NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30.

THIS DISSERTATION HAS BEEN MICROFILMED EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30.

LA THÈSE A ÉTÉ MICROFILMÉE TELLE QUE NOUS L'AVONS RECEUE
THE EFFECT OF AMP·PNP ON ATP HYDROLYSIS CATALYSED BY BEEF HEART SUBMITOCHONDRIAL PARTICLES AND F1-ATPASE

by

Tania Kastelic
B.Sc., Simon Fraser University, 1983

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE in the Department of Chemistry

Tania Kastelic 1987
SIMON FRASER UNIVERSITY
April 1987

All rights reserved. This work may not be reproduced in whole or in part, by photocopy or other means, without permission of the author.
Permission has been granted to the National Library of Canada to microfilm this thesis and to lend or sell copies of the film.

The author (copyright owner) has reserved other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without his/her written permission.

Name: Tania Kastelic

Degree: Master of Science

Title of THESIS:

The Effect of AMP·PNP on ATP Hydrolysis
Catalysed by Beef Heart Submitochondrial Particles and F1-ATPase

Examining Committee:

Chairman: Dr. P. Percival

Dr. M. J. Gresse
Senior Supervisor
Associate Professor
Chemistry

Dr. W. R. Richards
Associate Professor
Chemistry

Dr. A. N. Sessler
Professor
Chemistry

Dr. P. D. Bragg
External Examiner
Professor
Department of Biochemistry
University of British Columbia

Date Approved: 10 April 1987
PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

The Effect of AMP-PNP on ATP Hydrolysis Catalysed by

Beef Heart Submitochondrial Particles and F1-ATPase

Author:

Tania Kastelic

April 15, 1987

(signature)  (name)  (date)
ABSTRACT

The mechanism of adenosine-5'-triphosphate (ATP) hydrolysis catalysed by beef heart submitochondrial particles (SMP) and soluble F₁-ATPase was studied using the ATP analog, adenylyl imidodiphosphate (AMP•PNP). AMP•PNP inhibition of ATP hydrolysis by energized SMP was shown to be insensitive to increasing concentrations of the hydrolysis product, adenosine-5'-diphosphate (ADP), under the conditions used. Hyperbolic competitive inhibition was also observed which was consistent with a model in which binding of two AMP•PNP molecules occurred, both competitive with respect to ATP. The dissociation constants (Ki and Ki₂) were determined to be 0.58 μM and 19 μM, respectively.

Whereas AMP•PNP inhibition yielded linear steady-state rates of ATP hydrolysis when catalysed by energized SMP, time-dependent inhibition was observed with non-energized SMP and F₁-ATPase. The time-dependent inhibition was reversible as shown when treated enzyme was diluted into an assay medium, either directly or after the treated enzyme was centrifuge gel filtrated through a Sephadex G-50 column to remove free or loosely bound inhibitor. Whereas non-energized SMP recovered virtually all (99%) activity during the time course of the assay, F₁-ATPase recovered only 69% activity. The remaining inhibition is attributed to AMP•PNP bound at a high affinity site. The rate of ATP hydrolysis was shown to decrease exponentially during time-dependent AMP•PNP inhibition, and the recovery of activity also increased exponentially after removal or dilution of AMP•PNP.

AMP•PNP inhibition of F₁-ATPase activity also displayed hyperbolic competitive inhibition, consistent with the binding of a single AMP•PNP molecule with a Ki of 0.16 μM.
DEDICATION

To Mom, Dad, and Willy

Their love, encouragement, support, and guidance is my strength and foundation.
"The reasonable man adapts himself to the world; the unreasonable one persists in trying to adapt the world to himself. Therefore all progress depends on the unreasonable man."

George Bernard Shaw

"Of course women are the most unreasonable of all, n'est pas?"

Tania Kastelic
ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the people in the lab who gave their time unselfishly, and helped with the mitochondrial preparations. This work could not have been started without them.

To a special member of the lab group, Dr. Seeloch Beharry, who, in addition to helping with the F\textsubscript{i} preparations, was always there as a friend.

But most of all, I would like to thank Dr. Michael J. Gresser, who gave me the opportunity to work in the field of enzyme kinetics and membrane bioenergetics as an undergraduate. Throughout this entire experience, he was always available to offer encouragement and support, while providing assistance, and guidance with his knowledge and expertise.
# TABLE OF CONTENTS

Approval ......................................................................................................................... ii

ABSTRACT ........................................................................................................................ iii

DEDICATION ....................................................................................................................... iv

QUOTATIONS ................................................................................................................... v

ACKNOWLEDGEMENTS ........................................................................................................ vi

List of Tables ................................................................................................................... ix

List of Figures .................................................................................................................. x

I. ABBREVIATIONS ........................................................................................................... 1

II. INTRODUCTION ......................................................................................................... 3

III. OBJECTIVES ............................................................................................................. 10

IV. EXPERIMENTAL PROCEDURES .......................................................................... 11

MATERIALS ...................................................................................................................... 11

METHODS ........................................................................................................................ 12

V. RESULTS AND DISCUSSION ................................................................................... 21

Effect of ADP on AMP·PNP Inhibition of Energized SMP ............................................... 21

Determination of Ki for AMP·PNP Inhibition of Energized SMP .................................. 36

The Effect of AMP·PNP on Non-energized SMP .............................................................. 51

Effect of AMP·PNP on Soluble F₄-ATPase ..................................................................... 73

Determination of Ki for AMP·PNP Inhibition of Soluble F₄-ATPase ............................... 84

VI. SUMMARY AND FUTURE WORK .......................................................................... 99

VII. APPENDICES .......................................................................................................... 101

APPENDIX 1 ..................................................................................................................... 101

APPENDIX 2 ..................................................................................................................... 103

APPENDIX 3 ..................................................................................................................... 107
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reactivation of $F_1$-ATPase Activity</td>
<td>81</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>$F_0F_1$-ATPase</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Method of Preparing SMP and $F_1$</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Structures of ATP and AMP·PNP</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>Diagram of a Column Assembly Used for Reactivation Studies</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>Effect of PK on AMP·PNP Inhibition of ATP Hydrolysis by Energized SMP</td>
<td>22</td>
</tr>
<tr>
<td>6</td>
<td>Lineweaver-Burk Plot for PK Activity</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>Steady-State ADP Concentrations as a Function of PK Concentration</td>
<td>26</td>
</tr>
<tr>
<td>8</td>
<td>Effect of ADP on AMP·PNP Inhibition of ATP Hydrolysis</td>
<td>29</td>
</tr>
<tr>
<td>9</td>
<td>Effect of ADP on AMP·PNP Inhibition of ATP Hydrolysis Catalysed by Energized SMP</td>
<td>31</td>
</tr>
<tr>
<td>10</td>
<td>Possible Mechanism Involving IF, Interaction with $F_0F_1$-ATPase</td>
<td>34</td>
</tr>
<tr>
<td>11</td>
<td>Population of $F_0F_1$-ATPase Acting as a Coupling Enzyme</td>
<td>35</td>
</tr>
<tr>
<td>12</td>
<td>Lineweaver-Burk Plots for AMP·PNP Inhibition of the ATPase Activity of Energized SMP</td>
<td>37</td>
</tr>
<tr>
<td>13</td>
<td>Secondary Plot for AMP·PNP Inhibition of the ATPase Activity of Energized SMP</td>
<td>39</td>
</tr>
<tr>
<td>14</td>
<td>Proposed Mechanism for AMP·PNP Inhibition of ATPase Activity: Model 1</td>
<td>42</td>
</tr>
<tr>
<td>15</td>
<td>Non-Linear Regression of the Rate Expression Derived for Model 1 to Fig. 13</td>
<td>44</td>
</tr>
<tr>
<td>16</td>
<td>Proposed Mechanism for AMP·PNP Inhibition of ATPase Activity: Model 2</td>
<td>47</td>
</tr>
<tr>
<td>17</td>
<td>Non-Linear Regression of the Rate Expression Derived for Model 2 to Fig. 13</td>
<td>49</td>
</tr>
<tr>
<td>18</td>
<td>Effect of AMP·PNP on ATPase Activity of Non-Energized SMP</td>
<td>52</td>
</tr>
<tr>
<td>19</td>
<td>Sample Non-Linear Regression Fits for the Inactivation of ATPase Activity by AMP·PNP</td>
<td>55</td>
</tr>
<tr>
<td>20</td>
<td>Effect of Preincubation Time of AMP·PNP with Non-Energized SMP</td>
<td>57</td>
</tr>
</tbody>
</table>
### CHAPTER 1

**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP/ADP/ATP</td>
<td>adenosine 5'-mono-/di-/triphosphate</td>
</tr>
<tr>
<td>AMP·PNP</td>
<td>adenylyl imidodiphosphate</td>
</tr>
<tr>
<td>AP₃A</td>
<td>P₁, P₂ diadenosine pentaphosphate</td>
</tr>
<tr>
<td>β-NAD⁺/β-NADH</td>
<td>β-nicotinamide adenine dinucleotide (oxidized/reduced)</td>
</tr>
<tr>
<td>β-NADP⁺/β-NADPH</td>
<td>β-nicotinamide adenine dinucleotide phosphate (oxidized/reduced)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>ε-ATP</td>
<td>N¹, N² etheno-ATP</td>
</tr>
<tr>
<td>F₁</td>
<td>soluble ATPase</td>
</tr>
<tr>
<td>FCCP</td>
<td>carbonylcyanide p-trifluoromethoxy phenylhydrazone</td>
</tr>
<tr>
<td>G-6-PDH</td>
<td>glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>HK</td>
<td>hexokinase</td>
</tr>
<tr>
<td>IF₃</td>
<td>natural ATPase inhibitor</td>
</tr>
<tr>
<td>ITP</td>
<td>inosine 5'-triphosphate</td>
</tr>
<tr>
<td>LDH</td>
<td>L-lactate dehydrogenase</td>
</tr>
<tr>
<td>NAP₄-AMP·PNP</td>
<td>N-4-azido-2-nitrophenyl-7-aminobutyryl-AMP·PNP</td>
</tr>
<tr>
<td>PCP</td>
<td>pentachlorophenol</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenol pyruvate</td>
</tr>
<tr>
<td>Pₗ</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PK</td>
<td>pyruvate kinase</td>
</tr>
<tr>
<td>SMP</td>
<td>submitochondrial particles</td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>2', 3'-O-(2, 4, 6-trinitrophenyl)-ATP</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>tris (hydroxymethyl) aminomethane hydrochloride</td>
</tr>
<tr>
<td>Tris-Base/OAc</td>
<td>Tris (hydroxymethyl) aminomethane/acetate</td>
</tr>
</tbody>
</table>
CHAPTER II
INTRODUCTION

Much effort has been devoted to elucidating the mechanism of ATP synthesis catalysed by the mitochondrial ATP synthase complex. The ATP synthase complex, located on the inner membrane of the mitochondrion (matrix side), is an intricate oligomeric protein composed of a lipid soluble fraction, $F_o$, and a soluble fraction, $F_i$ (Fig. 1 - ref. 1). Both fractions are themselves of multisubunit complexity. The stoichiometry of $F_o$ is $a,b,c_3$, which varies depending upon the source and method of extraction. It is thought that not only does $F_i$ serve as an anchor for $F_o$, but that it also contains a pore through which protons pass during ATP synthesis and hydrolysis. The catalytic moiety, $F_i$, contains five subunits: $\alpha(55.2 \text{ kD})$, $\beta(51.6 \text{ kD})$, $\gamma(30.1 \text{ kD})$, $\delta(15.1 \text{ kD})$, and $\epsilon(5.7 \text{ kD})$ arranged in a $\alpha_3\beta\gamma\delta\epsilon$ stoichiometry (1, 2).

$F_i$, prepared as outlined in Fig. 2 (adapted from ref. 3), catalyses the $\text{Mg}^{2+}$-dependent hydrolysis of ATP to ADP and inorganic phosphate (Pi). Recently it has been shown that $F_i$ isolated from a number of sources can synthesize ATP, although the release of product does not occur (4 - 9). Although soluble $F_i$-ATPase cannot catalyse net ATP synthesis, when reconstituted into $F_i$ depleted submitochondrial particles, it is capable of synthesizing ATP in the presence of an oxidizable substrate e.g. succinate or NADH (10). Utilizing an electrochemical gradient (consisting of two components: the transmembrane potential—separation of charge, and the pH gradient) which is formed upon oxidation of electron transport chain substrates or the hydrolysis of ATP, SMP can couple this electrochemical gradient to net ATP synthesis.

Binding studies have revealed the existence of six nucleotide binding sites on $F_i$ (11). The catalytic sites, known to hydrolyse a number of nucleotide triphosphates
Figure 1

$F_0F_1$–ATPase
Figure 2

Method of Preparing SMP and $F_1$
(ATP, e-ATP, TNP-ATP, GTP, ITP, 2-azido-ATP, 8-azido-ATP) (12 – 15), have been localized to the β-subunits, whereas the "regulatory" sites are highly specific for adenine nucleotides (AD(T)P, 2-azido-AD(T)P, and AMP·PNP). Labelling studies using azido derivatives of adenine nucleotides, support the hypothesis that the regulatory sites are located at the α/β interfaces (16). Binding studies to the isolated β-subunit from Rhodospirillum rubrum F, have shown that the β-subunit contains two nucleotide binding sites; one high affinity site (Kd for ATP and ADP of 4.4 and 6.4 µM, respectively—dependent of Mg2+), and one low affinity site (Kd for ATP of 200 µM—Mg2+-dependent) (17). In contrast, the isolated α-subunit from E. coli, has been shown to contain only one adenine nucleotide binding site (Kd for ATP and ADP of 0.1 and 0.9 µM, respectively) (18). Kinetic and oxygen labelling experiments in the presence of Mg2+, show that adenine nucleotides exhibit negative cooperativity in binding to the catalytic sites and positive cooperativity in catalysis (19). It has also been shown that the cooperative effects of ATP can be eliminated by bicarbonate which is thought to accelerate ADP release once Pi has dissociated (20).

In order to elucidate the mechanism of mitochondrial ATP synthase complex, substrate analogs, photoactivatable substrate analogs, as well as radiolabelled substrates and analogs have been extensively used to study catalytic mechanism, binding properties and subunit interactions (21). One such ATP substrate analog is adenylyl imidodiphosphate (AMP·PNP), shown in Fig. 3.

Synthesized by Yount et al. (22) in order to study the mechanism of myosin ATPase in muscle (23), AMP·PNP is used extensively as an ATP analog to study mitochondrial ATPase. It was once thought that F1 was incapable of hydrolysing the phosphorimidate linkage, yet able to bind AMP·PNP and ATP, its normal substrate, with equal efficiency (24). However, recent studies have shown that not only is
Figure 3

Structures of ATP and AMP-PNP
AMP·PNP hydrolysed by Ca²⁺-ATPase from sarcoplasmic reticulum (25), and *E. coli* alkaline phosphatase (22), but also by F₁-ATPase, although slowly in the presence of Mg²⁺ (pH 8.0) (26).

Although inhibition studies with F₁ and SMP have shown AMP·PNP to be a strong competitive inhibitor of ATP hydrolysis (11, 27 - 31), with the dissociation constant (Kᵢ) for the enzyme–inhibitor complex reported to be as low as 14 nM (11), oxidative phosphorylation by SMP (29 - 32) is not inhibited. This has been interpreted as evidence supporting the existence of separate catalytic sites for the ATP synthesis and hydrolysis reactions (31). Another possibility consistent with the binding-change mechanism proposed by Boyer et al. (33, 34), is that AMP·PNP tightly bound at the catalytic site is released upon binding ADP at a high affinity site located on a second interacting β-subunit. AMP·PNP bound to F₁ is released upon ADP binding within one catalytic turnover of the enzyme. In this way, AMP·PNP is never observed to cause inhibition of ATP synthesis and would appear to be a one-way inhibitor of ATP synthase. Under typical ATP synthesis assay conditions, initial velocities are monitored at high ADP concentrations, which are kept constant using an ATP trap (HK, G-6-PDH, glucose, and NADP⁺). Depending on the efficiency of the coupled enzyme system, ADP concentrations maintained in such assays may overcome any possible inhibition by AMP·PNP. Contaminating ADP present in the assay medium, may also relieve AMP·PNP inhibition prior to initiation of ATP synthesis. Studies have shown that ADP has a significant effect on relieving AMP·PNP inhibition of ATP hydrolysis by SMP or F₁ (29, 31, 35). In other studies in which binding of AMP·PNP or its photoactivatable analog NAP⁺-AMP·PNP to F₁ was measured, it was shown that addition of ADP caused release of AMP·PNP (36, 37). Since synthesis of ATP by ATP synthase requires a transmembrane electrochemical potential (Δµ⁺) (38),
the lack of AMP·PNP inhibition may also be explained by a decreased affinity of the
ATP analog for SMP due to a conformational change of the enzyme upon energization
of the membrane. In view of these observations, the sensitivity of AMP·PNP inhibition
of ATP hydrolysis by energized SMP to ADP may be of fundamental importance in
explaining the absence of AMP·PNP inhibition of ATP synthesis.

In addition to not inhibiting ATP synthesis, another peculiar property observed
for the ATP analog AMP·PNP, was the wide range of Ki values reported for its
inhibition of ATP hydrolysis. In published studies, Ki values which vary over a
hundred fold range have been reported; 14 nM (11) to 0.2 - 1.3 µM (28, 30, 31).
This in itself is not surprising since F₁ possesses multiple nucleotide binding sites with
different affinities for AMP·PNP: one high affinity site (Kd = 18 nM) and two low
affinity sites (Kd = 1.0 µM) (11). The Ki value observed may represent the
dissociation of AMP·PNP from a site which is dependent on the assay conditions
used, i.e., AMP·PNP and ATP concentrations, dilution of the enzyme prior to assay,
etc.. The Ki values observed may also reflect the presence of ADP, which may lower
the affinity of the enzyme for AMP·PNP and lead to an increase in the Ki value.

Studies of AMP·PNP inhibition of ATP hydrolysis catalysed by F₁ (28, 31, 35)
have also revealed a time-dependent inhibition not observed with energized SMP.
Questions which must be raised are: does complete inhibition of ATP hydrolysis by
AMP·PNP occur or is a steady-state level of inhibition reached? Is this inhibition
reversible? Until these questions are answered, the conclusions based on F₁ studies
which are used to elucidate the mechanism of ATP synthesis, may not be directly
applicable to the mechanism of mitochondrial ATP synthase complex in vivo.
CHAPTER III
OBJECTIVES

1. Determine whether increasing ADP concentration reduces AMP•PNP inhibition of ATP hydrolysis by energized SMP. Determine the extent of AMP•PNP inhibition at various steady-state ADP concentrations.

2. Determine the Ki value(s) for AMP•PNP inhibition of ATP hydrolysis over a wide range of AMP•PNP concentrations.

3. Observe the kinetic effects of AMP•PNP on non-energized SMP.

4. Study the AMP•PNP inhibition of ATP hydrolysis catalysed by F_1 as a function of incubation time.

5. Determine the Ki value for AMP•PNP inhibition of F_1-ATPase.

6. Formulate mechanisms which can rationalize the results obtained, and relevant published results.
CHAPTER IV
EXPERIMENTAL PROCEDURES

MATERIALS

All reagents used were reagent grade, analytical reagent grade or enzyme grade quality. The following chemicals and enzymes were obtained from Boehringer Mannheim: β-NADP (crystallized monopotassium salt); PEP (monopotassium salt); AP₃A (trilithium salt); AMP-PNP (tetralithium salt); G-6-PDH (ammonium sulfate suspension, Grade 1 from yeast); and LDH (ammonium sulfate suspension, from rabbit muscle). The chemicals and enzymes obtained from Sigma Chemical Co. were as follows: rotenone; β-NADH (disodium salt, Grade III from yeast); Tris-HCl; Tris-Base; sucrose (Grade I); BSA (crystallized, lyophilized); ATP (disodium salt, from equine muscle); ADP (sodium salt, Grade IX from equine muscle); HK (ammonium sulfate suspension, Type C-300 from Bakers yeast); and PK (lyophilized, Type III from rabbit muscle).

Monobasic potassium phosphate (KH₂PO₄), dibasic potassium phosphate (KH₂PO₄ · 3H₂O), and D-glucose were obtained from Amachem Co. Sephadex G-50 (fine) was obtained from Pharmacia Fine Chemicals. EDTA was from BDH Chemicals. Succinic acid was obtained from two sources: Sigma Chemical Co. (disodium salt, hexahydrate) and McArthur Chemical Co. Ltd. Ammonium sulfate was from Schwarz/Mann, Inc. division of Mediscience. PCP was from ICN Pharmaceuticals.
**METHODS**

- Preparation of Beef Heart SMP

  Beef heart mitochondria (heavy fraction) isolated by the method of Smith (39) were used in the preparation of SMP as described by Beyer (40). SMP were stored at -70 °C until used.

- Preparation of Beef Heart Mitochondrial F₁-ATPase

  Beef heart mitochondria (both heavy and light fractions) were used to prepare F₁-ATPase by the method described by Knowles and Penefsky (41, 42). The F₁ was stored at 4 °C as an ammonium sulfate suspension until used.

- Desalting Ammonium Sulfate Suspensions of Coupling Enzymes

  Sephadex-G-50 (fine) was swollen overnight (4 °C) in 50 mM Tris-OAc pH 7.5 (5 g Sephadex : 100 ml buffer). A 1.0 ml tuberculin syringe fitted with a porous polyethylene frit was filled with swollen Sephadex. The columns were centrifuged in a I.E.C. tabletop centrifuge, swinging bucket rotor model 221, at 1050 x g (setting 5, 1 min). Unless otherwise stated, all Sephadex columns were centrifuged under these conditions. Ammonium sulfate suspensions were centrifuged at 27,000 x g, in a Sorvall RC-5B Centrifuge, (15,000 rpm, 4 - 6 °C, 15 min, Sorvall SS-34 rotor). The pellet was resuspended in cold buffer, aliquots (125 μl) were added to each packed Sephadex column (0.7 ml), and centrifuged in the I.E.C. tabletop centrifuge. The eluate was then collected and diluted with cold buffer to the appropriate concentration.
Desalting Ammonium Sulfate Suspensions of F₁

The procedure used to desalt F₁ was similar to that described for the coupling enzymes except Sephadex was swollen in F₁ resuspension buffer (0.25 M sucrose/1 mM MgCl₂/50 mM Tris-OAc pH 7.5); an aliquot of F₁ ammonium sulfate suspension was centrifuged at 17,000 x g (12,000 rpm, 4 - 6 °C, 15 min, Sorvall SS-34 rotor), the pellet was quickly dissolved in F₁ resuspension buffer (25 °C) to approximately 1.2 mg/ml, centrifuged through a packed Sephadex column, and the eluate diluted to 0.6 mg/ml with F₁ resuspension buffer (25 °C). The desalted F₁ solution was kept at room temperature (20 - 25 °C) during the experiment. Usually 5% activity loss occurred within 5 hours under these conditions.

Preparation of Reactivation Experiment Columns

Sephadex swollen in 50 mM Tris-OAc pH 7.5 (SMP reactivation) or F₁ resuspension buffer (F₁ reactivation) was added to a 1 ml tuberculin syringe fitted with a porous polyethylene frit, and centrifuged in the tabletop centrifuge. The packed Sephadex column (0.7 ml) was transferred to a clean conical centrifuge tube (15 ml) and fitted with a cut eppendorf tip (1000 µl: 250 µl capacity when cut), which would eventually contain the treated enzyme sample (100 - 125 µl). The set-up is shown in Fig. 4.

Protein Determinations

Protein concentrations were determined by the method of Lowry et al. (43) using BSA (ε₂₈₀nm = 0.667 ml·mg⁻¹·cm⁻¹) as standard.
Figure 4

Diagram of a Column Assembly Used for Reactivation Studies
Preparation of Stock Solutions

All stock solutions were made in Tris-OAc buffer (50 mM, pH 7.5), and adjusted to pH 7.5 with either Tris-OAc (50 mM, pH 4) or Tris-Base (50 mM, pH 9.8), unless otherwise stated.

A stock solution of rotenone (1 mM in 95% ethanol) was diluted twofold into a 1:1 solution of 95% ethanol/Tris-OAc (50 mM, pH 7.5). This solution was prepared fresh each day before use.

A stock solution of PCP (10 mM in 95% ethanol) was diluted to a concentration of 0.15 mM in a 1:1 solution of 95% ethanol/Tris-OAc (50 mM, pH 7.5). The diluted PCP solution was prepared fresh each day before use.

Stock PEP was adjusted to pH 7.5 using a small volume of KOH (5 M) in addition to Tris-OAc (50 mM, pH 7.5).

Statistics

Two computer programs used for the non-linear regression analysis. They were:

1. Biomedical Computer Programs (BMDP)
   Department of Biomathematics
   University of California
   Los Angeles, U.S.A.

2. ITERAT
   Dr. K. E. Newman
   Department of Chemistry
   Simon Fraser University
   Burnaby, B.C., Canada
Spectrophotometric Assays

All spectrophotometric assays were performed at 30 °C using a Cary 14 spectrophotometer which monitored the change in absorbance of NADH or NADPH at 340 nm \( (\varepsilon_{340nm} = 6.22 \text{ mM}^{-1}\text{cm}^{-1}) \) as a function of time.

ATP Hydrolysis Assay

The rate of ATP hydrolysis catalysed by SMP or F₁ was monitored by coupling the production of ADP to the oxidation of NADH using PEP, PK, and LDH. The reaction is shown in Scheme 1.

Monitoring ATP hydrolysis by SMP required special assay conditions in order to avoid complications in the interpretation of the data, produced by some of the other integral proteins associated with the vesicle membrane. Rotenone, an inhibitor of the NADH dehydrogenase complex of the mitochondrial respiratory chain, was added 1.5 min prior to the addition of NADH to the reaction medium in order to prevent energization of the SMP membrane by NADH oxidation. If left uninhibited, the observed rate of NADH oxidation would be greater than the true net rate of ATP synthesis. \( \text{ATP} \rightarrow 2\text{ADP} \). AMP was also included to inhibit adenylate kinase, which catalyses the reaction: \( 2\text{ADP} \rightarrow \text{ATP} + \text{AMP} \). If this reaction were not inhibited, ADP would bypass the coupled enzyme assay system, resulting in a net decrease in the ADP concentration in the assay medium.

Monitoring ATP hydrolysis under energized conditions required oxygenation of the buffer (50 mM Tris-OAc, pH 7.5), MgCl₂, succinate, and glucose solutions prior to initiation of the reaction in order to prevent O₂ depletion during the course of the reaction. Succinate, a respiratory chain substrate, was included to energize the
In order to study ATP hydrolysis by non-energized SMP, PCP was added to the standard assay medium. PCP, a proton ionophore, dissipates the transmembrane pH gradient ($\Delta p$H) and the electrical potential ($\Delta \Psi$) generated by the oxidation of succinate for hydrolysis of ATP. The minimum concentration of PCP which abolished ATP synthesis by dissipating the transmembrane electrochemical potential ($\Delta \mu_{H^+}$), was added to the hydrolysis medium. The residual rate of NADPH production observed when monitoring ATP synthesis was due to glucose oxidation in the presence of Pi catalysed by G-6-PDH.
ATP Synthesis Assay

The rate of ATP synthesis catalysed by energized SMP was monitored by coupling the synthesis of ATP to NADPH production using glucose, HK, and G-6-PDH. The reaction is shown in Scheme 2. Again ATP was included in the assay mixture to prevent ATP formation by adenylate kinase. The reaction mixture was also oxygenated prior to the assay.

PK Kinetic Parameters' Assay

The rate of ATP synthesis catalysed by PK was monitored by coupling the production of ATP to the appearance of NADPH using glucose, HK, and G-6-PDH. The reaction is shown in Scheme 3. Although the production of pyruvate by PK can also be measured directly using LDH and NADH, the reaction shown in this scheme maintains a constant ADP concentration in the assay medium, which is necessary when measuring initial velocities.
SUCCINATE + $\frac{1}{2}$O$_2$ \rightarrow FUMARATE + H$_2$O

\[ P_i + ADP \xleftrightarrow{SMP} ATP + H_2O \]

GLUCOSE-6-PHOSPHATE \xrightarrow{HK} GLUCOSE

$\beta$-NADP$^+$ \xrightarrow{G-6-PDH} $\beta$-NADPH + H$^+$

6-PHOSPHOGLUCONO-$\delta$-LACTONE

Scheme 2

ATP Synthesis: Coupled Enzyme Assay System
Scheme 3

ATP Synthesis—From ADP and PEP: Coupled Enzyme Assay System
CHAPTER V
RESULTS AND DISCUSSION

Effect of ADP on AMP·PNP Inhibition of Energized SMP

In order to observe the effect of ADP on AMP·PNP inhibition of ATP hydrolysis catalysed by energized SMP, without changing the total nucleotide concentration in the assay medium, the concentration of PK in the reaction medium was varied. As shown in Fig. 5, at saturating PK concentration, 0.5 \( \mu M \) AMP·PNP was able to inhibit ATP hydrolysis by 27%. As expected, lowering the PK concentration decreased the rate of NADH oxidation observed in the presence and absence of AMP·PNP.

As the concentration of PK is lowered from saturating levels where the production of ADP is rate limiting, to subsaturating conditions where the production of ADP by SMP catalysis is not, the steady-state concentrations of ADP were expected to increase. Using Eq. 3 from Appendix 1, the steady-state ADP concentration could be calculated given the concentration and kinetic parameters of the PK used, and the rate of NADH oxidation observed. Since the specific activity of the PK used varied greatly with the age and preparation of the enzyme, the kinetic parameters of this enzyme were determined for each experiment. From the experiment shown in Fig. 5, the \( K_m \) and \( V_{max} \) under saturating conditions of PEP, was determined to be 565 \( \mu M \) ADP and 295 \( \mu \text{mole} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \), respectively, as calculated from the Lineweaver–Burk plot shown in Fig. 6. As shown in Fig. 7, the steady-state ADP concentration did decrease as the concentration of PK in the assay medium was increased. Even at the lowest PK concentration used (4.1 \( \mu g/ml \)), only 4 \( \mu M \) ADP was present (20% of the total...
Effect of PK on AMP•PNP Inhibition of ATP Hydrolysis by Energized SMP.

SMP (0.16 mg) were incubated at 30 °C for 3 minutes in an assay medium containing 50 mM Tris-OAc pH 7.5, 6 mM glucose, 10 mM succinate, 10 mM MgCl₂, 20 mM PEP, 5 mM Pi, 0.4 mM AP₃A, 5 μM rotenone, 0.11 mM NADH, 30 μg LDH, and various concentrations of PK, in the presence (●) or absence (○) of 0.5 μM AMP•PNP. The assay was initiated with the addition of ATP (20 μM). The decrease in absorbance at 340 nm was monitored versus time. The total reaction volume was 1.0 ml.
Lineweaver-Burk Plot for PK Activity.

PK (90 μg) was incubated at 30 °C for 3 min in an assay medium containing 50 mM Tris-OAc pH 7.5, 6 mM glucose, 10 mM succinate, 10 mM MgCl₂, 20 mM PEP, 5 mM Pi, HK (0.36 mg), G–6–PDH (0.10 mg), and 1 mM NADP⁺. The reaction was initiated with the addition of ADP. The increase in absorbance at 340 nm due to NADP⁺ reduction was monitored versus time to determine the rate of ATP production. The total reaction volume was 1.0 ml.
Figure 7

Steady-State ADP Concentrations as a Function of PK Concentration.

Based on Eq. 3 from Appendix 1, the steady-state ADP concentration was determined in the presence (●) and absence (○) of 0.5 μM AMP-PNP.
adenine nucleotide present).

When the rate of NADH oxidation was plotted versus the steady-state ADP concentration (Fig. 8), no relief of AMP·PNP inhibition by ADP concentrations reaching 3.5 μM was observed. Fig. 8 (inset) which plots the percent inhibition versus steady-state ADP concentration, shows this insensitivity. The average inhibition was 28.8 ± 2.9%. The results of a similar experiment (Fig. 9) using the same concentration range of PK and ATP concentrations, but higher SMP concentration (0.31 mg/ml), again did not show a sensitivity to ADP. The AMP·PNP concentration was raised to 1.6 μM in order to observe a significant level of inhibition (33%) at 35.2 μg/ml PK. The sensitivity (or lack thereof) of AMP·PNP inhibition to ADP is shown in Fig. 9 (inset).

The higher steady-state ADP concentrations which may have been required to relieve AMP·PNP inhibition of energized SMP, were not reached in these experiments. Due to the rapid loss of PK activity at low concentrations and the very low rates of NADH oxidation which would have been difficult to monitor spectrophotometrically, the low PK concentrations necessary to substantially increase the steady-state ADP concentrations were not achieved in these and other experiments performed.

In addition to the level of steady-state ADP concentration, another possible explanation for why AMP·PNP inhibition of ATP hydrolysis by energized SMP is not sensitive to ADP, yet AMP·PNP is unable to inhibit ATP synthesis, is a possible heterogeneity of the enzyme population. Physiologically there is no benefit for the cell to hydrolyze newly synthesized ATP. The oxidation of substrates (NADH, succinate, etc.) necessary to "prime" the mitochondrial membrane for ATP synthesis would be futile. It is therefore possible that the hydrolytic activity observed using SMP, may in
Figure 8

Effect of ADP on AMP-PNP Inhibition of ATP Hydrolysis.

At the given PK concentration, and rate of ATP hydrolysis by energized SMP in the presence (●) and absence (○) of 0.5 μM AMP-PNP shown in Fig. 5, and the corresponding steady-state ADP concentration shown in Fig. 7, the rate of ATP hydrolysis can be plotted as a function of steady-state ADP concentration. The inset shows the sensitivity of AMP-PNP inhibition of ATP hydrolysis to steady-state ADP concentration.
Figure 9

Effect of ADP on AMP·PNP Inhibition of ATP Hydrolysis Catalysed by Energized SMP.

The experimental conditions were as those described in the legend to Fig. 5, except SMP (0.31 mg) were incubated in the presence (●) or absence (○) of 1.0 μM AMP·PNP. The inset shows the inhibition of ATP hydrolysis as a function of steady-state ADP concentration.
fact be a consequence of the SMP isolation procedure. The naturally occurring inhibitor protein (IF₁) which inhibits ATP hydrolysis, may either be dissociated from F₁, or still bound to F₁ but conformationally unable to inhibit ATP hydrolysis. Activity may also arise from F₁ bound to membrane fragments which may show a higher affinity for ATP than F₁ bound to membrane vesicles which have been energized. The physiological association of IF₁ to F₁ in vivo, may be a mechanism which prevents ATP (newly synthesized or endogenous) from binding to the catalytic site of F₁. Ordered binding of substrates, ADP and Pi, may prevent ATP or ATP analogs from binding to the same site.

Fig. 10 is such a model where IF₁ prevents the binding of a nucleotide triphosphate. This model would predict no AMP·PNP inhibition or product inhibition of ATP synthesis to occur. Pi binding prior to ADP is likely to occur at the third nucleotide binding site (not participating in the catalytic cycle) giving support to the stoichiometry of Pi binding to F₁ (approx. 1 : 1) found by Penefsky (44). The interaction of AMP·PNP with SMP i.e. its ability to inhibit the hydrolysis but not synthesis of ATP, which has been used to support the theory of two different sites on F₁: one for synthesis and the other for hydrolysis, may indicate the presence of different F₁ populations. It is therefore possible that AMP·PNP may only affect F₁ incapable of ATP synthesis. It is conceivable that although lowering the PK concentration did increase the steady-state ADP concentration, the population of F₁ "geared" to ATP synthesis may have kept the ADP concentration low by catalysing the synthesis of ATP from newly hydrolysed ADP and Pi present, as shown in Fig. 11, in this way acting as a coupling enzyme.
Figure 10

Possible Mechanism Involving IF$_1$ Interaction with F$_{0}$F$_{1}$-ATPase
Figure 11

Population of $F_1 F_1$-ATPase Acting as a Coupling Enzyme
Determination of $K_i$ for AMP·PNP Inhibition of Energized SMP

AMP·PNP inhibition of ATP hydrolysis catalysed by SMP has been studied by other workers (30, 31). Energization of the submitochondrial membrane either by NADH oxidation or ATP hydrolysis, yielded $K_i$ values of 0.16 $\mu$M (0 – 1.48 $\mu$M AMP·PNP – ref. 31) and 1.3 $\mu$M (0 and 1.92 $\mu$M AMP·PNP – ref. 30). In the presence of the uncoupler FCCP, a $K_i$ value of 0.20 $\mu$M was determined (0 – 6.3 $\mu$M AMP·PNP – ref. 30). The $K_i$ values determined for soluble F$_1$-ATPase also varied with the AMP·PNP concentration range used. Cross and Nalin (11) determined a $K_i$ value as low as 14 nM (0 – 50 nM AMP·PNP), while AMP·PNP concentrations in the $\mu$molar region gave $K_i$ values of 0.92 $\mu$M (30), 0.5 $\mu$M (28), 0.33 $\mu$M (31), and 0.32 $\mu$M (27).

Because of the variety of conditions used, it was decided to study AMP·PNP inhibition of ATP hydrolysis by energized SMP using a large range of AMP·PNP concentration. As seen in Fig. 12, the Lineweaver-Burk plots for AMP·PNP inhibition of ATPase activity show AMP·PNP to be a competitive inhibitor of energized SMP in agreement with other studies (11, 27 – 31). The kinetic parameters $K_m$ and $V_{max}$, for ATP hydrolysis by energized SMP determined in the absence of AMP·PNP, were calculated from the horizontal intercept ($-1/K_m$) and vertical intercept ($1/V_{max}$). The values of $K_m$ and $V_{max}$ were found to be 53.2 $\mu$M and 372 n mole·min$^{-1}$·mg$^{-1}$.

The replot of the slopes from the Lineweaver-Burk plots versus the inhibitor concentration shown in Fig. 13, was not linear, as expected for a competitive inhibitor (see Appendix 2 for explanation). The secondary plot is hyperbolic. As the concentration of AMP·PNP increases, the slope of the graph decreases.
Lineeweaver–Burk Plots for AMP·PNP Inhibition of the ATPase Activity of Energized SMP.

SMP (0.18 mg) were incubated for 3 min at 30 °C in an assay medium containing 50 mM Tris-OAc pH 7.5, 6 mM glucose, 10 mM succinate, 10 mM MgCl₂, 20 mM PEP, 5 mM Pi, 0.4 mM AP₅A, 5 μM rotenone, PK (0.18 mg), LDH (75 μg), 0.13 mM NADH, and AMP·PNP (0 – 40 μM). ATP (20 – 100 μM) was added at the third minute of incubation to initiate the hydrolysis reaction. The rate of NADH oxidation was monitored at 340 nm. The total reaction volume was 1.0 ml. The curve symbols (△, ●, ○, □, ■, ◊, X) represent AMP·PNP concentrations of (0, 1, 2, 5, 10, 25, and 40 μM), respectively. The vertical bars indicate a 5% error.
Secondary Plot for AMP·PNP Inhibition of the ATPase Activity of Energized SMP.

The slopes from the Lineweaver-Burk plots shown in Fig. 12 were plotted as a function of AMP·PNP concentration.
Because of the existence of three $\beta$-subunits per $F_1$-ATPase, a model which only involves one AMP•PNP binding competitively with respect to ATP, yet while bound allows two site catalysis to occur at the remaining two sites, was postulated (Fig. 14). If one assumes that AMP•PNP bound at a site does not affect the maximal velocity of the two sites involved in catalysis, then the secondary plot would obey Eq. 1.

$$\text{slope} = \frac{K_m\left\{1 + \frac{a \cdot [I]}{K_i}\right\}}{V_{\text{max}}\left\{1 + \frac{b \cdot [I]}{K_i}\right\}}$$

where $a$ and $b$ are constants defined in Appendix 3

Equation 1

Eq. 1 would predict that at the extremes of inhibitor concentration ($[I] = 0$ and $\infty$), the slope would increase from $K_m/V_{\text{max}}$ (at $[I] = 0$), to its limiting value of $(K_m/V_{\text{max}}) \cdot a/b$ or $K_m'/V_{\text{max}}'$ (at $[I] = \infty$). In order for the slope to increase, parameter $a$ must be greater than parameter $b$.

A non-linear regression analysis of Eq. 1 to the experimental data shown in Fig. 13, yielded Fig. 15. As can be seen from this graph, the model could predict the observed data at the low AMP•PNP concentrations, whereas large deviations occurred at the higher concentrations used.
Proposed Mechanism for AMP-PNP Inhibition of ATPase Activity: Model 1.

where

E - Free enzyme
T - ATP
D - ADP and Pi
I - AMP-PNP
- high affinity
- low affinity
- change in binding affinity

\[
\begin{align*}
D & \xrightarrow{k_5} TED \\
E \cdot T & \xleftarrow{k_1} \xrightarrow{k_2} TED \\
& \xleftarrow{k_3} T \\
TED & \xrightarrow{k_4} T \\
& \xrightarrow{k_6} IED \\
& \xrightarrow{k_7} I \cdot E \cdot T \\
\end{align*}
\]
Non-Linear Regression of the Rate Expression Derived for Model 1 to Fig. 13.

Slope expression was:

\[
\text{slope} = \frac{K_m \left(\frac{1 + P_1 \cdot [I]}{1 + P_2 \cdot [I]}\right)}{V_{\text{max}}}
\]

where

\begin{align*}
P_1 &= \frac{a}{K_i} \\
P_2 &= \frac{b}{K_i}
\end{align*}

\[a\] and \[b\] are defined in Appendix 3

Fit:

Correlation Matrix:

\[
\begin{pmatrix}
1.00 & 0.96 \\
0.96 & 1.00
\end{pmatrix}
\]

\[K_m/V_{\text{max}} = 0.14 \mu\text{mole}^{-1} \cdot \text{min}^{-1} \cdot \text{mg} \cdot \text{mM}\]

\[P_1 = 1.09 \pm 0.18\]

\[P_2 = 0.16 \pm 0.04\]
A second mechanism was therefore proposed which would allow the binding of a second molecule of AMP-PNP to F₁, again competitive with respect to ATP. This model is shown in Fig. 16. As in the previous model, if one assumes that the maximal velocity of two site catalysis is not affected by the presence of bound AMP-PNP, then the secondary plot would obey Eq. 2.

\[
\text{slope} = \frac{\text{Km}}{\text{Vmax}} \left\{ \frac{1 + a \cdot [I]/K_i}{1 + b \cdot [I]/K_i} \left(1 + c \cdot [I]/K_i\right) \right\}
\]

where a, b, and c are constants defined in Appendix 4.

Equation 2

Eq. 2 would predict that at the extremes of inhibitor concentration ([I] = 0 and infinity), the slope would increase from \(\text{Km}/\text{Vmax}\) (at [I] = 0), to infinity (at [I] = ∞). At high inhibitor concentrations, the slope should vary linearly with inhibitor concentration. As in the previous model, in order for the slope to increase, constant a must be greater than b.

A non-linear regression analysis of Eq. 2 was performed using the experimental data shown in Fig. 13. As can be seen from Fig. 17, the model involving the binding of multiple AMP-PNP molecules to ATP synthase, fit the experimental data over the entire AMP-PNP concentration range used. Under single-site catalysis conditions, the equilibrium constant for ATP hydrolysis by soluble F₁-ATPase, was found to be

46
Figure 16

Proposed Mechanism for AMP·PNP Inhibition of ATPase Activity: Model 2.

where
E - Free enzyme
T - ATP
D - ADP and Pi
I - AMP·PNP
* high affinity

low affinity

* change in binding affinity
Figure 17

Non-Linear Regression of the Rate Expression Derived for Model 2 to Fig. 13.

Slope expression was:

\[
\text{slope} = \frac{K_m}{V_{\max}} \left\{ \frac{1 + P_1 \cdot [I]}{1 + P_2 \cdot [I]} \right\} \left\{ \frac{1 + P_3 \cdot [I]}{1 + P_3 \cdot [I]} \right\}
\]

where

\[ P_1 = \frac{a}{K_i} \]
\[ P_2 = \frac{b}{K_i} \]
\[ P_3 = \frac{c}{K_i} \]

a, b, and c
are defined in
Appendix 4

Fit:

<table>
<thead>
<tr>
<th>Correlation Matrix:</th>
<th>( \frac{K_m}{V_{\max}} = 0.14 ) ( \mu \text{ mole}^{-1} \cdot \text{min} \cdot \text{mg} \cdot \text{mM} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>P1 = 1.72 ± 0.10</td>
</tr>
<tr>
<td>0.97 1.00</td>
<td>P2 = 0.41 ± 0.04</td>
</tr>
<tr>
<td>0.75 0.87 1.00</td>
<td>P3 = 0.18 x 10^{-1} ± 0.02 x 10^{-1}</td>
</tr>
</tbody>
</table>
0.5 (45) and approximately 0.5 with KCl-washed SMP (46 – 48). If the equilibrium constants for the ATP hydrolysis steps (k1/k2 and k12/k11) shown in Fig. 16, are set to 0.5, Ki and Ki values of 0.58 μM and 19 μM, respectively, could be calculated from the fit shown in Fig. 17.

In order to determine if the slope is in fact dependent on inhibitor concentration at the high AMP·PNP concentrations (consistent with Model 2) or approaches a limiting value which is independent of inhibitor concentration (Model 1), additional studies at very high AMP·PNP concentrations will need to be done.

The Effect of AMP·PNP on Non-energized SMP

Whereas AMP·PNP produced linear inhibited steady-state rates of ATP hydrolysis by energized SMP at AMP·PNP concentrations as high as 40 μM in the presence of 20 μM ATP, time-dependent inhibition was observed under non-energized conditions. This time-dependent loss in activity was not due simply to the SMP or any of the coupling enzymes losing activity over the course of the assay.

As compared to ATP hydrolysis in the absence of AMP·PNP (Fig. 18 curve 1) which showed a slight lag in hydrolysis before a linear steady-state rate was achieved (ΔA/min = 0.188), AMP·PNP added to the SMP three minutes prior to ATP (Fig. 18 curve 3) or with ATP (Fig. 18 curve 4), produced time-dependent inhibition. The initial rate of ATP hydrolysis monitored, was faster when AMP·PNP was not preincubated with the enzyme (Fig. 18 curve 4) as compared to a three minute preincubation (Fig. 18 curve 3), corresponding to 58% and 67% inhibition, respectively. A non-linear regression analysis to the equation describing the first order decay of ATPase activity (see Appendix 5) for the time-dependent inhibition shown in Fig. 18
Figure 18

Effect of AMP·PNP on ATPase Activity of Non-Engerized SMP.

SMP (0.18 mg) were incubated at 30 °C for 3 min in a medium which contained 50 mM Tris-OAc pH 7.5, 6 mM glucose, 10 mM MgCl₂, 22 mM PEP, 5 mM Pi, 0.4 mM AP₅, 0.18 mg PK, 75 µg LDH, 5 µM rotenone, 0.13 mM NADH, 7.5 µM PCP, and where indicated 5 µM AMP·PNP. The hydrolysis reaction was initiated with ATP (final concentration 40 µM). The total reaction volume was 1.0 ml. The change in absorbance due to NADH oxidation was monitored at 340 nm.

Curves:
- Curve 1 + PCP: no AMP·PNP
- Curve 2 - PCP: no AMP·PNP
- Curve 3 + PCP: + AMP·PNP (ATP initiates rxn)
- Curve 4 + PCP: + (AMP·PNP and ATP initiate rxn)
curves 3 and 4, gave apparent first order rate constants \( k_{\text{app, inact}} \) of 0.080 and 0.211 min\(^{-1}\), respectively, and the percent activity remaining at steady-state as 0 and 14%, respectively. Sample inactivation fits for curves 3 and 4 (Fig. 18), are shown in Fig. 19. The absence of PCP in the assay medium (Fig. 18 curve 2) resulted in a 17% decrease in ATP hydrolysis activity \( \Delta A/min = 0.156 \). Although succinate was not included in the ATP hydrolysis medium, energization of the membrane vesicle may have allowed for the synthesis of ATP. Because the observed net rate of ATP hydrolysis is equal to the difference between the ATP hydrolysis and synthesis rates, any synthesis of ATP would decrease the net hydrolysis rate observed.

As Fig. 20 shows, the initial rate of ATP hydrolysis was sensitive to the length of time AMP•PNP was preincubated with non-energized SMP. As the preincubation time increased, the initial rate of ATP hydrolysis decreased. AMP•PNP added with ATP caused a 56% inhibition of ATPase activity, whereas a 2 hours preincubation, resulted in 94% inhibition of the rate of ATP hydrolysis. Inhibition of ATP hydrolysis by AMP•PNP appears to be biphasic; that is, a rapid inactivation is followed by a slower rate of inactivation. Based on the constants \( k_{\text{app, inact}} \) determined for curves 3 and 4 (Fig. 18), a time course for AMP•PNP inhibition is shown in Fig. 21. Included in Fig. 21 is the data from Fig. 20. As can be seen from this graph, there is virtually no difference in the percent inhibition (±5%) within the first 20 min, but large deviations occur after this time. The fit of the first order rate equation to the data shown in Fig. 18 curve 4, requires that at steady-state \( (t \to \infty) \), the level of inhibition be 86%. A similar fit to the data of Fig. 18 curve 3, requires that at steady-state ATP hydrolysis be 100% inhibited. As can be seen from Fig. 21, the initial inhibition measured after a prolonged preincubation of non-energized SMP with AMP•PNP is intermediate between these two cases. The biphasic nature of
Sample Non-Linear Regression Fits for the Inactivation of ATPase Activity by AMP-PNP.

curve 1: fit to Fig. 18 (curve 4)

\[ \kappa_{\text{inact}} = 0.211 \text{ min}^{-1} \]

curve 2: fit to Fig. 18 (curve 3)

\[ \kappa_{\text{inact}} = 0.080 \text{ min}^{-1} \]
Figure 20

Effect of Preincubation Time of AMP·PNP with Non-Energized SMP.

SMP (0.18 mg) were preincubated at 30 °C for the indicated length of time prior to the initiation of the ATP hydrolysis reaction by the addition of 40 μM ATP, in a medium which contained 50 mM Tris-OAc pH 7.5, 6 mM glucose, 10 mM MgCl₂, 20 mM PEP, 5 mM Pi, 0.4 mM AP₃A, 7.5 μM PCP, and 5 μM AMP·PNP. Three minutes prior to the initiation of the assay, 0.18 mg PK, 75 μg LDH, and 5 μM rotenone were added to the assay medium followed by 0.13 mM NADH, 1.5 min prior to initiation. The total reaction volume was 1 ml. The absorbance change due to NADH oxidation was monitored at 340 nm. The initial rate of NADH oxidation was compared to the control rate which did not contain AMP·PNP in the assay medium.
Theoretical AMP\textperiodcentered PNP Inhibition Curves: A Function of Preincubation Time.

curve 1: Data from non-linear regression of Fig. 18

\[ \kappa_{\text{inact}} = 0.080 \text{ min}^{-1} \]
\[ \Delta A/\text{min}_0 = 0.082 \]
\[ \Delta A/\text{min}_\infty = 0 \]

curve 2: Data from non-linear regression of Fig. 18

\[ \kappa_{\text{inact}} = 0.211 \text{ min}^{-1} \]
\[ \Delta A/\text{min}_0 = 0.085 \]
\[ \Delta A/\text{min}_\infty = 0.027 \]

\[ \Delta A/\text{min}_\text{steady-state} \text{ for the uninhibited conditions} = 0.188 \]

The solid circles (●) represent data given in Fig. 20.
AMP•PNP inhibition of ATP hydrolysis may be a result of a heterogeneity in the enzyme population, or alternatively it may represent different processes: binding of additional AMP•PNP molecules to F₁ or slow conversion of the enzyme-inhibitor complex to a more inhibited form. If this is the case, the equation describing the time course of inactivation may be a function of two exponentials.

In order to test if the observed time-dependent inhibition of ATP hydrolysis catalysed by non-energized SMP using the ATP analog AMP•PNP was reversible, an aliquot of inhibited SMP was either diluted directly into a new assay medium or applied to a Sephadex G-50 column, which would remove free or loosely bound AMP•PNP from the enzyme, before reassaying for activity. Fig. 22 curve 1 shows the linear steady-state rate observed when ATP was added to non-energized SMP in the absence of inhibitor compared with curve 2 in which the SMP was incubated for 3 min with 5 μM AMP•PNP prior to the addition of ATP. The apparent rate constant for inactivation (k_{app inact}), ΔA/min₀ and ΔA/min were determined to be 0.078 min⁻¹, 0.088, and 0.007, respectively. The extent of inhibition at time zero was determined to be 63%. It should be noted that in calculating the initial level of AMP•PNP inhibition, it is assumed that the kinetics observed in the presence of ATP (and therefore during enzyme turnover) are the same as in its absence. After a 15 min preincubation of AMP•PNP with SMP, the level of inhibition calculated using the above values of k_{app inact}, ΔA/min₀, ΔA/min was 86%. An aliquot of this hydrolysis medium, first applied to a Sephadex column, then diluted into an assay medium, shows that relative to the control which contained no AMP•PNP in the preincubation medium and displayed a linear steady-state rate of ATP hydrolysis when initiated with 0.5 mM ATP (Fig. 23 curve 1), the presence of AMP•PNP showed a reactivation of ATPase activity (curve 2). The calculated percent inhibition on addition

61
SMP (0.37 mg) were incubated in the presence (curve 2) or absence (curve 1) of 5 μM AMP•PNP at 30 °C for 3 min. The assay medium also contained 50 mM Tris-OAc pH 7.5, 6 mM glucose, 10 mM MgCl₂, 10 mM PEP, 0.2 mM AP₅A, 5 mM Pi, 5 μM rotenone, 15 μM PCP, 0.14 mM NADH, 0.30 mg PK, and 90 μg LDH. The reaction was initiated by the addition of 20 μM ATP. The change in absorbance at 340 nm due to the oxidation of NADH, was monitored. The total reaction volume was 1.0 ml. The steady-state rate of ATP hydrolysis under these conditions (curve 1) was ΔA/min = 0.236 or 103 n mole•min⁻¹•mg⁻¹.
Reactivation of ATPase Activity by Non-Energized SMP: ATP Present in Preincubation Medium.

SMP (0.37 mg) were incubated at 30 °C for 15 min in a preincubation medium containing the presence (curve 2) or absence (curve 1) of 5 μM AMP•PNP, 50 mM Tris-OAc pH 7.5, 6 mM glucose, 10 mM MgCl₂, 10 mM PEP, 0.2 mM AP₃A, 5 mM Pi, 15 μM PCP, 20 μM ATP, and 0.30 mg PK. At the 15th min of preincubation, a 100 μl aliquot was added to a packed Sephadex column (0.7 ml) and centrifuged on a tabletop centrifuge. At the 17th min (time 0 for the activity assay), 75 μl of the eluate was added to an assay medium containing 50 mM Tris-OAc pH 7.5, 6 mM glucose, 10 mM MgCl₂, 10 mM PEP, 0.2 mM AP₃A, 5 mM Pi, 5 μM rotenone, 15 μM PCP, 0.12 mM NADH, 0.30 mg PK, and 90 μg LDH. ATP (0.5 mM) was added after a 3 min incubation to initiate ATP hydrolysis. The total assay volume was 1.0 ml. The rate of NADH oxidation was monitored at 340 nm. The reactivation activity of the control (AMP•PNP absent from the preincubation medium) was ΔA/min = 0.107 or 0.65 μmole•min⁻¹•mg⁻¹ (curve 1).
curves 1 & 2

2 min

ATP included in preincubation medium
of the eluate to the assay medium based on a non-linear regression analysis of the reactivation equation (see Appendix 5) to the data of Fig. 23 curve 2, was 92% ($\Delta A/\text{min} = \text{uninhibited rate} = 0.107$). Total recovery of ATPase activity (relative to the control) was achieved within 7 min of diluting the eluate.

The activity observed when the preincubation medium lacked ATP, again showed a linear steady-state rate of ATP hydrolysis when initiated with 0.5 mM ATP (Fig. 24 curve 1). The presence of 5 $\mu$M AMP·PNP in the preincubation medium resulted in an inhibition which was reversible (curve 2). Based on a non-linear fit, the calculated initial inhibition upon diluting the eluate into the assay medium was 83% ($\Delta A/\text{min} = \text{uninhibited rate} = 0.110$). Again, total recovery of ATPase activity curve 1 (relative to the control) was achieved within 7 min.

When an aliquot of the preincubation medium was added directly to an assay medium, a linear steady-state rate of ATPase activity was observed for SMP not preincubated with AMP·PNP (Fig. 25 curve 1). As anticipated, the enzyme when preincubated with 5 $\mu$M AMP·PNP, displayed a reactivation of ATPase activity (curve 2). Based on the non-linear regression fit for the recovery of activity shown in Fig. 25 (curve 2), the calculated initial level of inhibition upon diluting the inhibited enzyme directly into the assay medium, was 90% ($\Delta A/\text{min} = \text{uninhibited rate} = 0.122$). The percent inhibition remaining upon diluting the inhibited SMP into an assay medium after 9 min (30 s. prior to complete NADH oxidation), was 2%.

The average initial level of ATPase inhibition upon diluting the treated SMP, which was calculated based on the reactivation fits, was 88%. This compares with 86% inhibition which was expected after a 15 min incubation of AMP·PNP with SMP. Sample reactivation fits to Figs. 23 - 25 (curve 2), are shown in Fig. 26.
Reactivation of ATPase Activity Catalysed by Non-Energized SMP: ATP Omitted From Preincubation Medium.

The preincubation media were identical to those described in the legend to Fig. 23, except ATP was omitted. The assay media were identical to those described in the legend to Fig. 23. The reactivation activity measured for the control (AMP·PNP absent from the preincubation medium) was $\Delta A/\text{min} = 0.110$ or $0.66 \mu\text{mole}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ (curve 1).
ATP curves 1 & 2

2 min

ATP absent from preincubation medium
Reactivation of ATPase Activity: Inhibited SMP Added Directly into the Assay Medium.

The preincubation medium was identical to that given in the legend to Fig. 23. At the 15th min of preincubation, 75 μl of the solution was added directly into the assay medium described in Fig. 23. ATP (0.5 mM) was added after a 3 min incubation, to initiate ATP hydrolysis. The total assay volume was 1.0 ml. The rate of NADH oxidation was monitored at 340 nm. The reactivation activity of the control (AMP•PNP absent from the preincubation medium) was ΔA/min = 0.122 or 0.71 μmole·min⁻¹·mg⁻¹ (curve 1).
ATP included in preincubation medium
Figure 26

Sample Non-Linear Regression Fits to Figs. 23 - 25 (Curve 2).

A: Fit to Fig. 23 (curve 2)
   \( k_{\text{app, react}} = 0.146 \text{ min}^{-1} \)

B: Fit to Fig. 24 (curve 2)
   \( k_{\text{app, react}} = 0.160 \text{ min}^{-1} \)

C: Fit to Fig. 25 (curve 2)
   \( k_{\text{app, react}} = 0.169 \text{ min}^{-1} \)
It should be noted that the specific activity of the enzyme prior to the application of the non-energized SMP to the Sephadex column, was the same as the eluate.

**Effect of AMP-PNP on Soluble F$_i$-ATPase**

The time-dependent AMP·PNP inhibition of ATP hydrolysis was not only observed with non-energized SMP, but also with soluble F$_i$-ATPase. As can be seen from Fig. 27, in the presence of 20 μM ATP, concentrations of AMP·PNP less than 0.5 μM produced linear steady-state rates of ATP hydrolysis. AMP·PNP concentrations greater than this produced the typical time-dependent inhibition observed with non-energized SMP. It is likely that curvilinear rates were not seen at the lower concentrations of AMP·PNP, because complete NADH oxidation occurred before slow tight binding of AMP·PNP to F$_i$ took place to an observable extent. Fig. 27 curve 3 ([AMP·PNP] = 0.25 μM) does show a slight decrease in rate prior to depletion of NADH. Based on the constants obtained from fitting the first order rate equation to the data shown in Fig. 27, k$\text{app}^\text{act}$ and $\Delta$A/min$^\text{act}$ for ATP hydrolysis (20 μM ATP), the extent of inhibition at various AMP·PNP concentrations was plotted as a function of time (Fig. 28). As this graph shows, 1 μM AMP·PNP (5% of the total nucleotide concentration present) was able to inhibit the initial rate of ATP hydrolysis by 66%. At steady-state, the inhibition had reached a value of 98%.

As with non-energized SMP, it was interesting to determine if the inactivation of F$_i$ by AMP·PNP was reversible. F$_i$ preincubated with ATP (20 μM) and AMP·PNP (0 - 1.0 μM) for 9 min, was applied to a Sephadex G–50 column and reassayed for activity. Fig. 28 (■) shows the inhibition expected after a 9 min preincubation time.
Figure 27

Time-Dependent AMP-PNP Inhibition of ATP Hydrolysis Catalysed by F1.

F1 (11.6 µg) was added to an assay medium which had been incubated at 30 °C for 3 min to initiate the ATP hydrolysis reaction. The assay medium contained 50 mM Tris-OAc pH 7.5, 10 mM MgCl₂, 10 mM PEP, 0.2 mg PK, 0.11 mg LDH, 5 mM Pi, 20 µM ATP, AMP-PNP (0 - 1 µM), and 0.13 mM NADH. The change in absorbance at 340 nm due to oxidation of NADH, was monitored. The total reaction volume was 1.0 ml. The steady-state rate of ATP hydrolysis in the absence of AMP-PNP under these conditions (curve 1) was ΔA/min = 0.193 or 2.67 μmole·min⁻¹·mg⁻¹. Curves 1 - 6 contained 0, 0.1, 0.25, 0.5, 0.75, and 1 µM AMP-PNP, respectively.
curves 2-6

4 min

curve 1

1 min

ΔA = 0.1
Extent AMP-PNP Inhibition of F₁-ATPase Activity as a Function of Incubation Time.

Based on non-linear fits of the data from Fig. 27 to the equation given in Appendix 5, the percent inhibition as a function of incubation time was obtained.

Parameters calculated from regression analysis:

<table>
<thead>
<tr>
<th>[AMP·PNP] (µM)</th>
<th>kapp_{inact} (min⁻¹)</th>
<th>(ΔA/minₐ₀ - ΔA/minₕ)</th>
<th>ΔA/minₕ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0.193</td>
</tr>
<tr>
<td>0.25</td>
<td>0.099</td>
<td>0.897</td>
<td>0.049</td>
</tr>
<tr>
<td>0.50</td>
<td>0.150</td>
<td>0.592</td>
<td>0.019</td>
</tr>
<tr>
<td>0.75</td>
<td>0.152</td>
<td>0.514</td>
<td>0.007</td>
</tr>
<tr>
<td>1.00</td>
<td>0.167</td>
<td>0.374</td>
<td>0.004</td>
</tr>
</tbody>
</table>

The curve symbols (●, ○, ■, □) represent incubation times of (0, 5, 9, and ∞ min), respectively.
As shown in Fig. 29, recovery of ATPase activity was also observed. The results of non-linear fits to the reactivation curves, are shown in Table 1. As can be seen from this table, between 11 and 18% of the inhibition was lost upon centrifuging the preincubation medium through the Sephadex G-50 column, and prior to reassaying ATPase activity.

Whereas non-energized SMP were able to totally reverse the extent of inhibition developed in the preincubation period, $F_1$ inhibited from 78 to 91% was only able to reverse the level of inhibition to an average steady-state level of 31.4 ± 3.8%. The remaining steady-state hydrolysis activity of the $F_1$-ATPase (2/3 the control rate), may be due to AMP-PNP or its hydrolysis product AMP-PNH$_2$, still bound at a high affinity site, which is not released under these experimental conditions, or during the time course of the assay.

Fig. 30 shows apparent rate constants for inactivation of ATPase activity by AMP-PNP, as a function of ATP concentration. As can be seen by this graph, $k_{app}$ values at a given AMP-PNP concentration, appear to decrease with increasing inact ATP concentration. As was seen with energized SMP, AMP-PNP inhibition of ATPase activity, led to mechanistic models which predicted multiple subunit involvement. The curvilinear nature of the secondary plot (slope vs. AMP-PNP concentration), was ascribed to the existence of multiple catalytic sites present on the enzyme, each possessing different affinities for the ATP analog. The apparent dependence of $k_{app}$ on ATP concentration may be a result of ATP lowering the probability of inact AMP-PNP binding to the enzyme, as well as influencing which nucleotide binding site AMP-PNP is able to bind to.
Reactivation of ATPase Activity by F:\_ ATP in Preincubation Medium.

F\_ (11.6 \( \mu \)g) was added to a preincubation medium at 30 °C containing 50 mM Tris–OAc pH 7.5, 10 mM MgCl\_2, 10 mM PEP, 0.2 mg PK, 5 mM Pi, 20 \( \mu \)M ATP, and AMP\_ PNP (0 – 1 \( \mu \)M). Nine minutes after adding F\_, a 125 \( \mu \)l aliquot of the preincubation medium was added to a packed Sephadex column (0.7 ml) and centrifuged on a tabletop centrifuge. At the 11th min (time 0 for the activity assay), 70 \( \mu \)l of the eluate was added to an assay medium (incubated at 30 °C for 3 min) containing 50 mM Tris–OAc pH 7.5, 10 mM MgCl\_2, 10 mM PEP, 5 mM Pi, 2 mM ATP, 0.2 mg PK, 0.11 mg LDH, and 0.15 mM NADH. The total assay volume was 1.0 ml. The rate of NADH oxidation was monitored at 340 nm. The reactivation activity of the control (AMP\_ PNP absent from the preincubation) was \( \Delta A/\text{min} = 0.130 \) or 25.7 \( \mu \)mole\_min\(^{-1}\_mg\(^{-1}\) (curve 1). Curves 2 – 4 contained 0.5, 0.75, and 1.0 \( \mu \)M AMP\_ PNP in the preincubation medium, respectively.
Addition of $F_1$ eluate

curves 1–4

2 min

$\Delta A - 0.1$
Table 1

Reactivation of F$_1$-ATPase Activity

<table>
<thead>
<tr>
<th>[AMP·PNP] in preincubation (µM)</th>
<th>Inhibition (%) (9th min)</th>
<th>Inhibition (%) (initial)</th>
<th>Δ Inhibition (%)</th>
<th>$k_{app, react}$ (min$^{-1}$)</th>
<th>Inhibition (%) (steady-state) (reassay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.50</td>
<td>78</td>
<td>64</td>
<td>15</td>
<td>0.860</td>
<td>32</td>
</tr>
<tr>
<td>0.75</td>
<td>86</td>
<td>75</td>
<td>11</td>
<td>0.720</td>
<td>26</td>
</tr>
<tr>
<td>1.00</td>
<td>91</td>
<td>73</td>
<td>18</td>
<td>0.667</td>
<td>36</td>
</tr>
</tbody>
</table>

Data is from Fig. 28 and non-linear fits of the reactivation curves from Fig. 29.
Figure 30

Apparent Rate Constants for F₁ Inactivation: A Function of AMP·PNP and ATP Concentration.

The apparent rate constants for F₁ inactivation by AMP·PNP are shown as a function of AMP·PNP and ATP concentrations. The curve symbols (●, ○, ■, □) correspond to ATP concentrations of (20, 30, 45, and 60) μM, respectively. AMP·PNP concentration ranged from 0.25 - 1.0 μM. Rate constants were obtained under conditions given with Fig. 33 and were calculated as described in Appendix 5.
As the question of error and confidence in the determination of \( \kappa_{\text{inact}} \) and \( \kappa_{\text{react}} \) arises, a separate experiment was devoted to this problem. Errors of \( \pm 5.1\% \) and \( 14.0\% \) (the result of four determinations in each case) were assigned to the apparent rate constants of inactivation and reactivation, respectively.

Determination of \( K_i \) for AMP•PNP Inhibition of Soluble F,-ATPase

As mentioned earlier, many workers have determined \( K_i \) values for AMP•PNP inhibition of soluble F,-ATPase (11, 27, 28, 30, 31). The \( K_i \) values which varied from 14 nM to 0.92 \( \mu \)M, could be dependent on the experimental conditions used. AMP•PNP inhibition of F,-ATPase activity in a low inhibitor concentration range (50 - 150 nM), does show competitive inhibition with respect to ATP (Fig. 31). The \( V_{\text{max}} \) and \( K_m \) values determined for the range of ATP concentration used (20 - 60 \( \mu \)M), were 9.43 \( \mu \)mole min\(^{-1}\) mg\(^{-1}\) and 45.6 \( \mu \)M, respectively. The secondary plot of slope versus AMP•PNP concentration, shown in Fig. 32, is linear at these inhibitor concentrations. Since higher AMP•PNP concentrations (to 1 \( \mu \)M) caused significant time-dependent inhibition during the course of the assay, Lineweaver-Burk plots for a wide range of inhibitor concentration (0.05 - 1.0 \( \mu \)M), which are shown in Fig. 33, were obtained based on velocities calculated either on the initial rate determined from a fit of the first order rate equation to the data (when time-dependent inhibition was observed), or measured directly (when no time-dependent inhibition occurred). Fig. 33 shows that 1 \( \mu \)M AMP•PNP causes more inhibition than would be predicted at the lower ATP concentrations, as evident by the upward curvature of the plot. This phenomenon was also observed by Schuster et al. (27). Slopes calculated at the high ATP concentrations, were plotted versus inhibitor concentration and are shown in Fig. 34. As seen with energized SMP, F, also
Figure 31

Lineweaver-Burk Plots for AMP•PNP Inhibition of F$_i$-ATPase: Low AMP•PNP Concentrations.

F$_i$ (11.6 µg) was added to an assay medium incubating at 30 °C for 3 min which contained 50 mM Tris-OAc pH 7.5, 10 mM MgCl$_2$, 10 mM PEP, 0.2 mg PK, 0.11 mg LDH, 5 mM Pi, AMP•PNP (0 - 150 nM), 0.13 mM NADH, and ATP (20 - 60 µM) as indicated. F$_i$ added to the assay mixture initiated ATP hydrolysis. The change in absorbance due to the oxidation of NADH, was monitored at 340 nm. The total reaction volume was 1.0 ml. Symbols used were: (●, ○, ■, □) corresponding to AMP•PNP concentrations of (0, 50, 100, and 150 nM), respectively.
Secondary Plot for AMP·PNP Inhibition of F₁-ATPase Activity: Low AMP·PNP Concentrations.

The slopes from the Lineweaver-Burk plots shown in Fig. 31 were plotted versus AMP·PNP concentration. The apparent Kᵢ was determined to be 0.25 μM.
Lineweaver-Burk Plots for AMP·PNP Inhibition of F\textsubscript{1}-ATPase.

F\textsubscript{1} (10.2, 11.6, or 12.4 μg) was added to an assay medium incubating at 30 °C for 3 min which contained 50 mM Tris-OAc pH 7.5, 10 mM MgCl\textsubscript{2}, 10 mM PEP, 0.2 mg PK, 0.11 mg LDH, 5 mM Pi, AMP·PNP (0 – 1.0 μM), 0.13 mM NADH, and ATP (20 – 60 μM) as indicated. F\textsubscript{1} added to the assay mixture initiated ATP hydrolysis. The change in absorbance due to the oxidation of NADH was monitored at 340 nm. The total reaction volume was 1.0 ml. Symbols used were: (●, ○ , □, △, ⊕, ⊖) corresponding to AMP·PNP concentrations of (0, 0.05, 0.1, 0.15, 0.5, 0.75, and 1.0 μM), respectively. The initial velocities were either calculated from a non-linear fit (when time-dependent inhibition was observed) or directly (when no time-dependent inhibition was observed).
displayed a curvilinear secondary plot which was biphasic. A fit of the equation derived for the model shown in Fig. 14 to the data shown in Fig. 34 is shown in Fig. 35. Assuming that the equilibrium constants for the two ATP hydrolysis steps \( (k_1/k_2 \text{ and } k_{12}/k_{13}) \) are equal to 0.5, then the \( K_i \) value for binding a single AMP-PNP molecule to \( F_i \) would be 0.16 \( \mu M \). This value is in good agreement with those reported by the majority of workers, but it is an order of magnitude greater than the value determined by Cross and Nalin (11). The discrepancy in the \( K_i \) values may reside in the assay technique employed. In all other studies, \( F_i \) was added to the assay medium to initiate ATP hydrolysis, whereas Cross and Nalin initiated hydrolysis with the addition of ATP after a 10 min incubation. Studies by Kironde and Cross (49) have shown that beef heart \( F_i \) stored as a (NH₄)₂SO₄/ATP/EDTA/sucrose/Tris, pH 8.0 suspension, contains three bound adenine nucleotides after Sephadex gel filtration. Two of these adenine nucleotides are bound to non-exchangeable sites, and the third at an exchangeable site. It is possible that dilution of the enzyme into an assay medium not containing ATP would allow the nucleotide bound at the catalytic site to dissociate. The long preincubation (10 min) of \( F_i \) with AMP-PNP may allow AMP-PNP to bind to this high affinity site; it is very likely that it is this site for which Cross and Nalin measured a \( K_i \) value of 14 nM. The presence of ATP, which shows a high affinity for this site (\( K_a = 10^{12} \text{ M}^{-1} \)) (45), would effectively prevent AMP-PNP from interacting at this site. A comparison of the \( K_i \) values determined for the binding of a single AMP-PNP molecule to \( F_i \)-ATPase (0.16 \( \mu M \)) and energized SMP (0.58 \( \mu M \)), shows that energization lowers AMP-PNP affinity. This is in agreement with Penefsky (47) who showed that upon energization of KCl-washed SMP by succinate or NADH, ATP affinity decreased. Cherynak and Kozlov (50) also showed that succinate induced energization relieved AMP-PNP inhibition, consistent with a change in AMP-PNP affinity.
Secondary Plot for AMP·PNP Inhibition of F$_1$-ATPase Activity.

The slopes from the Lineweaver-Burk plots (obtained in the high ATP concentration region) shown in Fig. 33, were plotted versus AMP·PNP concentration.
Non-Linear Fit of the Rate Expression Derived for Model 1 to Fig. 34.

Slope expression was:

$$\text{slope} = \frac{K_m}{V_{\text{max}}} \left( \frac{1 + P_1 \cdot [I]}{1 + P_2 \cdot [I]} \right)$$

where

\[ P_1 = \frac{a}{K_i} \]
\[ P_2 = \frac{b}{K_i} \]

a and b are defined in Appendix 3

Fit:

<table>
<thead>
<tr>
<th>Correlation Matrix</th>
<th>Km/Vmax</th>
<th>P1</th>
<th>P2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.83 mmole⁻¹ min⁻¹ mg⁻¹ mM</td>
<td>6.38 ± 0.75</td>
<td>1.20 ± 0.25</td>
</tr>
</tbody>
</table>
slope, (mmole⁻¹ min mg mM)

[AMP-PNP], (μM)
Given the results of Cross and Nalin, and the fact that binding of AMP•PNP causes ADP release (24), the complete model for AMP•PNP interaction with F$_i$-ATPase may be best represented by the model shown in Fig. 36. This model shows that AMP•PNP binding to F$_i$ can result in the determination of three unique $K_i$ values, depending on the assay conditions. The ability of AMP•PNP to bind to the high affinity site measured by Cross and Nalin, is dependent on the level of ATP present during the preincubation, as well as the rate at which ADP dissociates from this site during single site catalysis.
Proposed Mechanism for AMP·PNP Inhibition of ATPase Activity: Single and Multiple Site Binding

where
E - Free enzyme
T - ATP
D - ADP and Pi
I - AMP·PNP
high affinity
• low affinity
• change in binding affinity
SUMMARY AND FUTURE WORK

As can be seen, the mechanism of ATP hydrolysis catalysed by mitochondrial ATP synthase and its inhibition by AMP-PNP, is very complex. AMP-PNP inhibition of ATP hydrolysis catalysed by energized SMP was shown to be insensitive to increasing concentrations of ADP under the conditions used.

Due to the existence of multiple catalytic sites, Ki values determined from secondary plots were expected to vary depending on experimental conditions used. Hyperbolic competitive inhibition was observed, which was consistent with a model in which binding of two AMP-PNP molecules occurred, both competitive with respect to ATP. The dissociation constants (Ki and Ki,) were determined to be 0.58 μM and 19 μM, respectively. AMP-PNP inhibition of F1-ATPase activity also displayed hyperbolic competitive inhibition, consistent with the binding of a single AMP-PNP molecule with a Ki of 0.16 μM.

AMP-PNP inhibition was shown to be time-dependent when ATP hydrolysis was catalysed by non-energized SMP or F1, in contrast to catalysis by energized SMP. This inhibition was shown to be totally reversible with non-energized SMP, whereas only 69% activity was recovered with F1-ATPase. The remaining inhibition is attributed to AMP-PNP bound at a high affinity site. Further studies are needed to explain time-dependent AMP-PNP inhibition of ATP hydrolysis catalysed by F1 or SMP. Ideally, radiolabeled AMP-PNP should be used to correlate the amount of AMP-PNP bound to F1 with the level of inhibition. Double labelling studies will make it possible to determine whether the remaining inhibition observed at steady-state is due to AMP-PNP bound at a catalytic site, or its hydrolysis product, AMP-PNH3.
Since the ATP analog AMP·PNP appears to be a one way inhibitor of the ATP synthase complex, it would be beneficial to synthesize the ADP analog AMP·PNH₂ and determine if it acts as a one way inhibitor of ATP synthesis. Future studies may also show that the ATPase activity observed using F₁ and SMP preparations is due to F₁ devoid of IF₁, or F₁ possessing IF₁ conformationally different than its in vivo counterpart. Possible ordered binding of substrates and the role of IF₁ may provide researchers with the key to elucidating the mechanism of the ATP synthase complex.
APPENDIX 1

The steady-state concentration of ADP in a ATP hydrolysis medium was determined knowing the specific activity and Km value for ADP at saturating PEP, for the PK used, and simple rearrangement of the Michaelis-Menten equation (Eq. 1) in terms of the ADP concentration (Eqs. 2 and 3).

\[
V = \frac{V_{\text{max}} \cdot [\text{ADP}]}{[\text{ADP}] + \text{K}_m}
\]

where \( V_{\text{max}} \) = maximum rate = kcat \cdot [total \ PK]

\( V \) = rate observed

\( \text{K}_m \) = [ADP] which gives half maximal velocity

Equation 1

\[
[\text{ADP}] = \frac{V \cdot \text{K}_m}{V_{\text{max}} - V}
\]

Equation 2
\[
\frac{[\text{ADP}]_{\text{v=}}}{SA \cdot [\text{stock PK}] \cdot \text{volume of stock PK used} - V} \cdot \frac{V \cdot \text{Km}}{V}
\]

where \(SA\) = specific activity of PK used

Equation 3
Dead-end inhibitors which bind to the same enzyme form that the substrate does, are known as competitive inhibitors. The simple reaction mechanism is shown in Fig. 1.

\[
E \overset{k_1}{\underset{k_2}{\rightleftharpoons}} ES \overset{k_3}{\rightarrow} E + P
\]

where
- \( E \) = free form of the enzyme
- \( S \) = substrate
- \( I \) = inhibitor
- \( P \) = product

Figure 1
Deriving the rate equation for product formation for the scheme shown above (steady-state approximation), yields Eq. 1.

\[ v = \frac{V_{\text{max}} \cdot [S]}{[S] + K_m \cdot (1 + [I]/K_i)} \]

where \( V_{\text{max}} \) = maximum velocity = \( k_1 \cdot e \)

\( K_m = [S] = (k_2 + k_1)/k_1 \)

\( 0.5 \cdot V_{\text{max}} \)

\( K_i = \) dissociation constant for enzyme-inhibitor complex

\( e = \) total enzyme concentration

Equation 1

A Lineweaver-Burk plot (1/V vs 1/[S]) for competitive inhibition would therefore obey Eq. 2.

\[ \frac{1}{V} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}} \cdot \frac{(1 + [I]/K_i)}{[S]} \]

Equation 2
Fig. 2 shows typical Lineweaver–Burk plots.

From Eq. 2, the slope from a Lineweaver–Burk plot would be given by Eq. 3.

slope = \frac{K_m \cdot \left(1 + \frac{[I]}{K_i}\right)}{V_{max}}

Equation 3

Or in other terms,

slope = \frac{K_m}{V_{max}} + \frac{K_m}{V_{max} \cdot K_i} \cdot [I]
This would predict a secondary plot (slope vs. [I]) to be linear as shown in Fig. 3.

![Figure 3](image-url)

The horizontal intercept would correspond to the negative of the dissociation constant (Ki) for the enzyme-inhibitor complex.
The derivation of the rate expression for the model (shown below) of AMP·PNP inhibition of ATP hydrolysis catalysed by SMP or F₁, is presented here.

Model 1: (binding of a single AMP·PNP molecule)

The steady-state rate expressions for the different enzyme species are:

1. \[
\frac{dT[ED]}{dt} = 0 = k_1[ET] + k_4[TED] + k_5[IED] - (k_3 + k_6[T] + k_7[I])[ED]
\]

2. \[
\frac{dT[TED]}{dt} = 0 = k_1[T][ED] - (k_4 + k_5)[TED]
\]

3. \[
\frac{dT[ET]}{dt} = 0 = k_1[ED] + k_2[TED] - k_1[ET]
\]

4. \[
\frac{dT[IED]}{dt} = 0 = k_4[I][ED] + k_9[1ED] + k_{12}[IET] - (k_3 + k_6[T] + k_{10})[IED]
\]

5. \[
\frac{dT^{T}[IED]}{dt} = 0 = k_9[T][IED] - (k_9 + k_{10})[IED]
\]
Simplification of the rate expression was as follows:

1. Setting $[I] = 0$, the rate equation becomes:

$$v = \frac{V_{\text{max}} \cdot [T]^{\infty}}{[T] + K_m}$$

where $V_{\text{max}} = \frac{k_1 k_2 e}{(k_1 + k_3)}$

$$K_m = \frac{(k_1 + k_2)(k_4 + k_5)}{k_3 (k_1 + k_3)}$$

2. Eq. 1 now reduces to:

$$v = \frac{V_{\text{max}} [T] \cdot \left\{ \frac{1 + k_8 k_{10} (k_4 + k_5) [I]}{k_3 k_5 (k_4 + k_{10}) [I]} \right\}}{[I] \left\{ \frac{1 + k_1 k_8 (k_4 + k_5) (k_{10} + k_{12}) [I]}{k_3 k_{12} (k_4 + k_5) (k_9 + k_{10})} \right\} + \frac{K_m \left\{ \frac{1 + k_1 (k_{11} + k_{12}) [I]}{K_I} \right\}}{k_{12} (k_1 + k_2)}}$$

Equation 2
Simplification of the rate expression was as follows:

1. Setting [I] = 0, the rate equation becomes:

\[ v = \frac{V_{\text{max}} \cdot [T]^x}{[T] + K_m} \]

where \( V_{\text{max}} = \frac{k_1 k_5 e}{k_1 + k_3} \)

\[ K_m = \frac{(k_1 + k_5)(k_4 + k_5)}{k_5(k_1 + k_3)} \]

2. Eq. 1 now reduces to:

\[ v = V_{\text{max}} [T] \frac{[1 + k_8 k_{10} (k_4 + k_5)^{[I]}]}{k_3 k_5 (k_9 + k_{10}) K_I} \]

\[ = \frac{[1 + k_1 k_8 (k_4 + k_5)(k_9 + k_{10}) [I] / K_I]}{k_3 k_5 (k_9 + k_{10}) K_I} + K_m \frac{[1 + k_1 (k_4 + k_5) [I] / K_I]}{k_{12} (k_1 + k_2)} \]

Equation 2
3. Setting \([\theta] = \infty\), Eq. 1 becomes

\[
v = \frac{k_1 k_5 [\theta]}{k_3 k_{12} (k_1 + k_5) (k_{10} + k_{12})} \left\{ \frac{k_8 k_{10} (k_{11} + k_{12})}{k_3 k_{10} (k_9 + k_{10})} \right\} + \frac{(k_1 + k_2) (k_4 + k_5)}{k_3 (k_1 + k_5)} \left\{ \frac{k_{11} (k_{11} + k_{12})}{k_{12} (k_1 + k_2)} \right\}
\]

This reduces to:

\[
v = \frac{k_8 k_{10} e^{[\theta]}}{(k_9 + k_{10})}
\]

\[
= \frac{[\theta] k_8 (k_{10} + k_{12})}{k_{12} (k_9 + k_{10})} + \frac{(k_{11} + k_{12})}{k_{12}}
\]

\[
v = \frac{k_{10} e^{[\theta]}}{(k_{10} + k_{12})}
\]

\[
= \frac{[\theta] + (k_9 + k_{10}) (k_{11} + k_{12})}{k_8 (k_{10} + k_{12})}
\]
or

\[
v = \frac{V_{\text{max}}'}{[T] + K_{\text{m'}}}
\]

where \(V_{\text{max}}' = \frac{k_{12}k_{13}e}{(k_{i0} + k_{i1})}\)

\(K_{\text{m'}} = \frac{(k_{i} + k_{14})(k_{1} + k_{13})}{k_{i}(k_{i1} + k_{i3})}\)

4. Simplification of Eq. 2

Let \(z = \frac{k_{1}k_{8}(k_{4} + k_{s})(k_{10} + k_{12})}{k_{3}k_{12}(k_{1} + k_{5})(k_{9} + k_{10})}\)

\[= \frac{k_{5}k_{10}(k_{4} + k_{s})}{k_{3}k_{5}(k_{9} + k_{10})} \times \frac{k_{1}(k_{10} + k_{12})k_{5}}{k_{10}(k_{1} + k_{5})k_{12}}\]

\[
\frac{k_{1}k_{5}e}{(k_{1} + k_{5})}\]

Since \(V_{\text{max}} = \frac{k_{1}k_{5}(k_{10} + k_{12})}{k_{10}k_{12}e}\)

\(V_{\text{max}}' = \frac{k_{1}k_{5}e}{(k_{10} + k_{12})}\)

if we assume \(V_{\text{max}} = V_{\text{max}}'\), then \(z\) reduces to:

\[
\frac{k_{s}k_{14}(k_{4} + k_{1})}{k_{i}k_{5}(k_{i1} + k_{i3})}
\]
Therefore Eq. 2 becomes:

\[ \nu = \frac{V_{\text{max}} [T] \left( 1 + \frac{k_1 k_{10} (k_4 + k_5) [1]}{k_3 k_5 (k_9 + k_{10})} \right)}{[T] \left( 1 + \frac{k_1 k_{10} (k_4 + k_5) [1]}{k_3 k_5 (k_9 + k_{10})} \right) + K_m \left( 1 + \frac{k_1 (k_{11} + k_{12}) [1]}{k_{12} (k_1 + k_2)} \right)} \]

Dividing the denominator and numerator by:

\[ \frac{k_1 k_{10} (k_4 + k_5) [1]}{k_3 k_5 (k_9 + k_{10})} \frac{k_1 k_{10} (k_4 + k_5) [1]}{k_3 k_5 (k_9 + k_{10})} \]

the rate expression becomes:

\[ \nu = \frac{V_{\text{max}} [T]}{[T] + K_m \left( 1 + \frac{k_1 (k_{11} + k_{12}) [1]}{k_{12} (k_1 + k_2)} \right) \left( 1 + \frac{k_1 k_{10} (k_4 + k_5) [1]}{k_3 k_5 (k_9 + k_{10})} \right)} \]

or

\[ \nu = \frac{V_{\text{max}} [T]}{[T] + K_m \left( 1 + a [1]/K_i \right) \left( 1 + b [1]/K_i \right)} \]

where

\[ a = \frac{k_1 (k_{11} + k_{12})}{k_{12} (k_1 + k_2)} \]

\[ b = \frac{k_1 k_{10} (k_4 + k_5)}{k_3 k_5 (k_9 + k_{10})} \]

\[ k_1 k_{10} (k_4 + k_5) [1]/K_i \]

Equation 3
The inverse of Eq. 3 is:

\[
\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}} \left( \frac{1 + a[I]/K_i}{1 + b[I]/K_i} \right)} \cdot \frac{1}{[I]}
\]

The mechanism predicts competitive inhibition with a slope which varies with inhibitor concentration by the following equation:

\[
\text{slope} = \frac{K_m \left\{ 1 + a \cdot \frac{[I]}{K_i} \right\}}{V_{\text{max}} \left\{ 1 + b \cdot \frac{[I]}{K_i} \right\}}
\]
APPENDIX 4

The derivation of the rate expression for the model (shown below) of AMP•PNP inhibition of ATP hydrolysis catalysed by SMP or F1, is presented here.

Model 2: (binding of two AMP•PNP molecules)

The steady-state rate expressions for the different enzyme species are the same as those given in Appendix 3, with the following change in $d[IED]/dt$, and addition of $d[1,ED]/dt$.

4. $d[IED] = 0 = k_4[I][IED] + k_5[I][TED] + k_9[I][IET] + k_{12}[I,ED] - \left( k_{10} + k_{11} + k_{14} \right) [IED]$ / $dt$

7. $d[I,ED] = 0 = k_{13}[I][IED] - k_{11}[I,ED]$ / $dt$
Enzyme conservation equation is given by:

\[ e = [ET] + [ED] + [TED] + [IED] + [I_{ED}] + [I_{ET} + I_{ED}] \]

Rate expression for product (D) formation is:

\[ v = \frac{d[D]}{dt} = k_1[TED] + k_{18}[IED] \]

Note: \( K_i = k_i/k_e = [ED][I] \)

\[ K_{i_3} = k_{i_3}/k_{i_4} = [IED][I] \]

After appropriate manipulation of the above equations (and those given in Appendix 3), the rate expression in terms of ([T], e, and [I]), was determined to be:

\[
v = \frac{k_1k_5e[T]}{(k_1 + k_2)^2} \left\{ 1 + \frac{k_8k_{10}(k_4 + k_5)[I]/K_1}{k_2k_6(k_9 + k_{10})} \right\} [T] \left\{ 1 + k_1k_8(k_4 + k_5)(k_{10} + k_{12})[I]/K_i \right\} + \frac{(k_1 + k_2)(k_4 + k_5)}{k_3(k_1 + k_4)} \left\{ 1 + k_1(k_{11} + k_{12})[I]/K_1 \left\{ 1 + \frac{(k_{12})}{k_{12}(k_1 + k_2)} \right\} \right\} + \frac{(k_1 + k_2)(k_4 + k_5)}{k_3(k_1 + k_4)} \left\{ 1 + k_1(k_{11} + k_{12})[I]/K_1 \left\{ 1 + \frac{(k_{12})}{k_{12}(k_1 + k_2)} \right\} \right\} \]

Equation 1
Using the simplification techniques used in Appendix 3, and again assuming 
\( V_{\text{max}} = V_{\text{max}'} \), then Eq. 1 reduces to:

\[
v = \frac{V_{\text{max}} [T]}{[T] + K_m \left( 1 + \frac{k_1 (k_{11} + k_{12}) [I] / K_i}{k_{12} (k_{11} + k_{12})} \left( 1 + \frac{k_{12} [I] / K_{i_2}}{k_{11} + k_{12}} \right) \right) \left( 1 + \frac{k_{8} k_{10} (k_{4} + k_{5}) [I] / K_i}{k_{3} k_{5} (k_{9} + k_{10})} \right)}
\]

where \( V_{\text{max}} \) and \( K_m \) are as defined in Appendix 3

Equation 2
Eq. 2 can also be written as:

\[ v = \frac{V_{\text{max}} [T]}{[T] + K_m \left( 1 + \frac{a[I]}{K_i(1 + \frac{c[I]}{K_i})} \right) \left( 1 + \frac{b[I]}{K_i} \right)} \]

where

\[ a = \frac{k_1(k_{11} + k_{13})}{k_{12}(k_1 + k_1)} \]
\[ b = \frac{k_4k_{10}(k_4 + k_4)}{k_3k_5(k_9 + k_{10})} \]
\[ c = \frac{k_{12}}{k_{11} + k_{12}} \]

Equation 3
The inverse of Eq. 3 is:

\[ \frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{K_m}{V_{\text{max}}} \left\{ \frac{1 + a[I]/K_i (1 + c[I]/K_i)}{1 + b[I]/K_i} \right\} \frac{1}{[I]} \]

The mechanism therefore predicts competitive inhibition, with the slopes from Lineweaver-Burk plots, to vary with inhibitor concentration by the following equation:

\[ \text{slope} = \frac{K_m}{V_{\text{max}}} \left\{ \frac{1 + a \cdot [I]/K_i \cdot (1 + c \cdot [I]/K_i)}{1 + b \cdot [I]/K_i} \right\} \]
APPENDIX 5

The equations which were used to describe the time-dependent inhibition or recovery of activity observed using non-energized SMP or F₁ was a simple first-order process with respect to the rate of ATP hydrolysis. The derivation of an expression which relates absorbance to time, is described here.

\[ V = (V_0 - V_\infty) e^{-kt} + V_\infty \]  - (inactivation)

\[ V = (V - V_0)(1 - e^{-kt}) + V_0 \]  - (reactivation)

where \( V \) = velocity at any time \( t \)
\( V_0 \) = initial rate
\( V_\infty \) = steady-state rate
\( k \) = apparent rate constant of inactivation or reactivation

\[ \frac{dA}{dt} = (V_0 - V_\infty) e^{-kt} + V_\infty \]

\[ \int_{A(0)}^{A} \left[ (V_0 - V_\infty) e^{-kt} + V_\infty \right] dt \]

\[ A \left| \begin{aligned} A(0) = & \left( V_0 - V_\infty \right) e^{-kt} + V_\infty \left|_0^A \\
A(0) = & \left( \frac{-k \infty}{-k \infty} \right) \end{aligned} \right. \]
where $A = \text{absorbance at any time } t$

$A = A_0 = (V_0 - V) \cdot (1 - e^{-kt}) + V \cdot t + A_0$

Note: The term $(V_0 - V)/k$ in the inactivation and reactivation processes, is negative and positive, respectively.
BIBLIOGRAPHY


121


