration step was, however, accompanied by a loss of protein of 10 to 30%. A method of desalting proteins, very similar to the one presented here, has been described by Neal and Florini (22).
RESULTS

Two independent methods of analysis revealed that incubation of F₁ with ²³⁰P₀ resulted in binding of F₁ by the enzyme. It may be seen in Fig. 2A that significant binding occurred at concentrations of P₀ below 50 μM, as indicated by either the Paulus pressure dialysis cell or the centrifuge column. P₀ binding was slightly underestimated by the latter procedure. The affinity of F₁ for P₀ under the conditions of the experiment, equilibrium was reached in about 15 min. Because the rate of the binding step could be influenced by various additions to the reaction mixture, the enzyme was routinely incubated for 30 min with "2Pi as indicated by either the Paulus pressure dialysis cell or the centrifuge column. P₀ was added last and incubation was continued overnight at room temperature. At the end of the incubation period, 0.2-ml aliquots were transferred to the centrifuge columns for measurement of ligand binding as described under "Experimental Procedures." Paulus cell, ○—○; centrifuge column, □—□.

Because only a small fraction of the total radioactivity in the reaction mixtures of experiments such as those shown in Fig. 2 became associated with the protein, it was necessary to establish that "2Pi was in fact the ligand rather than a radioactive contaminant of the "²³³P₀ preparation. Several experiments were designed to rule out contamination. The first was an experiment to show that bound radioactive ligand was displaced from the enzyme by nonradioactive inorganic phosphate. Fig. 3 illustrates a study of the rate at which radioactivity was bound to and released from F₁. It may be seen (Curve 2) that, when under the conditions of the experiment, equilibrium was reached in about 15 min. Because the rate of the binding step could be influenced by various additions to the reaction mixture, the enzyme was routinely incubated for 30 min with "²³³P₀ before measurements were made. The ATPase activity of F₁ was stable throughout the 30 min incubation.³ Curve 2 in Fig. 3 shows the rate at which radioactivity was displaced from F₁ following the addition of 5 mM P₀.

In order to obtain further support for the conclusion that only "²³³P₀ was bound to F₁, in those experiments, sample of F₁ were incubated with "²³³P₀ and buffer and then passed through centrifuge columns as described under "Experimental Procedures.

³ M. Kasahara and H. S. Penefsky, unpublished experiments.
dure." The effluent, which contained F₁ and associated radioactivity, was denatured with perchloric acid, treated with molybdate, and extracted with isobutyl alcohol/benzene as described by Lindberg and Ernster (24). At least 97% of the radioactivity appeared in the organic phase. A third experiment, not reported here, in which increasing amounts of Pᵢ were added to reaction mixtures containing F₁ and a constant amount of ³²Pᵢ, demonstrated that the ratio of mol of Pᵢ/mol of F₁ was independent of the specific radioactivity of the ³²Pᵢ over a 40-fold range. These properties of the radioactivity, which became associated with F₁ during the incubation, strongly support the conclusion that the radioactive ligand was indeed ³²Pᵢ.

Fig. 3 also demonstrates that Pᵢ binding to F₁ was reversible. Addition of a 12.5-fold molar excess of Pᵢ to a reaction mixture at binding equilibrium (Curve 2) led, after 48 min of incubation, to a 12-fold decrease in binding of ³²Pᵢ by the enzyme. It may also be seen in Fig. 3 that the half-time of the binding step (about 0.5 min) was considerably more rapid than that of the displacement step (about 2 min). A second test for the reversibility of Pᵢ binding by F₁ made use of an experimental design originally described by Wetlauffer and Stahmann (25) (Table II). In Line 1 of the table, the ratio of Pᵢ bound to F₁ was 0.1 mol of Pᵢ/mol of F₁ when 4.2 μM F₁ and 24 μM Pᵢ were incubated for 30 min (Control) and 0.16 mol of Pᵢ/mol of F₁ when 8.4 μM F₁ was incubated for 30 min with 48 μM Pᵢ (Experimental). Following the dilution of the Experimental reaction mixture with an equal volume of buffer, the ratio of Pᵢ bound to F₁ quickly returned to that of the undiluted control values (Lines 2 to 4).

The proportionality between Pᵢ binding and the amount of F₁ protein added to the reaction mixture is illustrated in Fig. 4. It will be evident from experiments described below that the concentration of ³²Pᵢ used in this experiment, 400 μM, was sufficient to saturate, or nearly saturate, the enzyme binding site at all concentrations of F₁ employed. Good linearity was obtained over a 17-fold range of protein concentration.

Specific anions also exerted a marked influence on Pᵢ binding by F₁. It may be seen in Table III that in a reaction mixture which contained only 5 μM F₁, 100 mM Tris/acetate, pH 7.5, and 44 μM F₁, the ratio of Pᵢ bound to F₁ was 0.094 to 0.037 mol/mol of protein. However, addition of Na₂SO₄ increased the

![Fig. 4. Proportionality between Pᵢ binding and the protein content of the reaction mixture.](http://www.jbc.org/)

**Fig. 4. Proportionality between Pᵢ binding and the protein content of the reaction mixture.** The reaction mixture contained 100 mM Tris/acetate (pH 7.5), 400 μM Pᵢ (1.2 × 10⁶ cpm of ³²Pᵢ), 0.4 mM MgSO₄, 1 mM K₂CrO₇, and F₁, as indicated in a final volume of 125 μl. The reaction was initiated by adding F₁ and incubation was continued at room temperature for 30 min. Aliquots of 100 μl were withdrawn and added to the tops of centrifuge columns for measurement of Pᵢ binding as described under "Experimental Procedures." The point corresponding to zero protein was obtained by incubating a reaction mixture without F₁. The total radioactivity found in the column effluent for this sample (54 cpm) was subtracted from the other values before calculation of Pᵢ bound to enzyme. The abscissa indicates total nanomoles of F₁ in the reaction mixture. The ordinate indicates total nanomoles of ³²Pᵢ bound to protein in the reaction mixture.

**TABLE III**

| Experiment | Additions | Pᵢ/F₁ |
|------------|-----------|-------|
|            | mM        | mol/mol |
| 1          | None      | 0.034 |
| Na₂SO₄     | 0.2       | 0.058 |
| 0.4        | 0.060     |
| 0.8        | 0.096     |
| 4          | 0.114     |
| 2          | None      | 0.037 |
| Na₂SO₄     | 2         | 0.102 |
| K₂CrO₇     | 0.5       | 0.142 |
| 2          | 0.150     |
| K₂SO₄      | 8         | 0.104 |
| Na₂BO₄     | 0.5       | 0.031 |
| 2          | 0.106     |
| NaHCO₃     | 1.6       | 0.028 |
| 24         | 0.021     |
| KCl        | 8         | 0.035 |
| NaCl       | 8         | 0.026 |

**TABLE II**

| Reversibility of Pᵢ binding to F₁ |
|-----------------------------------|
|                                    |
| The reaction mixture for the control (no dilution step) contained 100 mM Tris/acetate (pH 7.5), 0.8 mM MgSO₄, 4.2 μM F₁, and 24 μM Pᵢ (6.1 × 10⁶ cpm of ³²Pᵢ) in a final volume of 37 ml. The reaction mixture for the experimental was identical with that for the control except that the F₁ concentration was 8.4 μM and the concentration of Pᵢ was 49 μM. F₁ was added last and the reaction mixtures were incubated at room temperature for 30 min. At 30 min, aliquots of 80 μl were removed and applied to the tops of centrifuge columns for measurement of Pᵢ binding as described under "Experimental Procedures." The results obtained are designated "Before dilution." An aliquot of 250 μl was then withdrawn from the Experimental reaction mixture and added to 250 μl of a solution containing 100 mM Tris/acetate (pH 7.5), and 0.8 mM MgSO₄. The dilution thus reduced the concentrations of Pᵢ and F₁ to those of the Control reaction mixture. Pᵢ binding was measured on an 80-μl aliquot 15 s after dilution. Additional aliquots of 80 μl were analysed at 15 and 30 min after dilution.

| Time       | Control | Experimental | F₁/M | Pᵢ/F₁ |
|------------|---------|--------------|------|-------|
| "Before dilution" |          |              |      |       |
| [μM]       | [mol/mol] | [μM]       | [mol/mol] |       |
| 0.032      | 0.335   | 0.10        | 0.107 | 0.67  | 0.16  |
| After dilution of |          |              |      |       |
| "Experimental" |          |              |      |       |
| [μM]       | [mol/mol] | [μM]       | [mol/mol] |       |
| 0.032      | 0.335   | 0.10        | 0.093 | 0.335 | 0.11  |
| 0.032      | 0.335   | 0.095       | 0.037 | 0.335 | 0.11  |
Experimental Procedures. The pH was determined in a separate 100-μl aliquots were withdrawn and transferred to the tops of centrifuge columns for measurement of P$i$ binding as described under "Experimental Procedures." The pH was determined in a separate series of tubes which were identical except that $^{32}$P$i$ was replaced by water.

It was established that the hydrolysis of adenine nucleotides by F$i$ was dependent on the addition of divalent metal ions such as Mg$^{2+}$, Mn$^{2+}$ or Co$^{2+}$ (1), although hydrolysis of ATP, at very low ratios, occurred even in the presence of EDTA (28). In addition, the affinity of at least one binding site for ADP on beef heart F$i$ was greater in the presence of Mg$^{2+}$, 0.28 μM, than in the presence of EDTA, 11 μM (11). It was found that EDTA also reduced the binding of P$i$ by F$i$ (Fig. 6). Incubation of F$i$ with 400 μM P$i$ and 1 mM chromate led to a binding ratio of 0.48 mol of P$i$/mol of F$i$. Addition of EDTA resulted in a decrease in the ratio. Virtually no binding of P$i$ to F$i$ was observed at concentrations of EDTA greater than about 1 mM and, at 4 mM EDTA, the radioactivity in the effluent of the centrifuge column binding method ("Experimental Procedures") did not differ significantly from the radioactivity observed when F$i$ was omitted from the reaction mixture.

Direct evidence for the participation of a divalent metal ion in the binding of P$i$ to F$i$ was demonstrated at lower concentrations of P$i$. It may be seen in Fig. 7 that incubation of 4.1 μM F$i$ with 32 μM P$i$ and 1 mM chromate resulted in a binding ratio of 0.11 mol of P$i$/mol of F$i$. Addition of magnesium acetate to the reaction mixture raised the binding ratio about 2-fold to a value of 0.26. It is significant that no more than 10 μM Mg$^{2+}$ was sufficient to satisfy the requirement of the system for a metal ion. Cobalt and calcium appeared to be less effective than Mg$^{2+}$, at least with regard to the maximum observed binding ratio, over the range of concentrations tested. Mn$^{2+}$ also stimulated P$i$ binding by F$i$. However, the effects were variable and difficult to reproduce. Whereas the stimulation of P$i$ binding by metals was readily observed at low concentrations of F$i$, the stimulation was considerably smaller at higher ligand concentrations. In view of the fact that 10 μM, or less, metal ion concentrations produced maximal binding, it

![Figure 5](http://www.jbc.org/)

**Fig. 5 (left).** Effect of pH on P$i$ binding to F$i$. The reaction mixture in A contained 100 mM Tris/acetate of the pH indicated, 60 μM P$i$ (3.2 × 10$^6$ cpm of $^{32}$P$i$), 1 mM MgSO$_4$, and 5.2 μM F$i$. The reaction mixture in B contained 100 mM Tris/acetate of the pH shown, P$i$ of the concentration indicated, 2.26 × 10$^6$ cpm of $^{32}$P$i$, and 4.2 μM F$i$ (pH 7.5, ▲, pH 6, ●). The final volume was 125 μl for all reaction mixtures. F$i$ was added last and incubation was continued for 30 min at room temperature. At the end of the incubation period, 100-μl aliquots were withdrawn and transferred to the tops of centrifuge columns for measurement of P$i$ binding as described under "Experimental Procedures." The pH was determined in a separate
seemed probable that low level metal ion contamination of
both the enzyme solution and other reagents accounted for the
absence of a complete dependency on metals for $P_i$ binding as
well as for the reduced stimulation at high ligand concentra-
tions.

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Fig. 7. Stimulation of $P_i$ binding to $F_i$ by divalent metal ions. The
reaction mixture contained 100 mM Tris/acetate (pH 7.5), $32 \mu M$ $P_i$
($1.16 \times 10^6$ cpm of $^{32}P_i$), 1 mM $K_2CrO_7$, 4.1 $\mu M$ $F_i$, and the acetate salts
of the metals indicated in a final volume of 125 $\mu l$. The reaction was
initiated by adding $F_i$ and incubation was continued for 30 min at
room temperature. At the end of the incubation period, 100-$\mu l$
aliquots were withdrawn from each reaction mixture and transferred to
the tops of centrifuge columns for measurement of $P_i$ binding as
described under "Experimental Procedures." Solutions of cobalt ace-
tate were prepared fresh from the dry salt for each experiment.
Magnesium acetate, $\square$; cobalt acetate, $\triangle$; calcium acetate,
$\Delta$.

Fig. 8. The effects of aurovertin and 2,4-dinitrophenol (DNP) on
$P_i$ binding by $F_i$. A, the reaction mixtures contained 100 mM Tris/
acetate (pH 7.5), $55 \mu M$ $P_i$ ($3.6 \times 10^6$ cpm of $^{32}P_i$), 1 mM $MgSO_4$, 10 $\mu M$
$F_i$, and aurovertin or 2,4-dinitrophenol as shown in a final volume of
0.1 ml. Aurovertin was added from a concentrated solution in metha-
nol. The final concentration of methanol in each experiment was 0.25
mm. The reactions were initiated by adding $F_i$ and incubation was
continued for 30 min at room temperature. At the end of the incuba-
tion period, aliquots of 50 $\mu l$ were removed and transferred to centri-
fuge columns for measurement of ligand binding as described under
"Experimental Procedures." B, Scatchard-type plots (23) of $P_i$ bind-
tate were prepared fresh from the dry salt for each experiment.

Fig. 9. Inhibition of $P_i$ binding to $F_i$ by adenine nucleotides. The
reaction mixtures contained 100 mM Tris/acetate (pH 7.5), 1
mM $MgSO_4$, 1 $\mu M$ $F_i$, 24 $\mu M$ $P_i$ ($2.14 \times 10^6$ cpm $^{32}P_i$), and, where
indicated, 48 $\mu M$ ADP or 48 $\mu M$ ADP and $6.07 \times 10^6$ cpm of $[^3H]ADP$
in a final volume of 125 $\mu l$. Aurovertin was added from a concen-
trated solution in methanol. The concentration of methanol in the
reaction mixture, 0.25 mm, did not influence $P_i$ or ADP binding.
Reaction mixtures in B contained $P_i$ but no radioactivity whereas all
$P_i$ was omitted from C. The reactions were started by adding $F_i$
and incubation was continued for 20 min at room temperature. At the
end of the incubation period, 100-$\mu l$ aliquots were transferred to the
tops of centrifuge columns for measurement of ligand binding as
described under "Experimental Procedures."

TABLE IV
Prevention of ADP inhibition of $P_i$ binding to $F_i$ by aurovertin

| Additions | $P_i/P_i/F_i$ | ADP/$P_i/F_i$ |
|-----------|--------------|---------------|
| A. Complete | 0.115 | 0.344 |
| $+17 \mu M$ aurovertin | 0.91 | 1.88 |
| B. $-30P_i + 48 \mu M [^3H]ADP$ | 1.90 | 1.93 |
| $-30P_i + 48 \mu M [^3H]ADP + 17 \mu M aurovertin$ | | |
| C. $-P_i + 48 \mu M [^3H]ADP$ | 0.056 | 0.354 |
| $-P_i + 48 \mu M [^3H]ADP + 17 \mu M aurovertin$ | | |

$P_i$ binding to $F_i$ was influenced by a variety of substances
which are known to exert profound effects on the ATPase
activity of $F_i$ or on energy coupling reactions or both. The
effects of aurovertin and 2,4-dinitrophenol are shown in Fig.
8A. It may be seen that both compounds enhanced $P_i$ binding
by $F_i$, although aurovertin was considerably more effective. In
order to determine the mode of the enhancement of $P_i$ binding
by $F_i$, titrations were carried out, similar to those shown in
Fig. 2, in the absence and in the presence of aurovertin. The
results in the presence of aurovertin are presented in the form
of a Scatchard-type (23) plot in Fig. 8B. F<sub>i</sub> exhibited only one Pi binding site/molecule whether in the absence or the presence of aurovertin. However, whereas the K<sub>0</sub> in the absence of aurovertin was 60 μM (Fig. 2), in the presence of aurovertin the K<sub>0</sub> was 20 μM.

Adenine nucleotides partially inhibited Pi binding by F<sub>i</sub>. In Fig. 9 it is shown that ATP was considerably less effective than the ATP analog AMP-P(NH)P. Approximately 50% of the total observable inhibition occurred at an ATP concentration of 45 μM, whereas only 4 μM AMP-P(NH)P was sufficient to produce 50% inhibition. Moreover, the extent of the inhibition also was greater with the analog (Fig. 9). ADP appeared to be about as effective an inhibitor as ATP under these experimental conditions.

The inhibition of Pi binding by ADP was prevented when aurovertin was included in the reaction mixture (Table IV). It may be seen in A that aurovertin stimulated Pi binding under the conditions of the experiment. Moreover, in the presence of Mg<sup>2+</sup> aurovertin was without effect on ADP binding to the enzyme, either in the absence of Pi, as shown earlier with rat liver F<sub>i</sub> (29) or in the presence of Pi (B). In the presence of 17 μM aurovertin, however, ADP was completely without effect on Pi binding by F<sub>i</sub> (D).

Although arsenate has long been recognized as an inhibitor of oxidative phosphorylation (30), the mechanism of the inhibition remains unclear. Because arsenate is an analog of Pi, it was of considerable interest to determine if arsenate and Pi competed for a binding site on F<sub>i</sub>. The results of a competition type experiment are shown in Fig. 10. At low concentrations of Pi, 40 μM (Curve A), arsenate inhibited Pi binding. The maximum inhibition observed under these conditions (84%) occurred at 4.6 mM arsenate. However, when Pi binding to F<sub>i</sub> was measured in the presence of 2 mM Pi (Curve B, Fig. 10), the same concentrations of arsenate were considerably less effective as inhibitor. The inhibition observed with 4.8 mM arsenate in this case was 12%.

**DISCUSSION**

The central finding in this study was the observation that F<sub>i</sub> exhibited a single binding site for Pi. The binding was reversible and characterized by a dissociation constant at pH 7.5 of 80 μM. It should be emphasized that the dissociation constants were calculated on the assumption that all of the Pi in the reaction mixture was capable of binding to F<sub>i</sub>. If the binding species was only one of the two major charged forms of Pi, at pH 7.5, the correct dissociation constant would be about one-half that given here. The strong pH dependency of Pi binding (Fig. 5) suggests, moreover, that only one ionic species of Pi participated in the binding reaction, that a specific ionic group on the protein was being titrated or that both effects were important. These values obtained with F<sub>i</sub> may be compared with a dissociation constant of 1.5 mM estimated for the myosin·Pi complex at pH 8 from analysis of ATP displacement kinetics (31). The F<sub>i</sub>-phosphate complex described in this paper also should be distinguished from the acyl phosphate compounds which could be formed from Pi and the (Na<sup>+</sup>-K<sup>+</sup>)-ATPase (32) and from Pi and the sarcoplasmic reticulum ATPase (33).

Considerable experimental evidence indicated that agents which influenced oxidative phosphorylation or ATPase activity, or both, also influenced Pi binding by F<sub>i</sub>. A summary comparing the effects on Pi binding and ATPase of most of the agents examined in this study is presented in Table V. Table V. Thus, at low concentrations of Pi, aurovertin enhanced Pi binding by F<sub>i</sub> (Table V and Fig. 8). The enhancement by aurovertin reflected an increased affinity of F<sub>i</sub> for Pi which was induced by the antibiotic since only one binding site for Pi was observed while the dissociation constant decreased from 80 to 20 μM.

Several anions enhanced Pi binding by F<sub>i</sub>, at low concentrations of Pi. These included SO<sub>4</sub><sup>2-</sup>, SO<sub>3</sub><sup>2-</sup>, and chromate. The latter appeared to be most effective. Bicarbonate, which stimulated ATPase activity of beef heart F<sub>i</sub> (26) to a greater extent than chromate (27) appeared to be without effect on Pi binding even at concentrations of 24 mM (Table III). The mode of enhancement of Pi binding by dinitrophenol was not determined, although in this connection it is relevant to point out that dinitrophenol stimulated the ATPase activity of preparations of F<sub>i</sub>, derived from a number of tissues (34) and that
several weak binding sites for this uncoupler were present on
the beef heart enzyme (35). Since uncouplers such as carbonyl
cyanide p-trifluoromethoxyphenylhydrazone were without effect
on 
binding under the conditions used for the experiment of
Fig. 8, it seems probable that the dinitrophenol effect was
similar to that of other anions and unrelated to its activity as
an uncoupler.
Sulfhydryl reagents such as N-ethylmaleimide, iodoacetic
acid, and mercurial as well as reducing agents such as dithio-
reitol were without effect on P binding to F, whether prein-
cubated with the enzyme alone or included in the reaction
mixture for P binding. On the other hand, inhibitors of F,
ATPase activity inhibited P binding (Table V). Thus, batho-
phosphanthroline, which was a poor inhibitor of ATPase activity
(36), also did not influence P binding whereas the 1:1 nickel-
bathophosphanthroline complex, which inhibited ATP-
ase 50% at a concentration of about 2 \( \mu M \) (36), inhibited P
binding 50% at a concentration of 10 \( \mu M \). The protein ATPase
inhibitor of Pullman and Monroy (15) also inhibited P binding
(Table V). Incubation of the inhibitor with F, under conditions
which led to 50% inhibition of ATPase activity also led to a
50% reduction in P binding by the enzyme.

The observation that P binding was inhibited by EDTA
(Table V and Fig. 6) suggested participation of divalent metal
ions in the binding reaction. However, it was not at all clear
that EDTA inhibited P binding by chelating metal ions free in
solution since measurements using the centrifuge column pro-
cedure under the conditions of Fig. 6 indicated that \( ^{14} \)CJEDTA
was bound by F,. The chelator may thus have interfered with
P binding through steric or other effects. The possibility that
EDTA might bind to F, was raised by Adolfson et al. (28).

Stimulation of P binding by Mg\(^{2+}\), Ca\(^{2+}\), and Co\(^{2+}\), Fig. 7, did
however provide direct support for the conclusion that a metal
ion was an important component of the binding reaction. In
view of the fact that 5 to 10 \( \mu M \) metal ion concentrations
provided maximal binding at a P concentration of 32 \( \mu M \) and
of the dissociation constant of the Mg\(^{2+}\) and Ca\(^{2+}\)-phosphate
complexes, about 5 \( \mu M \) (37), it seems likely that a metal-protein complex rather than a metal-phosphate complex
was the participating species in the binding reaction.

Adenine nucleotides formed an additional class of com-
ounds of interest with regard to P binding by F, (Table V).
The method of preparing F, for these binding studies produced
samples of the enzyme in which the two sites which engaged in
readily reversible binding were apparently unoccupied ("Ex-
perimental Procedures"). Thus, incubation of F, with 48 \( \mu M \)
\([\text{H}]\)ADP resulted in binding of almost 2 mol of ADP/mol of F,
(Table III) as shown previously (8, 11). Incubation of F, with P,
and ATP, ADP, or AMP-P(NH)P resulted in dramatic reduc-
tions in P binding (Fig. 9). The observation that ATP and
ADP were equally effective may well reflect the fact that the
large excess of enzyme added quickly converted ATP to ADP in
the reaction mixture. However, AMP-P(NH)P which was not
hydrolyzed by F, (38) was a particularly effective inhibitor.

Several considerations suggest that the observed interac-
tions of P, with F, occurred at a specific binding site. Thus, no
more than one site/molecule was detected under a variety of
experimental conditions. It would seem that binding also did
not occur at possible anion binding sites in which dinitrophenol
was bound (35) since dinitrophenol and selected anions
enhanced P binding by F,. More than one site of interaction of
P, with F, however, was detected in studies with nucleotide-
depleted F, (8). In these experiments, P, binding did not
approach saturation even at high concentrations of ligand and it
appeared that denaturation of the protein, which occurred readily in the complete absence of ATP or other stabilizing
agents, exposed new and nonspecific anion binding sites on the
molecule.

One of the most interesting aspects of P, binding to F, lies in
the possibility that the P, binding site is the site to which P,
binds for ATP synthesis in oxidative phosphorylation. In view
of the likelihood that F, does participate in the terminal transphosphorylation reaction of oxidative phosphorylation
(2), it was perhaps to be expected that the enzyme would
exhibit such a site. It is, however, conceivable that F, binds to
another component of the oligomycin-sensitive ATPase complex (39). Thus, the observations presented in this paper,
that a variety of agents which modulate ATPase and oxidative
phosphorylation also modulate P, binding, lend support to but
do not establish the proposal that F, is the P, binding protein
of oxidative phosphorylation. If the P, site does indeed partici-
participate in oxidative phosphorylation, the experiments with
arsenate could provide an explanation for the mechanism of uncou-
pling of oxidative phosphorylation by this compound. Arsenate
uncoupling could be expressed via a competition with P, for a
binding site on F, This suggestion is consistent with the
conclusions of Mitchell et al. (40) which were based on other
kinds of evidence. The fact that high concentrations of arse-
enate were needed to inhibit P, binding to F, appreciably is
consistent with observations that 50% inhibition of the ADP/O
ratio in mitochondria required molar ratios of arsenate to P, of
4 to 10 (40, 41).

The relationship between the P, binding site and other
known binding sites on F, is a matter of considerable interest.
It is possible that P, was bound in a nucleotide binding site. It
is not ruled out, however, that binding occurred in a distant
site since the enhancement and inhibition of binding caused
by a variety of agents which altered ATPase activity could
have been a reflection of allosteric or other effects. If P, did
bind in a nucleotide binding site, a distinction would have to
be made between binding in the position occupied by the
phosphate of ATP and in an ADP subsite. The observation that
ADP was completely without effect on P, binding in the
presence of aurovertin, even though the two readily reversible
nucleotide binding sites were virtually completely filled with
ADP, argues against P, binding in an ADP subsite. Further-
more, the fact that AMP-P(NH)P was a far more effective
inhibitor of P, binding than ADP raises the possibility that the
P, binding site was in fact in the position occupied by the
phosphate group of ATP. These points are currently under
investigation.

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