AN ABSTRACT OF THE THESIS OF

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Title: Isolation, Characterization, and Kinetics of Mitochondrial F_1-ATPase from Crayfish (Orconectes nais) Gills.

Abstract Approved: Gaylen Henzel

A soluble F_1-ATPase was isolated from the mitochondria of crayfish (Orconectes nais) gill tissue. The maximal mitochondrial disruption rate (95%) was obtained by sonicating for 4 min at pH 8.6. A purification of 15-fold was estimated.

The F_1-ATPase preparation was stable at 4°C to -70°C when kept in 20% glycerol. The pH optimum for soluble F_1-ATPase (7.0-7.2) was slightly lower and more narrow than for membrane-bound enzyme (7.0-7.8). Both membrane-bound and soluble F_1-ATPase were stimulated by HCO_3^-, SO_4^{2-}, and Cl^-; the highest stimulated activity for all was obtained at 35 mM. The activity was in the order $HCO_3^- > SO_4^{2-} > Cl^-$. In addition, the apparent K_a for soluble enzyme was 10.1, 10.7, and 11.1 mM, respectively.

Oligomycin and DCCD inhibited the membrane-bound F_1-ATPase with I_{50} of 0.019 μg/ml and 2.2 μM, respectively, but were ineffective in inhibiting the soluble enzyme. Both were similarly sensitive to DIDS and vanadate. Soluble

ATPase was significantly more sensitive to pCMB and NO_3^- than the membrane-bound enzyme. In addition, soluble F_1-ATPase was slightly more sensitive to azide and NBD-Cl than membrane-bound enzyme. The differences between soluble and membrane-bound enzyme in the general properties as well as inhibitor and modulator sensitivities suggest conformational change transmission between F_0 and F_1 sectors, and slight conformational differences between soluble F_1 and membrane-bound F_1.

Kinetic studies on the membrane-bound F_1-ATPase indicated that the MgATP complex was the true substrate for the ATPase activity and had a K_m value of 0.327 mM. Free ATP was a competitive inhibitor (K_i = 0.77 mM), and free Mg^{2+} was a mixed inhibitor (K_i = 0.81 mM, K_i' = 5.89 mM). However, free ATP also acted as an activator. Lineweaver-Burk plots for MgATP hydrolysis at high free Mg^{2+} concentration exhibited apparent negative cooperativity. This was not the case for high free ATP level. These results suggest that, although free ATP inhibited the enzyme by binding to catalytic sites, it stimulated the ATPase activity by binding to noncatalytic sites.

ISOLATION, CHARACTERIZATION, AND KINETICS OF MITOCHONDRIAL F_1-ATPASE FROM CRAYFISH (Orconectes nais) GILLS

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INTRODUCTION

F_0F_1ATPase, also called ATP synthase, is a multisubunit complex in mitochondria, chloroplasts, and bacteria that catalyzes the synthesis of ATP from ADP and inorganic phosphate. The reaction is driven by the electrochemical potential of protons present across the membrane derived from photo- or respiratory oxidation processes (Futai et al., 1989; Senior, 1990; Penefsky and Cross, 1991; Pedersen and Amzel, 1993). In some conditions the enzyme may also establish an electrochemical H^+ gradient through the hydrolysis of ATP (Tuena de Gomez-Puyou et al., 1988). In bacteria growing under anaerobic conditions, the enzyme functions in the reverse direction, generating an electrochemical gradient of H^+ by the hydrolysis of ATP (Futai and Kanazawa, 1983; Schneider and Altendorf, 1987).

In animals, yeast, and Neurospora, F_0F_1-ATPase complexes are located in the inner membranes of mitochondria. In plants, they are localized both in the inner mitochondrial membrane and in the thylakoid membrane of chloroplasts and in bacteria they are found in the cytoplasmic membrane (Penefsky, 1979; Futai and Kanazawa, 1983; Futai et al., 1989; Senior, 1990; Pedersen and Amzel, 1993).

ATP synthase from different sources have in common their overall structure (Penefsky and Cross, 1991). They

consist of two parts: the hydrophilic and extramembranous F_1 portion which is also called F_1-ATPase, and the hydrophobic membrane-bound F_0 complex. F_1 is the catalytic sector concerned with ATP synthesis and hydrolysis (Futai and Kanazawa, 1983; Tuena de Gomez-Puyou et al., 1993). In all species it is composed of five unlike subunits with the stoichiometry $\alpha_3\beta_3\gamma\delta\epsilon$. F_0 functions as a proton channel and is concerned with energy transfer to or from F_1 (Schneider and Altendorf, 1987). F_0 composition varies among species. The simplest is the E.coli F_0 with three unlike subunits having the stoichiometry $a_1b_2c_{10\pm1}$ (Schneider and Altendorf, 1987; Senior, 1990). The most complex, so far as known, is the mitochondrial F_0 with seven unlike subunits. These are b, c, d, F_6, OSCP, subunit 6, and A_6L (Penefsky and Cross, 1991). In addition, ATP synthase contains an additional polypeptide designated e (Walker et al., 1991). There is in mitochondria an ATPase inhibitor protein (IF_1) which at low proton-motive force binds to the β subunits of F_1 and inhibits ATP hydrolysis (Pullman and Monroy, 1963; Schwerzmann et al., 1982; Rouslin, 1987; Tuena de Gomez-Puyou et al., 1988). In bovine mitochondria the stoichiometries of several F_0 subunits and of IF_1 are one mole each of d, OSCP, A_6L, and IF_1, and two moles each of b and F_6 per mole of F_1 (Hekman et al., 1991). It is also known that subunit c (DCCD-binding proteolipid) is present

in mammalian F_0 complex in multiple copies (Kiehl and Hatefi, 1980).

There are six nucleotide-binding sites on F_1-ATPases from different sources (Cross and Nalin, 1982; Wise et al., 1983; Xue et al., 1987; Girault et al., 1988). Three of the sites exchange bound nucleotide rapidly during catalytic turnover. They are believed to be directly involved in catalysis and are referred to as catalytic sites (Cross et al., 1984; Boyer, 1989; Duncan and Cross, 1992). The three remaining sites are referred to as noncatalytic sites (Cross, 1988). Studies on isolated α and β subunits from F_1 (Dunn and Futai, 1980; Rao et al., 1988; Senior, 1988) suggest that catalytic sites are on β subunits and noncatalytic sites on α. However, more recent evidence indicate that at least the noncatalytic sites are positioned at α-β subunit interfaces (Verburg and Allison, 1990; Zhuo et al., 1992).

The F_1-ATPase has been isolated and studied in a wide variety of sources such as bacteria, yeast, chloroplasts and mitochondria of plants, and a number of mammalian sources (Pedersen and Amzel, 1993). The methods for isolation of the enzyme generally require mechanical disruption or ultrasonic treatment of mitochondria (Penefsky, 1979). F_1 is bound to the membrane by interaction with F_0 and in most cases it is easily released into the medium by treatment with either ethylenediamine-tetraacetic acid (EDTA) at low ionic

strength (Munoz, 1982) or chloroform (Linnett et al., 1979; Penefsky, 1979). The F_1-ATPase can be further purified by two steps: isolelectric precipitation of impurities and column chromatography on DEAE-sephadex (Randall et al., 1985; Spitsberg et al., 1985; Yoshihara et al., 1991). In general, purified enzyme is cold-labile, and the activity is increased by some anions such as HCO_3^-, Cl^-, and SO_4^{2-} (Cohen and MacPeek, 1980; Du and Boyer, 1990; Divita et al., 1992; Itoh et al., 1993). In addition, a wide variety of inhibitors of the F_1-ATPase activity have been identified. Besides the native inhibitor protein (IF_1), they also include adenine nucleotides and analogs such as ADP, AMP-P(NH)P, and BzATP (Penefsky, 1979; Ackerman et al., 1987) that are all competitive inhibitors, some anions such as azide that is a specific inhibitor for F_1-ATPase (Bowman, 1983; Itoh et al., 1993), and group-specific reagents such as 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and dicyclohexyl-carbodiimide (DCCD) that chemically modify specific amino acid residues in or near catalytic sites (Satre et al., 1982; Matsuno-Yagi and Hatefi, 1984; Bullough and Allison, 1986). In addition, F_0 inhibitors such as oligomycin and DCCD inhibit membrane-bound ATPase activity but do not inhibit the soluble enzyme (Smith et al., 1985; Archinard et al., 1986; Hicks and Krulwich, 1990; Matsuno-Yagi and Hatefi, 1993a and b).

Disruption of mitochondria into submitochondrial

particles (SMP) is important for the removal of the protein inhibitor and other contaminants (Horstman and Racker 1970; Penin et al., 1979). In addition, the SMP are obtained by sonication of mitochondria and are used for F_1-ATPase extraction in most purification methods (Beechey et al., 1975; Serrano et al., 1976; Tyler and Webb, 1979; Spitsberg et al., 1985). The sonication conditions usually include mitochondrial concentration, pH of medium, power, and length of sonication. However, the optimum sonication conditions have not been reported. Detailed studies will provide an effective method to disrupt mitochondria and provide the basis for isolation and purification of the F_1-ATPase.

Although F_1-ATPase has been purified and studied in a wide variety of sources, the purification and characterization of crustacean F_1-ATPase have not been described. Many investigations of Na^+,K^+-ATPase in the gills of various crustaceans have been reported and indicate the activity of the enzyme is high in order to meet their needs for ion and osmoregulation (Neufeld and Pritchard, 1979; Kosiol et al., 1988). Thus, these cells require a high ATP level and the activity of the F_1-ATPase should be high as well. In addition, some purification methods for F_1-ATPase have been described (Penin et al., 1979; Unitt and Lloyd, 1981; McEnery et al., 1984; Spitsberg et al., 1985; Moriyama et al., 1991) and the crystalline form has been obtained from some species (Lutter et al., 1993;

Pedersen et al., 1995). However, the enzymes from different species and tissues require different purification procedures and different studies require different levels of purity. Therefore, studying the method for soluble F_1-ATPase preparation from crayfish gills, which is stable and completely isolated from F_0 complex, is useful to characterize the enzyme. Additionally, it is useful to compare the soluble F_1-ATPase with its membrane-bound form and with the enzymes from other sources.

The mechanisms of synthesis and hydrolysis of ATP by F_1-ATPase are still not well understood. However, one way to obtain information about the mechanism of an enzyme is to study the effects of inhibitors. A vanadate-insensitive ATPase could be interpreted as a type of phosphohydrolase that does not form a covalent phosphoenzyme intermediate in the reaction mechanism (Futai et al., 1989). The sensitivities of group-specific reagents could be used to determine whether there are essential amino acid residues for catalysis (Senior, 1990). In addition, chemical modification of any one of the three potential catalytic sites by NBD-Cl causes complete inhibition of the enzyme (Ferguson et al., 1975). It could suggest that a cyclical three-site mechanism normally operates (Cross, 1988; Boyer, 1989; Weber et al., 1993). Therefore, detailed studies on the effects of inhibitors are significant for understanding the enzymatic mechanism.

Little is known about how F_0 interacts with F_1, and how the energy contained within the electrochemical proton gradient is transduced to catalytic sites of F_1. In all F_0F_1-ATPase complexes, there is a free carboxyl group in subunit c that appears to be involved in transmembrane proton conduction (Fillingame, 1990; Penefsky and Cross, 1991). DCCD reacts with this carboxyl group (Fillingame, 1990; Senior, 1990; Penefsky and Cross, 1991). Oligomycin also binds to a region in F_0 that includes subunit c (Kiehl and Hatefi, 1980; Nagley et al., 1986). These reagents block proton translocation through F_0 and energy transfer between F_1 and F_0 (Penefsky and Cross, 1991). As a result, ATP hydrolysis by SMP is greatly inhibited by DCCD or oligomycin (Matsuno-Yagi et al., 1985; Matsuno-Yagi and Hatefi, 1993a). These data suggest that a conformational change in F_0 is communicated to the β subunits of F_1. By comparison with isolated F_1, however, the effects of unmodified F_0 on F_1 of F_0F_1 complex are not described. Studies on the influences of inhibitor and modulator sensitivities between the soluble F_1-ATPase and membrane-bound F_1-ATPase will help to understand more about the mechanism of oxidative phosphorylation. Up to now, no detailed comparisons of the properties of membrane-bound and soluble F_1-ATPase from any species have been reported.

Regulation of F_1-ATPase activity in the cell is not understood. The intercellular regulation of F_1-ATPase may

include the natural F_1 inhibitor protein that powerfully inhibits ATP hydrolysis (Cintron and Pedersen, 1979; Krull and Schuster, 1981), but that also is an essential component for achieving maximal ATP synthesis at high ATP concentrations (Tuena de Gomez-Puyou et al., 1988; Martins et al., 1992) and cooperativity between multiple catalytic sites (Rao and Senior, 1987; Weber et al., 1994). In addition, the regulation may also involve the components of oxidative phosphorylation such as ADP, ATP, and P_i as well as Mg^{2+} (Martins et al., 1988b; Guerrero et al., 1990; de Vicente et al., 1991; Murataliev et al., 1991; Burgard et al., 1994; Jault et al., 1994). Therefore, studies on the effects of ATP and Mg^{2+} will help to understand both the regulation mechanism in the cell and more about the kinetic properties.

Finally, the role of the noncatalytic nucleotide sites is unknown. The results of studies of their specificity and interactions with catalytic sites are controversial. The noncatalytic sites may play a regulatory role in the mitochondrial and chloroplast enzyme (Di Pietro et al., 1980; Milgrom et al., 1990) while the degree of regulation seems to be slight or nonexistent for the E.coli enzyme (Wise and Senior, 1985). Further studies indicate that ATP must be bound to noncatalytic sites of CF_1 and MF_1 to observe maximal steady-state rates of ATP hydrolysis (Milgrom et al., 1990; Milgrom et al., 1991;

Murataliev, 1992; Jault and Allison, 1993; Milgrom and Cross, 1993; Wang et al., 1993). The binding of ADP and GTP to these sites appears to inhibit ATP hydrolysis (Di Pietro et al., 1980; Milgrom et al., 1991; Milgrom and Cross 1993; Jault and Allison, 1994). These results suggest that the noncatalytic sites may have a similar regulatory role in ATP hydrolysis but such a role has not been determined since the noncatalytic sites fill rapidly under conventional ATPase assay conditions (Milgrom et al., 1990; Jault and Allison, 1993). If catalytic and noncatalytic sites specifically bind with different nucleotides, however, the role noncatalytic sites might be discerned and studied.

The objectives of my study were threefold. First, I attempted to optimize sonic disruption of mitochondria and to describe a simple, rapid, effective method of isolation and partial purification of the soluble mitochondrial F_1-ATPase from crayfish (Orconectes nais) gills. Second I characterizated and compared the membrane-bound and soluble forms of the enzyme concerning physical and enzymatic properties as well as inhibitor and modulator sensitivities. Third, my study provides direct kinetic evidence for MgATP as the true substrate and for the regulatory mechanism of ATP and Mg^{2+}. My study also provides indirect evidence for the regulatory role of noncatalytic sites.

MATERIALS AND METHODS

Animals: Crayfish (Orconectes nais) were obtained from various ponds on the campus of Emporia State University, Emporia, Kansas.

Chemicals: All reagents were analytical grade. ATP, Tris, Hepes, BSA, and all inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were from Fisher Scientific Co.

Isolation of Mitochondria: The gill tissues were washed and homogenized in isolation medium containing 10 mM Tris/Hepes (pH 7.4), 250 mM sucrose, 1 mM DTT, 0.5 mM Na_2EDTA, at a volume to tissue ratio of 10 ml/g fresh weight at 4^0C. The homogenate was centrifuged for 10 min at 800 g to remove nuclei and debris. The resulting supernatant was collected and mitochondria pelleted by centrifugation for 20 min at 10,000 g. The pellet was washed once with isolation medium, resuspended in glycerol buffer containing 10 mM Tris/Hepes (pH 7.6), 0.5 mM Na_2EDTA, 1 mM DTT, and 20% (v/v) glycerol, and stored at -70^0C. For most experiments, freshly prepared mitochondria were used for sonication treatment.

Sonication of Mitochondria: To study the optimal conditions for sonication, the mitochondrial samples were suspended in glycerol buffer at different concentrations and at different pH values, and sonicated for different time

periods. A W-385 Sonicator with #418 microtip (Heat Systems Ultrasonic Inc.) was used. The cycle time was set for 1 sec, duty cycle at 50%, with output control at 4. The procedure was carried out at 0-8^{0}C. The sonicated samples were centrifuged at 10,000 g for 20 min and the pellet and supernatant separated for enzymatic assay. The treatment that resulted in maximal total ATPase activity in the supernatant was considered the optimal condition.

Isolation of Submitochondrial Particles (SMP): The mitochondrial sample was suspended in glycerol buffer at about 5.0 mg of protein per ml at pH 8.6 and sonicated for 4 min at 0-8^{0}C. The sonicated volume was about 50 ml. After sonication, the sample was centrifuged at 10,000 g for 20 min to sediment broken mitochondria and large fragments. The resulting supernatant was centrifuged for 1 hr at 100,000 g to sediment the submitochondrial particles. Pelleted SMP were resuspended in glycerol buffer (pH 7.6) and stored at -70^{0}C. All procedures were carried out at or near 4^{0}C.

Preparation of Soluble F_1-ATPase: The F_1 was extracted from submitochondrial particles utilizing established procedures (Beechey et al., 1975). The SMP sample was suspended in glycerol buffer (pH 7.6) at about 5.0 mg protein/ml at room temp (20-25^{0}C). One volume of chloroform was added to two volumes of SMP suspension. The mixture was stirred vigorously for 15-25 sec and centrifuged for 10 min

at 2,000 g. The upper aqueous phase was collected and further centrifuged for 1 hr at 100,000 g to sediment membrane-bound fragments. The supernatant contained the soluble F_1-ATPase preparation. The preparation was stored at -70^0C.

Protein Analysis: Protein concentration was determined with a Bio-Rad protein assay (Bradford, 1976) using bovine serum albumin as the standard. All data represent the mean values of six different observations.

Determination of ATPase Activity: In general, the reaction mixtures contained the following: 10 mM Tris, 3 mM $MgCl_2$, 50 mM $NaHCO_3$, and 5 mM Na_2ATP (final concentration, adjusted to pH 7.2 with Hepes) in a final assay volume of 0.5 ml. Control assays were done in the absence of HCO_3^-. The reaction mixtures contained 10-12 μg of protein for mitochondria, about 8 μg protein for SMP, and about 1 μg for soluble F_1-ATPase preparation. Enzyme samples were incubated at $37-38^0$C for 15 min in the reaction mixtures without Na_2ATP. The reaction was started by adding Na_2ATP and terminated by the addition of 1.0 ml of 2% ammonium molybdate in 1.15 N H_2SO_4 and 1.0 ml of 8% $FeSO_2$ in 1.15 N H_2SO_4. A 15 min time period was allowed for color development. Absorbancies were read at 700 nm that represented released inorganic phosphate (P_i). The ATPase activity was defined as the difference between activity obtained with and without 50 mM $NaHCO_3$ (HCO_3^- stimulated

ATPase activity). Enzyme specific activity (S.A) was expressed as micromoles P_i released per mg protein per hr. All data represent the mean values of six different observations.

In experiments where ATP concentration was varied, ATPase activity was determined by the addition of different concentration of Na_2ATP (adjusted to pH 7.2 with Tris). In experiments where $NaHCO_3$ concentration was varied, the pH of the reaction mixtures was adjusted with citric acid. In the experiments for determination of pH optimum, the 10 mM Bis-Tris buffer was used for pH range 5.8-7.15 and the pH was adjusted with citric acid; 10 mM Tris buffer was still used for pH range 7.15 to 8.85 as in other experiments.

In the kinetic studies for ATP and Mg^{2+} regulation, the enzymatic assay was measured in the absence of bicarbonate, since HCO_3^- could affect the calculation of [MgATP], [ATP_f], and [Mg^{2+}_f]. The ATPase activity was expressed as the azide-sensitive activity (calculated as the difference in the presence and absence of 5 mM azide).

Inhibitor Dissociation and pH Adjustment: KNO_3, NaN_3, PCMB, DIDS, and Na_3VO_4 were dissolved in water. Oligomycin, DCCD, and NBD-Cl were dissolved in 50% alcohol. In the studies for oligomycin, DCCD, and NBD-Cl, the reaction mixtures contained a final concentration of 0.95% alcohol. Before use, the solutions of NaN_3 and Na_3VO_4 were adjusted to pH 7.2 with acetic acid. The solutions of PCMB and DIDS were

adjusted to pH 7.2 with NaOH.

Determination of MgATP, Free ATP, and Free Mg^{2+} Concentration: The concentrations of MgATP complex, free Mg^{2+}, and free ATP were calculated using the stability constant estimated by Adolfsen and Moudrianakis (1978a). The stability constant that must be used was the apparent stability constant. This was the intrinsic stability constant corrected for the effects of pH, temperature, ionic strength, and the monovalent cation concentration of the reaction mixture. In the experimental conditions (pH 7.2, Temp 37-38^{0}C), the apparent stability constant for MgATP was estimated by the following equation (Adolfsen and Moudrianakis, 1978a):

$$K_{MgATP} = (1.4\times10^{(5-3.1[NaCl])})/(1.631+17[Na_t]) \qquad (1)$$

In the experiments, the [NaCl] and $[Na_t]$ varied with addition of different amounts of Na_2ATP and $MgCl_2$. On the other hand, the stability constant of MgATP complex may be expressed as follows:

$$K_{MgATP} = [MgATP]/[Mg^{2+}_f][ATP_f] \qquad (2)$$

The equations describing the equilibrium between the total concentrations $[ATP_t]$ and $[Mg^{2+}_t]$ and the free concentrations $[ATP_f]$ and $[Mg^{2+}_f]$ are:

$$[ATP_t] = [ATP_f] + [MgATP] \quad (3)$$

$$[Mg^{2+}_t] = [Mg^{2+}_f] + [MgATP] \quad (4)$$

By solving equations 2, 3, and 4, the [MgATP], $[ATP_f]$, and $[Mg^{2+}_f]$ were obtained.

RESULTS

Isolation of Soluble F_1-ATPase

Isolation of Mitochondria: About three mg of mitochondrial protein were obtained from 1 g wet weight gill tissue. The specific activity of ATPase from intact mitochondria was about 26 μMol P_i/mg protein/hr.

Optimum Conditions for Sonic Disruption of Mitochondria: The breakage of mitochondria was markedly sensitive to sonication time periods below 4 min (Fig. 1). A longer sonication increased the breakage of mitochondria. However, the amount of broken mitochondria remained almost the same between 2 to 4 min. Therefore, the optimum sonication time of 2 to 4 minutes resulted in about 80% of the mitochondria being broken. However, mitochondrial concentration did not seem to affect the sonic disruption over the range 0.7-6.0 mg protein/ml (Fig. 2).

Fig. 3 shows the effect of pH on mitochondrial disruption. The breakage was sensitive to the pH over the range 7.0-8.6, increasing at higher pH values. The highest yield of broken mitochondria was obtained at pH 8.6. Almost 90% of the mitochondria was broken.

Summarizing these experiments, sonication treatment of mitochondria suspended in glycerol buffer at pH 8.6 and at 5 mg protein per ml for 4 min was adopted as a standard procedure. It was very effective for obtaining the

Fig. 1. Effect of sonication time periods on disruption of mitochondria. The mitochondrial samples were suspended in glycerol buffer at pH 7.4 and 2.5 mg protein per ml. The volume was 4 ml. Supernatant and pellet represented broken and unbroken mitochondria. The glycerol buffer and sonication power as well as determination of ATPase activity and protein concentration are described in "Materials and Methods".

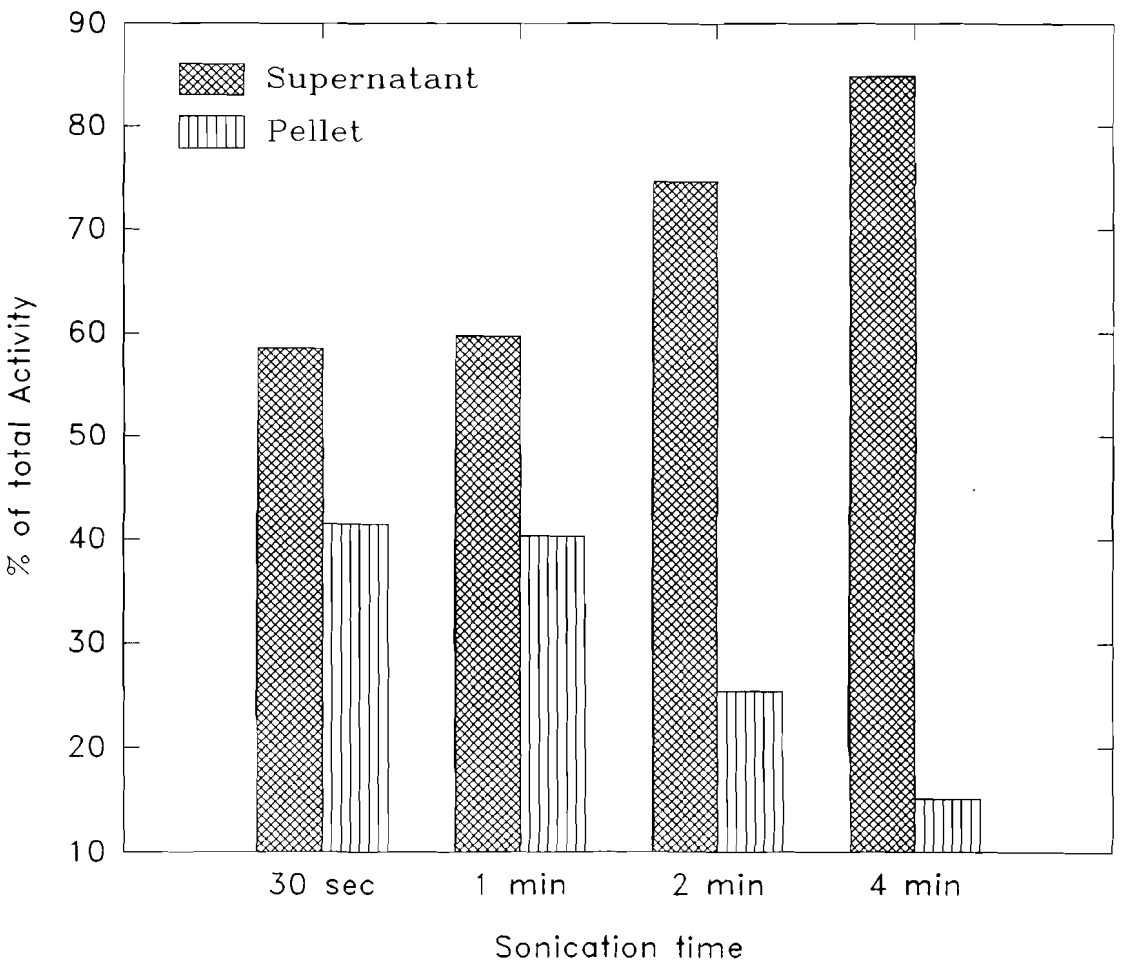

90
80
70
60
50
40
30
20
10
% of total Activity
Supernatant
Pellet
30 sec
1 min
2 min
4 min
Sonication time

Fig. 2. Effect of concentration on sonic disruption of mitochondria. Mitochondrial samples were sonicated for 2 min at pH 7.4. Other conditions were the same as in Fig. 1.

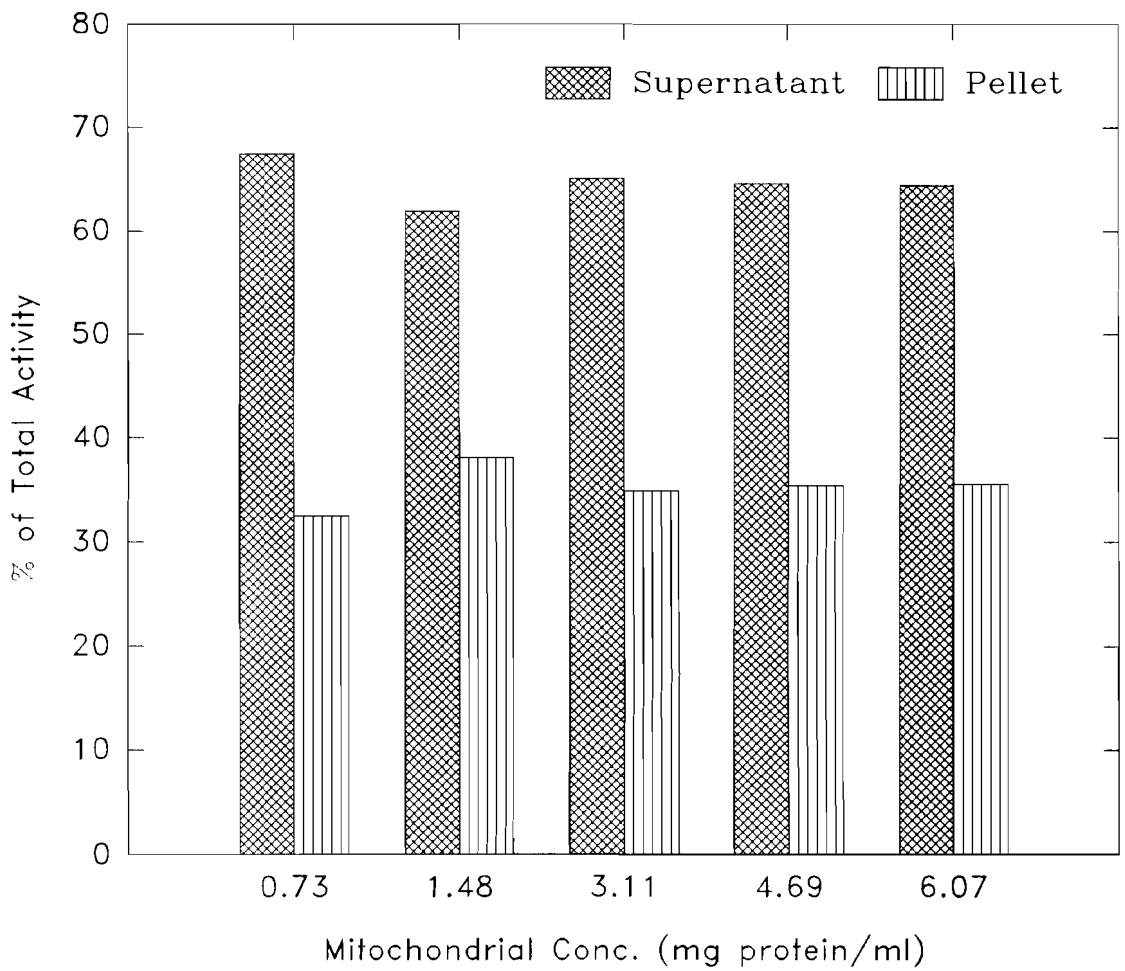
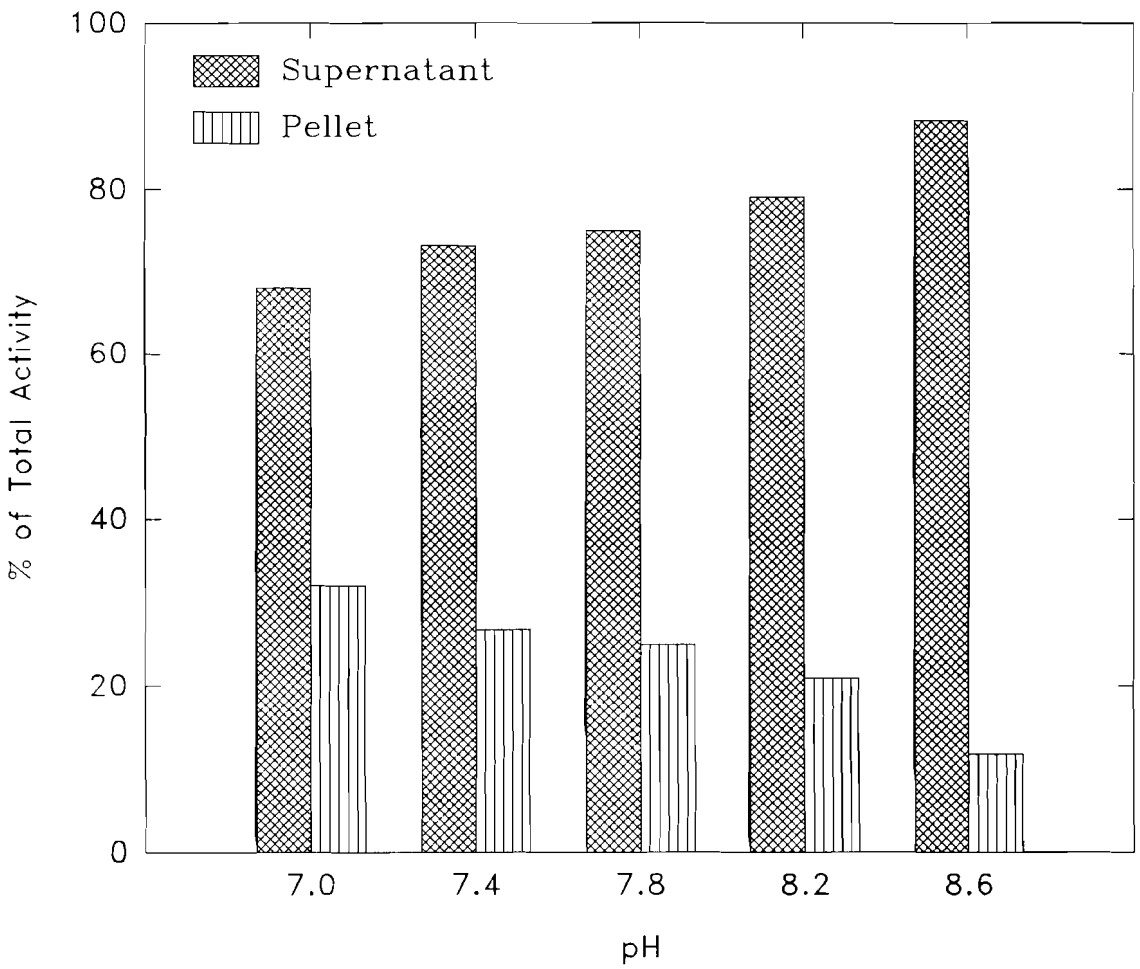

80
70
60
50
40
30
20
10
0
% of Total Activity
Supernatant
Pellet
0.73
1.48
3.11
4.69
6.07
Mitochondrial Conc. (mg protein/ml)

Fig. 3. Effect of pH on sonic disruption of mitochondria. Mitochondrial samples were suspended in glycerol buffer at 3.5 mg of protein per ml and sonicated for 2 min. Other conditions were the same as in Fig. 1.


100
80
60
40
20
0
% of Total Activity
Supernatant
Pellet
7.0
7.4
7.8
8.2
8.6
pH

submitochondrial particles. There was little ATPase activity remaining in the unbroken mitochondria and large membrane fragments. More than 95% of the total activity was obtained in the supernatant that contained about 70% of total protein.

Preparation of Soluble F_1-ATPase: The standard procedures for preparation of soluble F_1-ATPase are summarized in Fig. 4. The soluble F_1-ATPase isolated by this procedure showed a 17.4-fold increase in specific activity over intact mitochondria. It comprised 1.55% of the mitochondrial protein and 28.4% of mitochondrial total activity (Table 1). Sonication treatment of mitochondria activated the ATPase about 21.5% as seen by the increase in total activity. Considering this activation, a purification of 15-fold was estimated.

There were two major losses of activity in this procedure. One occurred after sedimentation of submitochondrial particles by high-speed centrifugation. About 40% of broken mitochondrial activity remained in the supernatant. This probably represented the soluble F_1 portion released from membranes after sonication. Another occurred after chloroform treatment. Just 42% of SMP ATPase activity was released into the aqueous phase, while almost 60% remained in the membrane. In addition, the major enhancement (7.5-fold increase) of specific activity occurred after the chloroform treatment by the removal of

Fig. 4. Schematic outline of the procedure for preparation of soluble F_1-ATPase.

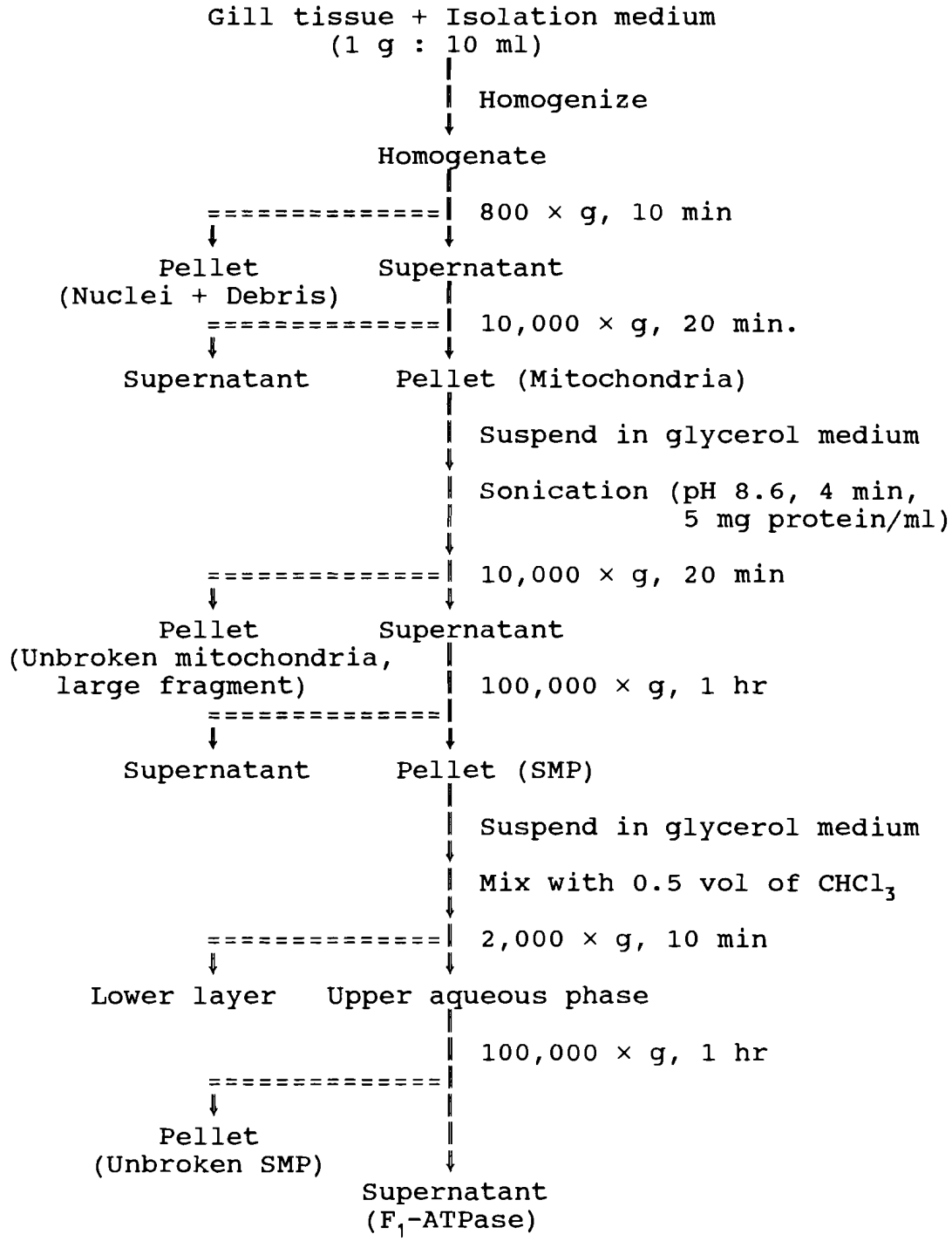

Gill tissue + Isolation medium
(1 g : 10 ml)
Homogenize
Homogenate
800 × g, 10 min
Pellet
(Nuclei + Debris)
Supernatant
10,000 × g, 20 min.
Supernatant
Pellet (Mitochondria)
Suspend in glycerol medium
Sonication (pH 8.6, 4 min,
5 mg protein/ml)
10,000 × g, 20 min
Pellet
(Unbroken mitochondria,
large fragment)
Supernatant
100,000 × g, 1 hr
Supernatant
Pellet (SMP)
Suspend in glycerol medium
Mix with 0.5 vol of CHCl3
2,000 × g, 10 min
Lower layer
Upper aqueous phase
100,000 × g, 1 hr
Pellet
(Unbroken SMP)
Supernatant
(F1-ATPase)

Table 1. Preparation of soluble F_1-ATPase. Extent of purification was estimated considering apparent activation by sonication.

Fraction	Total Protein (mg)	Specific Activity (μMol P_i/mg/hr)	Total Activity (μMol P_i/hr)	Purification (x-fold)
Mitochondria	180.87	26.31	4758.7	----
After sonication	190.92	30.28	5781.1	1.00
Broken Mitochondria	140.75	40.62	5717.3	1.34
SMP	55.31	60.91	3370.5	2.01
Aqueous phase (extraction)	15.10	93.21	1407.6	3.08
Final Preparation	2.95	457.59	1349.9	15.11

the majority of protein with the high-speed centrifugation step. Therefore, the chloroform extraction technique was an effective method for the solubilization of the F_1-ATPase from SMP.

General Characteristics

In general, the properties of soluble F_1-ATPase were similar to those of the membrane-bound enzyme. Both the membrane-bound and soluble ATPase hydrolyzed ATP (MgATP), but did not hydrolyze ADP or MgADP.

Effect of Temperature: The membrane-bound and soluble mitochondrial ATPase activities were compared at different temperatures ranging from 10-60^{0}C (Figures 5 and 6). The activities for both displayed similar temperature dependence. The maximum activity for soluble ATPase occurred at about 40^{0}C. Just 10% of maximum activity occurred at 10^{0}C and almost no activity at 60^{0}C.

Effect of pH: The membrane-bound and soluble mitochondrial ATPase activities were compared at pH values ranging from 5.8-8.85 (Figures 7 and 8). The pH dependencies for both membrane-bound fractions, mitochondria and SMP, were similar (Fig. 7), but soluble F_1-ATPase activity shows a slightly higher pH dependence than those from membrane-bound forms. The membrane-bound enzyme displayed a broad pH optimum (7.0-7.8), with a maximum at pH 7.4. The soluble F_1-ATPase activity displayed a slight shift to a

Fig. 5. Effect of temperature on activity of soluble F_1-ATPase.

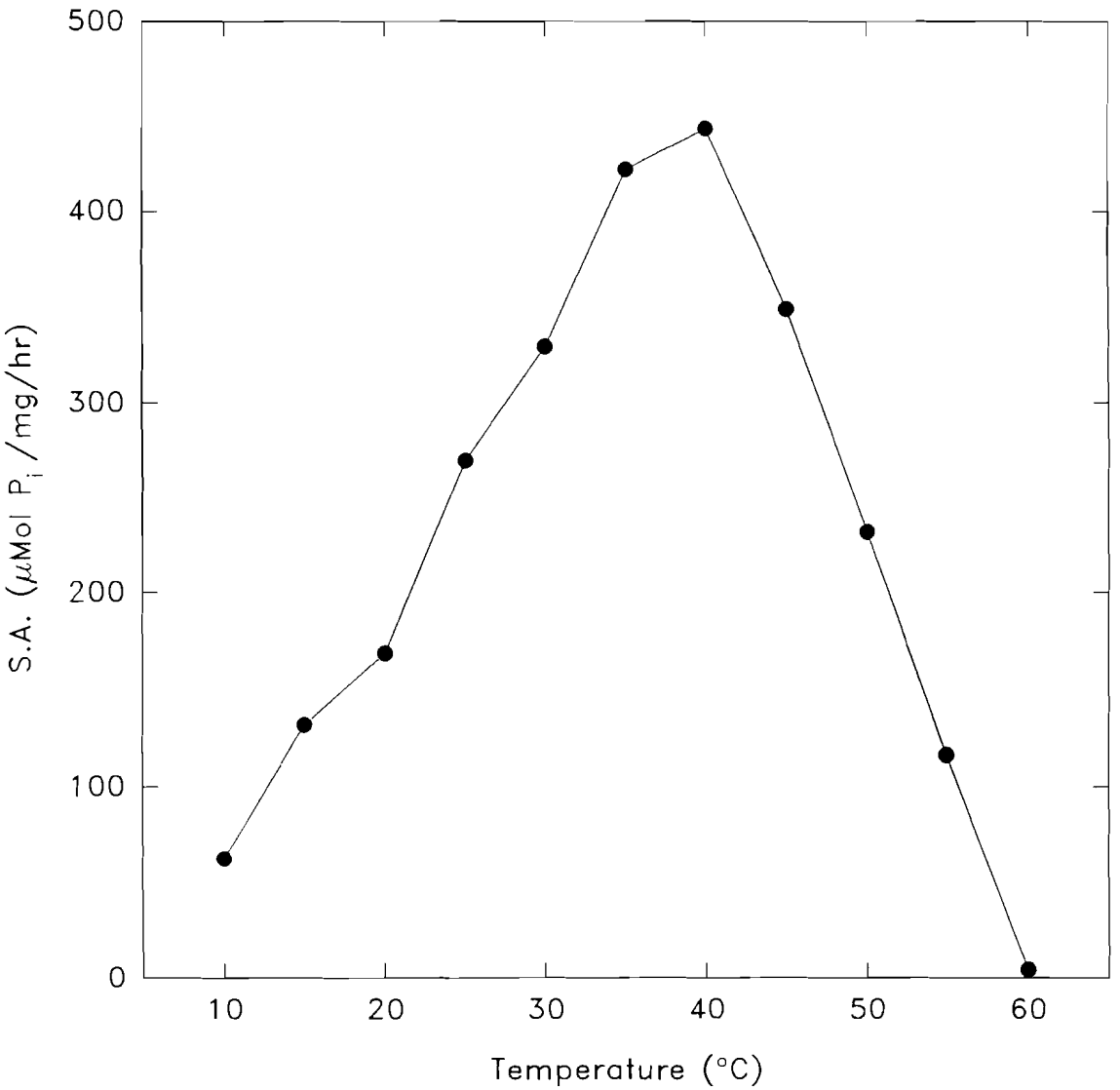

500
400
300
200
100
0
S.A. (μMol P_i /mg/hr)
10
20
30
40
50
60
Temperature (°C)

Fig. 6. Effect of temperature on activity of membrane-bound mitochondrial ATPase: mitochondria (■), submitochondrial particles (●).

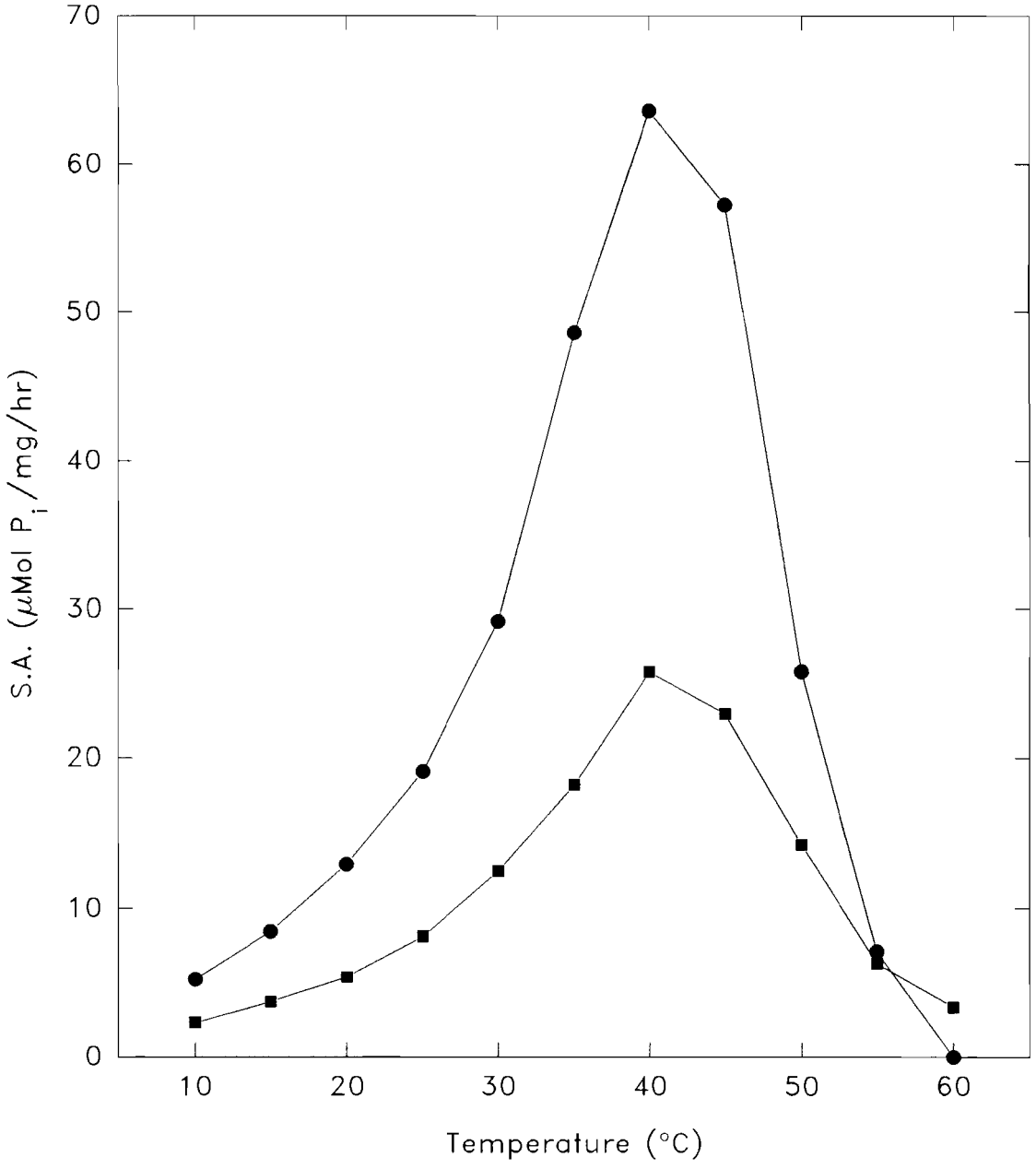

70
60
50
40
30
20
10
0
S.A. (μMol P_i /mg/hr)
10
20
30
40
50
60
Temperature (°C)

Fig. 7. Effect of pH on soluble F_1-ATPase activity. The buffers were 10 mM Bis-Tris/citric acid (pH 5.8-7.15) and 10 mM Tris/Hepes (pH 7.15-8.85).

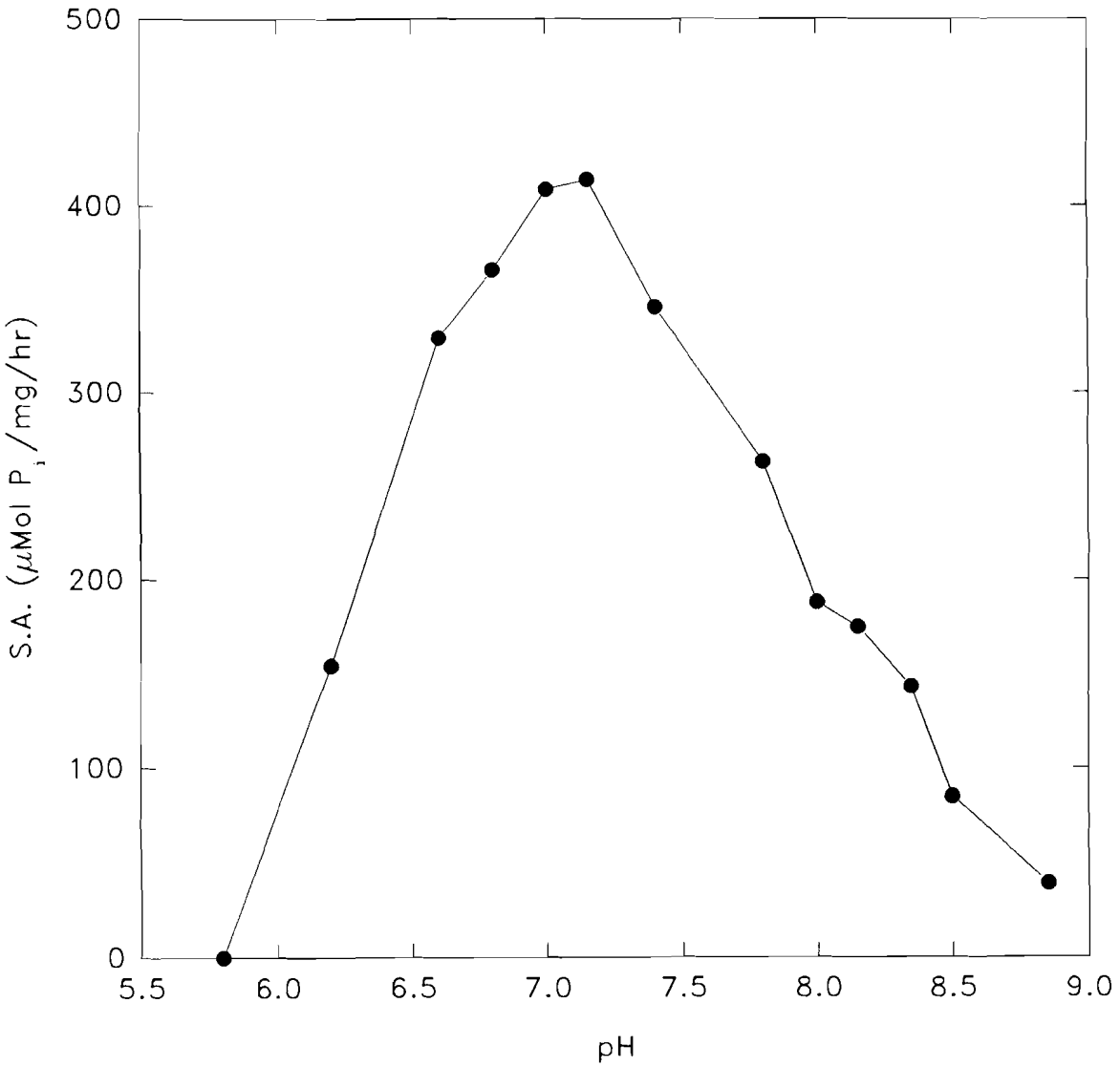
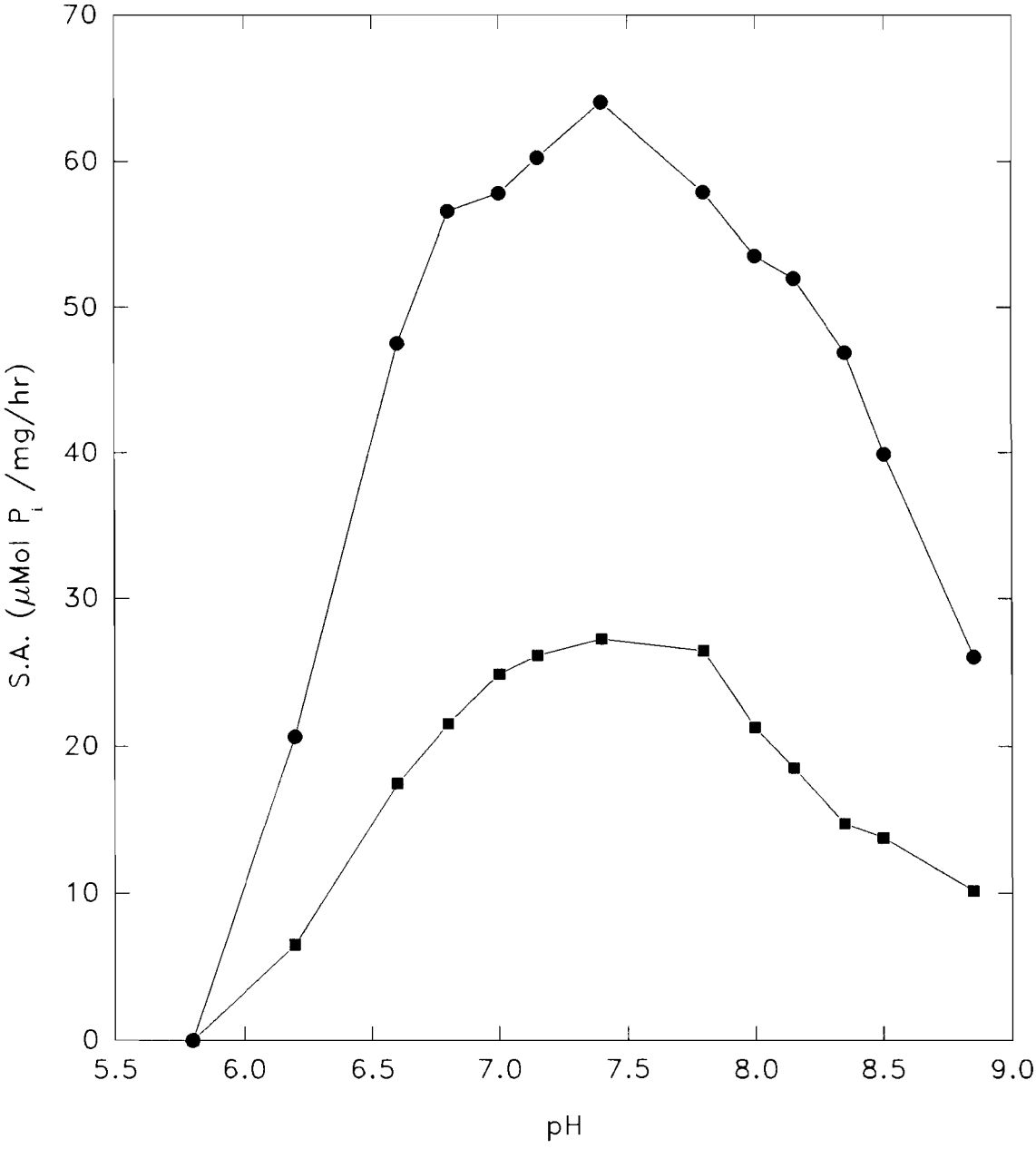

500
400
300
200
100
0
S.A. (μMol P_i /mg/hr)
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

Fig. 8. Effect of pH on membrane-bound ATPase activity. Mitochondria (■), submitochondrial particles (●). The buffers were the same as in Fig. 7.


70
60
50
40
30
20
10
0
S.A. (μMol P_i /mg/hr)
5.5
6.0
6.5
7.0
7.5
8.0
8.5
9.0
pH

more acid and relative narrow pH optimum (7.0-7.2), with a maximum at pH 7.15. In addition, activities for both soluble and membrane-bound enzymes were almost zero at pH 5.8. About 40% of maximum membrane-bound enzyme activity occurred at pH 8.85, while soluble enzyme showed less than 10%.

Stability and Storage: Figures 9, 10, and 11 show the effect of storage temperature on ATPase activity of the different fractions. Mitochondria, SMP, and soluble enzymes all were quite stable at -70^0C, -17^0C, and 4^0C in 20% glycerol buffer. Soluble F_1-ATPase did not show cold-lability in the glycerol buffer. Soluble F_1-ATPase was not very stable at 25^0C, but it was inactivated more slowly than membrane-bound enzyme at the same temperature. About 40% of the initial soluble F_1-ATPase activity was retained at 25^0C after one week, while just 20% remained for membrane-bound enzymes.

For long term storage, the mitochondria, SMP, and soluble F_1-ATPase were suspended in glycerol buffer containing 10 mM Tris/Hepes (pH 7.6), 0.5 mM Na_2EDTA, 1 mM DTT, and 20% (v/v) glycerol, and stored at -70^0C. In this condition, more than 90% of initial activity was retained for up to 6 months. All experiments reported here were done on enzyme stored in this manner.

Anion-Stimulated Activity

The influence of HCO_3^- on ATPase activity of both

Fig. 9. Effect of storage temperature on the stability of soluble F_1-ATPase. The enzyme was stored in 20% glycerol buffer (pH 7.6) at a concentration of 0.1 mg per ml, at -70°C (●), -17°C (■), 4°C (▲), and 25°C (▼).

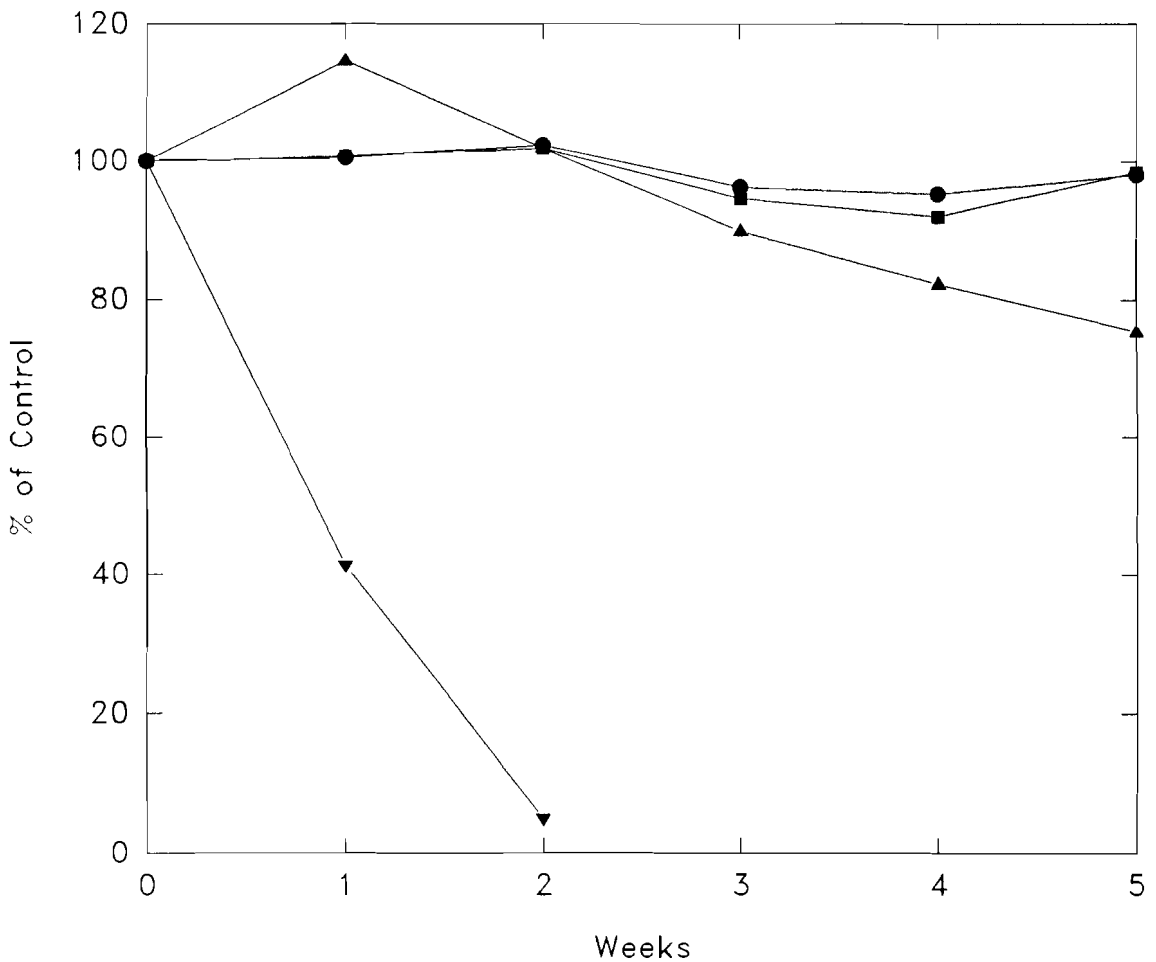

120
100
80
60
40
20
0
% of Control
0
1
2
3
4
5
Weeks

Fig. 10. Effect of storage temperature on the stability of SMP ATPase. Except for the concentration (0.8 mg/ml), all conditions were the same as in Fig. 9.

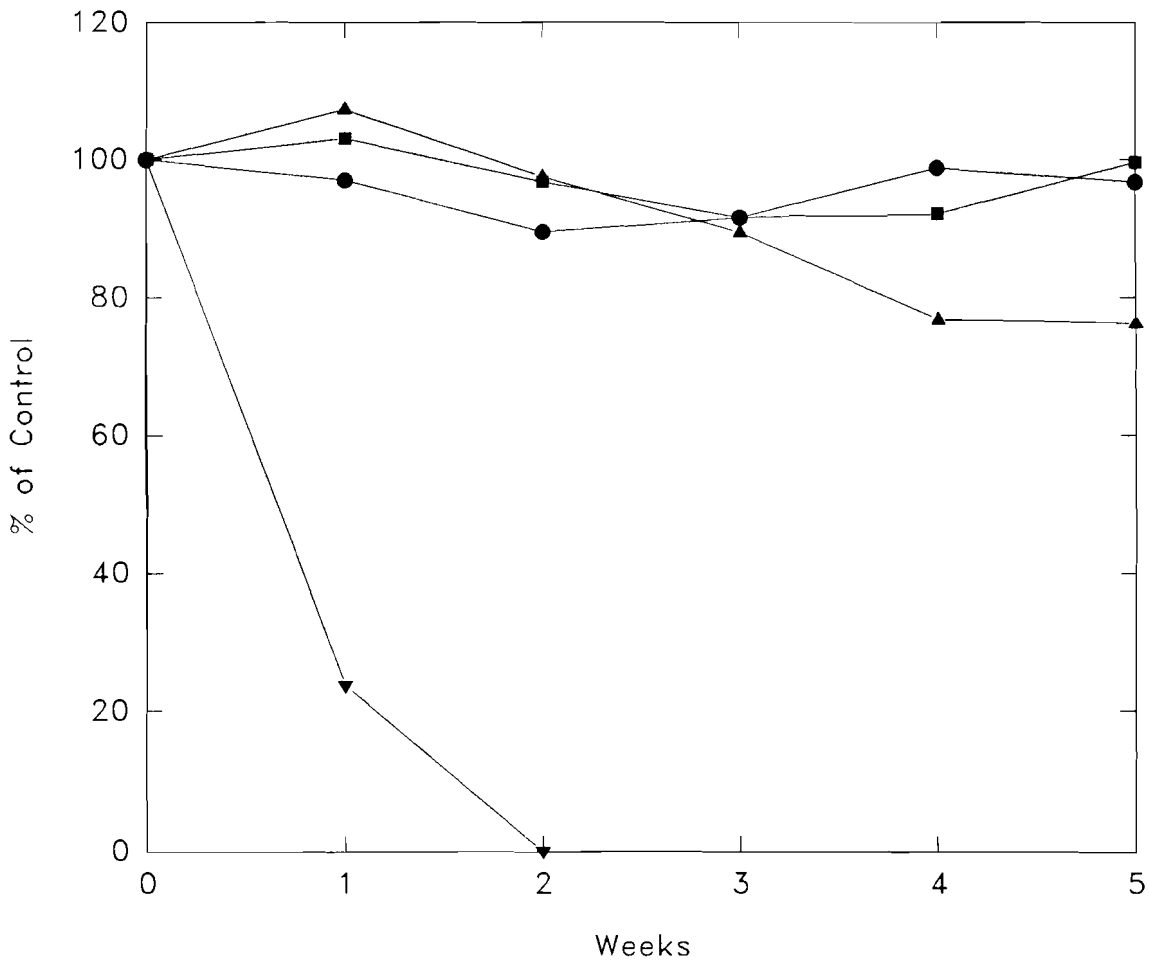
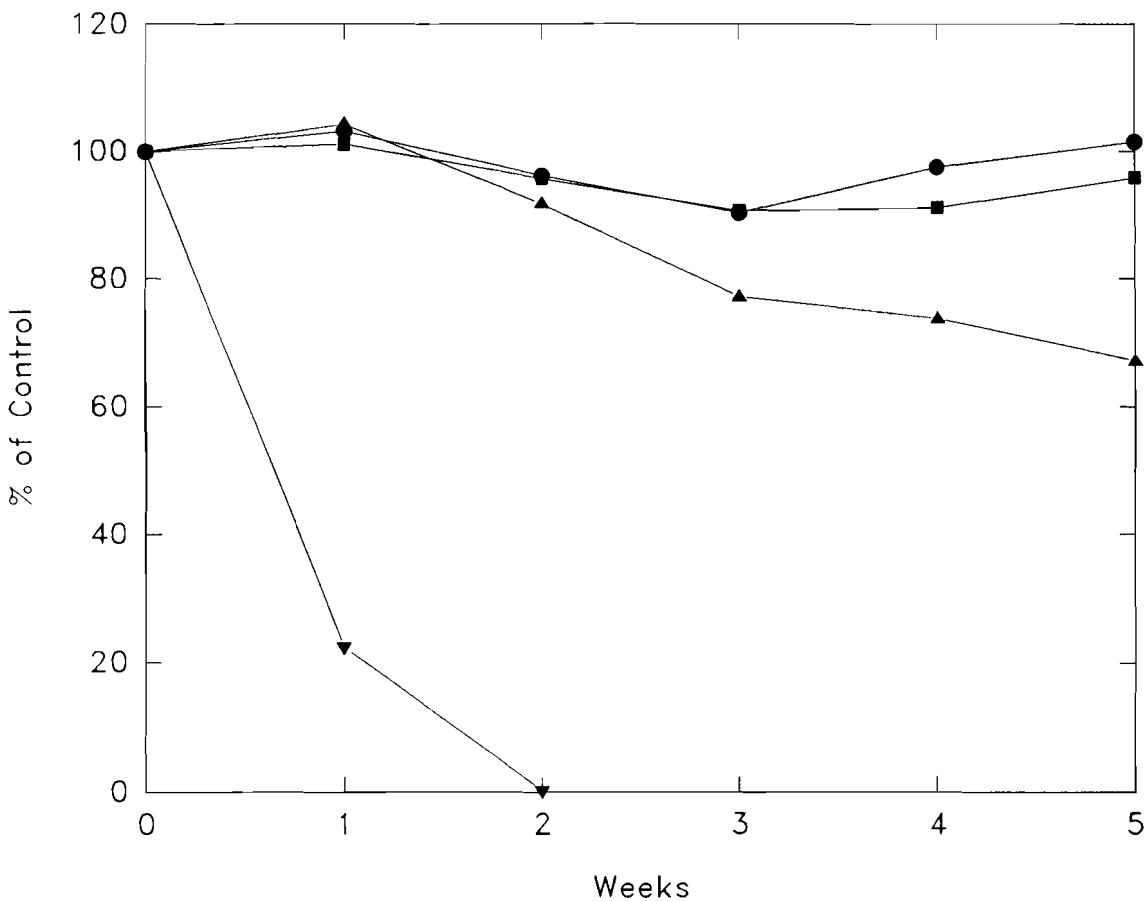

120
100
80
60
40
20
0
% of Control
0
1
2
3
4
5
Weeks

Fig. 11. Effect of storage temperature on the stability of mitochondrial ATPase. Except for the concentration (1.0 mg/ml), all conditions were the same as in Fig. 9.


120
100
80
60
40
20
0
% of Control
0
1
2
3
4
5
Weeks

soluble and membrane-bound enzymes is shown in Fig. 12. Highest activities for both enzyme forms were observed in the presence of 35 mM HCO_3^-. The activity decreased as the concentration of HCO_3^- exceeded 35 mM. Membrane-bound enzyme retained about 40% of highest activity at 150 mM HCO_3^-, while soluble enzyme retained just 24%. From the Lineweaver-Burk plot for HCO_3^-, the apparent K_a values for soluble F_1-ATPase and membrane-bound ATPase were 10.1 and 15.8 mM, respectively. This suggested that soluble F_1-ATPase affinity for HCO_3^- was higher than for membrane-bound enzyme. The theoretical V_{max} was 613.2 μMol P_i/mg protein/hr for soluble enzyme and 94.0 μMol P_i/mg/hr for membrane-bound enzyme.

Soluble F_1-ATPase activity was differentially sensitive to anions (Figures 12a and 13). It was stimulated optimally by HCO_3^- and slightly by SO_4^{2-} and Cl^- (Table 2). The anion-stimulated activities were in the order $HCO_3^- > SO_4^{2-} > Cl^-$. SO_4^{2-} and Cl^- stimulated activities were 10% and 8% of the HCO_3^- stimulated activity, respectively (Table 2). However, the apparent K_a values for HCO_3^-, SO_4^{2-} and Cl^- were close and were 10.1, 10.7, and 11.1 mM, respectively (Table 2). In addition, for these anions, the highest activities of both membrane-bound (data not shown) and soluble enzymes (Fig. 12a) all were observed at 35 mM. Therefore, 35 mM was considered the optimal anion concentration.

Fig. 12. Effect of [HCO_3^-] on bicarbonate stimulated activity of soluble (a) and membrane-bound (SMP) (b) enzymes. Reaction mixture consisted of 10 mM Tris, 3 mM $MgCl_2$, 5 mM Na_2ATP/Tris (pH 7.2), and was adjusted to pH 7.2 with citric acid. The ATPase activity was defined as the difference between activity obtained with and without HCO_3^-.

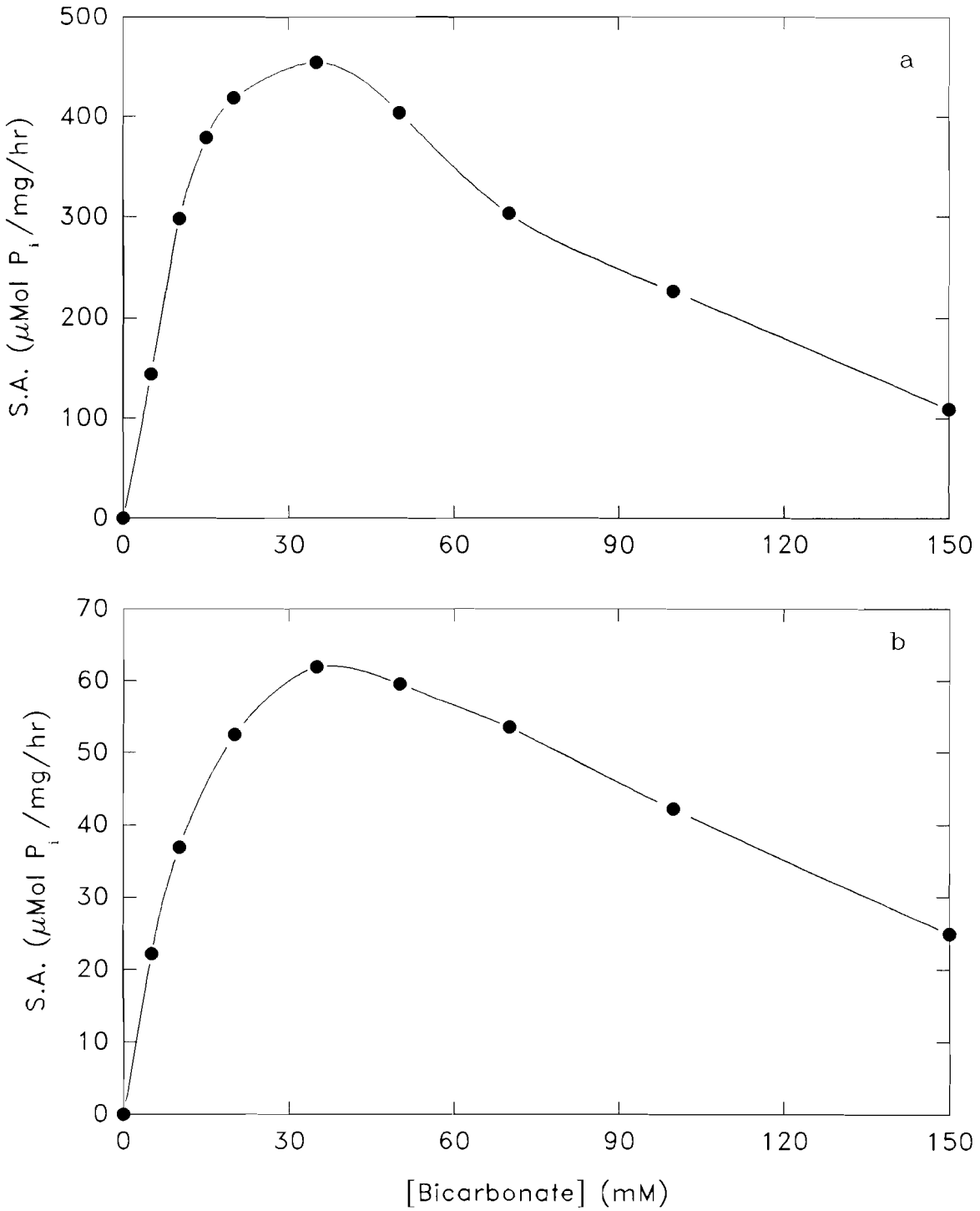
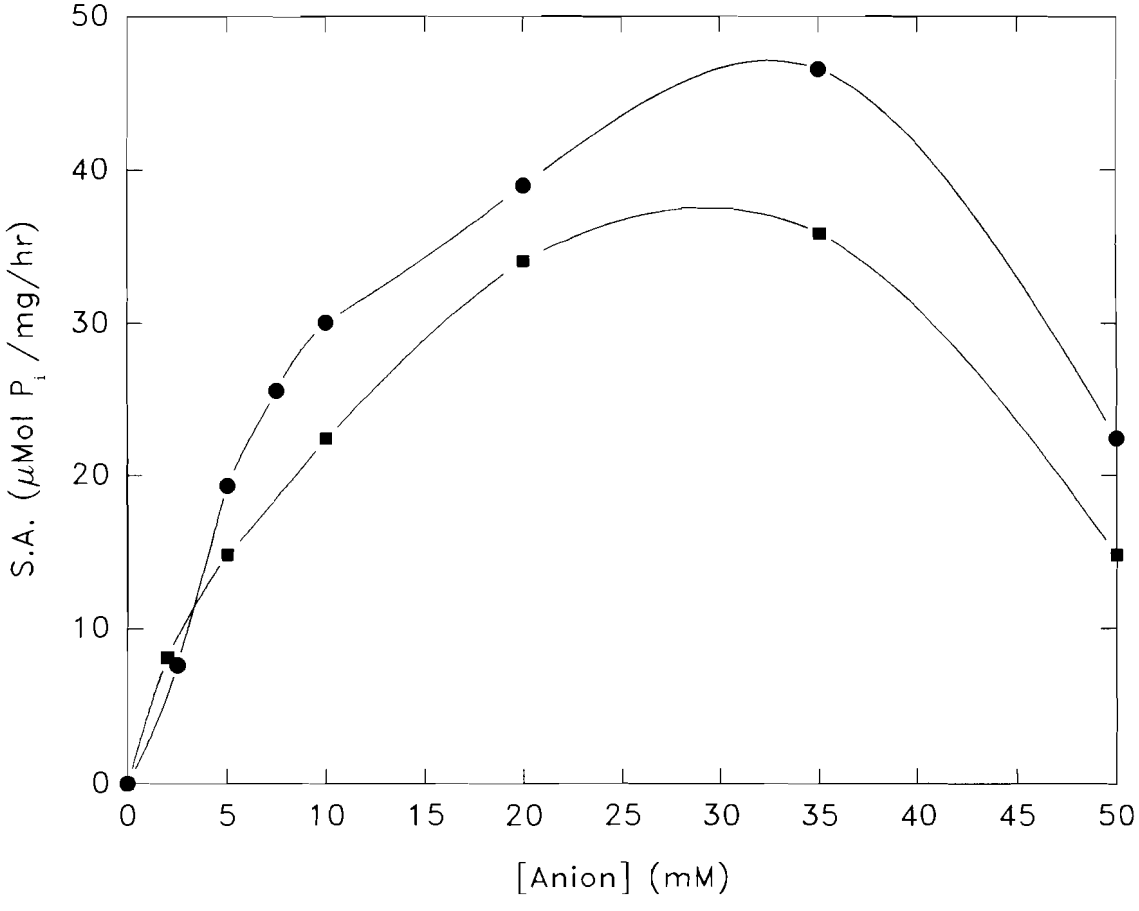

a
b
S.A. (μMol P_i /mg/hr)
S.A. (μMol P_i /mg/hr)
[Bicarbonate] (mM)
0
30
60
90
120
150
0
100
200
300
400
500
0
10
20
30
40
50
60
70

Table 2. Kinetic parameters of anion stimulation for soluble F_1-ATPase activity. Apparent K_a and theoretical V_{max} were determined by Lineweaver-Burk plot analysis in anion concentration range of 2-35 mM. Maximal activities were determined from Figures 12 and 13. All experimental conditions were as described in Figures 12 and 13.

Anions	Maximal Activity (μMol P_i/mg/hr)	Apparent K_a (mM)	Theoretical V_{max} (μMol P_i/mg/hr)
HCO_3^-	455.2	10.1	613.2
SO_4^{2-}	46.6	10.7	61.3
Cl^-	35.8	11.1	46.9

Fig. 13. Effect of anion, sulfate (●) and chloride (■), on soluble F_1-ATPase activity. The reaction mixture was as described in Fig. 12. The ATPase activity was expressed as the anion stimulated activity that was calculated as the difference in the presence and absence of the anion. In the chloride effect, the 6 mM Cl^- in the reaction mixture was not included in the indicated Cl^- concentration.


50
40
30
20
10
0
S.A. (μMol P$_i$ /mg/hr)
0
5
10
15
20
25
30
35
40
45
50
[Anion] (mM)

Effects of Inhibitors

Effect of Nitrate: As seen in Fig. 14, the activities of both membrane-bound and soluble ATPase were inhibited by NO_3^-. At a concentration of 200 mM KNO_3, 97% of soluble enzyme activity was inhibited; 50% inhibition was observed at 28 mM (Table 2). For membrane-bound enzyme, 74% of activity was inhibited at 200 mM, and the I_{50} increased to 85 mM (Table 3). The soluble F_1-ATPase was 3-fold more sensitive to NO_3^- than the membrane-bound enzyme.

Effect of NaN_3: Azide is a specific inhibitor for F_1-ATPase. Both membrane-bound and soluble ATPase activities were inhibited by NaN_3 (Fig. 15). Inhibition was virtually complete at 2 mM. Both enzyme forms share a similar sensitivity to azide. The I_{50} for soluble enzyme (93 μM) was just slightly lower than that of membrane-bound enzyme (I_{50} = 110 μM) (Table 3).

Effect of pCMB: F_1-ATPase was extremely sensitive to pCMB (Fig. 16). The sensitivity was very different between soluble and membrane-bound enzymes. Soluble F_1-ATPase activity was completely inhibited at 5 μM pCMB. Increasing the concentration to 100 μM, 96% and 90% of activities were inhibited for SMP and mitochondria, respectively. 50% inhibition was observed at 0.15, 1.0, and 1.5 μM pCMB for soluble enzyme, SMP, and mitochondria, respectively (Table 3). The soluble F_1-ATPase was 7-fold more sensitive to pCMB than SMP and 10-fold more sensitive to pCMB than

Fig. 14. Effect of nitrate on activity of F_1-ATPase from crayfish gills. (●) soluble enzyme and (■) membrane-bound enzyme (SMP). Enzyme samples were incubated in reaction medium with KNO_3 at 37-38°C for 15 min. Reaction conditions were as described in "Materials and Methods". Activity in the absence of NO_3^- was set to 100%. KNO_3 concentration was varied from 5 to 200 mM.

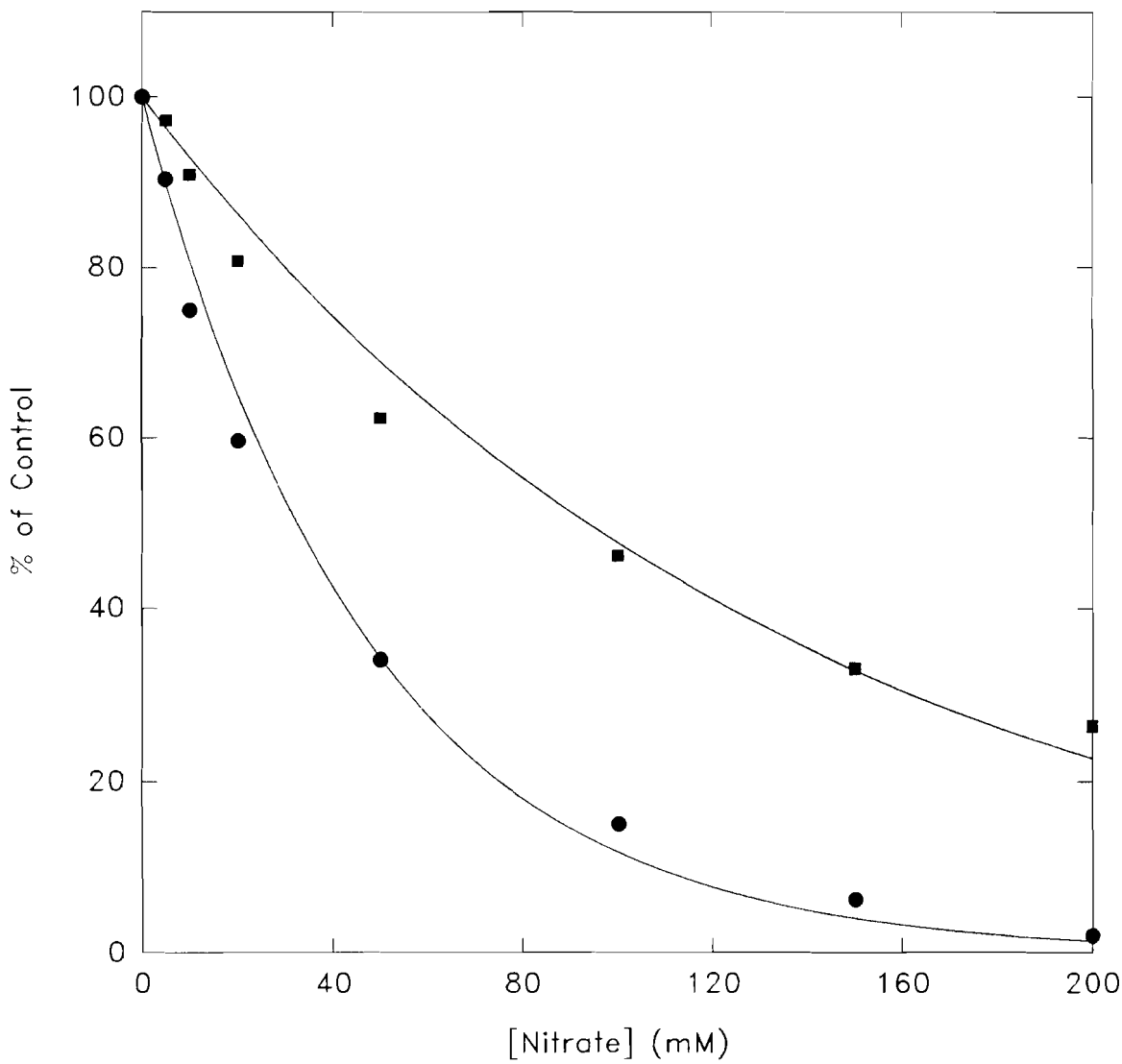

100
80
60
40
20
0
% of Control
0
40
80
120
160
200
[Nitrate] (mM)

Table 3. Half maximal inhibitory concentrations (I_{50}) of some inhibitors for soluble and membrane-bound F_1-ATPase. All data were obtained by analysis of Figures 14-21.

Inhibitor	Soluble F_1-ATPase	Membrane-bound F_1-ATPase
Azide	93 μM	110 μM
NO_3^-	28 mM	85 mM
Oligomycin	ineffective	0.018 μg/ml
DCCD	ineffective	2.2 μM
pCMB	0.15 μM	1.0 μM (SMP) 1.5 μM (Mit)
NBD-Cl	9.3 μM	13.0 μM
DIDS	12.5 μM	12.5 μM
VO_4^{3-}	10.8 mM	10.8 mM

Fig. 15. Effect of NaN_3 concentration on soluble (●) and membrane-bound (SMP, ■) ATPase activity. Enzyme samples were preincubated in reaction medium with NaN_3 (pH was adjusted to 7.2 with acetic acid) at 37-38°C for 15 min. Activity in absence of NaN_3 was set to 100%. NaN_3 concentration was varied from 5 μM to 2 mM.

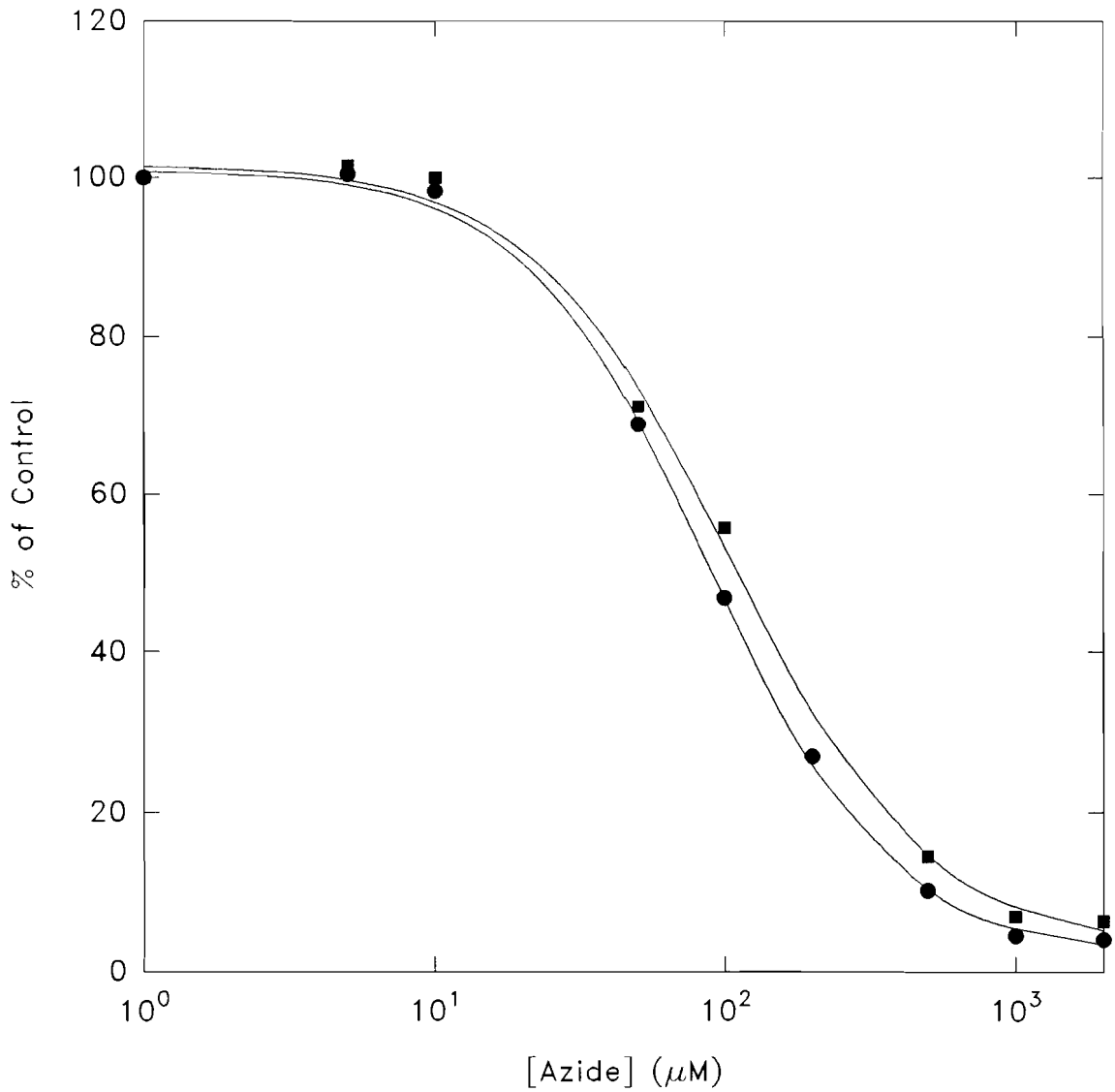
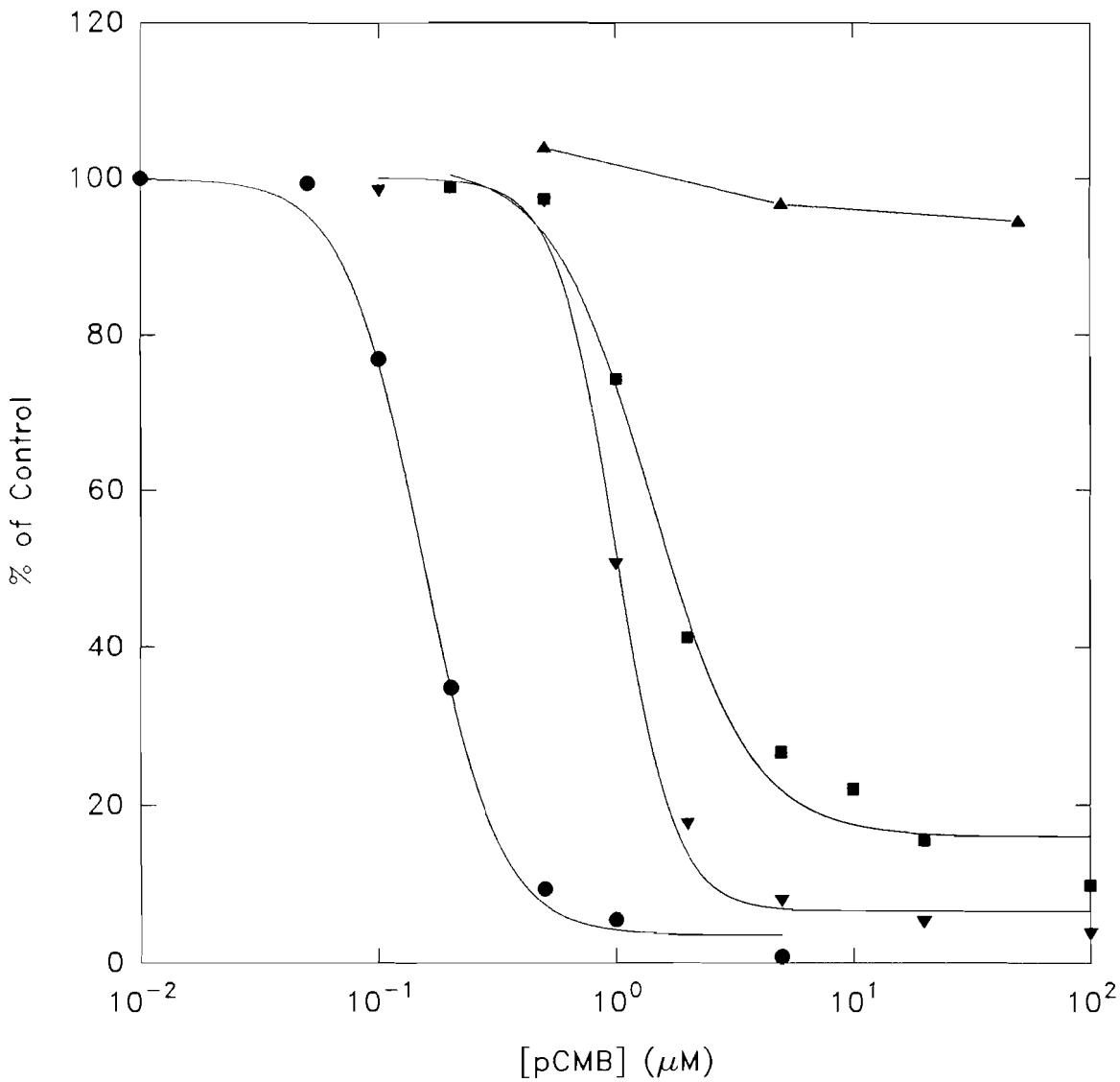

120
100
80
60
40
20
0
% of Control
10^0
10^1
10^2
10^3
[Azide] (μM)

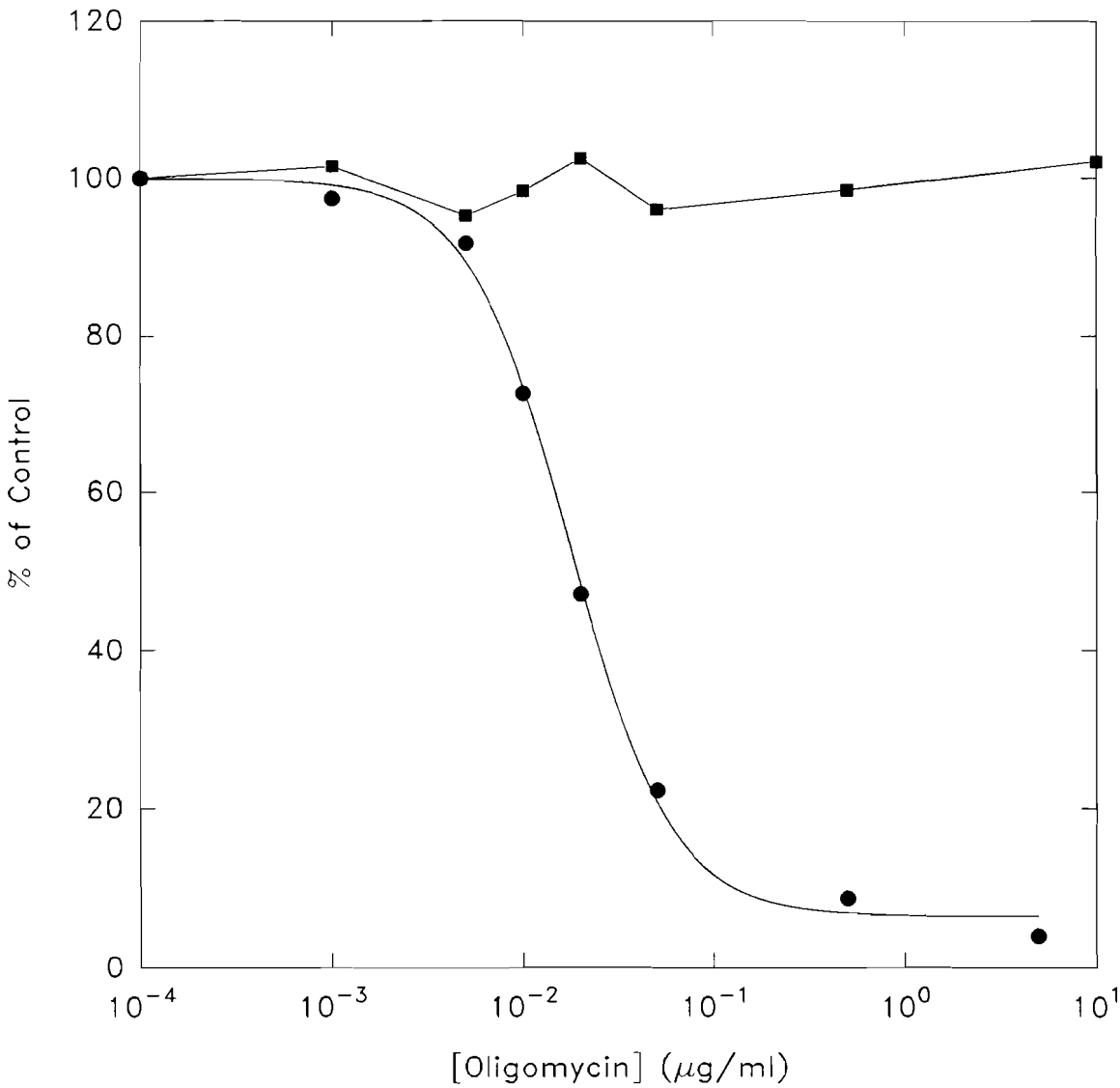
Fig. 16. Effect of pCMB concentration on F_1-ATPase activity. Soluble enzyme (●), SMP (▼), and mitochondria (■) were preincubated in reaction medium with pCMB (adjusted to pH 7.2 with NaOH) at 37-38°C for 15 min. In another experiment (▲), soluble enzyme was preincubated with pCMB plus 0.1 mM DTT. Activity in absence of pCMB was set to 100%. pCMB concentration was varied from 0.05 to 100 μM.


120
100
80
60
40
20
0
% of Control
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
[pCMB] (μM)

mitochondria. pCMB had no significant effect on the soluble ATPase activity with prior incubation with 0.1 mM DTT. At 5 and 50 μM pCMB, the soluble enzyme activity was 96 and 94%, respectively. This suggested that DTT protected F_1-ATPase against pCMB inactivation. To study the reversibility of pCMB inhibition, soluble enzyme was preincubated with 0.5 μM and 5 μM pCMB for 7 min and then 0.1 mM DTT was added for 8 more min. The activities (data not shown) were 40% of control for 0.5 μM pCMB and 4% for 5 μM pCMB. These values were higher than those in absence of DTT at same pCMB concentration. This result suggested that pCMB inhibition was partially reversible by DTT.

Effect of Oligomycin: The influence of oligomycin on F_1-ATPase activity is shown in Fig. 17. Oligomycin was an effective inhibitor of the membrane-bound enzyme. At a concentration of 0.5 μg/ml oligomycin, 92% of activity was inhibited. 50% of inhibition was observed at 0.019 μg/ml oligomycin (Table 3). In contrast, oligomycin was ineffective in inhibiting the soluble F_1-ATPase. Even increasing the concentration to 10 μg/ml, no significant effect was detected. Because oligomycin acts at a small peptide (OSCP) which is associated with the F_0 portion of the F_0F_1-ATPase (Walker et al., 1984), these results suggest that the F_1-ATPase was solubilized and separated from the F_0 segment the procedure used in the my work.

Fig. 17. Effect of oligomycin concentration on soluble (■) and membrane-bound (SMP, ●) ATPase activity. Enzyme samples were preincubated in reaction medium with oligomycin (dissolved in 50% alcohol) at 37-38°C for 15 min. The reaction mixture contained a final concentration of 0.95% alcohol. Activity in the absence of oligomycin was set to 100%. Oligomycin concentration was varied from 0.005 to 10 μg/ml.


120
100
80
60
40
20
0
% of Control
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
10^{1}
[Oligomycin] (μg/ml)

Effect of DCCD: Soluble and membrane-bound ATPase also showed a differential response to DCCD (Fig. 18). Membrane-bound enzyme was inhibited by DCCD with an I_{50} of 2.2 μM (Table 3). It appears only partially inhibitory. The highest inhibition (64%) was observed at 5 μM DCCD. The level of inhibition reached with 5 μM DCCD was not increased by increasing the DCCD concentration to 20 μM. Soluble F_1-ATPase appeared virtually insensitive to DCCD in the concentration range 0.1 to 100 μM. At a concentration 20 times greater than the highest inhibitory concentration (5 μM) for membrane-bound enzyme, the soluble F_1-ATPase activity was 96% of the control.

Effect of NBD-Cl: Both the membrane-bound and soluble F_1-ATPase were inhibited by the adenine analog, NBD-Cl (Fig. 19). At 100 μM NBD-Cl, the membrane-bound and soluble ATPase activities were 3% and 1%, respectively, of the control. 50% inhibition was observed at 9.3 and 13.0 μM NBD-Cl, respectively, for soluble and membrane-bound enzymes (Table 3). Soluble F_1-ATPase was slightly more sensitive to inhibition by NBD-Cl than the membrane-bound enzyme.

Effect of DIDS: Mitochondrial F_1-ATPase was extremely inhibited by DIDS (Fig. 20). At the concentration range from 1 to 200 μM, soluble and membrane-bound enzymes were equally sensitive to DIDS. About 92% of ATPase activity was inhibited at 100 μM DIDS. 50% inhibition was observed at 12.5 μM DIDS for both enzyme forms (Table 3).

Fig. 18. Effect of DCCD concentration on soluble (▪) and membrane-bound (SMP, ●) ATPase activity. Enzyme samples were preincubated in reaction medium with DCCD (dissolved in 50% alcohol) at 37-38°C for 15 min. The final reaction mixture included 0.95% of alcohol. Activity in the absence of DCCD was set to 100%. DCCD concentration was varied from 0.1 to 20 μM.

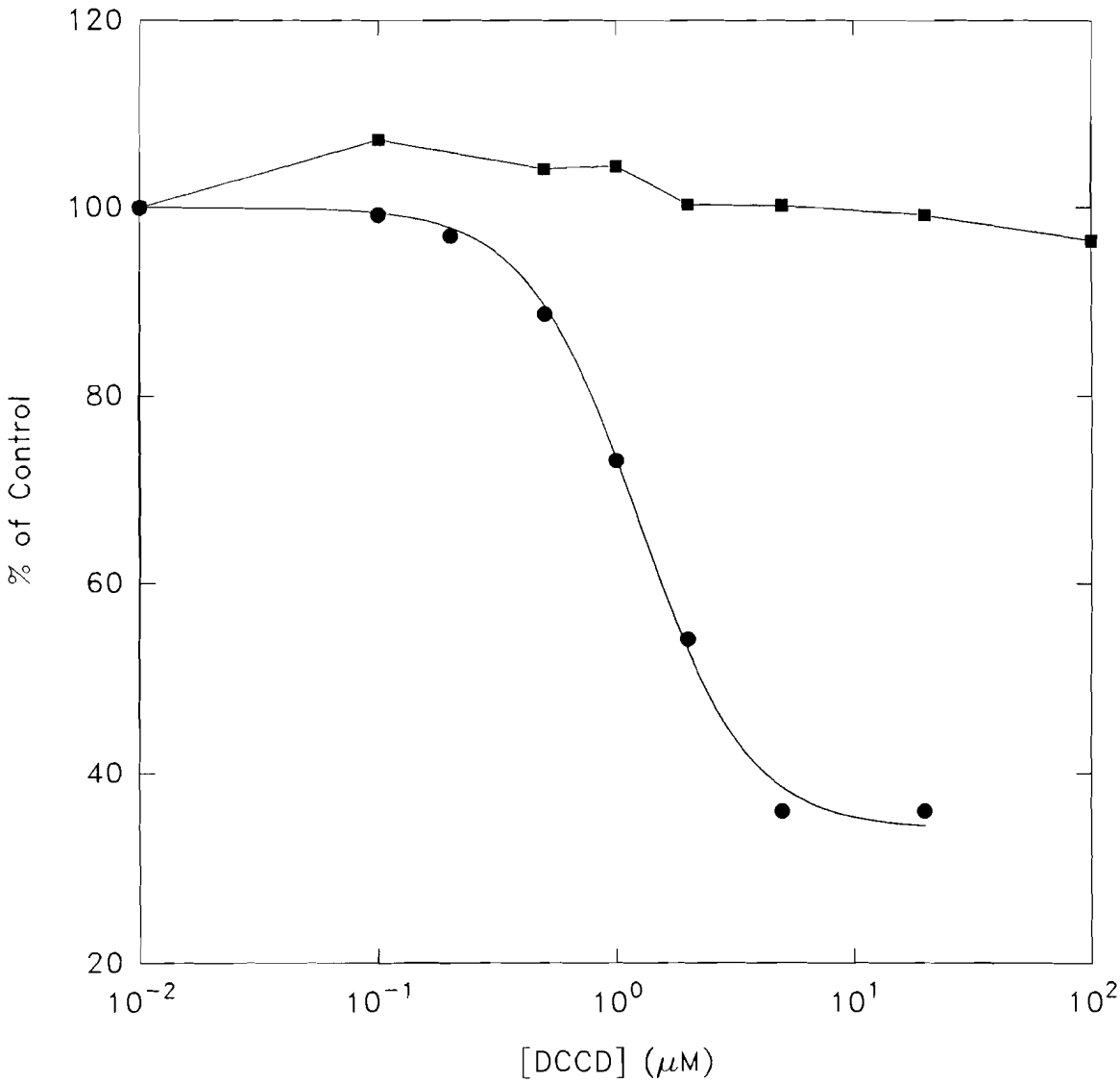
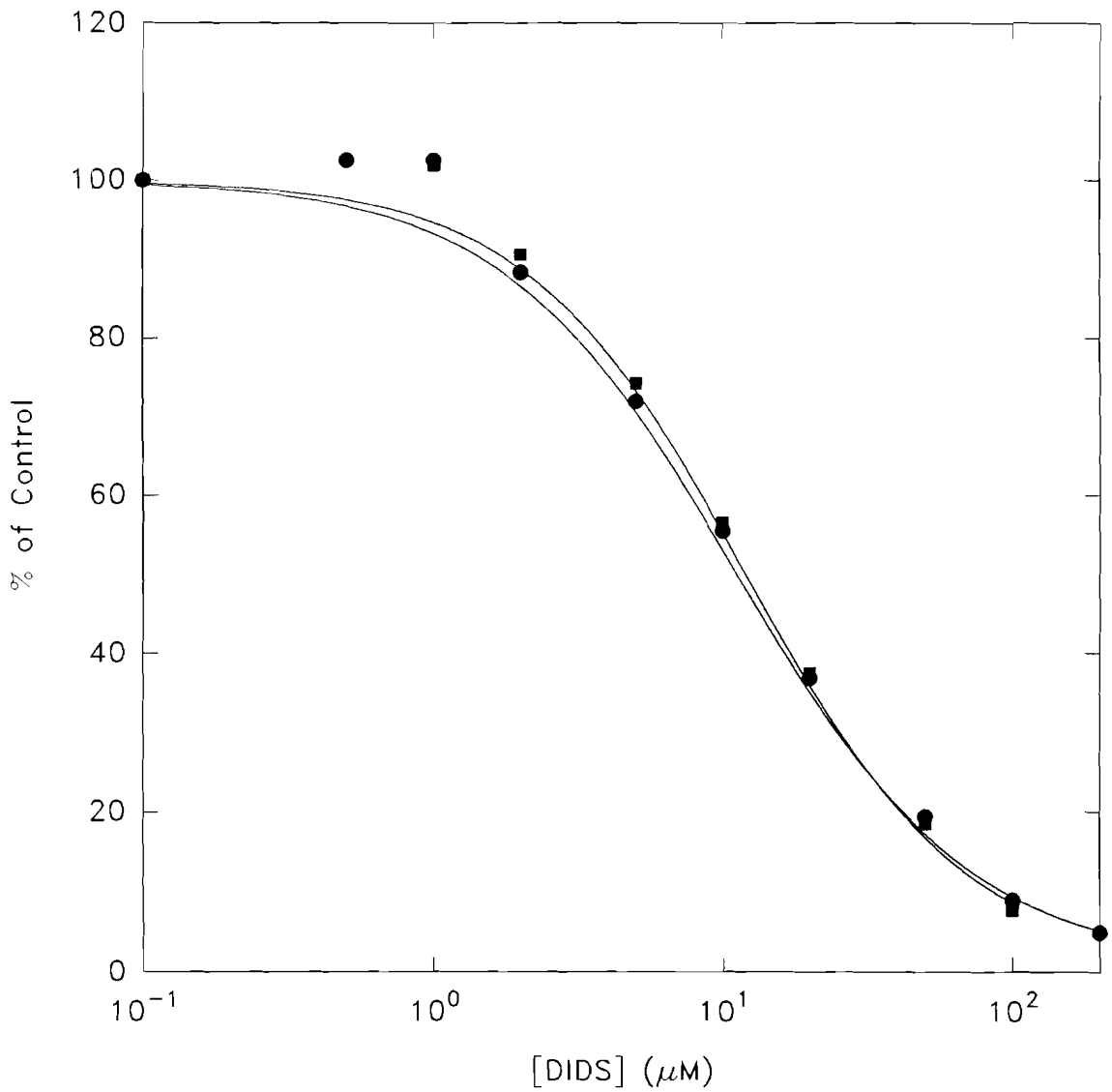

120
100
80
60
40
20
% of Control
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
[DCCD] (μM)

Fig. 19. Effect of NBD-Cl concentration on soluble (●) and membrane-bound (SMP, ■) ATPase activity. Enzyme samples were preincubated in reaction medium with NBD-Cl (dissolved in 50% alcohol) at 37-38°C for 15 min. The final reaction mixture included 0.95% alcohol. Activity in the absence of NBD-Cl was set to 100%. NBD-Cl concentration was varied from 1 to 200 μM.

Fig. 20. Effect of DIDS concentration on soluble (●) and membrane-bound (SMP, ■) ATPase activity. Enzyme samples were preincubated in reaction medium with DIDS (pH was adjusted to 7.2 with NaOH) at 37-38°C for 15 min. Activity in absence of DIDS was set to 100%. DIDS concentration was varied from 1 to 200 μM.


120
100
80
60
40
20
0
% of Control
10^{-1}
10^{0}
10^{1}
10^{2}
[DIDS] (μM)

Effect of Vanadate: Mitochondrial F_1-ATPase was sensitive to high concentrations of VO_4^{3-} (Fig. 21). At the concentration range from 0.5 to 50 mM, membrane-bound and soluble enzymes were equally sensitive to VO_4^{3}. They shared a same half maximal inhibition of 10.8 mM (Table 3). At a concentration of 50 mM VO_4^{3-}, soluble and membrane-bound ATPase activities were 10.4% and 7.4% of the control, respectively.

To summarize inhibitor sensitivities of both soluble and membrane-bound F_1-ATPase, oligomycin and DCCD inhibited the membrane-bound enzyme activity, but were ineffective in inhibiting the soluble enzyme. Even though both enzyme forms shared identical sensitivity to DIDS and vanadate, soluble ATPase was significantly more sensitive to pCMB and NO_3^-, and slightly more sensitive to azide and NBD-Cl than the membrane-bound enzyme. These results suggested interaction between F_1 and F_0 portions, and slight conformational differences between soluble F_1 and membrane-bound F_1.

Kinetics of Membrane-bound F_1-ATPase

Effect of ATP: The membrane-bound F_1-ATPase activity was determined over a wide range of total ATP concentrations (0.2-20 mM). The velocity curve failed to conform to Michaelis-Menten kinetics at high ATP concentration (Fig. 22). Further studies showed that the activity depended upon not only ATP but also Mg^{2+} concentration. Fig. 22 shows

Fig. 21. Effect of VO_4^{3-} concentration on soluble (●) and membrane-bound (SMP, ■) ATPase activity. Enzyme samples were preincubated in reaction medium with Na_3VO_4 (pH was adjusted to 7.2 with acetic acid) at 37-38°C for 15 min. Activity in absence of Na_3VO_4 was set to 100%. Na_3VO_4 concentration was varied from 0.5 to 50 mM.

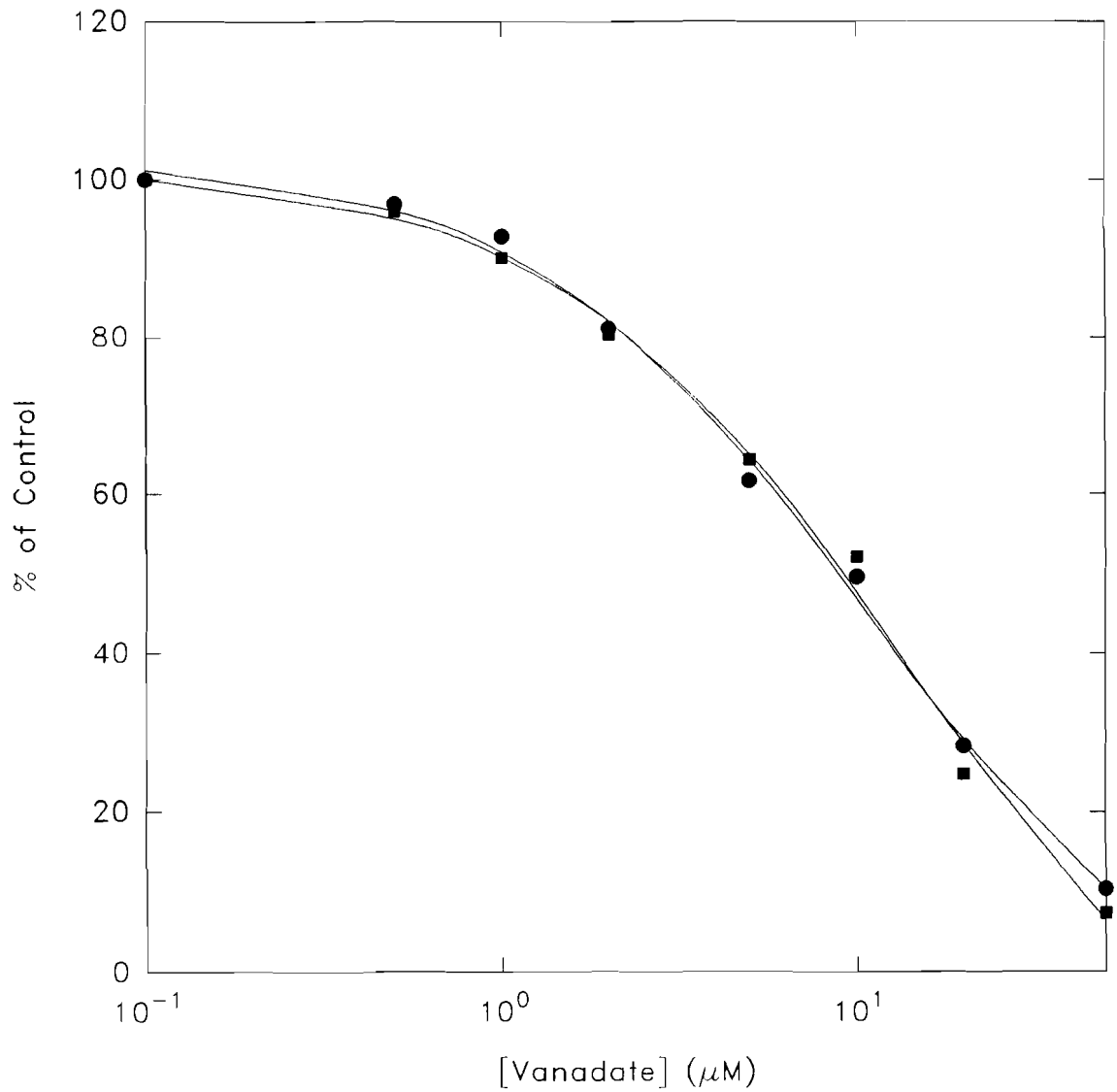
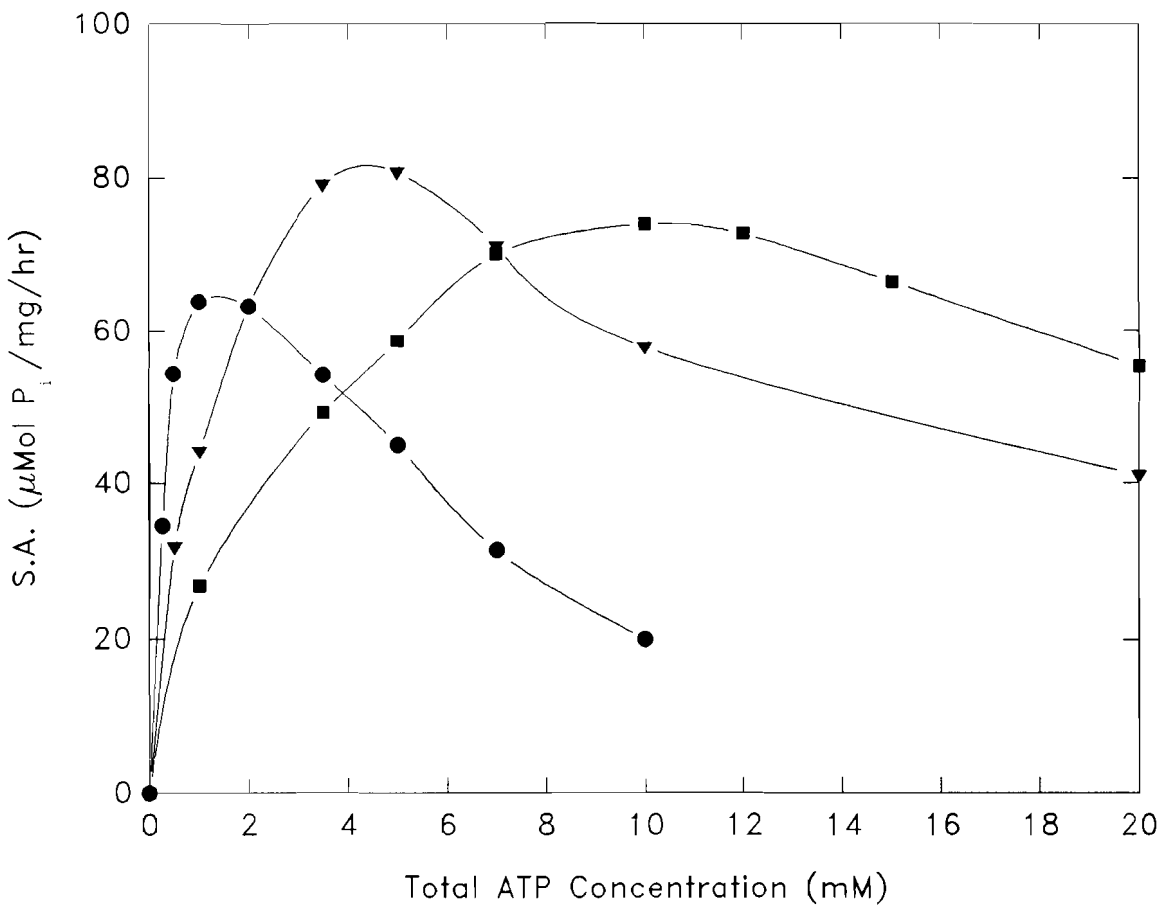

120
100
80
60
40
20
0
% of Control
10^{-1}
10^{0}
10^{1}
[Vanadate] (μM)

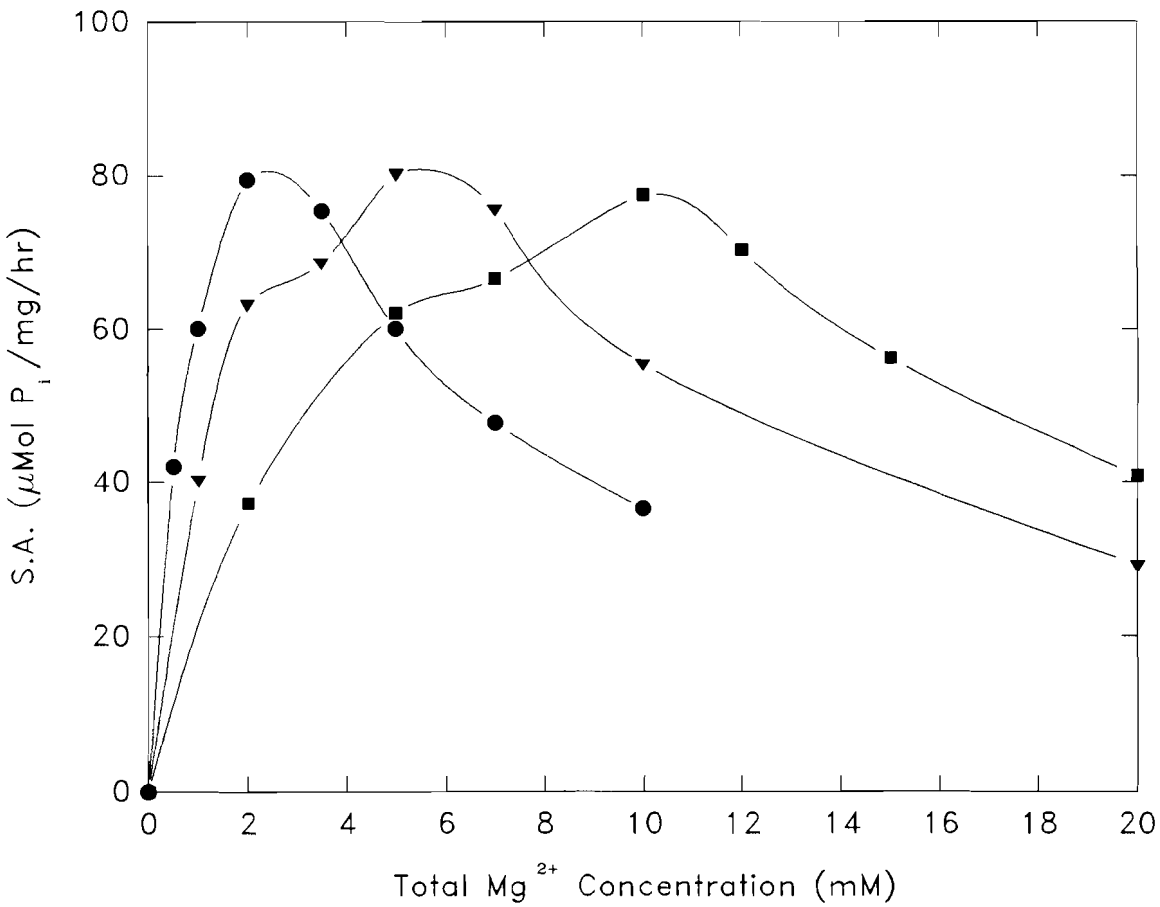
Fig. 22. Effect of varying total ATP concentration on the activity of membrane-bound (SMP) F_1-ATPase from crayfish gills. Except for the ATP and Mg^{2+} concentrations, all other reaction conditions were described as in "Materials and Methods". In the reaction mixture, the total Mg^{2+} concentration was 1 (●), 5 (▼), and 10 (■) mM.


100
80
60
40
20
0
S.A. (μMol P_i/mg/hr)
0
2
4
6
8
10
12
14
16
18
20
Total ATP Concentration (mM)

the velocity data in which $[ATP_t]$ was varied at three different values of $[Mg^{2+}{}_t]$. At each fixed Mg^{2+} concentration, ATPase activity rose to maximum and then fell again. The peak activities were shifted to higher ATP concentrations with an increase in the total Mg^{2+} concentration. Peak activities occurred when $[Mg^{2+}{}_t] \simeq [ATP_t]$. At this point, the concentration of MgATP complex was high. However, when $[ATP_t] > [Mg^{2+}{}_t]$, ATPase activity was strongly inhibited. This suggested that the excess free ATP inhibited the ATPase activity.

Effect of Mg^{2+}: Fig. 23 shows how total Mg^{2+} concentration was varied at three different values of total [ATP]. ATPase activity was zero when Mg^{2+} was absent, suggesting that the enzyme could not hydrolyze ATP. At each ATP concentration, the activity also rose to maximum and then fell. The peak activities were shifted to higher Mg^{2+} concentration with increasing of the ATP concentration and occurred when $[ATP_t] \simeq [Mg^{2+}{}_t]$. ATPase activity was also strongly inhibited when $[Mg^{2+}{}_t] > [ATP_t]$. These results suggested that excess free Mg^{2+} inhibited the enzyme. In addition, a small peak exists before the maximum (Fig. 23). Because the activity was defined as the bicarbonate stimulated activity (calculated as the difference in presence and absence of 50 mM $NaHCO_3$), the activity curve was a combination of two curves (the velocity curves in presence and absence of bicarbonate). In fact, in absence of

Fig. 23. Effect of varying total Mg^{2+} concentration on the activity of membrane-bound F_1-ATPase. Except for ATP and Mg^{2+} concentration, all other reaction conditions were as described in "Materials and Methods". In the reaction mixture, the total ATP concentration was 2 (●), 5 (▼), and 10 (■) mM.


100
80
60
40
20
0
S.A. (μMol P_i /mg/hr)
0
2
4
6
8
10
12
14
16
18
20
Total Mg^{2+} Concentration (mM)

bicarbonate there was a peak activity at $[Mg^{2+}_t] < [ATP_t]$ (data not shown), while the peak velocity occurred at $[Mg^{2+}_t] \approx [ATP_t]$ in the presence of bicarbonate. The possible explanation for this difference was that bicarbonate was also binding with Mg^{2+} to form $MgHCO_3^+$ and $Mg(HCO_3)_2$ in the reaction mixture (Greenwald, 1941; Sillen and Martell, 1964) and it caused a decrease of $[Mg^{2+}_t]$. Therefore, a small peak was formed. These results suggest that a certain concentration of free ATP stimulated the ATPase activity. With this scenario, a small peak should also occur after the maximum in the graph of activity versus [ATP]. However, it was not detected (Fig. 22). The possible explanation was that the inhibition by excess Mg^{2+} was stronger than by excess ATP and that the intervals between points were large.

Mechanism of Inhibition by Free ATP: To explain these results, it was assumed that MgATP complex was the actual substrate and that both free Mg^{2+} and ATP were inhibitors. When free ATP was in relatively high concentrations (such as 3 mM and 5 mM), almost all of the Mg^{2+} was as MgATP and free Mg^{2+} concentration was very low so that it might be omitted from the calculations (Table 4). In this situation, the free ATP inhibition might be studied alone without Mg^{2+} effect. The effect of high free ATP concentration on membrane-bound F_1-ATPase activity in absence of bicarbonate is shown in Fig. 24. The double-reciprocal plots show two straight lines intersecting on the 1/v axis, and indicated that free ATP

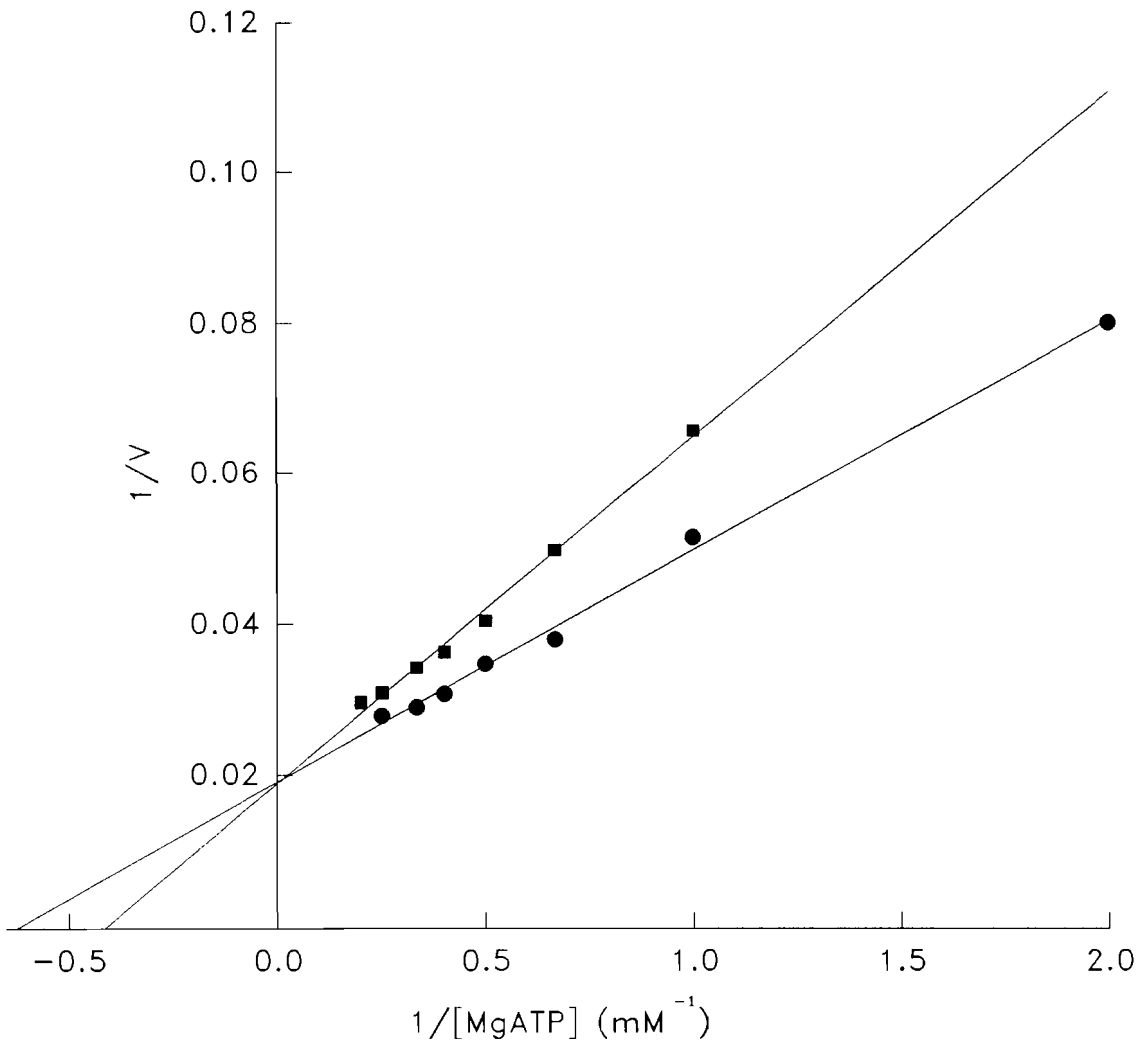
Table 4. The apparent stability constants for MgATP complex (K_{MgATP} = [MgATP] / [Mg^{2+}_f] [ATP_f]), [MgATP], [ATP_f], and [Mg^{2+}_f] under different added [$MgCl_2$] and [Na_2ATP]. The data were calculated by the method as described in "Materials and Methods".

[Mg^{2+}_f] (mM)	[ATP_f] (mM)	K_{MgATP} (M^{-1})	[MgATP] (mM)	[ATP_f] (mM)	[Mg^{2+}_t] (mM)
0.3	0.25	85088	0.2406	0.0094	0.5406
0.3	0.5	84347	0.4810	0.0190	0.7810
0.3	1.0	82892	0.9613	0.0387	1.2613
0.3	1.5	81471	1.4410	0.0590	1.7410
0.3	2.0	80082	1.9201	0.0799	2.2201
0.3	3.0	77398	2.8761	0.1239	3.1761
0.3	4.0	74833	3.8294	0.1706	4.1294
3.0	0.5	84347	0.4980	0.0020	3.4980
3.0	1.0	82892	0.9960	0.0040	3.9960
3.0	1.5	81471	1.4939	0.0061	4.4939
3.0	2.0	80082	1.9917	0.0083	4.9917
3.0	3.0	77398	2.9871	0.0129	5.9871
3.0	4.0	74833	3.9823	0.0177	6.9823
3.0	5.0	72379	4.9771	0.0229	7.9771
3.0	6.0	70031	5.9712	0.0289	8.9265
3.0	7.0	67783	6.9657	0.0343	9.9657
4.0	1.0	82892	0.9970	0.0030	4.9970
4.0	2.0	80082	1.9938	0.0062	5.9938
4.0	3.0	77398	2.9903	0.0097	6.9903
4.0	4.0	74833	3.9867	0.0133	7.9867
4.0	5.0	72379	4.9828	0.0172	8.9828
4.0	6.0	70031	5.9787	0.0213	9.9787
4.0	7.0	67783	6.9743	0.0257	10.9743
4.0	8.0	65628	7.9696	0.0304	11.9696

Cont. Table 4.

$[ATP_f]$ (mM)	$[ATP_t]$ (mM)	$[Mg^{2+}{}_t]$ (mM)	K_{MgATP} (M^{-1})	[MgATP] (mM)	$[Mg^{2+}{}_f]$ (mM)
0.3	0.5	0.2079	84709	0.2	0.0079
0.3	1.0	0.7280	83248	0.7	0.0280
0.3	1.5	1.2489	81820	1.2	0.0489
0.3	2.0	1.7705	80426	1.7	0.0705
0.3	3.0	2.8158	77730	2.7	0.1158
0.3	4.0	3.8641	75154	3.7	0.1641
0.3	5.0	4.9155	72690	4.7	0.2155
3.0	3.5	0.5021	79431	0.5	0.0021
3.0	4.0	1.0043	78107	1.0	0.0043
3.0	4.5	1.5065	76813	1.5	0.0065
3.0	5.0	2.0088	75546	2.0	0.0088
3.0	5.5	2.5112	74308	2.5	0.1112
3.0	6.0	3.0137	73096	3.0	0.0137
3.0	7.0	4.0188	70749	4.0	0.0188
5.0	5.5	0.5013	76460	0.5	0.0013
5.0	6.0	1.0027	75213	1.0	0.0027
5.0	6.5	1.5041	73992	1.5	0.0041
5.0	7.0	2.0055	72798	2.0	0.0055
5.0	7.5	2.5070	71629	2.5	0.0070
5.0	8.0	3.0085	70484	3.0	0.0085
5.0	9.0	4.0117	68265	4.0	0.0117
5.0	10.0	5.0151	66137	5.0	0.0151

Fig. 24. Lineweaver-Burk plots 1/V versus 1/[MgATP], showing the mechanism of inhibition of membrane-bound (SMP) F_1-ATPase by high free ATP concentration. The ATPase activity was expressed as the azide-sensitive activity, and determined in absence of bicarbonate at different levels of free ATP: 3 mM (●) and 5 mM (■), The [MgATP] and [ATP_f] were calculated by the method as described in "Materials and Methods" and shown in Table 4. Other reaction conditions were as described in "Materials and Methods".


0.12
0.10
0.08
0.06
0.04
0.02
1/V
−0.5
0.0
0.5
1.0
1.5
2.0
1/[MgATP] (mM^{-1})

increased the K_m value of the enzyme for MgATP, but did not alter the V_{max}. This is a classical competitive pattern. Free ATP was a competitive inhibitor. Therefore, the inhibition could be described as follows (Webb, 1963; Dixon and Webb, 1979):

$$E + MgATP \overset{K_s}{\rightleftharpoons} EMgATP \overset{K_p}{\Rightarrow} E + products$$

$$E + ATP \overset{K_i}{\rightleftharpoons} EATP$$

where K_i is the dissociation constant for competitive inhibition by free ATP, K_s is the substrate dissociation constant, K_p is the rate constant for the breakdown of the complex into products. The rate equation for this model is as follows:

$$V = \frac{V_{max}}{1 + (1 + [ATP]/K_i)K_m/[MgATP]}$$

where V_{max} is the highest rate of reaction, K_m is Michaelis constant. The intercepts of reciprocal plots were:

$$1/V_{max} \quad \text{and} \quad 1/K_m(1 + [ATP_f]/K_i)$$

on vertical and horizontal axes, respectively. From these relationships, the V_{max}, K_m and K_i were estimated as

52.7 μMol P_i/mg protein/hr, 0.320 mM and 0.77 mM, respectively (Table 5).

Mechanism of Inhibition by Free Mg^{2+}: As with high ATP_f concentration, free Mg^{2+} inhibition might be studied alone without the effect of ATP with high $[Mg^{2+}_f]$, such as 3 mM and 4 mM. The effect of high fixed free Mg^{2+} concentration on membrane-bound F_1-ATPase activity in absence of bicarbonate is shown in Fig. 25. The double-reciprocal plots were straight lines as MgATP concentration ranged from 1 mM to 4 mM that intersected to the left of the vertical axis and above the base line. Further analysis showed that free Mg^{2+} caused a decrease in both the highest activity and the enzyme's affinity for MgATP. Therefore, free Mg^{2+} showed a mixed inhibitory pattern. The inhibition could be described as follows (Webb, 1963; Dixon and Webb, 1979):

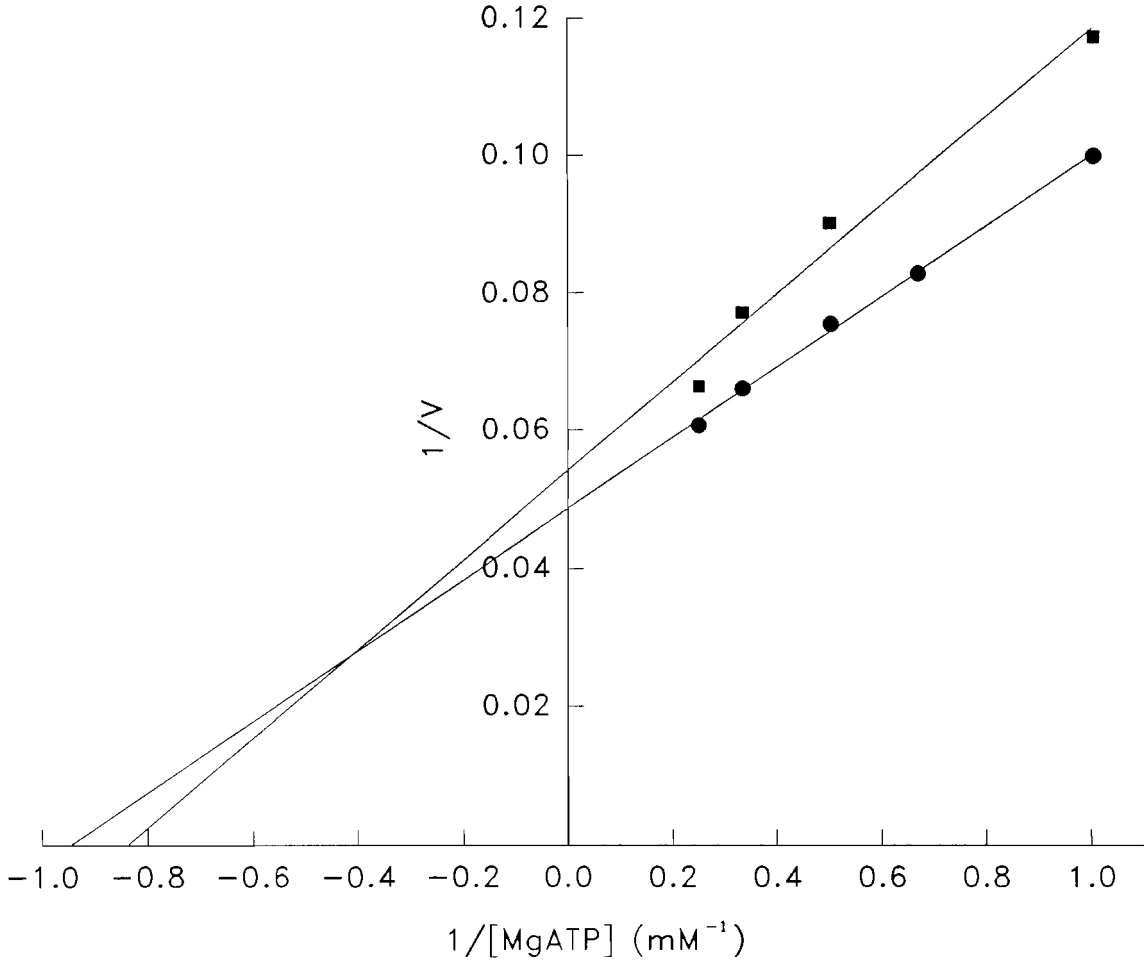
$$\begin{array}{ccc} E & \overset{K_s}{\rightleftharpoons} & E\text{-}MgATP \overset{K_p}{\Longrightarrow} E + \text{products} \\ \| K_i & & \| K_i' \\ E\text{-}Mg^{2+} & \overset{K_s'}{\rightleftharpoons} & Mg\text{-}E\text{-}MgATP \end{array}$$

where K_i is the dissociation constant for Mg^{2+} from E-Mg^{2+} complex, K_i' is the dissociation constant for Mg^{2+} from enzyme-substrate complex, and K_s and K_p are the same as in the previous case. The equation for this model is as follows:

Table 5. Kinetic parameters for inhibition of membrane-bound F_1-ATPase by ATP and Mg^{2+}. The data were estimated by analysis of Figures 24 and 25.

Inhibitor	Substrate	Inhibition type	K_i (mM)	K_m (mM)	V_{max} (μMol P_i/mg/hr)
ATP	MgATP	Competitive	0.77	0.320	52.7
Mg^{2+}	MgATP	Mixed	0.81	0.334	31.2
			5.89 (K_i')		

Fig. 25. Lineweaver-Burk plots 1/V versus 1/[MgATP], showing the mechanism of inhibition of membrane-bound (SMP) F_1-ATPase by high free Mg^{2+} concentration. The ATPase activity was expressed as the azide-sensitive activity and determined in absence of bicarbonate at different levels of free Mg^{2+}: 3 mM (●) and 4 mM (■). The [MgATP] and [Mg^{2+}_f] were calculated by the method as described in "Materials and Methods", and shown in Table 4. Other reaction conditions were as described in "Materials and Methods".


0.12
0.10
0.08
0.06
0.04
0.02
1/V
−1.0
−0.8
−0.6
−0.4
−0.2
0.0
0.2
0.4
0.6
0.8
1.0
1/[MgATP] (mM^{-1})

$$V = \frac{V_{max}/(1 + [Mg^{2+}_f]/K_i')}{1 + (K_m/[MgATP])(1 + [Mg^{2+}_f]/K_i)/(1 + [Mg^{2+}_f]/K_i')}$$

The intercepts of reciprocal plots were:

$$(1 + [Mg^{2+}_f]/K_i')/V_{max} \quad \text{and}$$

$$(1 + [Mg^{2+}_f]/K_i')/K_m(1 + [Mg^{2+}_f]/K_i)$$

on vertical and horizontal axes, respectively. From these relationships, the V_{max}, K_m, K_i and K_i' were estimated as 31.2 μMol P_i/mg protein/hr, 0.334 mM, 0.81 mM, and 5.89 mM, respectively (Table 5).

Comparison of two K_m values for MgATP determined by above reaction systems, high free ATP and high free Mg^{2+}, showed good agreement (0.320 mM and 0.334 mM). Therefore, the true substrate for ATP hydrolysis reaction of crayfish membrane-bound F_1-ATPase was the MgATP complex (Segel, 1975; Dixon and Webb, 1979), and a mean K_m value of 0.327 mM was estimated.

Stimulation of ATPase Activity by Low Free ATP: From the above analysis, it was found that the two V_{max} values were very different (Table 5). The V_{max} value determined by high free ATP reaction system was nearly twice as high as the V_{max} with high free Mg^{2+}. In the MgATP concentration range indicated in Fig. 23, the free ATP level was very low (< 18 μM, see Table 4). This suggests that the difference

was caused by the presence or absence of free ATP. In addition, the maximum reaction rate obtained when $[ATP_t] > [Mg^{2+}_t]$ in absence of HCO_3^- also suggests that free ATP was essential for maximum activity.

The stimulation of ATPase activity by low free ATP was explored further by fixing free Mg^{2+} or ATP at 0.3 mM and varying the MgATP concentration from 0.2 to 4.7 mM (Fig. 26). The plot indicates the activity was always higher at 0.3 mM free ATP, even though free ATP by itself was not hydrolyzed. Furthermore, free ATP stimulated the enzyme at low substrate level but had little effect at high substrate level. It should be noted free ATP levels increase with increasing levels of MgATP in fixing free Mg^{2+} concentration (Table 4). So, the free ATP levels for the two curves (Fig. 26) were close at high MgATP level. This result again suggests that free ATP stimulated ATPase activity.

In addition, the double-reciprocal plots in Fig. 27 show ATPase activity at 3 mM free Mg^{2+} displayed apparent negative cooperativity. The same results were obtained at 4 mM and 6 mM free Mg^{2+} (data not shown). In contrast, the activity obtained at 3 mM or 5 mM free ATP did not display apparent negative cooperativity (Fig. 22 and 27, linear). These results suggest that free ATP bound to non-catalytic sites.

To summarize the above results, free ATP significantly stimulated membrane-bound F_1-ATPase activity. This was

Fig. 26. Lineweaver-Burk plot showing the effect of low levels of free ATP or Mg^{2+} on membrane-bound ATPase activity. 0.3 mM free ATP (■) and 0.3 mM free Mg^{2+} (●), Reaction conditions were the same as in Figures 24 and 25.

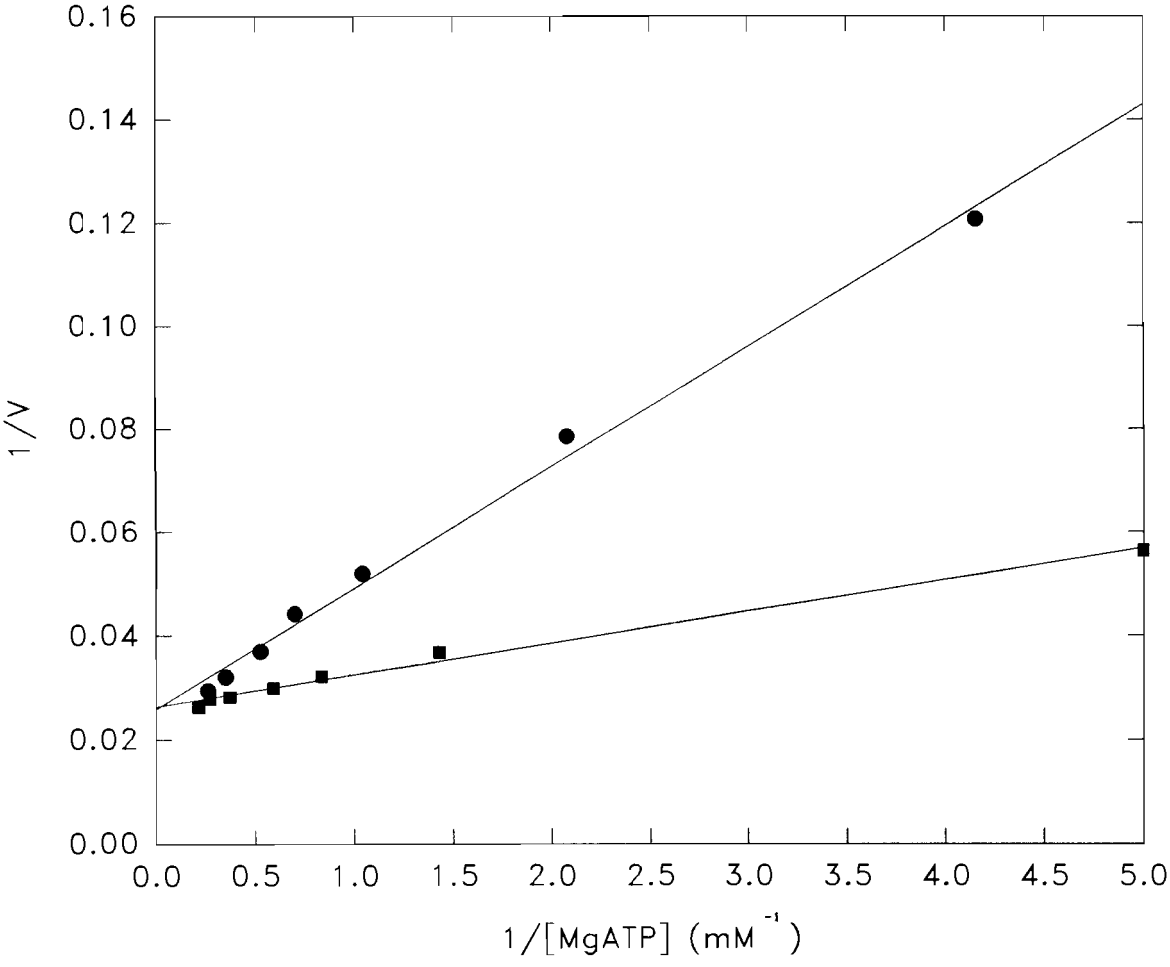
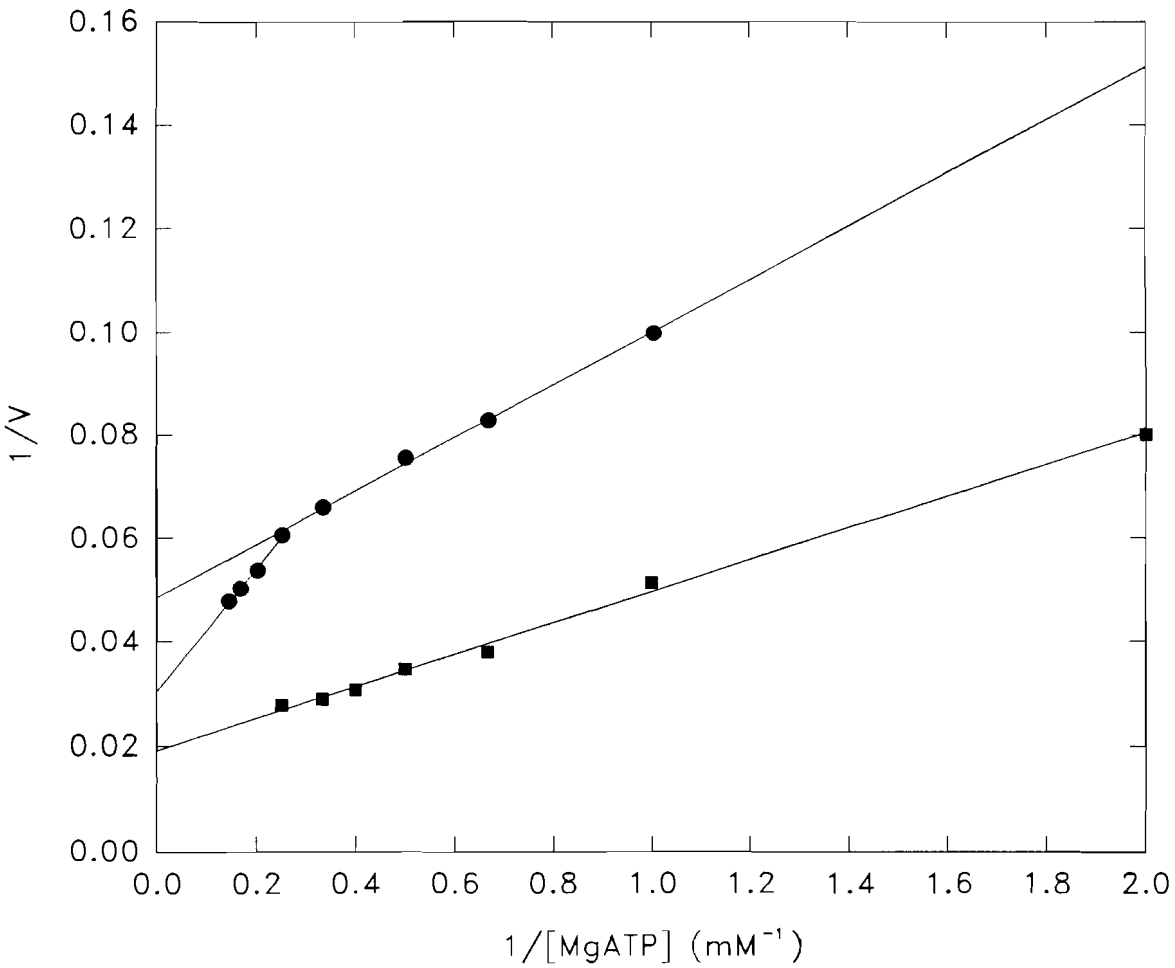

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1/V
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
1/[MgATP] (mM -1)

Fig. 27. Lineweaver-Burk plot, showing the comparison of ATP hydrolysis catalyzed by membrane-bound (SMP) F_1-ATPase at 3 mM free ATP (■) and 3 mM free Mg^{2+} (●). These two curves were replotted from Figures 24 and 25.


0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
1/V
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
1/[MgATP] (mM^{-1})

probably due to ATP binding to non-catalytic sites. This binding of ATP to non-catalytic sites is essential for the maximum activity.

ATPase Activity When [ATP] Equal to [Mg^{2+}]: To determine whether the enzyme conforms to Michaelis-Menten kinetics, membrane-bound F_1-ATPase activity was determined when total ATP concentration was equal to Mg^{2+} concentration in the presence of bicarbonate. Fig. 28 shows that ATPase activity rose to maximum and then decreased. The maximum value was observed at 3.5 mM of both ATP and Mg^{2+}. Similar results were obtained with soluble enzyme (data not shown). The decrease was greater than with membrane-bound enzyme. Comparing with those at fixed [ATP_t] or [$Mg^{2+}{}_t$] situations, the decrease was much less when [ATP_t] = [$Mg^{2+}{}_t$]. Further studies indicated that the activity decreased at high substrate concentration was probably due to the NaCl buildup in the reaction mixture. Addition of one molecule of Na_2ATP and $MgCl_2$ produces two molecules of NaCl. Fig. 29 shows the effect of NaCl concentration on membrane-bound enzyme activity. The data were identical with Fig. 28. At 20 mM of both Na_2ATP and $MgCl_2$, that contained 40 mM NaCl, the activity was about 83% of the maximum value (Fig. 28). On the other hand, the activity at 40 mM NaCl was about 76% of the highest value (Fig. 29). The effect of NaCl was greater with soluble enzyme. These results suggest that the

Fig. 28. Effect of ATP concentration on membrane-bound (SMP) F_1-ATPase activity when $[ATP_t] = [Mg^{2+}{}_t]$. With the variance of $[ATP_t]$, the same concentrations of $MgCl_2$ and Na_2ATP were maintained in the reaction mixture. The reaction conditions were described as in "Materials and Methods".

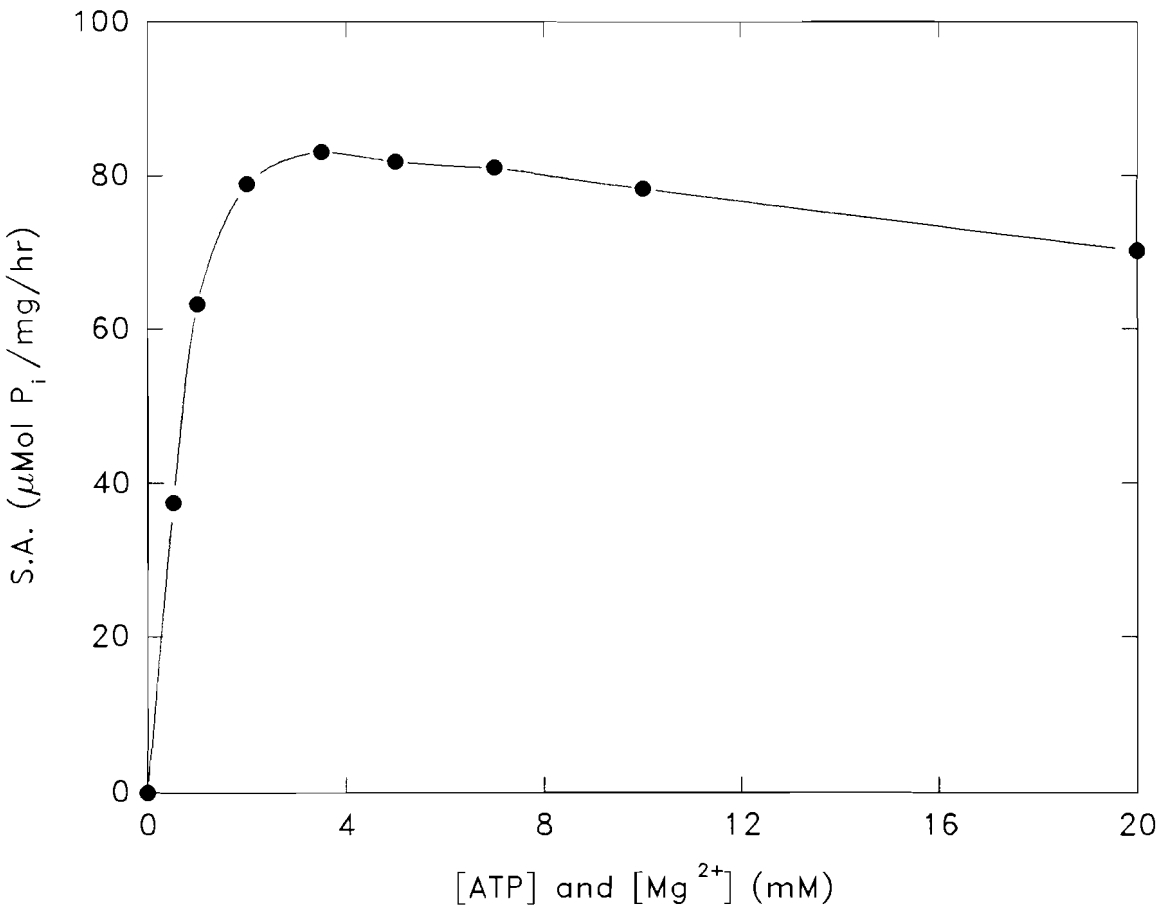
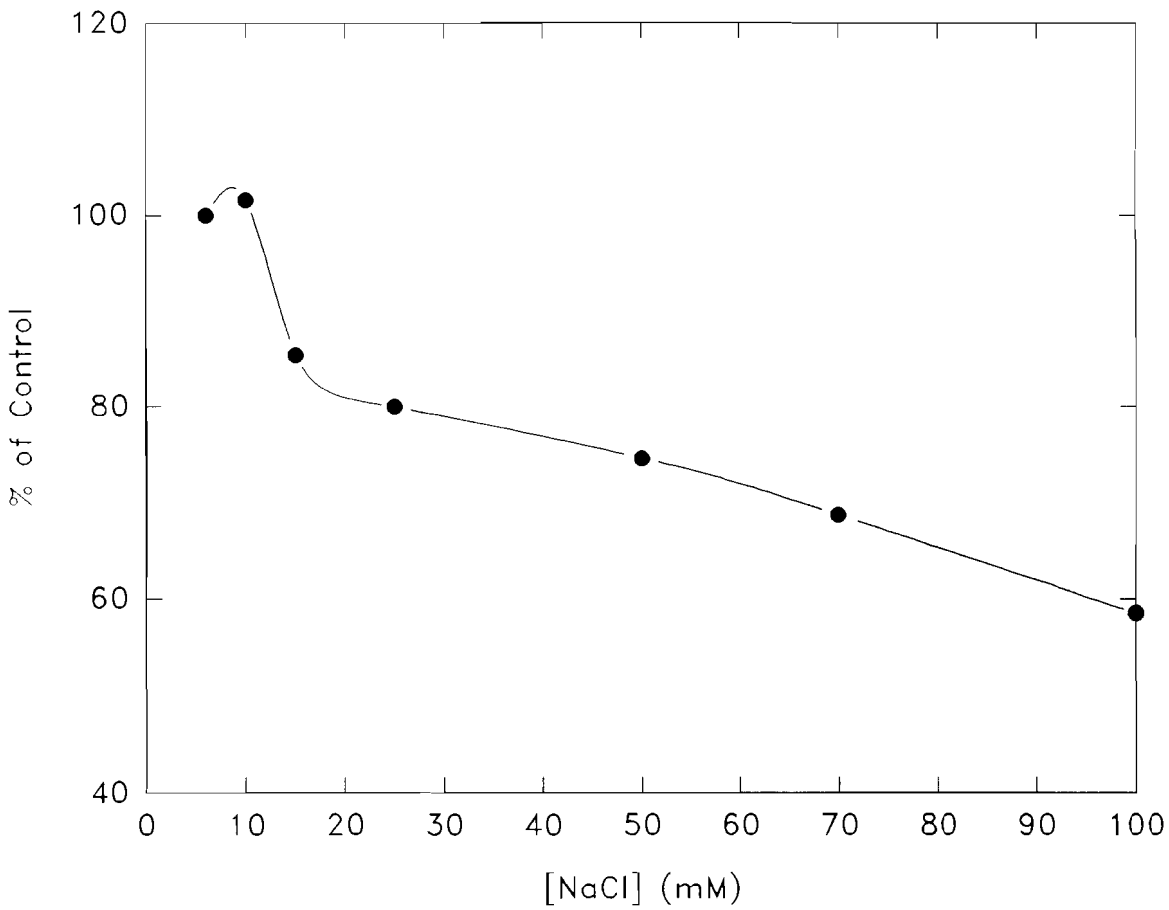

100
80
60
40
20
0
S.A. (μMol P_i /mg/hr)
0
4
8
12
16
20
[ATP] and [Mg^{2+}] (mM)

Fig. 29. Effect of NaCl concentration on membrane-bound (SMP) F_1-ATPase activity. Reaction conditions were described as in "Materials and Methods". The activity determined with the general reaction mixture (6 mM NaCl) was set to 100%.


120
100
80
60
40
% of Control
0
10
20
30
40
50
60
70
80
90
100
[NaCl] (mM)

hydrolysis reaction catalyzed by the enzyme was sensitive to NaCl.

DISCUSSION

Preparation of Soluble F_1-ATPase

In my study, a simple and rapid method for isolation of soluble F_1-ATPase from crayfish gills was developed. It is the first report of isolation of F_1-ATPase from a crustacean. The F_1-ATPase was isolated from mitochondria in about 3 hours. This method was shown to be reproducible. Since glycerol and no ATP were used, the preparation described in my paper offered some advantages over previously reported preparations: the enzyme possessed high stability and it eliminated the tightly bound nucleotides. Therefore, this preparation could be directly used to characterize the enzyme and to study the binding of externally added nucleotides.

My study showed that the disruption of mitochondria was sensitive to length of sonication. Similar results were reported by Tyler and Webb (1979) and Lambeth and Lardy (1971). Mitochondria were not completely broken with low power and shortened sonication treatment. However, when a more prolonged sonication treatment was used, more ATPase activity was lost, perhaps owing to the removal of ATPase into the medium (Knowles and Penefsky, 1972; Ferguson et al., 1976). In contrast, mitochondrial concentration did not affect the sonic disruption over the range 0.7-6.0 mg protein per ml. A much higher concentration of mitochondria

was used in previously reported methods (Catterall and Pedersen, 1971; Penin et al., 1979). The effect of concentration was not reported. Therefore, a possible effect could show at higher concentrations.

My work indicated a high yield of broken mitochondria were obtained at alkaline pH (8.6). In fact, a high pH (above 8.0) was used for sonication treatment in most of reported methods (Horstman and Racker, 1970; Lambeth and Lardy, 1971; Penin et al., 1979). This was probably due to disruption of mitochondrial structure at high pH (Horstman and Racker, 1970; O'Rourke and Wilson, 1992).

The extent of purification, the amount of protein, and the total activity for the preparation described by my paper were similar to the results carried out with rat liver mitochondria (Williams et al., 1984). This procedure showed sonication treatment markedly increased the ATPase activity. Similar results were obtained in other reports (Penin et al., 1979; Williams et al., 1984; Randall et al., 1985). A possible explanation is that the native inhibitor protein was released into the sonication medium. There were two situations that were good for release of the inhibitor in this step: 1) SMP is an inside-out membrane preparation that may favor the dissociation of the inhibitor protein from the enzyme (Tuena de Gomez-Puyou et al., 1988); 2) the sonication medium was at alkaline pH that also favors dissociation of the inhibitor from the enzyme (Horstman and

Racker, 1970; Feinstein and Moudrianakis, 1984; O'Rourke and Wilson, 1992).

Approximately 40% of ATPase activity was lost from membranes after sedimentation of SMP from sonication medium. Similar results have been reported (Knowles and Penefsky, 1972; Ferguson et al., 1976). It is possible some of the F_1-ATPase was removed from the membrane by sonication treatment.

The yield of ATPase activity released by chloroform treatment was about 42% of SMP activity. Similar losses of ATPase activity during chloroform extraction were observed by Beechey et al. (1975), Penin et al. (1979), Tyler and Webb (1979), Williams et al. (1984), and Hicks and Krulwich (1987). A possible explanation is that only a portion of the ATPase released from SMP escaped denaturation by chloroform.

The final preparation described in my study could contain some contaminating protein, but F_1-ATPase was fully isolated from F_0F_1 complex. This is supported by the following: all the tested properties were different from the properties of membrane-bound enzyme and similar to the properties of purified F_1-ATPase from other sources (Penefsky, 1979; Futai and Kanazawa, 1983; Yoshihara et al., 1991).

General Characteristics

Effect of Temperature: For any enzyme, both reaction

rate and inactivation rate increase with the temperature. The optimum temperature is determined by the balance between these two effects. Thus, there are different apparent optimum temperatures with different reaction time intervals. In addition, the optimum temperature also depends on other reaction conditions. Therefore, optimum temperature is not constant and has only relative significance.

The optimum temperature for soluble F_1-ATPase was slightly higher than that for membrane-bound enzyme. This suggests that soluble F_1-ATPase was more stable at higher temperatures. This was supported by the stability experiments in my paper. The optimum temperature ranged from 37-60^{0}C for other F_1-ATPases (Patil et al., 1979; Martins et al., 1988a; Garza-Ramos et al., 1990). My data are within this range.

Effect of pH: Soluble F_1-ATPase displayed a narrower and more acid pH optimum than the membrane-bound enzyme. Similar behavior has been reported for plant mitochondrial F_1-ATPase (Iwasake and Asahi, 1983; Randall et al., 1985). These results suggest conformational differences between the two enzyme forms.

Alkaline pH optima ranged from 8.0 to 10.0 with the enzymes from different sources (Selwyn, 1967; Patil et al., 1979; Iwasaki and Asahi, 1983; Martins et al., 1988a; Itoh et al., 1993). The pH optimum presented here was much lower than this range. Whether the activity was expressed as anion

stimulated activity or inhibitor-sensitive activity, the pH optima were within the reported range. Therefore, this behavior suggests that crayfish F_1-ATPase has a different structure than the enzyme from other sources. In contrast, the pH optimum was similar to that of Na^+,K^+-ATPase (7.25) from crayfish (Orconectes limosus) gills (Kosiol et al., 1988).

The reaction:

$$H^+ + HCO_3^- \dashrightarrow H_2O + CO_2\uparrow$$

probably occurred in the assay medium (Sillen and Martell, 1964). When the assay medium was at acid pH, the bicarbonate would be lost by release of CO_2. Therefore, it may be a reason for the steep decrease of activity in acid pH.

Enzymic stability: A important property of soluble F_1-ATPase is its cold-labile property. There is a rapid rate of ATPase inactivation at 0-4^{0}C; it is more stable at 25^{0}C than ice bath temperature (Penefsky, 1979; Senior, 1990). This inactivation apparently occurs with the dissociation of the enzyme into individual subunits (Penefsky and Warner, 1965; Senior, 1990). However, glycerol, methanol, ATP, and sucrose protect the F_1-ATPase from inactivation (Iwaski and Asahi, 1983; Senior, 1990). When kept in glycerol, the enzyme is quite stable from -70^{0}C to room temperature (Penin et al., 1979). On the other hand, combination with the

inhibitor protein (Pullman and Monroy, 1963; Higashi et al., 1975) or OSCP (Horak et al., 1989) also confers cold-stability on F_1-ATPase.

The soluble F_1-ATPase preparation in this study exhibited good stability at 4^0C. Later experiments showed that it was not sensitive to oligomycin. Therefore, the apparent cold-stability was due to either keeping the enzyme in 20% glycerol or the combination of the enzyme with the inhibitor protein. According to the specific activity and the property of the final preparation, the former is more probable.

In contrast, the results presented here show that soluble F_1-ATPase was more stable at room temperature than membrane-bound enzyme. Similar data have been reported by Iwasaki and Asaki (1983). These results suggest a more rapid dissociation of membrane-bound F_1-ATPase into individual subunits than the soluble enzyme at room temperature.

Stimulatory Effect of Anion on F_1-ATPase Activity

In the F_1-ATPase of mammalian mitochondria (Ebel and Lardy, 1975; Pedersen, 1976), yeast mitochondria (Divita et al., 1992), plant mitochondria (Partridge et al., 1985; Wang and Sze, 1985; Itoh et al., 1993), and plant chloroplast (Cohen and MacPeek, 1980), it has been shown that a number of anions stimulated hydrolysis of ATP when they were included in the assay medium. However, the data for

comparison of soluble and membrane-bound enzyme are lacking. My study indicates that anion sensitivities and highest anion stimulated concentration were similar between the two enzyme forms. Similar results were observed in oat root mitochondria (Randall et al., 1985). This suggests similar regulatory mechanisms operate in soluble and membrane-bound ATPase.

Although the K_a values for SO_4^{2-} and Cl^- are lacking, the K_a for HCO_3^- presented here was larger than that (5.8 mM) obtained in rat liver mitochondrial ATPase (Ebel and Lardy, 1975). The anion-stimulated activity in my study was in the following order: $HCO_3^- > SO_4^{2-} > Cl$. It is similar to the order $HCO_3^- > Cl^- > SO_4^{2-}$ in rat liver mitochondria (Lambeth and Lardy, 1971) and in bulbs of a monocotyledonous plant (Itoh et al., 1993).

Anion stimulation mechanisms have been studied. Some reports (Cross et al., 1982; Wong et al., 1984) indicate HCO_3^- stimulates the mitochondrial ATPase from beef heart by decreasing K_m as well as by increasing V_{max}. In fact, the mechanism for different anions is similar. There is an anion-binding site on the enzyme (Ebel and Lardy, 1975). When the anion binds to the site, F_1-ATPase conformation is altered (Cohen and MacPeek, 1980). The competitive interactions between the anion (Lambeth and Lardy, 1971) suggest that anions compete for same binding site(s) on the ATPase. The activity may be influenced by the type of anion

at the binding site(s) and its influence on the hydrolysis of ATP at the catalytic site. This mechanism also partially explains why Na_2SO_4 (data not shown) and NaCl (Fig.29) inhibited the bicarbonate stimulated activity. Guerrero et al. (1990) showed that anions bind in place of P_i that is near the ADP binding site and causes a different conformation change. Activating anions release ADP formed by ATP hydrolysis; inhibitory anions block the ADP release.

It was noted HCO_3^- competes with ATP to bind Mg^{2+} in assay medium. When bicarbonate concentration increases, the substrate MgATP level decreases and free ATP (inhibitor) concentration increases. This partially explains why the enzyme activity markedly decreases with high bicarbonate. Other probable reasons will be discussed later within my thesis.

Inhibitor Sensitivities

Effect of NO_3^-: The tonoplast-type ATPase is specifically inhibited by NO_3^- at low level (Churchill and Sze, 1984; Lew and Spanswick, 1985; Griffith et al., 1986). Mitochondrial F_1-ATPase was also sensitive to this anion. The enzyme from plant mitochondria was inhibited at low NO_3^- level with I_{50} of 7-8 mM (Grubmeyer and Spencer, 1977; Randall et al., 1985; Wang and Sze, 1985). The mammalian mitochondrial ATPase was sensitive to high concentration of NO_3^- (I_{50} = 110 mM) (Ebel and Lardy, 1975). The present data

(I_{50} = 28 mM) were between these two.

The mechanism of NO_3^- action is not clear. It is generally thought that NO_3^- binds to the anion-binding site(s) and interacts with the active sites (Penefsky, 1979; Randall et al., 1985). Although comparison of NO_3^- inhibition between soluble and membrane-bound F_1-ATPase is lacking, my results clearly indicate that soluble F_1-ATPase was 3-fold more sensitive to NO_3^- than the membrane-bound enzyme. This significant difference suggests greater accessibility of NO_3^- to the anion binding site(s) of soluble enzyme.

Both tonoplast ATPase and F_1-ATPase were sensitive to NO_3^- and they also have an alkaline pH optimum (Fricker and Willmer, 1990). However, crayfish F_1-ATPase presented in my study was an exception. It was strongly inhibited by NO_3^-, but it had a neutral pH optimum.

Effect of NaN_3: Azide is a specific inhibitor for F_0F_1 type ATPase. Although tonoplast ATPase and mitochondrial ATPase share similarities in the inhibitor sensitivities, tonoplast type ATPase was not inhibited by azide like plasma membrane ATPase (Bowman, 1983; Randall and Sze, 1986; Nelson, 1988). Therefore, azide was used to distinguish between F_1-ATPase and other ATPases. My data show that azide strongly inhibited crayfish mitochondrial ATPase. But the sensitivity was slightly less than that of membrane-bound or purified F_1-ATPase from other sources (Bowman, 1983; Wang

and Sze, 1985; Itoh et al., 1993). This suggests that anion-stimulated activity is less sensitive to azide than basal activity (absence of stimulator). In addition, my data also show that soluble enzyme was slightly more sensitive to azide than membrane-bound enzyme. Similar results were obtained with oat F_1-ATPase by Randall et al (1985). These results suggest slight structural differences between the two enzyme forms.

The interaction of azide with the F_1-ATPase has been widely studied (Ebel and Lardy, 1975; Yoshida et al., 1977; Guerrero et al., 1990; Senior, 1990; Murataliev et al., 1991; Al-Shawi and Senior, 1992), and has given convincing evidence that azide combines at the anion binding site. This explains the decreased sensitivity of anion-stimulated activity compared to basal activity because of antagonistic effects of azide and activating anions. The combination of azide stabilizes the inactive Mg^{2+}-F_1-ADP complex, blocks the release of products, and further inhibits catalytic site cooperativity. Therefore, azide inhibits multisite (steady-state cooperative) ATPase activity, but does not inhibit unisite (single-site) activity.

Effect of pCMB: The mercurial reagent, pCMB, specifically acts on SH groups of many proteins, resulting in a loss of biological activity. Inhibition of F_1-ATPase by pCMB has been reported (Pedersen, 1976; Azocar and Munoz, 1978). pCMB modifies both thiol groups and disulfide

bonds in the enzyme. Although SH groups probably are not located in the active site of F_1-ATPase (Penefsky and Cross, 1991), the modification induces a conformational change in the enzyme and causes dissociation of the enzyme into subunits. There are different degrees of accessibility to the SH groups. The number of modified SH groups is determined by pCMB concentration. Inactivation of the enzyme is increased with an increase in modification (Azocar and Munoz, 1978). In addition, Pedersen (1976) thought SH groups had a relationship to anion-binding sites.

The present data show that crayfish F_1-ATPase was more sensitive to pCMB than that from rat liver and E. coli (Pedersen, 1976; Azocar and Munoz, 1978). In addition, pCMB inhibition of the soluble F_1-ATPase was greater than for the membrane-bound enzyme. Azocar and Munoz (1978) obtained similar results with E. coli F_1. These results suggest slight structural differences between crayfish F_1-ATPase and that from other sources. It also suggests that SH groups were more accessible in soluble enzyme than in membrane-bound enzyme. The present data indicate that SMP was more sensitive to pCMB than intact mitochondria. Because the ATPase is inside the intact mitochondria and outside the SMP, these results suggest that the inner membrane of mitochondria limits movement of pCMB. However, the more probable explanation is that pCMB also inhibits the permeases in mitochondrial inner membrane and affects the

movement of ATP, ADP, and phosphate across the membrane that are involved in hydrolysis of ATP. The fact that DTT prevented F_1-ATPase against pCMB inactivation and that the inhibition was partially reversible by DTT suggested DTT reduction of SH groups and chelation with pCMB.

Effect of Oligomycin: Oligomycin is a specific inhibitor of membrane-bound F_1-ATPase. It acts at a small peptide in the F_0 sector known as the oligomycin sensitivity conferral protein (OSCP) (Penefsky, 1979; Liang and Fisher, 1983). OSCP links F_1 and F_0 sectors like a stalk (Dupuis et al., 1985b; Archinard et al., 1986). F_1 is insensitive to oligomycin. However, in the reconstituted F_1-F_0 complex (Dupuis et al., 1985a) or in the reconstituted F_1-OSCP complex (Horak, et al., 1989), F_1 becomes sensitive to oligomycin. Further study (Dupuis and Vignais, 1987) indicated that maximal inhibition of oligomycin was obtained in combination of one mole of OSCP with one mole of F_1.

It is clear from the present results that crayfish membrane-bound F_1-ATPase was sensitive to oligomycin but soluble enzyme was not. This is identical with the previous reports and suggests that F_1 undergoes conformational changes when it binds to the F_0 sector. In addition, the membrane-bound enzyme studied here was much more sensitive to oligomycin than that from plant (Randall et al., 1985) and mammal (Matsuno-Yagi and Hatefi, 1993a). This suggests stronger interaction between F_1 and OSCP in crayfish

mitochondria.

Effect of DCCD: DCCD is a carboxyl group reagent. It reacts covalently with an essential carboxyl group in subunit c of F_0 sector (Sebald et al., 1979; Smith et al., 1985; Hermolin and Fillingame, 1989; Fillingame et al., 1991; Kluge and Dimroth, 1993; Matsuno-Yagi and Hatefi, 1993b). This reaction causes inhibition of proton translocation though F_0 and conformational change of subunit c (Sebald et al., 1979; Sussman and Slayman, 1983). The conformational change is transmitted to the catalytic sites of F_1 part by ϵ subunit (Schneider and Altendorf, 1987; Mendel-Hartvig and Capaldi, 1991), and ATPase activity is inhibited. Some studies indicated that two mole of DCCD/mole of F_0 is required for maximal inhibition of H^+ translocation, but only one mole of DCCD/mole of F_0 for maximal inhibition of F_1F_0-ATPase (Hermolin and Fillingame, 1989).

In contrast to oligomycin, crayfish membrane-bound F_1-ATPase was much less sensitive to DCCD than that from other sources (Bowman, 1983; Randall et al., 1985; Wang and Sze, 1985). This suggests either weaker interaction between F_1 and F_0 or less accessibility of DCCD to c subunit in crayfish F_1F_0 ATPase.

My data show that even with increasing DCCD concentration to 100 μM, 20-fold higher than the maximal inhibitory concentration for membrane-bound enzyme, the

soluble F_1-ATPase from crayfish still was not sensitive to DCCD. However, it has been reported that DCCD was also able to bind to and inactivate a number of purified F_1-ATPases when applied at about a 10-fold higher concentration and at an slightly acidic pH (Esch et al., 1981; Satre et al., 1982; Khananshvili and Gromet-Elhanan, 1983b; Tommasino and Capaldi, 1985). This also suggests less accessibility of DCCD to β subunit in crayfish soluble F_1-ATPase.

Effect of NBD-Cl: NBD-Cl, a nucleotide analog, is a group-specific reagent. It reacts covalently with tyrosine or lysine in the β subunit of F_1-ATPase (Kohlbrenner and Boyer, 1982; Wang et al., 1987). These essential residues are located at or near the catalytic sites (Ting and Wang, 1980; Khananshvili and Gvoment-Elhanan, 1983a; Weber et al., 1992). The modification with NBD-Cl depends on pH and it tends to bind to tyrosine at neutral pH and lysine at alkaline condition (Andrews et al., 1984a and b). In addition, reaction of one mole of NBD-Cl/mole of F_1 is sufficient to fully inhibit the F_1-ATPase (Weber et al., 1994).

The half inhibition concentrations (I_{50}) of NBD-Cl (ranging from 3 to 50 μM) have been reported in F_1-ATPase from different sources (Bowman, 1983; Randall et al., 1985; Wang and Sze, 1985; Yoshida and Allison, 1990). My data were within this range and showed that soluble F_1-ATPase was slightly more sensitive to NBD-Cl than membrane-bound

enzyme. This was similar to that found in oat mitochondrial ATPase (Randall et al., 1985).

Effect of DIDS: DIDS is a specific anion channel blocker in human red blood cells (Cabantchik et al., 1978), corn protoplasts (Lin, 1981), and Chara (Keifer et al., 1982). It inhibited tonoplast-type ATPase with a high sensitivity (Churchill and Sze, 1984; Randall and Sze, 1986). Wang and Sze (1985) showed that mitochondrial ATPase was equally sensitive to DIDS as the tonoplast-type ATPase in oat roots. Studies with mammalian F_1-ATPase are lacking, The present study showed that crayfish F_1-ATPase was also sensitive to DIDS and just slightly less sensitive than tonoplast-type and F_1F_0-type ATPase from plants (I_{50} = 4-10 μM) (Churchill and Sze, 1984; Wang and Sze, 1985; Randall and Sze, 1986).

The mode of action of DIDS on the mitochondrial ATPase is not understood. DIDS can directly interact with anion-binding sites of the tonoplast-type ATPase (Wang and Sze, 1985). DIDS inhibits anion transport in red blood cells by covalently reacting with the ϵ-amino groups of two lysine residues on the transport protein (Cabantchik and Rothstein, 1974). The data presented here show that both DIDS and activating anions acted similarly with both soluble and membrane-bound F_1-ATPase. This suggests that DIDS and activating anions might bind to similar anion-sensitive sites on the F_1 sector of the enzyme.

Effect of Vanadate: VO_4^{3-} is a specific inhibitor for plasma membrane ion-motive ATPase, such as Na^+,K^+-ATPase, Ca^{2+}-ATPase, and plasma membrane H^+-ATPase, which have aspartyl phosphate intermediates (Nelson, 1988; Futai et al., 1989). In these enzymes, a pentacovalent phosphorus was assumed to be a transition state intermediate since they were readily inhibited by the P_i analogue vanadate. Recent research (Hochman et al., 1993) showed that vanadate also inhibits the activity of chloroplast F_1-ATPase. This suggested the involvement of a pentacovalent phosphorus as a transition state intermediate in reactions catalyzed by CF_1-ATPase. However, vanadate failed to inhibit the activity of mitochondrial F_1-ATPase (Bowman, 1983; Wang and Sze, 1985), and its inhibition on mitochondrial ATPase has not been reported.

My results show that crayfish mitochondrial ATPase was sensitive to high concentrations of vanadate with I_{50} at 10.8 mM. Such sensitivity is much lower than that from plasma membrane H^+-ATPase (I_{50} = 1-100 μM) (Bowman, 1983; O'Neill and Spanswick, 1984; Cruz-Mireles and Ortega-Blake, 1991; Lin and Randall, 1993) and from chloroplast F_1-ATPase (I_{50} = 0.5 mM) (Hochman et al., 1993). The results presented in my paper probably should not be interpreted as involvement of a pentacovalent phosphorus as a transition state intermediate but rather that vanadate acts as a inhibitory anion.

Taken together, the sensitivities to inhibitors distinguishes crayfish mitochondrial F_1-ATPase from all those previously examined and outlines a probable reaction mechanism. These results also support the suggestion of a transmission of conformational change between F_0 and F_1 sectors and of slight F_1 sector conformational differences between soluble and membrane-bound enzyme forms. That is, conformational change in F_0 is transmitted to F_1 and causes a conformational change in F_1 that reduces the catalytic activity. In contrast, an unmodified F_0 stabilizes F_1 and resistance to inhibitors is increased.

Kinetics of Membrane-Bound F_1-ATPase from Crayfish Gills

Figures 22 and 23 show that crayfish F_1-ATPase did not hydrolyze ATP but instead hydrolyzed MgATP. Both free ATP and Mg^{2+} were potential inhibitors. Similar kinetic results were widely obtained in many Mg^{2+}-dependent ATPases (Dixon and Webb, 1979). These results were also reported in fungal (de Vicente et al., 1991) and bacterial (Adolfsen and Moudriankis, 1978b) F_1-ATPase.

The interpretation of these kinetic data is difficult because of the equilibrium between the substrate, MgATP, and the potential inhibitors Mg^{2+} and ATP. This means these three compounds cannot be varied independently. No studies have reported the kinetic regulatory mechanisms for ATP and Mg^{2+} in F_1-ATPase. It is now clear from my study that free

ATP was a classical competitive inhibitor and that free Mg^{2+} was a mixed inhibitor. These results strongly suggest that free ATP competed for catalytic sites on the enzyme with MgATP complex and that Mg^{2+} probably bound to both catalytic sites and other metal ion binding site(s). Previous studies indicated Mg^{2+} may bind to both catalytic and non-catalytic sites (Senior 1979, 1981; Kironde and Cross, 1987; Urbauer et al., 1987; Murataliev, 1992). In the catalytic sites, Mg^{2+} binds with the E-ADP complex forming inactive enzyme-ADP-Mg^{2+} complex and resulting in F_1-ATPase inhibition (Guerrero et al., 1990; Murataliev et al., 1991; Murataliev, 1992). In the noncatalytic sites, binding of ATP with Mg^{2+} increases the release of P_i from the catalytic site to give the E-ADP complex required for formation of inactive E-ADP-Mg^{2+} complex (Guerrero et al., 1990; Murataliev, 1992). In addition, Mg^{2+} also binds to a site near the interface between the F_0 and F_1 sectors (Senior, 1981) that is involved in F_1 inactivation (Pedersen et al., 1987).

The K_m values for ATP or MgATP from 0.1 to 1 mM have been exhibited by F_1-ATPase from animals (Patil et al., 1979; Tyler and Webb, 1979; Laikind and Allison, 1983; Gruys et al., 1985; Aloise et al., 1991; Murataliev, 1992; Murataliev and Boyer, 1994), plants (Partridge et al., 1985; Randall et al., 1985), fungi (de Vicente, et al., 1991), and bacteria (Wise et al., 1983; Hicks and Krulwich, 1987). The K_m value of 0.327 mM for MgATP in my study was estimated

from the kinetic analysis. This is within the above range. Although reported inhibitor constants for ATP or Mg^{2+} are lacking, the K_i value of 1 mM for Mg^{2+} has been reported for Vigna sinensis (Peter et al., 1981). It is similar to my datum (0.81 mM). In addition, the K_i value of 0.77 mM for ATP in my study is also within the above reported K_m range for ATP or MgATP.

My results clearly indicate activation of crayfish F_1-ATPase by free ATP. Because free ATP competitively inhibited the enzyme, one can hypothesize that ATP also binds to the site(s) other than catalytic sites, stimulating the ATPase activity. Further studies indicated that pronounced negative cooperativity was exhibited when the enzyme hydrolyzed MgATP at high free Mg^{2+} concentration, but it was not observed at high free ATP concentration. According to recent studies (Jault and Allison, 1993, 1994; Jault et al., 1994), these results were explained as follows: at high free ATP level, saturation of noncatalytic sites with ATP stimulated crayfish F_1-ATPase activity to an optimal hydrolysis level. At high fixed free Mg^{2+} concentration, free ATP level was very low, and slightly increased with increasing of MgATP complex (see Table 4). At a certain free ATP level, the occupancy by ATP of the noncatalytic sites increased the ATPase activity. Therefore, apparent negative cooperativity at high fixed free Mg^{2+} level was due to binding of ATP to noncatalytic sites (Jault

and Allison, 1993). These results and explanation are consistent with the above hypothesis.

Although the regulatory degree of noncatalytic sites seems to be slight or nonexistent for the E. coli F_1-ATPase (Wise and Senior, 1985), recent evidence obtained from mitochondrial and chloroplast enzymes indicates that noncatalytic sites play a regulatory role, and that binding of ATP to these sites is essential for a high catalytic rate (Yoshida and Allison, 1986; Bullough et al., 1988; Melese et al., 1988; Milgrom et al., 1991; Shapiro et al., 1991; Vogel and Cross, 1991; Peinnequin et al., 1992; Wang et al., 1993; Burgard et al., 1994). Further study (Milgrom et al., 1990) indicated that the rate of ATP binding to noncatalytic sites of F_1-ATPase occurred at relatively low ATP concentration. At 5-20 μM, ATP binds at the last two of the three noncatalytic sites. My results support this suggestion.

The activation mechanism by binding of ATP to noncatalytic sites has been studied. Wang et al. (1993) indicated that binding of ATP to non-catalytic sites greatly enhances the structural stability of CF_1. Milgrom et al. (1991) and Divita et al. (1992) thought that non-catalytic sites filled by ATP interact with catalytic sites, causing the activity increase. The mechanism was further explained by Murataliev (1992) and Milgrom and Cross (1993). They indicated that ATP binding at non-catalytic sites decreases the stability of the inactive enzyme-ADP-Mg^{2+} complex,

resulting in the activation of the enzyme.

In addition, noncatalytic sites are very selective for adenine nucleotides (Kironde and Cross, 1986; Milgrom et al., 1990). Although noncatalytic sites also can bind ADP, MgADP, MgATP, GTP and PP_i, the occupancy inhibited the hydrolytic activity of the enzyme (Milgrom et al., 1991; Murataliev, 1992; Milgrom and Cross, 1993). These results explain why, in my experiments, MgATP at such a high level could not replace the ATP at noncatalytic sites to activate the enzyme.

The effects of free ATP and Mg^{2+} on crayfish F_1-ATPase probably reflect a physiological regulatory mechanism in the cells. Mg^{2+} is essential for the ATPase activity by forming the real substrate MgATP, but also is a strong inhibitor for ATP hydrolysis. However, ATP has three distinct effects on the enzyme activity. First, when complexed with Mg^{2+}, it is the substrate of the enzyme. ATP by itself is not hydrolyzed. Second, ATP behaves as a competitive inhibitor of the enzyme. Thus, free ATP appears to bind to the catalytic sites, although with lower affinity than MgATP. Third, ATP is an activator when it binds at the noncatalytic sites. In addition, ATP is a strong chelator of another effector, Mg^{2+}. So any single experiment that shows an effect of free ATP can also be interpreted in terms of an effect of lower free Mg^{2+}. Therefore, the effects of both free ATP and Mg^{2+} are interrelated. However, F_1 functions as

an ATP synthase in the cells. The physiological significance of ATP and Mg^{2+} remain for further discussion.

It is clear from my results that the activity decreases at high substrate concentration (Fig. 28) was mainly due to the NaCl buildup. So, an explanation for the effect of salt is also the interpretation for the activity decline. NaCl probably has three different effects on the enzyme activity. First, NaCl decreases the formation of the substrate, MgATP complex (Adolfsen and Moudrianakis, 1978a). The stability constant at 3.5 mM of both Na_2ATP and $MgCl_2$ (76,100 M^{-1}) is much higher than at 20 mM (45,500 M^{-1}). As $[MgATP]/[Mg^{2+}_f][ATP_f]$ decreases, and the inhibitor concentrations, [ATP] and $[Mg^{2+}]$, relatively increase. Thus, NaCl indirectly inhibited the enzyme. Second, Cl^- competes for the anion-binding site with HCO_3^-. Replacement of HCO_3^- by Cl^- decreases the enzyme activity (discussed in the "Effect of Anions" section). These two reasons cannot by themselves explain the different effects of NaCl between soluble and membrane-bound enzyme. Third, the enzyme probably is sensitive to ionic strength, even more so in the soluble form. The soluble enzyme activity determined in 60 mM Tris/Hepes buffer was about half as in 10 mM Tris/Hepes buffer (data not shown). These results support the suggestion that the enzyme is sensitive to ionic strength and that NaCl decreases the ATPase activity by increasing the ionic strength. The above three reasons also

explain why the enzyme activity decreased at high activating anion levels.

In conclusion, the purification procedure is a simple and rapid method for isolation of soluble F_1-ATPase from crayfish gills. The final preparation was useful to characterize the enzyme and to study the binding of externally added nucleotides. There were slight differences between the soluble and membrane-bound F_1-ATPase in the general properties as well as inhibitor and modulator sensitivities. These results suggest the conformational change transmission between F_0 and F_1 sectors and slight conformational differences between soluble F_1 and membrane-bound F_1. In addition, the kinetic studies indicated that 1) MgATP complex was the real substrate for crayfish F_1-ATPase, 2) both free ATP and Mg^{2+} were the inhibitors, and 3) free ATP also increased the activity by binding to the noncatalytic sites of the enzyme.

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Appendix A

The follow abbreviations are used:

Bis-Tris, (1,3-bis[tris(Hydroxymethyl)methylamino]propane;

BSA, bovine serum albumin;

BzATP, 3'-o-(4-benzoyl)-benzoyl ATP;

CF_1-ATPase, Chloroplast F_1-ATPase;

DCCD, N,N'-dicyclohexylcarbodiimide;

DIDS, 4,4' diisothiocyano-2,2'-stilbene disulfonic acide;

DTT, dl-dithiothreitol;

EDTA, ethylenediaminetatraacetate;

Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

K_a, activator constant;

K_i, dissociation constant for inhibitor from enzyme-inhibitor complex;

K_i', dissociation constant for inhibitor from substrate-enzyme-inhibitor complex;

K_m, Michaelis constant;

NBD-Cl, 4-chloro-7 nitrobenzofurazan;

OSCP, oligomycin-sensitivity-conferring protein;

pCMB, ρ-chloromercuri-benzoic acide;

P_i, inorganic phosphate;

S.A., specific activity;

SMP, Submitochondrial particle;

TNP-ATP, 2'(3')-o-2,4,6-trinitrophenyl ATP;

Tris, tris(hydroxymethyl)aminomethane.

Appendix B

Parameters, properties and mechanisms of inhibitors that were studied in this paper.

1. Nitrate: an inhibitory anion; significantly different sensitivity between membrane-bound (I_{50} =85 mM) and soluble (I_{50} = 28 mM) enzymes; probably reacts with anion-binding site; causes F_1 conformational change.
2. Azide: a specific inhibitor; slightly different sensitivity between membrane-bound (I_{50} = 110 μM) and soluble (I_{50} = 93 μM) enzymes; binds with anion-binding site; stabilizes the inactive Mg^{2+}-E-ADP complex; inhibits multisite ATPase activity but not unisite activity.
3. pCMB: a strong mercurial inhibitor; significantly different sensitivity between membrane-bound (I_{50} = 1.0 μM for SMP, 1.5 μM for mitochondria) and soluble (I_{50} = 0.15 μM) enzymes; acts on sulfhydryl group; causes conformational change and enzyme dissociation into subunits.
4. Oligomycin: a specific inhibitor for F_0 sector; inhibits membrane-bound (I_{50} = 0.019 μg/ml) but not soluble enzyme; acts on OSCP in F_0; causes conformational change in F_1.
5. DCCD: a carboxyl-group inhibitor; inhibits membrane-bound (I_{50} = 2.2 μM) but generally not soluble enzymes; modifies an essential carboxyl group in

subunit c of F_0 sector; causes F_0 and then F_1 conformational change.

6. NBD-Cl: a nucleotide analog and a group-specific inhibitor; slightly different sensitivity between membrane-bound (I_{50} = 13.0 μM) and soluble (I_{50} = 9.3 μM) enzymes; reacts covalently with tyrosine or lysine in catalytic sites.
7. DIDS: a strong inhibitor which had a relationship to anion channel; identical sensitivity (I_{50} = 12.5 μM) between the two enzyme forms; probably interacts with anion-binding site; causes F_1 conformational change.
8. Vanadate: a P_i analogue anion and a specific inhibitor for plasma membrane ion-motive ATPase; identical sensitivity (I_{50} = 10.8 mM) between the two enzyme forms; probably acts with anion-binding site; causes F_1 conformational change.
9. Free ATP: a competitive inhibitor; inhibitor constant K_i = 0.77 mM for membrane-bound enzyme; binds with catalytic site; competitively blocks the binding of the true substrate MgATP on catalytic sites.
10. Free Mg^{2+}: a mixed inhibitor; inhibitor constant K_i = 0.81 mM and K_i' = 5.89 mM for membrane-bound enzyme; binds to both the catalytic and noncatalytic sites; inhibits the enzyme by causing or increasing the formation of inactive E-ADP-Mg^{2+} complex.

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Signature of Major Advisor

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