THE EFFECTS OF VANADATE AND ARSENATE ON MITOCHONDRIAL ATP SYNTHASE

by

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B.Sc. Simon Fraser University 1984

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
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"The Effects of Vanadate and Arsenate on Mitochondrial ATP Synthase"

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ABSTRACT

The effect of vanadate on beef heart mitochondrial ATP synthase was studied. The studies included the effect of vanadate and other anions on: ATP synthesis catalyzed by submitochondrial particles (SMP); ATP hydrolysis catalyzed by SMP and by soluble coupling factor 1 (F1); and the binding of [32P]phosphate to F1.

The pattern of inhibition of ATP synthesis by vanadate indicated mixed inhibition with a $K_i$ of 0.18 mM and a $K_i'$ of 0.8 mM.

Vanadate inhibited ATP hydrolysis catalyzed by SMP. So also did pyrophosphate and potassium chloride but these latter required much greater concentrations for the same effect.

Phosphate, arsenate and possibly molybdate accelerated ATP hydrolysis.

Vanadate also inhibited ATP hydrolysis catalyzed by F1. Phosphate and pyrophosphate both accelerated hydrolysis.

Vanadate inhibited the binding of [32P]phosphate to F1 much more effectively than did arsenate. The data for arsenate suggested that the binding of arsenate and phosphate was mutually exclusive whereas the data for vanadate required a model in which vanadate and phosphate could bind simultaneously.

Vanadate apparently inhibits by stabilizing the enzyme in the conformation which binds the transition state (i.e. pentacoordinate phosphate).
Pyrophosphate inhibited SMP-catalyzed ATP hydrolysis and SMP-catalyzed ATP synthesis. In both processes, 21 mM pyrophosphate caused total inhibition of the enzyme. At least part of the inhibition resulted from the chelation of Mg$^{2+}$ by phosphate, thus depleting MgATP$^{2-}$ in hydrolysis and MgADP$^{-}$ in synthesis.

Pyrophosphate initially activated F$_i$-catalyzed ATP hydrolysis 3.5 fold, but this activation continuously decreased with time. This same pattern was seen with phosphate, but the activation by phosphate was much weaker.

The activating effects of phosphate and pyrophosphate are explained in terms of: the binding change mechanism; the decrease in chloride ion concentration (KCl being used to maintain constant ionic strength); and the effect of bound inhibitory ADP.
I thank Dr. Michael Gresser, my Senior Supervisor, for his guidance and inspiration. I thank my father, Dr. George Bramhall, for his many hours of assistance in typing and editing this thesis. I thank Marcia Craig and Dr. Paul Stankiewicz for many stimulating discussions. I also thank the rest of our research group: Seeloch Beharry, Mildred Johnson, Tania Kastelic and Kevin Doherty whose teamwork in the isolation of the mitochondria made this research possible.
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ABBREVIATIONS

ADP

ADPV

AMP-PNP

ATP

AP₅A

BSA

EDTA

Fo

F₁

G6PDH

HK

I

IP

Kᵢ

Kᵢₜ

LDH

NAD⁺/NADH

NADP⁺/NADPH

PEP

³²Pi

Pi/Asi/Vi

PK

SMP

adenosine 5’-diphosphate

adenosine 5’-diphosphovanadate

5’-adenyllyl-β,γ-imidodiphosphate

adenosine 5’-triphosphate

P',P²-diadenosine pentaphosphate

bovine serum albumin

ethylene diamine tetracetic acid

proton channel

soluble coupling factor 1

glucose 6-phosphate dehydrogenase

hexokinase

inhibitor

inhibitor protein (naturally occurring endogenous to the ATP synthetase complex).

the dissociation constant of the enzyme-inhibitor complex.  \( Kᵢ = [IE]/[I][E] \)

concentration of substrate which gives half maximal velocity

lactate dehydrogenase

nicotinamide adenine dinucleotide/reduced form

nicotinamide adenine dinucleotide phosphate/reduced form

phosphoenol pyruvate

\(^{32}\)Pphosphate (ortho)

inorganic phosphate/inorganic arsenate/inorganic vanadate

pyruvate kinase

submitochondrial particles
<table>
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<tr>
<td>Tris-Cl</td>
<td>tris(hydroxymethyl)aminomethane chloride</td>
</tr>
<tr>
<td>Tris-OAc</td>
<td>tris(hydroxymethyl)aminomethane acetate</td>
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<tr>
<td>v</td>
<td>velocity</td>
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<tr>
<td>[45V]</td>
<td>[45V]vanadate (ortho)</td>
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<tr>
<td>V_s</td>
<td>the velocity at saturating substrate concentration</td>
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CHEMICALS

Acetic acid Fisher Scientific
Adenosine 5'-diphosphate sodium salt, from equine muscle, Grade IX Sigma Chemicals
Adenosine 5'-triphosphate disodium salt, from equine muscle, Grade IX Sigma Chemicals
Ammonium hydroxide American Scientific and Chemical
Ammonium sulfate (special enzyme grade) Canadian Scientific Products
Arsenic acid monopotassium salt Sigma Chemicals
Bovine serum albumin Sigma Chemicals
DEAE-Sephadex G-50-120 Pharmacia Fine Chemicals
5’-diadenosine pentaphosphate Sigma Chemicals
P’P’s-di(adenosine-5’)-pentaphosphate lithium salt Boehringer Mannheim
Ethylene diamine tetracetic acid British Drug Houses (Canada)
Glucose 6-phosphate dehydrogenase (from yeast) ammonium sulfate suspension Grade I Boehringer Mannheim
Hexokinase (Type c-300 from yeast) Sigma Chemicals
L-lactate dehydrogenase (from rabbit muscle) ammonium sulfate suspension Boehringer Mannheim
Magnesium chloride American Scientific and Chemical
Magnesium sulfate Matheson Coleman and Bell
β-nicotinamide adenine dinucleotide phosphate monosodium salt Sigma Chemicals
β-nicotinamide adenine dinucleotide (reduced form, Grade III from yeast) Sigma Chemicals
³²Phosphorus labelled orthophosphate Amersham Radiochemicals
Phosphoenol pyruvate monopotassium salt Boehringer Mannheim
Potassium chromate K₂CrO₇ British Drug Houses (Canada)
Potassium phosphate monobasic $\text{KH}_2\text{PO}_4$
American Scientific and Chemical

Potassium phosphate dibasic $\text{K}_2\text{HPO}_4$
American Scientific and Chemical

Pyrophosphate, tetrascodium $\text{Na}_4\text{P}_2\text{O}_7$
Sigma Chemicals

Pyruvate kinase (from rabbit muscle) Type III
Sigma Chemicals

Rotenone
Sigma Chemicals

Sephadex Fine
Pharmacia Fine Chemicals

Sodium molybdate $\text{Na}_2\text{MoO}_4$
Allied Chemical

Succinic acid disodium salt
Sigma Chemicals

Sucrose Grade 1 crystalline
Sigma Chemicals

Tris(hydroxymethyl)aminomethane
Sigma Chemicals

Vanadium oxide ($\text{V}_2\text{O}_5$)
Aldrich Chemical Company
THE EFFECTS OF VANADATE AND ARSENATE ON MITOCHONDRIAL ATP SYNTHASE

INTRODUCTION

ATP synthase is an enzyme complex which catalyzes the synthesis of ATP from ADP and orthophosphate (Pi). It is located in the inner membrane of the mitochondria. Mitochondria are subcellular organelles consisting of two membranes. The outer membrane is permeable to solutes of molecular weight less than 10,000 (1). It contains several enzymes. The inner membrane is impermeable to nearly all ions and to most uncharged molecules. It contains the enzyme systems required for the transport of ions, substrates and nucleotides, the enzymes required for oxidative phosphorylation as well as pyridine nucleotide transhydrogenase and ß-hydroxybutyrate dehydrogenase (2).

The main function of mitochondria is oxidative phosphorylation. They contain the enzymes of the four respiratory complexes which comprise the electron transport chain. The transfer of electrons along this chain results in the pumping of protons out of the mitochondrial matrix, thus lowering the matrix proton concentration and forming both a proton concentration gradient and an electrical potential (negative inside) across the inner mitochondrial membrane. The flow of protons down this electrochemical potential gradient drives the formation of ATP by ATP synthase.

Submitochondrial particles (SMP) are formed by sonicating
mitochondria. They have an "inside-out" orientation with respect to intact mitochondria such that the ATP synthase complex protrudes outward instead of inward. SMP catalyze both ATP synthesis and ATP hydrolysis. Experiments using SMP permit access to the substrate binding sites of both the ATP synthase complex and the respiratory chain (3). Because of the association of a variety of protein complexes with the inner membrane and the association of an intrinsic inhibitor protein (IP) (4) with ATP synthase, this system is very complex and thus the results are difficult to analyze.

ATP synthase contains two main components, F₀ and F₁. F₀ is the proton channel. F₁, coupling factor 1, is the catalytic portion. For ATP synthesis to occur both components must be present since energy-releasing transport of protons down the electrochemical gradient must be coupled to energetically unfavorable synthesis of ATP from ADP and Pi. The mechanism by which the flow of protons through F₀ results in ATP synthesis by F₁ has not yet been determined.

F₁ can be detached from F₀. It is water-soluble and cold-labile. It has a molecular weight of approximately 347 000 (5). F₁, isolated by methods used here, likely has three moles of bound nucleotide per mole, two bound at non-catalytic sites, and one at a catalytic site (6). Soluble F₁ catalyzes ATP hydrolysis, i.e. the reversal of ATP synthesis (7) since this is an energetically favorable reaction. F₁ isolated from chloroplast (8,9) and from thermophilic bacteria PS3 (10) catalyzes ATP synthesis. But
even under thermodynamically favorable conditions, ATP synthesis catalyzed by soluble mitochondrial F₁ has been observed only in the presence of dimethylsulfoxide (11-13). Using isolated F₁, one can study the effects of substrates and their analogs on F₁-catalyzed reactions and on the binding ability of F₁. The results of these studies may help to elucidate the mechanism of ATP synthesis.

Studies of F₁-catalyzed ATP hydrolysis have determined the turnover time to be approximately 5 milliseconds (14). They also showed that the net rate of single-site catalysis is limited by the rate of product release (7).

Studies of phosphate bound to F₁ were initiated by Penefsky (14). If F₁ is incubated with ^32Pi under appropriate conditions, the molar binding ratio (^32Pi/F₁) is close to 2 (15).

Studies by Hutton and Boyer (16) showed that the presence of ATP or ADP in the reaction mixture results in release of previously bound phosphate from F₁.

Studies of the nucleotide substrate analog AMP-PNP have revealed that F₁ has six nucleotide binding sites, three exchangeable and three non-exchangeable (17), and that there is cooperativity between the three exchangeable sites (7).

Studies with phosphate substrate analogs have been less rigorous. Beef heart mitochondrial F₁ exhibits two types of phosphate binding: nonsaturable (loose) binding, and high affinity (tight) binding. The tight binding represents a single site, likely the site occupied by the γ-phosphate of ATP.
(14). $F_i$ appears to bind the monovalent ion. Phosphate binding and ATP hydrolysis by $F_i$ are influenced by specific anions, cations, adenine nucleotides and other substances (14, 15, 18). Though it has been shown that arsenate inhibits the binding of phosphate to $F_i$ (14), there has been little study of the effects of arsenate or other phosphate analogs on the phosphate binding or ATP hydrolysis activity of $F_i$.

Arsenate is a phosphate analog which uncouples oxidative phosphorylation (19, 20). This uncoupling has been interpreted as resulting from SMP-catalyzed formation of ADP-arsenate which hydrolyzes non-enzymically much faster than does ATP (21, 22). The spontaneous formation of arsenate esters in solutions containing arsenate and compounds containing hydroxyl groups has been inferred because the compounds thus formed act as substrate analogs for enzymes which normally use the corresponding phosphorylated compounds as substrates (23-26). These arsenate esters hydrolyze non-enzymically several orders of magnitude faster than the corresponding phosphate esters.

Vanadate is another phosphate analog. As with arsenate, experimental evidence implies the spontaneous formation of vanadate esters in solutions containing vanadate and compounds containing hydroxyl groups (27). There is also evidence that vanadate undergoes some enzymic reactions comparable to those of phosphate and arsenate and their esters (28, 29). Vanadate esters hydrolyze several orders of magnitude faster than arsenate esters (29).
Vanadium may be an essential element of mammals (30,31). Though its function is still not known, it is involved in lipid metabolism (32), causes an insulin-like response in cells (33-37), and suppresses cholesterol biosynthesis (38). There is evidence that it may also prevent dental caries (39,40). Vanadium is essential to some marine life. In seawater, vanadate (V$^{5+}$) is the principal form of vanadium. Tunicates assimilate vanadium against more than a ten million fold concentration gradient to concentrations of the order of 1 M (41). There is evidence that it may function in tunicates as an anti-feedant. Other evidence suggests that it may have a biochemical role in the formation of the tunic shell (42).

The focus of this study is to determine the effect of vanadate on oxidative phosphorylation through its ability to act as a phosphate analog. The molecular mechanism of ATP formation by ATP synthase in oxidative phosphorylation is not yet known. Because the electronic structure of vanadate is similar to that of phosphate, and because vanadate is much more stable than phosphate in a pentacoordinate structure analogous to that of the phosphate transition state of phosphoryl transfer reactions (43,44), it is hoped that vanadate may serve as a useful probe into the mechanism of ATP formation.

The effects of vanadate on various aspects of the ATP synthase system must be determined before vanadate can be successfully used as a probe. The study of these effects forms the body of this thesis. The hypothesis that the
formation of ADP-vanadate causes these effects has been tested. The following facts suggest that this mechanism is probable: the electronic structure of the valence shell is similar to that of phosphate and arsenate (42); there is evidence for SMP-catalyzed formation of ADP-arsenate (21); and there is evidence that vanadate undergoes some of the enzymic and non-enzymic reactions undergone by phosphate and arsenate and their esters (28, 29). The effects of arsenate and pyrophosphate were also studied to assist in determining the mechanism by which vanadate produces its effects.
METHODS

Isolation of Beef Heart Mitochondrial Adenosine Triphosphate

Fresh beef hearts were obtained from J&L Meats Slaughterhouse in Surrey, British Columbia. The mitochondria were isolated as described by Smith (45). F₁ was isolated from both the light and heavy fractions of mitochondria as described by Knowles and Penefsky (46,47). The purified F₁ was stored at 4°C as a suspension in ammonium sulfate (48,49).

Enzyme Preparation

Both the F₁ and most of the commercially obtained enzymes were stored as an ammonium sulfate suspension at 4°C. In preparation for use, the ammonium sulfate suspension was desalted (i.e. the ammonium sulfate removed) by a procedure similar to that described by Penefsky (14). The required amount of ammonium sulfate suspension was measured into a capped 2 ml polyethylene microcentrifuge tube and centrifuged at 17 400 x g for 10 minutes at 4°C. The supernatant was decanted and the inner walls of the tube were dried with tissue. The pellet was dissolved in 50 mM Tris-OAc, pH 7.5 at 25°C. This solution was further desalted using a Penefsky Sephadex centrifuge column. The centrifugate was then diluted with 50 mM Tris-OAc, pH 7.5, to the desired concentration.
When $F_i$ was to be used to catalyze ATP hydrolysis, the centrifugate was resuspended in 50 mM Tris-OAc, 2 mM MgCl$_2$ and 0.2 mM EDTA.

Sephadex Centrifuge Column Technique

Sephadex was swollen in the same buffer as the sample, 5 grams Sephadex per 100 ml buffer. A porous polyethylene frit (7) was placed in the bottom of a 1 ml tuberculin syringe and the syringe was filled with Sephadex, placed in a 15-ml conical centrifuge tube, and allowed to stand until it no longer dripped. The column was centrifuged 1 minute at room temperature at setting 5 (approximately 1100 x g) of a table-top I.E.C. Model CL clinical centrifuge with a swinging bucket rotor No. 221. The column was transferred to a clean conical centrifuge tube. A loading tip was placed on the top of the column. (This was obtained from about 2 cm cut off a 1000 µl Eppendorf micropipette tip.) An 80-100 µl aliquot of the sample was pipetted into the loading tip and the column was centrifuged 1 minute as before. (Note: When desalting $F_i$, the procedure was the same except that the spins were 2 minutes each instead of 1 minute.)

ATP Hydrolysis Assay

The rates of ATP hydrolysis catalyzed by $F_i$ or SMP were determined by coupling the hydrolysis reaction to the lactate
dehydrogenase (LDH)-catalyzed oxidation of NADH (Fig. 1). Pyruvate kinase (PK) catalyzes the conversion of the ADP formed and phosphoenol pyruvate (PEP) to pyruvate and ATP, thus regenerating ATP. LDH catalyzes the oxidation of NADH by pyruvate. The rate of oxidation of NADH was determined by measuring the decrease in absorbance at 340 nm., a wavelength at which NADH absorbs light and NAD⁺ does not.

\[ \text{ATP} \xrightarrow{\text{V}_{\text{hyd}}} \text{ADP} + \text{Pi} \]
\[ \text{PK} \rightarrow \text{PEP} \]
\[ \text{pyruvate} \]
\[ \text{LDH} \rightarrow \text{NADH} \]
\[ \text{lactate} \]

Fig. 1. LDH-catalyzed NADH oxidation coupled to SMP-catalyzed ATP hydrolysis.

The coupled enzyme assay system used in determining the F₁-catalyzed ATP hydrolysis activity contained 5 mM MgCl₂, 3.7 mM ATP, 3.7 mM PEP, 50 µg/ml PK, 50 mM Tris-Cl, 50 mM CH₃COOK, 175 µM NADH, 5-100 µl F₁ solution, and pH 8.0. The assay conditions used in studying SMP- and F₁-catalyzed ATP hydrolysis varied and are given in the figure legends. All components of the assay system except ATP were mixed in a cuvette and incubated at least 3 minutes at 30°C. A baseline was established. ATP was added to initiate the reaction and the cuvette inverted several times to mix the solution. The
absorbance reading was started within 15 seconds of addition of the ATP. The activity was determined using the molar extinction coefficient of NADH at 340 nm, which is $6.22 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$.

ATP Synthesis Assay

The rates of ATP synthesis catalyzed by SMP were determined by coupling the synthesis reaction to the glucose 6-phosphate dehydrogenase (G6PDH) catalyzed reduction of NADP$^+$ (Fig. 2). Hexokinase (HK) catalyzes the conversion of glucose and the ATP formed to glucose 6-phosphate and ADP, thus regenerating ADP. G6PDH catalyzes the reduction of NADP$^+$ by glucose 6-phosphate. The rate of reduction of NADP$^+$ is determined by measuring the increase in absorbance at 340 nm, as already noted. The concentrations of reagents used in the SMP catalyzed ATP synthesis assay system are given in the figure legends. The reaction components were incubated at 30°C for at least 3 minutes and the reaction was initiated by the addition of Pi.

\[
\text{ADP} + X \xrightarrow{\text{energized SMP}} \text{ADP} - X \xrightarrow{\text{HK}} \text{ADP} + \text{glucose} 6-X
\]

\[
\text{H}_2\text{O} \xrightarrow{\text{glucose}} \text{ADP} + X \xrightarrow{\text{G6PDH}} \text{NADP}^+ \xrightarrow{\text{NADPH}} 6-X-\text{gluconate}
\]

\[X = \text{phosphate or arsenate or vanadate}\]

Fig. 2. ATP synthesis assay coupled to reduction of NADP$^+$. 
Phosphate Binding Assay

The ability of F₁ to bind ³²Pᵢ under various assay conditions was determined as follows. F₁ was incubated 10 minutes at 30°C with all the components of the reaction mixture except ³²Pᵢ. Appropriate aliquots of the reaction mixture were added to ³²Pᵢ, mixed gently and incubated an additional 20 minutes at 30°C. 100 µl aliquots of this final reaction mixture were loaded onto a Sephadex centrifuge column as described in a previous section. The centrifugate was transferred to a polyethylene scintillation vial and 10 ml of water was added. Each sample was counted (Cerenkov radiation) for 5 minutes using an LKB Wallac 17 liquid scintillation counter.

Protein Determination

The Biorad microassay was used for protein determination in most of the experiments. The Lowry protein assay was used when determining the protein concentration for calculating enzyme activity (50). Bovine serum albumin (BSA) was used as the standard. The protein concentration of the BSA standard was determined by measuring absorbances at 290 nm using the extinction coefficient of 0.677 ml mg⁻¹ cm⁻¹.
RESULTS

ATP Synthesis Catalyzed by SMP

The ATP synthesis assay shown in Figure 2 was used to detect the formation of ADP-vanadate. Unlike ATP and ADP-arsenate, ADP-vanadate formation cannot be detected directly. This is not surprising since, as mentioned in the introduction, arsenate esters hydrolyze non-enzymically several orders of magnitude faster than phosphate esters (27) and vanadate esters hydrolyze several orders of magnitude faster still (29). Because of this instability, evidence for the existence of ADP-vanadate must be found indirectly.

The first method involves an inhibition study between vanadate and phosphate for ATP synthesis. If ADP-vanadate is synthesized by SMP, then vanadate would be expected to compete with phosphate in the formation of ATP. The pattern of inhibition expected could be indicative of either competitive or mixed inhibition. If vanadate were to compete for the same site as phosphate, forming and releasing ADP-vanadate from the catalytic site (Fig. 3), a competitive inhibition pattern would result. If vanadate were to compete for the same site as phosphate, and the resulting ADP-vanadate were to remain tightly bound, blocking one of the three catalytic sites and affecting the interaction between the enzyme sites, the V₃ may be affected and a mixed inhibition pattern would result (Fig. 4).
Fig. 3. A model showing how vanadate may compete for the same site as phosphate during ATP synthesis. A simplification of the model and the derivation of the rate of ATP synthesis are given in Appendix 1.

The latter is likely, since phosphate is pentacoordinate in the transition state and pentacoordinate vanadate is stable. Vanadate may lock the catalytic site into a conformation which binds tightly to the transition state, blocking its catalytic activity. The results obtained suggest a pattern indicative of mixed inhibition with a $K_i$ of 0.18 mM and $K_i'$ of 0.8 mM (Fig. 5). Thus, if ADP-vanadate is formed, it might affect the enzyme by the mechanism shown in Figure 4.
Fig. 4. A model showing how ADP-vanadate may bind tightly at a catalytic site of ATP synthase. If due to interaction between the catalytic sites, the \( V_m \) of the enzyme is changed when ADP-vanadate is bound, an inhibition pattern indicative of mixed inhibition would result.
Figure 5

The effect of vanadate on SMP-catalyzed ATP synthesis. The reaction volumes are 1.0 ml and contain 50 mM Tris-OAc, 3.0 mM glucose, 10 mM succinate, 2.0 mM MgCl₂, 1.0 mM NADP⁺, 50 μM ADP, 0.1 mM AP₅A, approximately 20 μg G6PDH, approximately 70 μg HK, 180 μg SMP, pH 8.0. The phosphate concentration is varied in the presence of 0.4 M (▲), 0.2 M (●) and no vanadate (■). The lines are the least squares fit of the data.
ATP Hydrolysis Catalyzed by SMP and F₁

Another assay used is LDH-catalyzed NADH oxidation coupled to SMP-catalyzed ATP hydrolysis (Fig. 1).

If the phosphate concentration is increased, by the law of mass action one would expect the net rate of hydrolysis \((v_{\text{hydrolysis}} - v_{\text{synthesis}})\) to decrease. Thus, due to the decrease in the steady state ADP concentration, one would expect a decrease in the rate of oxidation of NADH. If one were to add vanadate to this reaction, and if SMP catalyze ADP-vanadate formation, one would expect that the rapid hydrolysis of ADP-vanadate would result in an increase in steady state ADP concentration and thus the inhibitory effect of phosphate would be decreased.

These hypotheses could not be tested because phosphate increased the rate of SMP-catalyzed ATP hydrolysis at low phosphate concentrations \(<15 \text{ mM}\) (Fig. 6) rather than causing the expected decrease in rate.

Effects of phosphate

Ebel and Lardy (18) found that phosphate increased the rate of ATP hydrolysis catalyzed by rat liver SMP. Moyle and Mitchell (51) found that this increase was caused by phosphate decreasing the \(K_m\) for ATP in complete ATPase (from 500 μM to 106 μM) and in F₁ ATPase (from 440 μM to 210 μM). Huang and Mitchell (52) found that phosphate and arsenate activate ATP-driven energy-linked reduction of NAD⁺ by succinate catalyzed by beef heart muscle SMP. When the phosphate and
Figure 6

The effect of Pi on SMP-catalyzed ATP hydrolysis. The reaction volumes are 1.0 ml and contain 50 mM Tris-OAc, 10 mM succinate, 2.0 mM MgCl₂, 87.5 µg NADH, 4.0 mM PEP, approximately 40 µg PK, approximately 40 µg LDH, 1.0 µM rotenone, 10 µM ATP, 100 µM AP₅A, pH 7.5, phosphate concentrations as given on the graph, and 180 µg SMP either immediately after thawing (O), or after incubating on ice for 5 hours (●). At 100% activity, the rate of ATP hydrolysis is 0.38 µmol min⁻¹ mg⁻¹.
arsenate concentrations were below approximately 2 mM they observed activation similar to that seen in Figure 6; but when phosphate and arsenate concentrations were higher (20 mM), unlike the results in Figure 6, they found phosphate activation decreased significantly. This may result, at least in part, from the increase in ionic strength of the solution, as will be discussed later.

The phosphate concentration giving the greatest stimulation in these experiments was less than 50 mM (Fig. 6), and this optimal concentration varied with experimental conditions. Because of this variation, steps were taken to compare all experimental conditions.

Over the duration of the experiment, activation of SMP-catalyzed ATP hydrolysis by phosphate decreased. One possible factor affecting the potential of SMP to be activated is the time that the SMP had been thawed and were incubating at 0°C after removal from dry ice. Several hydrolysis assays of varying phosphate concentrations were prepared and the hydrolytic activity of the SMP was assayed immediately after the SMP had been thawed and after incubation at 0°C for five hours. The results in Figure 6 show diminished activation by phosphate after the SMP had been incubated.

One possible mechanism for phosphate activation of SMP-catalyzed hydrolysis which may explain this diminished activation over time was investigated. Anion interaction may cause conformational changes of F, resulting in changes in catalytic activity. One such possible change may involve
total or partial dissociation of the naturally occurring F$_i$-ATPase inhibitor protein (IP) during incubation at 0°C after removal from dry ice. It is possible that IP dissociates from the ATP-synthase complex of thawed SMP and that phosphate accelerates this release of IP. Thus, once the SMP have been thawed, the amount of IP associated with them should diminish over time, and so should the potential for activation by phosphate. Pullman and Monroy (4) found that mitochondrial IP increases cold stability of ATPase, and thus one may predict that with increasing time over which the SMP are thawed there will be a decrease in enzyme activity. As predicted, SMP assayed after being thawed for five hours in the manner described had a lower enzyme activity than SMP assayed immediately after thawing (Fig. 6). One might also predict that due to variation in the amount of IP associated with any one batch of SMP, the activity and amount of activation by phosphate could also vary with each batch of SMP. This too has been seen (compare Fig. 6 and the phosphate curve in Fig. 7).

Washing the SMP with KCl increases their hydrolytic activity, presumably by removing endogenous IP (53). To ensure that Pi did not produce its effects by inducing the release of IP, the effect of Pi on catalysis by SMP washed with KCl was studied. If this release of IP were the mechanism by which Pi produces its effects, phosphate activation should decrease after the SMP have been washed with KCl. This was not seen. Though the hydrolytic activity was
Figure 7
The effect of ionic strength on SMP-catalyzed ATP hydrolysis. The reaction volumes are 1.0 ml and contain 50 mM Tris-OAc, 10 mM succinate, 2.0 mM MgCl₂, 175 μM NADH, 100 μM ATP, 4.0 mM PEP, approximately 80 μg PK, approximately 80 μg LDH, 100 μM AP₅₉A, 2.0 μM rotenone, 9 μg 'KCl-pretreated' SMP, pH 7.5, and phosphate (●) or KCl (▲). Phosphate concentrations are as given, and the KCl concentrations are such that the ionic strength is the same as for the corresponding phosphate concentration of that x-coordinate value. This was calculated assuming that the second pKa of phosphate to be 6.5. At 100% activity, the rate of ATP hydrolysis is 2.7 μmol min⁻¹ mg⁻¹. The dashed lines show the calculated effect of phosphate on SMP-catalyzed ATP hydrolysis if ionic strength were maintained constant at 0 M (O) and 0.56 M (X).
increased approximately seven fold and this activity was stable over time, Pi activated SMP washed with KCl to about the same degree as SMP not washed with KCl.

Experiments were conducted to ensure that the activation of SMP-catalyzed ATP hydrolysis by phosphate did not result from interaction with the coupling enzymes or from differences in ionic strength, and to determine the specificity of this activation to phosphate.

Pi did not affect PK or LDH activities at the concentrations used in these assays. Thus, all effects were due to interactions with SMP.

Constant ionic strength was maintained with varying phosphate concentrations by adding necessary amounts of KCl. The amount of KCl required was calculated assuming the second pKa of phosphate to be 6.5. In Figures 6 and 7, where ionic strength was not kept constant, there was an increase in hydrolysis rate with increasing phosphate concentration at concentrations less than approximately 50 mM, but there was a decrease in rate with increasing phosphate concentrations at concentrations greater than 50 mM. This decrease appears to be caused by the increase in ionic strength, since it did not occur when ionic strength was kept constant (Fig. 8). The KCl concentration dependence shows that increasing the ionic strength decreases the rate of hydrolysis (Fig. 7).

If one subtracts the inhibitory effect of ionic strength from the activation caused by increasing the phosphate concentration, one sees that the phosphate activating effect
Figure 8

The effect of phosphate (○) and arsenate (■) on SMP-catalyzed ATP hydrolysis when the ionic strength is maintained constant. The reaction volumes are 1.0 ml and contain 50 mM Tris-OAc, 10 mM succinate, 2.0 mM MgCl₂, 175 μM NADH, 100 μM ATP, 4.0 mM PEP, approximately 80 μg PK, approximately 80 μg LDH, 100 μM AP₅A, 2.0 μM rotenone, 9.0 μg 'KCl-pretreated' SMP, pH 7.5. KCl was added to maintain an ionic strength of 0.56 M. The phosphate and vanadate concentrations are as given on the graph. At 100% activity, the rate of ATP hydrolysis is 1.6 μmol min⁻¹ mg⁻¹.
was greater at lower ionic strength than at higher ionic strength. This is also seen by comparing phosphate activation at low phosphate concentrations in Figures 7 and 8. In the experiments of Figure 7, no KCl was added to adjust the ionic strength, so at low phosphate concentrations the ionic strength of the assay was low and phosphate activated significantly. In Figure 8, KCl was added to increase the ionic strength to 0.56 M, and at low phosphate concentrations, phosphate had no effect.

In the study of the effect of phosphate on SMP-catalyzed ATP hydrolysis at constant high ionic strength (0.56 M), phosphate did not cause an increase in hydrolytic rate at concentrations less then 25 mM, but caused a linear increase as concentrations were increased from 50 to 100 mM (Fig. 8).

If one subtracts the effect of ionic strength from the phosphate curve in Figure 7, one would expect the result to show the effect of phosphate on hydrolysis if ionic strength had no effect. If one adds the inhibitory effect caused by increasing the ionic strength to 0.56 M (the ionic strength of 100 mM phosphate), one would expect the result to show the effect of phosphate when the ionic strength was maintained at 0.56 M. Both results show the rate of hydrolysis increases with phosphate concentration at low phosphate concentrations and levels off at phosphate concentrations above 20 mM.

The difference between the calculated curve of Figure 7 and the experimental curve of Figure 8 may be a result of the method by which the ionic strength was maintained, i.e.
addition of KCl. If chloride can bind to the site at which phosphate binds and inhibit hydrolysis, a decrease in the chloride concentration would result in a decrease in inhibition. In Figure 8, when the phosphate concentration is zero, the chloride concentration is maximum and thus so is inhibition by chloride. At low phosphate concentrations (less than 25 mM), the chloride concentration is high and phosphate cannot compete effectively. As the phosphate concentration is increased, phosphate competes more effectively. In addition, there is a decrease in chloride concentration which results in a decrease in inhibition by chloride; as a result, activation by phosphate appears to be much greater than was predicted without considering inhibition by chloride.

A study of the effect of phosphate on F1-catalyzed ATP hydrolysis at constant high ionic strength gave similar results to those of SMP-catalyzed ATP hydrolysis.

Penefsky (54) also observed this phosphate activation of F1-catalyzed ATP hydrolysis. He found that at 1 μM ATP, incubation of F1 with 10 mM phosphate resulted in an over sixfold acceleration in the hydrolysis rate and that this activation could be reversed by removal of the phosphate and regained by addition of phosphate. Preincubation with ATP in the presence of chelator also activated F1. There was a long lag in the initiation of hydrolysis by the unactivated enzyme and the rate of activation was dependent on the ATP concentration. When the hydrolysis assay was done in the presence of high ATP concentrations (6 mM) the hydrolysis
catalyzed by unactivated enzyme was indistinguishable from that catalyzed by activated enzyme, i.e., the activation was only observed at low ATP concentrations.

Penefsky (54) also found that activated F₁ binds ATP at a much faster rate than unactivated F₁. He suggested that in the absence of activator, ATP is required at a high affinity site for some minimal time period for the inactive to active transition to take place.

Another effect of phosphate was a change in the curvature of the time chart lines. When the SMP were not washed with KCl, the ATP hydrolysis time charts showed an initial lag in the rate of ATP hydrolysis. The rate of hydrolysis slowly increased until all of the substrate, NADH, was oxidized. When phosphate was included in the assay, this lag was absent. When the SMP were pretreated with KCl (as described in Methods) the rate of hydrolysis was constant when phosphate was omitted from the assay. When phosphate was included in the assay, the initial rate of hydrolysis was fast and this rate continuously decreased until all the NADH had been oxidized. When the ionic strength was kept constant by addition of KCl, the rate of hydrolysis was constant with a slightly faster rate at the beginning and a small decrease in rate just before the NADH was used up. The curvature was independent of the concentration of phosphate. Using F₁, when phosphate was omitted from the assay, there was a lag in the rate of hydrolysis, but when phosphate was included, hydrolysis was very fast initially and this rate continuously
decreased until all the NADH was oxidized. This phosphate effect was seen when the ionic strength was maintained constant by addition of KCl.

**Effects of vanadate**

Though phosphate activation complicated the results, studies of inhibition by vanadate were carried out as proposed, using both energized SMP and soluble F1.

The dependence of SMP-catalyzed ATP hydrolysis on vanadate concentration (in the absence of phosphate using energized SMP) is shown in Figure 9. Vanadate inhibits significantly even at very low concentrations (<10 μM), but at higher concentrations the rate becomes less sensitive to changes in the vanadate concentration, i.e., even though the activity is 68% at 50 μM Vi, it only drops to approximately 58% at 100 μM Vi and approximately 39% at 1.0 mM Vi. There is little change in ionic strength over this concentration range and, as shown in the KCl curve in Figure 7, no measurable effect caused by this change. For example, since the pKa's of vanadate and phosphate are similar one can follow the inhibition caused by the increase in ionic strength resulting from the increase in vanadate concentration as was done for phosphate in Figure 7. When the vanadate concentration is increased from 0 to 10 μM the inhibition caused by this increase in ionic strength is approximately 1%, i.e., less than experimental error. As the vanadate concentration is increased from 0 to 1 mM the inhibition due to the increase in ionic strength is at most 5%.
Figure 9

The effect of vanadate on SMP-catalyzed ATP hydrolysis. The reaction volumes were 1.0 ml and contained 50 mM Tris-OAc, 10 mM succinate, 2.0 mM MgCl₂, 175 µg NADH, 100 µM ATP, 4.0 mM PEP, approximately 80 µg PK, approximately 80 µg LDH, 100 µM AP₅A, 2.0 µM rotenone, 9.0 µg 'KCl-pretreated' SMP, pH 7.5. At 100% activity, the rate of ATP hydrolysis is 2.5 µmol min⁻¹ mg⁻¹ (2.3 µmol min⁻¹ mg⁻¹ for insert).
From Michaelis-Menten kinetics, one knows that the relation of \( 1/y \) vs \( 1/x \) of a rectangular hyperbola (a simple enzyme saturation curve) is linear. To determine if inhibition by vanadate follows simple saturation kinetics, the hyperbola in Figure 9, the \% activity vs the vanadate concentration, was replotted as a double reciprocal plot. In this plot, \( 1/(100 - \% \text{ Activity}) \) vs \( 1/[V_i] \) (Fig. 10), one sees a linear relationship at low vanadate concentrations, but at vanadate concentrations greater than approximately 250 \( \mu M \) the relationship deviates from this curve. This supports the hypothesis that the effects of vanadate are biphasic and it may bind tightly at the first site but it binds with a lower affinity at additional site(s). Extrapolating this line to \( 1/(100 - \% \text{ Activity}) = 0 \) one obtains the vanadate concentration which gives half maximal inhibition, \([V_i]_{0.5} = .043 \text{ mM}\).

A study of the effect of vanadate on F₁-catalyzed ATP hydrolysis at constant high ionic strength in the absence of phosphate shows similar results to those seen in the SMP-catalyzed system, but the sensitivity of the hydrolytic rate to vanadate concentration was much less at low vanadate concentrations, i.e. 100 \( \mu M \) \( V_i \) results in 87\% activity as compared to 58\% activity in the SMP system (Fig. 11). No firm conclusion can be drawn since these experiments were done at different ionic strengths.

Unlike phosphate, vanadate did not have any apparent effect on the curvature of the time chart lines. This may be
Figure 10

A double reciprocal plot \((1/(100 - \% \text{ activity}) \text{ vs } 1/[Vi])\) of the data of Figure 9.
Figure 11
The effect of vanadate on F\textsubscript{i}- (●) and SMP- (O) catalyzed ATP hydrolysis. The data from the SMP-catalyzed hydrolysis was taken from Figure 9. The reaction volumes of F\textsubscript{i}-catalyzed hydrolysis were 1.0 ml and contained 50 mM Tris-OAc, 2.0 mM MgCl\textsubscript{2}, 17.5 μM NADH, 0.2 mM EDTA, approximately 80 μg PK, approximately 80 μg LDH, 100 μM ATP, 4.0 mM PEP, approximately 5 μg F\textsubscript{i} and enough KCl to maintain the ionic strength at 0.28 M. Vanadate concentrations are as given. Note: there is no KCl in the assays of SMP-catalyzed ATP hydrolysis. At 100% activity, the rate of ATP hydrolysis is 3.9 μmol min\textsuperscript{-1} mg\textsuperscript{-1}. 
partially due to the fact that there is little change in ionic strength over the vanadate concentration range used.

The activation/inhibition by phosphate/vanadate may be non-specific. It may be due to non-specific anion effects such as those seen by Ebel and Lardy in beef heart F$_i$ (18), by Rectenwald and Hess in yeast mitochondrial F$_i$ (55) and by Slooten and Nuyten in *Rhodospirillum rubrum* chromatophores (56).

**Effects of arsenate, molybdate and pyrophosphate**

The effects of arsenate, molybdate and pyrophosphate were studied to help determine the specificity of the effects caused by phosphate and vanadate. Arsenate activation of SMP-catalyzed ATP hydrolysis had the same concentration dependence as phosphate whether or not ionic strength was maintained constant (Fig. 9,12). The slightly lower rates with arsenate when ionic strength was not maintained constant are within experimental error, but may also be due to its lower pKa (at 1.0 M ionic strength As$_i$ = 6.47 and Pi = 6.70) (57) and thus its higher ionic strength as compared to phosphate at the same concentration.

The effect of molybdate was studied only in experiments in which the ionic strength was not maintained constant. Molybdate appeared to cause a very slight increase in hydrolysis rate at low concentrations and then a decrease in rate typical of that seen for phosphate and arsenate at higher concentrations. If one subtracts the ionic strength effect, one sees that molybdate has an activating effect similar to
Figure 12

The effect of various anions on SMP-catalyzed ATP hydrolysis. The conditions are the same as in Figure 8. The data for the phosphate curve are taken from Figure 7. Phosphate (○), arsenate (▲) and molybdate (■) concentrations are as given. Ionic strength is not maintained constant. At 100% activity, the rate of ATP hydrolysis is 2.7 μmol min⁻¹ mg⁻¹.
that of phosphate and arsenate, but to a much lesser extent.

Pyrophosphate (PPI) inhibits both SMP-catalyzed ATP hydrolysis (Fig. 13) and synthesis (Fig. 14) very strongly. At 21 mM PPI, ATP hydrolysis was completely inhibited. This is at least partially due to the strong ability of PPI to chelate Mg$^{2+}$.

Since the concentration of Mg$^{2+}$ is 2.0 mM in the reaction assay system, at PPI concentrations greater than 2.0 mM, most of the Mg$^{2+}$ which is required for the substrate (MgATP$^{2-}$) will be chelated. Addition of Mg$^{2+}$ to the PPI-inhibited assay increased the rate of hydrolysis, but total reversal of PPI inhibition could not be observed since the Mg$^{2+}$ concentration could not be increased above 4 mM due to its precipitate interfering with the assay.

The effect of PPI on F$_i$-catalyzed ATP hydrolysis (at constant ionic strength) was very different from that seen using SMP (Fig. 15). A PPI concentration of only 1.0 mM results in an almost 3.5 fold increase in the rate of hydrolysis. Increasing the PPI concentration above 1.0 mM decreased this hydrolysis rate. At 21.2 mM PPI no significant hydrolysis occurred.

Fleury et al (58) found Mg$^{2+}$ and free ATP$^{4-}$ to be competitive inhibitors towards MgATP$^{2-}$ during F$_i$-catalyzed hydrolysis. At 0.125 mM ATP, in the absence of MgCl$_2$ no ATP was hydrolyzed. As the MgCl$_2$ concentration was increased, the rate of hydrolysis increased up to a maximum and then slowly decreased. The rise in activity corresponded to the increase
Figure 13

The effect of pyrophosphate on SMP-catalyzed ATP hydrolysis. The reaction volumes were 1.0 ml and contained 50 mM Tris-OAc, 10 mM succinate, 2.0 mM MgCl₂, 175 μM NADH, 100 μM ATP, 4.0 mM PEP, approximately 80 μg PK, approximately 80 μg LDH, 100 μM AP₃A, 2.0 μM rotenone, 9.0 μg 'KCl-pretreated' SMP, pH 7.5. KCl was added to maintain the ionic strength at 0.56 M. The pyrophosphate concentrations are as given. At 100% activity, the rate of ATP hydrolysis is 0.9 μmol min⁻¹ mg⁻¹.
The effect of pyrophosphate on SMP-catalyzed ATP synthesis. The reaction volumes were 1.0 ml and contained 50 mM Tris-OAc, 6.0 mM glucose, 10 mM succinate, 2.0 mM MgCl₂, 10 mM Pi, 1.0 mM NADP⁺, 100 μM APSA, approximately 20 μg G6PDH, approximately 70 μg HK, 50 μM ADP, 720 μg SMP, pH 7.5. KCl was added to maintain the ionic strength at 0.56 M. The pyrophosphate concentrations are as given. At 100% activity, the rate of ATP synthesis is 27 nmol min⁻¹ mg⁻¹.
Figure 15

The effect of pyrophosphate on F₁-catalyzed ATP hydrolysis. The assay conditions were as given in Figure 11. The ionic strength was maintained constant. The pyrophosphate concentrations are as given. Because the rate of hydrolysis was not constant over the period of the assay (see text p. 37), the initial rate (●) and final rate (■) were plotted for each assay. At 100% activity, the rate of ATP hydrolysis is 2.6 μmol min⁻¹ mg⁻¹.
in MgATP\textsuperscript{2-} concentration and the decrease corresponded to the excess of free Mg\textsuperscript{2+}. The same pattern was seen at 0.15 mM MgCl\textsubscript{2} as the concentration of ATP\textsuperscript{4-} was increased from 0 to 1.5 mM. The decrease in hydrolytic rate started at about 0.4 mM ATP.

Activation at low P Pi concentrations (less than 2.0 mM) may result, at least in part, from P Pi chelating excess Mg\textsuperscript{2+}, and thereby decreasing inhibition by Mg\textsuperscript{2+}. Inhibition at higher P Pi concentrations (greater than 2.0 mM) may result, at least in part, from depletion of the substrate, MgATP\textsuperscript{2-}, as was suggested with the SMP system.

The shape of the time charts showing the change of absorbance over time may also give clues to the mechanism of the effects of P Pi. During ATP hydrolysis in the absence of P Pi there was an initial lag before the rate of hydrolysis became constant (which was mentioned when discussing the effect of P i), but in the presence of P Pi the rate of hydrolysis was very high initially and slowed down continuously until all the NADH was oxidized. As was seen with phosphate, this effect was most prominent for the F\textsubscript{i}-catalyzed reaction.

This curvature of the time charts may be due to an increase in the amount of F\textsubscript{i} molecules containing bound inhibitory ADP. As ATP hydrolyzes, ADP and Pi are formed at the catalytic site. As discussed earlier, ADP inhibits hydrolysis when it is bound at the catalytic site. At the ATP concentrations used here (0.1 M) it is likely that only two of
the three catalytic sites are utilized during catalysis. Initially PPi may bind at the third site resulting in catalytic activity similar to that seen for three-site catalysis. As ATP is hydrolyzed, some ADP may remain bound at the catalytic site, preventing ATP or PPI from binding at this site. Thus, only two sites are available at which ATP can bind and PPI competes with ATP for these two sites. Even if the dissociation constant of PPI were large, during the period in which PPI is bound ATP would not be able to bind at that site and thus the rate of hydrolysis would decrease.

**F1 Binding Studies**

A study of the effect of vanadate on the binding of $^{32}$Pi to F1 was made to find evidence to test the hypothesis that vanadate competes with phosphate for the catalytic site. Competition studies showed that though vanadate inhibits the binding of $^{32}$Pi to F1 (Fig. 16), even very high vanadate concentrations (500 µM) do not totally prevent phosphate from binding to F1 (Fig. 17). This implies that both phosphate and vanadate can bind to F1 simultaneously, or some F1 molecules are in a conformation which prevents vanadate from displacing Pi.

An experiment was done to determine if phosphate irreversibly binds to some F1 molecules. This was done by incubating the F1 molecules with 75 µM $^{32}$Pi for 15 minutes at 30°C and then adding cold Pi (to a final concentration of 1
The effect of vanadate on the binding of $^{32}$Pi to $F_1$. The assays contained 90 mM Tris-OAc and 2.0 mM MgSO$_4$ at pH 7.5. The vanadate concentration are as given. The experiments were done at both 50 µM phosphate (■) and 75 µM phosphate (○) as shown by the solid line. Using the model in Scheme 1 and the data in this figure, no reasonable fit could be made if one assumed $K_{v} = 0$. The least squares fit resulted in: $K_p = 35$ µM, $K_v = 4.8$ µM, $K_{vp} = 0.66$ mM. The experimental values were calculated using these constants for both 50 µM phosphate (■) and 75 µM phosphate (○). At 100% binding, the binding ratio, $^{32}$Pi/$F_1$, is 0.14 for 50 µM Pi and 0.17 for 75 µM Pi.
Figure 17
The effect of 500 μM vanadate on the binding of $^{32}$Pi to F.<sup>1</sup>. The assay conditions were as described in Figure 16. The phosphate concentration was varied. 100% binding is represented by the binding ratio when the assay contains 150 μM phosphate and no vanadate. The curves show the relative binding of $^{32}$Pi phosphate concentrations in the absence (O) and presence (●) of 500 μM vanadate. At 100% binding, the binding ratio, $^{32}$Pi/F<sub>1</sub>, is 0.348.
mM) to determine its effect on the binding of $^{32}$Pi. The binding ratio ($^{32}$Pi/F$_i$) was determined by the assay procedure described in Methods. All $^{32}$Pi was displaced by the cold chase, thus no phosphate was irreversibly bound.

A model was proposed and equilibrium constants were derived to best fit the data (Scheme 1).

\[ \begin{align*}
\text{EXX} & \quad \text{XX} \\
\text{XX} & \quad \text{E} \\
\text{E} & \quad \text{E.P} \\
\text{X} & \quad \text{K}_r \\
\text{X} & \quad \text{K}_x \\
\text{EX} & \quad \text{EXP} \\
\end{align*} \]

X - arsenate or vanadate
P - phosphate
E - F$_i$

Scheme 1. A model depicting a possible mechanism for the binding of phosphate and arsenate or vanadate to F$_i$.

One can use the binding ratios at various phosphate [P] and inhibitor [X] concentrations, to calculate the equilibrium constants ($K_r$, $K_x$ and $K_{xp}$), for each reaction step in Scheme 1. This is done by assuming the system is at equilibrium. For this model, the total enzyme concentration ($e$) is equal to the sum of the concentrations of all enzyme species present, equation (1).

\[(1) \quad e = [E] + [EP] + [EX] + [EXP] \]
If all the steps are at equilibrium then equations (2), (3) and (4) hold true.

(2) \( K_r = \frac{[E][P]}{[E]P} \) \[ E = [EP]K_p \]

(3) \( K_x = \frac{[E][X]}{[EX]} \) \[ EX = \frac{[E][X]}{K_x} \]

(4) \( K_{xp} = \frac{[EX][P]}{[EXP]} \) \[ EXP = \frac{[EX][P]}{K_{xp}} \]

Substituting equation (2) into (3), and equation (3) into (4), one obtains equation (5).

(5) \[ EXP = \frac{[EP]K_p[X]}{K_xK_{xp}} \]

Substituting equations (2), (3) and (5) into the conservation equation, equation (1), one obtains equation (6).

(6) \[ e = \frac{[EP]}{[P]}(\frac{K_r + 1 + K_x[X] + K_{xp}[X]}{[P]K_xK_{xp}}) \]

\[ = \frac{[EP]}{[P]}(K_rK_xK_{xp} + K_{xp}[P] + K_xK_{xp}[X] + K_{xp}[X][P]) \]

\[ \frac{K_xK_{xp}[P] + K_{xp}[X][P]}{K_{xp}K_x[P] + K_{xp}[X] + [P](K_xK_{xp} + K_{xp}[X])} \]

\[ y = \frac{K_xK_{xp}[P] + K_{xp}[X][P]}{K_{xp}K_x[P] + K_{xp}[X] + [P](K_xK_{xp} + K_{xp}[X])} \]

\[ y = \frac{[P]}{K_xK_{xp}(K_x + [X]) + [P]} \]

\[ K_xK_{xp} + K_{xp}[X] \]

\( y \) is the ratio of enzyme molecules with phosphate bound over the total concentration of enzyme i.e., the 'binding ratio'.

(7) \[ y = \frac{[EP] + [EXP]}{e} \]

(8) Substituting equation (5) and (6) into equation (7) and rearranging:
A reasonable fit of the data could be made only if vanadate and phosphate could bind simultaneously \((K_{v.p} < \infty)\). The calculated curve is shown in Figure 16 (broken lines).

A study of the effect of arsenate on the binding of \(^{32}\text{Pi}\) to F, was made to help determine the specificity of F, to inhibition by vanadate. If vanadate exerts its effects through its stability as a pentacoordinate structure which binds tightly to the enzyme, one would expect that it would exhibit a different inhibitory pattern than arsenate which is not stable in this state. Figure 18 shows the effect of arsenate on the binding of \(^{32}\text{Pi}\) to F,. One can see that arsenate inhibits the binding of \(^{32}\text{Pi}\) to F, to a much lesser extent than does vanadate. This is supported by a similar competition study done by Penefsky (53). It showed that 1.0 mM arsenate was required to cause 50% inhibition of 40 \(\mu\text{M}\) Pi. Because Penefsky had a large quantity of data, the data could be fitted to Scheme 1. A least squares fit using these data is shown in Figure 19. The data could be explained by a model (simpler than that required to explain inhibition by vanadate), in which phosphate and arsenate do not bind simultaneously \((K_{v.p} = \infty)\). This supports the hypothesis that vanadate and arsenate inhibit by different mechanisms and thus that inhibition by vanadate could involve its stability as a pentacoordinate structure.
Figure 18

The effect of arsenate on the binding of $^{32}$Pi to F$_1$. The assay conditions were as described in Figure 16. The arsenate concentration was varied at two phosphate concentrations: 50 μM (■), and 100 μM (●).
\[ 32P^{1}/F^{1} \text{ (Fraction Bound)} \]
Figure 19

The effect of arsenate on the binding of $^{32}$Pi to $F_1$. Using the model in Scheme 1 and the data from Penefsky (14) shown in this figure by (■), a simplification could be made by assuming $K_{aP} = \infty$. The least squares fit (O) resulted in $K_r = 0.1$ mM, $K_A = 2.5$ mM, $K_{AP} = \infty$. At 100% binding, the binding ratio, $^{32}$Pi$/F_1$, is 0.21.
DISCUSSION

Phosphate activated SMP- and F₁-catalyzed ATP hydrolysis. This activation was sensitive to the ionic strength of the solution. At low ionic strength, phosphate activated at low phosphate concentrations (5 mM), but as the ionic strength was increased (to about 0.5 M), higher phosphate concentrations (>25 mM) were required before any activation occurred (Fig. 8).

It is possible that this phosphate activation is associated with the release of tightly bound inhibitory ADP (59). ADP binds to one of the catalytic sites and inhibits the initial rates of ATP hydrolysis. This inhibition is reversed by 1 mM ATP during ATP hydrolysis (60). Addition of phosphate also reverses this initial inhibition (61). Though ATP reverses ADP inhibition at 1 mM ATP, ATP may not have a significant effect at the concentration used in our experiments (0.1 mM ATP). If the addition of phosphate reverses the ADP inhibition as was observed by Kozlov (60), and if 0.1 mM ATP does not completely reverse ADP inhibition, Pi may accelerate the entire rate, not just the initial rate. Evidence for this is seen on the time charts of the effect of phosphate on ATP hydrolysis catalyzed by SMP (when the ionic strength was not maintained constant and the SMP had not been pretreated). When phosphate is absent and no KCl is added, the rate of change of absorbance is not constant, but continually increases until all the NADH is oxidized.
phosphate is present, there is a linear change of absorbance over time, i.e. no increase in the rate of hydrolysis. If the SMP are pretreated with KCl, the rate of hydrolysis is constant in the absence of additional anions, but continuously decreases if phosphate, arsenate, molybdate or KCl are included in the assay. The effect of phosphate and pyrophosphate on the time charts of hydrolysis catalyzed by F$_i$ at constant ionic strength, is similar to that of hydrolysis catalyzed by KCl pretreated SMP. In the absence of phosphate and pyrophosphate, the rate of hydrolysis is constant but in the presence of phosphate or pyrophosphate the rate continuously decreases. This decrease in rate is much more significant during F$_i$-catalyzed hydrolysis than SMP-catalyzed hydrolysis.

The effects of phosphate and pyrophosphate on the time charts of SMP-catalyzed ATP hydrolysis may result only from differences in ionic strength, since they appear to be quite small and appear similar for all the anions studied (Pi, Asi, MoO$_4^{2-}$, PPI and KCl); but since the effects of phosphate and pyrophosphate on the time charts of F$_i$-catalyzed ATP hydrolysis are seen even when the ionic strength is kept constant, they must be attributed to something else.

At the higher ionic strength, chloride may compete for the site at which phosphate binds and thus block it from binding, and inhibit its activation. This concept may be tested using the F$_i$ binding assay. If chloride reduces phosphate activation by binding at the same site, it should be
possible to inhibit $^{32}$Pi from binding to soluble F$_i$ by addition of sufficient chloride. This will not be conclusive evidence, since KCl may inhibit $^{32}$Pi from binding to F$_i$ by indirect means to yield similar results.

Pyrophosphate inhibits SMP-catalyzed ATP synthesis and hydrolysis. It also inhibits F$_i$-catalyzed ATP hydrolysis at high concentrations (above 2 mM) but activates F$_i$-catalyzed ATP hydrolysis at low pyrophosphate concentrations. The inhibitory effect was decreased by addition of small amounts of MgCl$_2$ (results not shown) and thus is at least partially due to the strong chelating ability of pyrophosphate which depletes Mg$^{2+}$ from the reaction requiring Mg-ATP as substrate. The activation may be explained in terms of the binding change mechanism.

The binding change mechanism for ATP hydrolysis by ATP synthases (62,63) has much evidence supporting it. In this mechanism ATP binds tightly at a catalytic site, and it is reversibly hydrolyzed to tightly bound ADP and Pi. ATP binds to another catalytic site. The resulting conformational changes reduce the binding of ADP and Pi at the first site. Thus substrate binding at one site greatly increases the rate of product release at another, so that when the substrate concentration is subsaturating (less than about 500 μM) the product release can be accelerated by addition of more substrate. 100 μM PPI accelerates F$_i$-catalyzed ATP hydrolysis. One explanation is that PPI acts as an ATP analog. At low ATP concentrations, PPI can bind at an empty
catalytic site, thus accelerating product release and the net hydrolysis rate. When the PPI concentration is high, it competes with ATP and the net result is a decrease in hydrolytic rate. Similar activation has been seen by Kasho and Boyer using inosine triphosphate (64). The effects were also attributed to accelerated product release. They also found further support for this hypothesis in that ATP decreased intermediate 31O exchange during ATP hydrolysis. A similar experiment could be done using PPI to further support this explanation for the effects of PPI. This model can also be tested by determining whether PPI can accelerate hydrolysis at saturating ATP concentrations, and by determining if PPI accelerates release of ATP from F\textsubscript{i}. It is interesting that PPI accelerates hydrolysis catalyzed by soluble F\textsubscript{i} but not hydrolysis or synthesis catalyzed by energized SMP. It is possible that in the energized system, ATP hydrolysis proceeds by a compulsory ordered sequential mechanism (64). When PPI binds, it must stay bound for the entire cycle and even though it accelerates the product release step, it competes with ATP for the substrate binding site, thus decreasing the number of sites accessible to ATP. The net result is inhibition. Research done at other laboratories supports this model. Roveri et al (65) found PPI reduces nucleotide binding to mitochondrial F\textsubscript{i}. Girault et al (66) and Teifert et al (67) found that PPI binds to at least two sites on chloroplast F\textsubscript{i}. The mechanism by which vanadate affects the ATP synthase complex is different from that of arsenate, phosphate,
molybdate, potassium chloride and pyrophosphate. Arsenate, phosphate and possibly molybdate activate SMP- and F₁-catalyzed ATP hydrolysis. Pyrophosphate activates F₁-catalyzed ATP hydrolysis at low concentrations and inhibits at higher concentrations. The unique inhibition by vanadate of ATP synthesis, ATP hydrolysis and ³²Pi binding to F₁ supports a model in which vanadate forms a stable pentacoordinate transition state analog and blocks one site of the enzyme by stabilizing it in its transition state.

Results from the study of inhibition of ATP synthesis by vanadate at various fixed phosphate concentrations showed a pattern of mixed inhibition (i.e. not simple competition for Pi). Thus, even large concentrations of phosphate (as seen by extrapolating 1/Pi to 0) will not overcome the inhibition by vanadate. This would be expected if vanadate binds tightly to a catalytic site and thus prevents access of phosphate to that site. Inhibition of SMP- and F₁-catalyzed hydrolysis show a biphasic pattern of inhibition, especially clearly for SMP-catalyzed hydrolysis. It appears that very low vanadate concentrations cause approximately one third of the enzyme to be inhibited (i.e. one of the three active sites). As the vanadate concentration is increased, the second vanadate competes less strongly, possibly due to competitive inhibition with ATP for the other two sites.

Studies of the effect of vanadate on the binding of ³²Pi to F₁ show a biphasic inhibition pattern and give further support for this mechanism. Vanadate inhibits strongly at low
concentrations, but at higher concentrations the binding is less sensitive to changes in the vanadate concentration. Penefsky did not find this biphasic pattern for arsenate inhibition of the binding of \(^{32}\)Pi to F\(_i\), again showing the uniqueness of the inhibitory mechanism of vanadate.

In summary, results here support the hypothesis that the effects of vanadate result from its ability to act as a phosphate analog. Supporting results include the ability of vanadate to: (1) inhibit ATP synthesis; (2) inhibit ATP hydrolysis and (3) inhibit the binding of \(^{32}\)Pi to F\(_i\). Though vanadate likely binds at a phosphate binding site, it affects the enzyme very differently than does phosphate. This is probably due to its stability as a pentacoordinate structure which binds tightly to the enzyme. This hypothesis is supported by the fact that: (1) inhibition of ATP synthesis by vanadate does not follow a pattern indicative of simple competitive inhibition; (2) unlike phosphate or arsenate which are not stable as pentacoordinate structures, vanadate inhibits rather than activates SMP- and F\(_i\)-catalyzed hydrolysis; (3) low concentrations of vanadate inhibit ATP hydrolysis; (4) low concentrations of vanadate inhibit the binding of \(^{32}\)Pi to F\(_i\), and (5) the inhibitory pattern of vanadate is different from that of phosphate or arsenate.

A study of the effect of phosphate and arsenate on the binding of \(^{40}\)Vi to F\(_i\) may add further support to this hypothesis if both phosphate and arsenate inhibit this binding, and the data fit the models and the constants derived
Phosphate and arsenate have the same activating effect on SMP-catalyzed ATP hydrolysis. Arsenate inhibits the binding of $^{32}$Pi to $F_i$, but this inhibition is weak. This is not unexpected since arsenate is not stable as a pentacoordinate structure and thus cannot bind tightly to the enzyme by stabilizing it in the conformation which binds the transition state.

Phosphate activates $F_i$-catalyzed hydrolysis, but at 0.5 M ionic strength, greater than 25 mM phosphate is required before activation is apparent. This requirement for a high phosphate concentration may be explained if chloride competes for phosphate at the catalytic site such that high phosphate concentrations are required to compete effectively.

Further experiments could be done to better define these effects. Another anion which has less effect could be used to maintain constant ionic strength. Different sources of chloride which result in different ionic strengths could be compared to determine whether inhibition results from increases in ionic strength or increase in chloride concentration. The effect of the binding of $^{32}$Pi to $F_i$ and the effect of chloride concentration on SMP-catalyzed ATP synthesis could also be tested.

Pyrophosphate strongly inhibited SMP-catalyzed ATP hydrolysis. A concentration of 21 mM pyrophosphate totally inhibits ATP hydrolysis. This inhibition may be caused by pyrophosphate chelating Mg$^{2+}$. Pyrophosphate activates
Fi-catalyzed ATP hydrolysis very strongly. Addition of 1.0 mM pyrophosphate results in a 3.5 fold increase in the rate of hydrolysis. When the pyrophosphate concentration is increased above 1.0 mM the rate of hydrolysis decreases until hydrolysis is completely inhibited at 21 mM pyrophosphate.

Acceleration of Fi-catalyzed ATP hydrolysis by pyrophosphate has been attributed to pyrophosphate binding at a catalytic site thus accelerating release of products from an alternate catalytic site. Phosphate and arsenate may also accelerate ATP hydrolysis by this mechanism. This hypothesis must be tested by studying the effects of these anions on ATP hydrolysis at saturating ATP concentrations. If they accelerate the dissociation of products by binding at an alternate catalytic site, this acceleration should not be seen at saturating ATP concentrations. The effect of these anions on $^{18}O$ exchange during Fi-catalyzed ATP hydrolysis could also be studied. If they accelerate the release of products from an alternative catalytic site, fewer reversals of hydrolysis can occur and thus less $^{18}O$ can be incorporated into the phosphate.

Pyrophosphate inhibits SMP-catalyzed ATP hydrolysis but accelerates Fi-catalyzed ATP hydrolysis. A study of the effect of pyrophosphate on hydrolysis of ATP catalyzed by uncoupled SMP may help to determine the reason. If pyrophosphate does not accelerate hydrolysis catalyzed by uncoupled SMP then the process is related to a membrane-associated mechanism.
The effect of pyrophosphate on the binding of \(^{32}\text{P}\) to \(F_i\) and of \(^{32}\text{PPI}\) to \(F_i\) should both be studied. If PPI activates \(F_i\)-catalyzed ATP hydrolysis by binding at a catalytic site and accelerating the release of products from a second site, one would expect it to bind tightly to \(F_i\) and compete with \(^{32}\text{P}\) and ATP for a binding site.

A study should also be made to determine if SMP or \(F_i\) catalyzes hydrolysis of PPI. If hydrolysis is observed, it would be quite conclusive evidence that PPI acts at the catalytic site.

The effect of the different anions (Pi, Asi, MoO\(_4\)\(^{-2}\), PPI, Cl\(^{-}\)), on uncoupled SMP-catalyzed ATP hydrolysis should be studied. If the difference in curvature of the time charts for SMP- and \(F_i\)-catalyzed hydrolysis results from an effect specific to the energized system, one would expect the results from uncoupled SMP-catalyzed hydrolysis to be similar to those of \(F_i\)-catalyzed hydrolysis.

There was variation in the error associated with data in this thesis. After being thawed, there was a continuous decrease in the activity of the SMP which had not been pretreated by washing with KCl. Though there was high reproducibility of data when assays were repeated within a short period of each other, as the time between assays became greater, the deviations increased. When the SMP were pretreated by washing with KCl, their activity was more stable and a maximum of approximately 10% activity was lost over the period of the experiment. For this reason, though data
obtained using KCl pretreated SMP was within about 10% error, some data values obtained using untreated SMP may be out by as much as 20%.
Simplification of scheme in Figure 3:

Assume that the system is in a steady state. Since \([Pi]\) is large and \([Vi]\) is large, their concentrations do not change significantly over the period of the experiment. Product inhibition is negligible since hexokinase traps the product and converts it back into ADP.

**Steady state approximation:**

1. \[ \frac{\Delta [EV]}{\Delta t} = 0 = k,E[Vi] - k,[EV] \]
2. \[ \frac{k_1}{k_r} = \frac{[E][Vi]}{[Ev]} \]
3. Assuming dissociation of products is rate limiting:
   \[ V_{syn} = k_s[EP] \]
4. Total enzyme, \(e\), is the sum of all species:
   \[ e = [E] + [EP] + [EV] \]
5. \[ \frac{\Delta [EP]}{\Delta t} = 0 = k_1[E][Pi] - (k_2 + k_3)[EP] \]
6. \[ [E] = \frac{(k_2 + k_3)[EP]}{k_1[Pi]} \]
7. \[ [EV] = \frac{[E][Vi]}{K_v} \]
Substituting equations (6) and (7) into equation (4):

\[ e = \frac{[EP] (1 + k_2 + k_3 (1 + [Vi]))}{k_i [Pi] K_v} \]

Substituting equation (9) into equation (3):

\[ V = k_s [EP] = \frac{k_s e}{1 + k_2 + k_3 (1 + [Vi])} \]

Multiplying top and bottom by [Pi]:

\[ V = \frac{k_s e [Pi]}{[Pi] + k_2 + k_3 (1 + [Vi])} \]

If one plots 1/V vs 1/[Pi]:

\[ \frac{1}{V} = \frac{k_i}{k_s e [Pi]} + \frac{k_2 + k_3 (1 + [Vi])}{k_s e k_i} \]

\[ \text{Slope} = \frac{k_2 + k_3 (1 + [Vi])}{k_s e k_i K_v} \]

\[ \text{y-intercept} = \frac{1}{k_s e} \]

i.e. The slope increases with [Vi], and the lines representing data at constant inhibitor (vanadate) concentration and varying phosphate concentration all intercept on the Y-axis.
REFERENCES


