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Effects of Zn^{2+} on the Activity and Binding of the Mitochondrial ATPase Inhibitor Protein, IF_1

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Zn^{2+} caused a noninhibitory binding of IF_1 to mitochondrial membranes in both rabbit heart SMP and intact rabbit heart mitochondria. This Zn^{2+}-induced IF_1 binding required the presence of at least trace amounts of MgATP and was essentially independent of pH between 6.2 and 8.2. Addition of Zn^{2+} after the formation of fully inhibited IF_1-ATPase complexes very slowly reversed IF_1-mediated ATPase inhibition without causing significant IF_1 release from the membranes. When Zn^{2+} was added during the state 4 energization of ischemic mitochondria in which IF_1 was already functionally bound, it slowed somewhat energy-driven ATPase activation. This slowing was probably due to the fairly large depressing effect Zn^{2+} had upon membrane potential development, but Zn^{2+} did not decrease the degree of ATPase activation eventually reached at 20 min of state 4 incubation. Zn^{2+} also preempted normal IF_1 release from the membranes, causing what little inhibitor that was released to rebind to the enzyme in noninhibitory IF_1–ATPase complexes. The data suggest that IF_1 can interact with the ATPase in two ways or through two kinds of sites: (a) a noninhibitory interaction involving a noninhibitory IF_1 conformation and/or an IF_1 docking site on the enzyme and (b) an inhibitory interaction involving an inhibitory IF_1 conformation and/or a distinct ATPase activity regulatory site. Zn^{2+} appears to have the dual effect of stabilizing the noninhibitory IF_1–ATPase interaction and possibily a noninhibitory IF_1 conformation while concomitantly preventing the formation of an inhibitory IF_1–ATPase interaction and possibly an inhibitory IF_1 conformation, regardless of pH. While the data do not rule out direct effects of Zn^{2+} on either free IF_1 or the free enzyme, they suggest that Zn^{2+} cannot interact readily with either the inhibitor or the enzyme once functional IF_1–ATPase complexes are formed.

KEY WORDS: Heart muscle mitochondria; mitochondrial ATPase; ATPase inhibitor subunit; IF_1; Zn^{2+}; noninhibitory IF_1 binding; myocardial ischemia.

INTRODUCTION

The F_1–ATPase inhibitory subunit, IF_1, was first isolated form bovine cardiac muscle mitochondria nearly 30 years ago (Pullman and Monroy, 1963). Bovine cardiac muscle IF_1 is a water-soluble, heat-stable polypeptide containing 84 amino acid residues whose combined residue molecular weights add up to a polypeptide molecular weight of 9578 (Frangione *et al.*, 1981). The inhibitor binds to the ATPase, 1 mole of inhibitor per mole of enzyme, under non-energizing conditions at low mitochondrial matrix pH such as occurs during anoxia or ischemia (Rouslin, 1987a; Rouslin and Pullman, 1987). Glycolysis-driven cell acidification (Rouslin, 1988; Rouslin and Broge, 1989b; Rouslin and Broge, 1990; Rouslin *et al.*, 1990) characaterizes most systems under hypoxia or anoxia. The lack of oxygen in ischemia presumably produces a gradual mitochondrial inner membrane deenergization possibly resulting in a relaxed, IF_1-receptive conformation of the

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F_1-ATPase to which the inhibitor can bind (Rouslin, 1987a; Rouslin and Pullman, 1987). Moreover, the acidosis that occurs may cause IF_1 protonation, possibly resulting in an active or inhibitory conformation of the inhibitor protein. IF_1-mediated ATPase inhibition during ischemia significantly diminishes a wasteful hydrolysis of glycolytically produced ATP by the deenergized mitochondrial ATP synthase (Rouslin *et al.*, 1986, 1990). Inhibitor binding thus slows net rates of tissue ATP depletion during ischemia, thereby delaying cell injury and death (Rouslin *et al.*, 1986, 1990; Rouslin, 1991).

Earlier studies by Chernyak *et al.* (1985) demonstrated that either Zn^{2+} or Cd^{2+} and, to lesser extents, Mn^{2+}, Co^{2+}, and Ni^{2+}, slowed the rate of inhibition of isolated bovine heart F_1-ATPase or of the ATPase in bovine heart submitochondrial particles (SMP) by the ATPase inhibitor protein. Zn^{2+} and Cd^{2+} were also shown to prevent the pH-dependent inhibition of the ATPase in deenergized mitochondria from rat hepatoma (Chernyak *et al.*, 1987, 1991) and from normal liver in ground squirrels (Bronnikov *et al.*, 1990). Another related study by these workers showed that Zn^{2+} accelerated the activation of the ATPase in bovine heart SMP in which the inhibitor was presumably already functionally bound (Khodjaev *et al.*, 1990). A mechanism involving a possible direct effect of Zn^{2+} and the other divalent metal ions studied on the inhibitor protein was proposed by these workers although the mechanism remains unconfirmed experimentally at this time.

These earlier studies on Zn^{2+} and other metal ions on IF_1-mediated ATPase inhibition did not examine the effects of these ions on inhibitor binding *per se* using an independent assay for the amount of inhibitor actually bound to membranes. In the present study we examined the effects of Zn^{2+} on IF_1-mediated changes in ATPase activity and also on concomitant changes in IF_1 binding to membranes in rabbit heart SMP and intact mitochondria. The results demonstrate that Zn^{2+} had a dual effect. First, it caused IF_1 to bind nearly fully to the mitochondrial membranes in intact mitochondria regardless of the pH of the incubation medium or of the pH-equilibrated matrix compartment. Second, the IF_1 that was bound was noninhibitory. That is, while the inhibitor was nearly fully bound, the ATPase remained largely uninhibited. The implications of this dual and paradoxical effect of Zn^{2+} on IF_1-ATPase interaction are discussed in terms of module of IF_1-ATPase interaction.

EXPERIMENTAL PROCEDURES

Preparation of Intact Control-Reenergized and Ischemic Rabbit Heart Mitochondria, Sonicated Mitochondria, Submitochondrial Particles, IF_1-Depleted Submitochondrial Particles, and IF_1-Containing Extracts

Male New Zealand white rabbits weighing approximately 1 kg were fully anesthetized with sodium pentobarbital (i.v. to effect) and then sacrificed by the rapid removal of their hearts. The hearts were either placed immediately in ice-cold 180 mM KCl and 10 mM ethylenebis (oxyethylenenitrilo)tetraacetic acid (EGTA) (KE solution) (control samples), or first made ishemic for 20 min by placing them in sealed Ziploc plastic bags immersed in a circulating water bath at 37°C as described earlier (Rouslin, 1983a,b, 1987a,b,c, 1988, 1991; Rouslin *et al.*, 1986, 1990) before being placed in ice-cold KE solution. The hearts were then minced finely in ice-cold 180 mM KCl, 10 mM EGTA, 0.5% bovine serum albumin, and 10 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS)-KOH, pH 7.2 (KEAM solution), and mitochondria were prepared from the cardiac muscle minces by Polytron homogenization as described earlier (Rouslin, 1983a,b, 1987a,b,c, 1988, 1991; Rouslin *et al.*, 1986, 1990). For the experiments depicted in Figs. 1–3 and 6, the control mitochondria were reenergized by shaking them vigorously for 10 min at 37°C in 0.25 M sucrose, 1 mM EGTA, and 20 mM MOPS-KOH, pH 7.2 (SEM solution), with 6.25 mM glutamate, 6.25 mM malate, and 2.5 mM Pi. This procedure served to maximally activate the mitochondrial ATPase at the beginning of the experiment before the start of subsequent incubations described below.

For the experiment presented in Fig. 6A, sonicated mitochondria were used. These were prepared by sonication of intact mitochondrial samples using three 10 s bursts with time allowed for sample recooling to ice-bucket temperatures. Submitochondrial particles (SMP) were prepared from mitochondrial samples by sonication and centrifugation as described earlier (Rouslin, 1987a,b; Rouslin and Pullman, 1987; Rouslin and Broge, 1989a). IF_1-depleted rabbit heart SMP used in the experiment depicted in Table I were prepared essentially by the same method used to prepare "regular" SMP except that sonification was carried out at pH 9.0 in the presence of 1.0 mM MgATP and they were centrifuged after soni-

cation for 60 min at 226,000 × g. This procedure served to strip away at least 90% of the endogenous IF_1 present on the particles. Also, the low-speed centrifugation step normally employed for the removal of large membrane fragments was eliminated from the procedure so that the yield of SMP protein would be effectively 100%. This made the calculation of the ratio of IF_1-depleted SMP to IF_1 to be added later a relatively simple matter. IF_1-containing extracts used either for rebinding to IF_1-depleted SMP as in the experiment presented in Table I, or for the determination of the amount of IF_1 bound to the membranes, as in the experiments depicted in Table I and Figs. 1–4, were prepared by alkaline extraction of intact mitochondria as described earlier (Pullman and Monroy, 1963; Frangione *et al.*, 1981, Rouslin, 1987a,b, 1988; Rouslin and Pullman, 1987).

In vitro Incubation Conditions

Except where noted, *in vitro* incubations of isolated intact and sonicated mitochondria and of IF_1-depleted SMP were carried out for 20 min at 37°C at protein concentrations of approximately 2 mg/ml for intact and sonicated mitochondria and 0.2 mg/ml for IF_1-depleted SMP. In the experiments depicted in Table I, IF_1-depleted rabbit heart SMP at approximately 0.2 mg/ml were incubated in the absence and presence of 50 μM Zn^{2+}, in the absence and presence of 0.5 mM MgATP, and in the absence and presence of an amount of IF_1-containing rabbit heart mitochondrial extract derived from the same quantity of mitochondria as were the IF_1-depleted SMP. In the experiments depicted in Figs 1–6, intact mitochondria were incubated at 37°C for the times and conditions indicated in the individual experiments. At the conclusion of the incubations, an aliquot of each mitochondrial sample was pelleted by centrifugation at approximately 17,000 × g for 10 min, resuspended in 0.25 mM sucrose and 10 mM MOPS-KOH, pH 7.2 (SM solution), and sonicated as described above for the subsequent assay of ATPase activity. In the experiments depicted in Figs. 1–4, an additional aliquot of each mitochondrial sample was used for the preparation of SMP as described above. These SMP samples were resuspended in SM solution and later alkaline-extracted as described below for the assay of IF_1 content. In the experiments presented in Table I, an aliquot of each SMP sample was used for alkaline extraction for the assay of IF_1 content.

For the MgATP-free samples in the experiments presented in Table I, the SMP samples prepared by sonication in the absence of added MgATP were then preincubated for 10 min at 37°C at pH 8.5 with 10 mM glucose and 10 U/ml of hexokinase (Sigma type C-3401) to deplete them of traces of ATP that might be present. Such MgATP-depleted SMP failed to bind additional IF_1 even in the presence of Zn^{2+}.

In the experiments presented in Fig. 3, 50 μM Zn^{2+} was added either at zero time, in which case it produced nonfunctional IF_1 binding (solid circles), or after 20 min of incubation at pH 6.4. In the experiments presented in Figs. 4 and 5, mitochondria from 20 min ischemic myocardium were used in which the inhibitor was already functionally bound to the ATPase. In the experiments presented in Fig. 4, the mitochondria were energized by vigorous shaking for the times indicated at 37°C in 0.25 M sucrose and 20 mM MOPS-KOH, pH 7.2, with 6.25 mM glutamate, 6.25 mM malate, and 4.0 mM Pi in the absence and presence of 50 μM Zn^{2+} and/or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). At the conclusion of the state 4 incubations, the shaking of the samples was stopped and they were quickly chilled to ice-bucket temperature. SMP were prepared by sonication in 0.25 M sucrose and 20 mM tris(hydroxymethyl)methylaminopropanesulfonic acid (TAPS)-KOH, pH 8.2, and after their final centrifugation, the SMP were resuspended in SM solution for assay of ATPase and IF_1 content. In the experiments presented in Fig. 5, the rates of the safranin dye response and the extents of membrane potential development were monitored during the state 4 energization of 20-min ischemic mitochondria using the safranin dye method of Akerman and Wikstrom (1976) employing the same basic medium used in the experiments presented in Fig. 4. In the membrane potential experiments, aliquots of mitochondria which were already in state 4 incubation at 37°C were sampled at the times indicated and placed in a cuvette which was also at 37°C. Safranin dye was then added to a concentration of 10 nmol/mg mitochondrial protein and the initial rate of the safranin dye response (Fig. 5B) and the extent of membrane potential developed and held after 1 min (Fig. 5A) were measured. In the experiments presented in Fig. 6A, intact mitochondria were incubated at the Zn^{2+} concentrations indicated in the presence of 4 mM Pi, and sonicated mitochondria were incubated in the presence of 0.5 mM MgATP without Pi. In the experiments presented in Fig. 6B, 3 μM ruthenium red was added to the mitochondria approximately 6 min before the addition

Table I. Effects of Zn^{2+} on the IF1-Mediated Inhibition of the Mitochondrial ATPase and on the Binding of 1 × IF_1 to IF_1-Depleted Rabbit Heart SMP at pH 6.4 in the Absence and Presence of MgATP[a]

Additions	ATPase specific activity[b]	IF_1 bound (IU/mg)[c]
No IF_1, no MgATP, no Zn^{2+}	6.14 ± 0.20	1.15 ± 0.12
No IF_1, no MgATP, + Zn^{2+}	6.17 ± 0.23	1.08 ± 0.02
No IF_1, + MgATP, no Zn^{2+}	5.26 ± 0.09	1.63 ± 0.11
No IF_1, + MgATP, + Zn^{2+}	5.31 ± 0.08	1.44 ± 0.08
+ IF_1, no MgATP, no Zn^{2+}	6.12 ± 0.22	1.28 ± 0.12
+ IF_1, no MgATP, + Zn^{2+}	6.08 ± 0.17	1.20 ± 0.12
+ IF_1, + MgATP, no Zn^{2+}	0.60 ± 0.03	5.77 ± 0.32
+ IF_1, + MgATP, + Zn^{2+}	3.99 ± 0.06	5.25 ± 0.27

[a] All data are averages ± SE of four separate determinations.
[b] ATPase specific activity is expressed as μmol/min/mg.
[c] One I.U. (inhibitory unit) is that amount of inhibitor which fully inhibits one international unit of enzyme activity by 100%, i.e., 1 mol ATP hydrolyzed/min. Where present, Zn^{2+} was at 50 μM and MgATP at 0.5 mM.

of Zn^{2+}, and the samples were incubated for an additonal 20 min at 37°C.

Other Procedures

Mitochondrial ATPase activity was measured in sonicated mitochondria or in SMP at 30°C as described previously (Rouslin, 1983a,b, 1987a,b,c, 1988, 1991; Rouslin *et al.*, 1986, 1990; Rouslin and Broge, 1989a,b, 1990; Rouslin and Pullman, 1987). The IF_1 content determinations presented in Table I and in Figs. 1–4 were carried out as described previously using our rat heart SMP titration procedure (Rouslin, 1987a,b, 1988; Rouslin and Broge, 1989a,b, 1990; Rouslin and Pullman, 1987). Protein was estimated by the Lowry procedure (Lowry *et al.*, 1951).

RESULTS

Table I shows the effects of the absence of MgATP, on the one hand, and of the presence of 50 μM Zn^{2+}, on the other, on both IF_1-mediated mitochondrial ATPase inhibition and on rabbit heart IF_1 binding to IF_1-depleted rabbit heart SMP. As can be seen, the noninhibitory binding of IF_1 observed in the presence of Zn^{2+} and the inhibitory binding of IF_1 in the absence of Zn^{2+} were both strictly dependent upon the presence of MgATP. This suggests that the noninhibitory IF_1 binding observed in the presence of Zn^{2+} resembled normal functional IF_1 binding and that, in both cases, the inhibitor becomes bound to the ATPase.

Percents IF_1 released in the experiments presented in Figs. 1–4 were calculated relative to the maximal amount of IF_1 that was bound in the experiments presented in a given figure. Thus, the sample containing the most IF_1 bound for each set of experiments was used as a reference sample for the set of experiments presented in that figure. For the experiments presented in Figs. 1–4 the average maximal IF_1 binding was 4.50 ± 0.26 I.U./mg ($n = 4$), where one inhibitory unit (I.U.) is the amount of inhibitor required to inhibit one international unit of enzyme activity by 100% (Rouslin, 1987; Rouslin and Pullman, 1987).

Figure 1A shows the effects of varying pH on IF_1-mediated ATPase inhibition in intact rabbit heart mitochondria in the absence and presence of Zn^{2+}. Figure 1B presents the degrees of IF_1 release observed for the same samples. In the absence of Zn^{2+}, a lowering of the pH of the incubations resulted in a graded increase in both ATPase inhibition and IF_1 binding, with most of the effect occurring between pH 7.4 and 6.6. These pH-dependent effects in the absence of Zn^{2+} are essentially identical to those that have been reported earlier both by others (e.g., Horstman and Racker, 1970; Galante *et al.*, 1981) and by ourselves (Rouslin, 1983a, 1987a; Rouslin and Broge, 1989a,b). In contrast, in the presence of 50 μM Zn^{2+}, there was a pH-independent, noninhibitory binding of IF_1 in whch ATPase activity and IF_1 binding both remained essentially maximal at all pH values examined.

Figure 2A shows the effects of varying Zn^{2+} concentration on IF_1-mediated ATPase inhibition in intact rabbit heart mitochondria at pH 8.2 and 6.4.

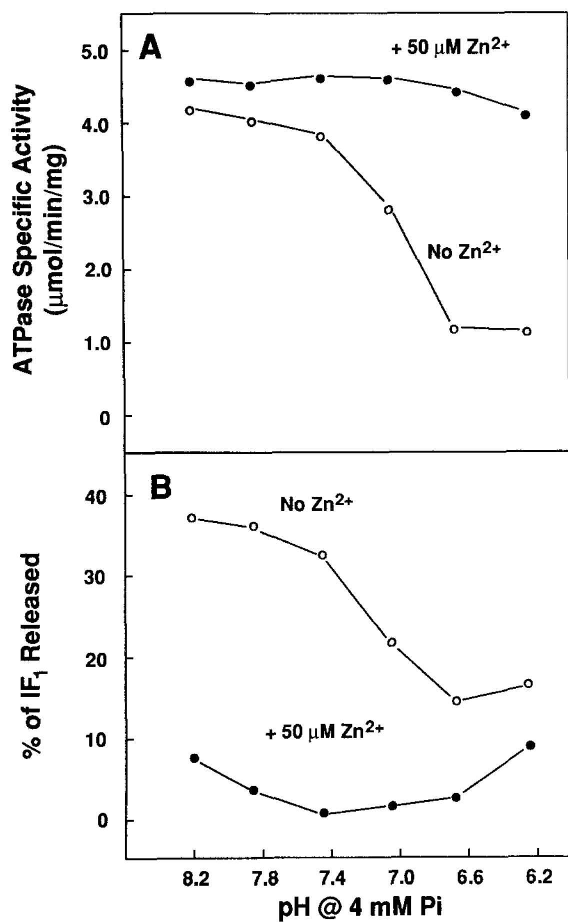

Fig. 1. The effect of varying pH on the formation of inhibitory IF_1-ATPase complexes as indicated by ATPase activity (A) and on IF_1 release (B) in intact rabbit heart mitochondria in the absence and presence of 50 μM Zn^{2+}. Intact rabbit heart mitochondria were incubated for 20 min at 37°C at the pH values indicated in the presence of 4 mM Pi and in the absence and presence of 50 μM Zn^{2+}. SMP were prepared from each sample and assayed for ATPase activity and IF_1 content.

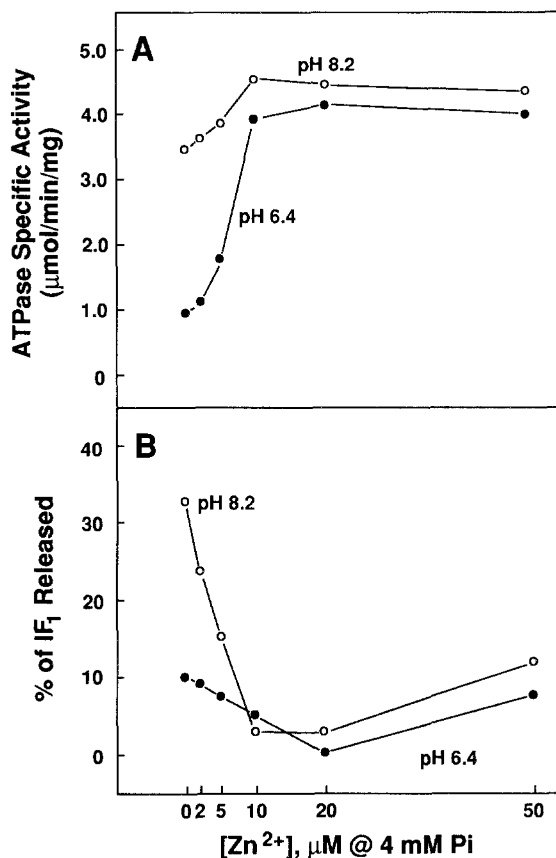

Fig. 2. The effect of varying Zn^{2+} concentration on the formation of inhibitory IF_1-ATPase complexes as indicated by ATPase activity (A) and on IF_1 release in intact rabbit heart mitochondria incubated at pH 6.4 and 8.2. (B) Intact rabbit heart mitochondria were incubated for 20 min at 37°C at the Zn^{2+} concentrations indicated at pH 6.4 or 8.2 in the presence of 4 mM Pi. SMP were prepared from each sample and assayed for ATPase activity and IF_1 content.

Figure 2B presents the degrees of IF_1 release observed for the same samples. At pH 8.2 there was a small but significant increase in ATPase activity caused by increasing the concentration of Zn^{2+}, with the maximal effect occurring at 10–15 μM Zn^{2+}. In the same samples at pH 8.2, there was a concomitant marked increase in noninhibitory IF_1 binding with increasing Zn^{2+} concentration, with the maximal effect also occurring at 10–15 μM Zn^{2+}. At pH 6.4 there was a marked increase in ATPase activity caused by increasing the concentration of Zn^{2+}, with the maximal effect occuring at 15–20 μM Zn^{2+}. In the same samples at pH 6.4 there was a small but significant increase in nonfunctional IF_1 binding caused by increasing the concentration of Zn^{2+}, with the maximal effect again occurring at 15–20 μM Zn^{2+}.

Figures 3A and 3B present time courses of change in ATPase activity and IF_1 binding, respectively, upon the addition of 50 μM Zn^{2+} after inhibitory IF_1-ATPase complexes had already been formed in intact rabbit heart mitochondria. In these experiments a 20-min incubation at pH 6.4 occurred before the addition of Zn^{2+}. For the points shown as solid circes, the Zn^{2+} was added at the outset before inhibitory IF_1 binding had occurred. As can be seen, the addition of Zn^{2+} after inhibitory IF_1-enzyme complex formation caused a very slow reversal of IF_1-mediated ATPase inhibition which was far from complete after 60 min at pH 6.4, but there was no significant reversal of the physical binding of IF_1 to the ATPase.

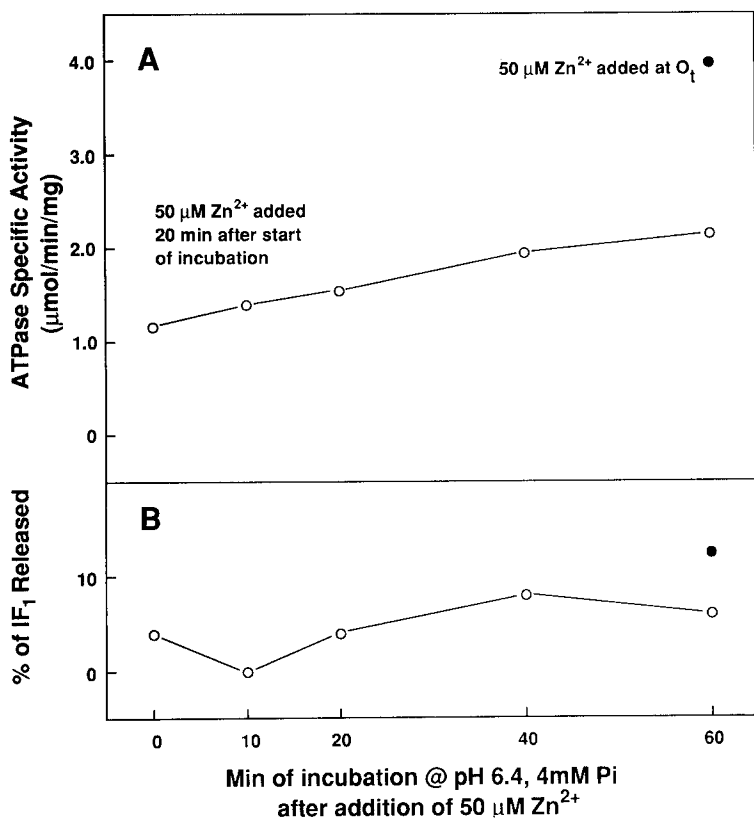

Fig. 3. The effect of adding 50 μM Zn^{2+} 20 min after the formation of inhibitory IF_1-ATPase complexes in intact rabbit heart mitochondria at pH 6.4 on ATPase activity (A) and on IF_1 release (B). Intact rabbit heart mitochondria were incubated for 20 min at 37°C in the presence of 4 mM Pi. 50 μM Zn^{2+} was added 20 min later and the mitochondria then incubated further for the times indicated. SMP were prepared from each sample and assayed for ATPase activity and IF_1 content. The solid circles in A and B show the effects of the addition of 50 μM Zn^{2+} at zero-time.

Figures 4A and 4B present time courses of ATPase activation and IF_1 release, respectively, during the state 4 energization of intact rabbit heart mitochondria prepared from 20-min ischemic rabbit hearts. In the "No Additions" protocol, energization by vigorous shaking with glutamate, malate, and Pi was accompanied by rapid and parallel increases in both ATPase activity and IF_1 release essentially identical to those that have been reported earlier by others (Schwertzmann and Pedersen, 1981) and by ourselves (Rouslin, 1987a, Rouslin and Pullman, 1987; Rouslin and Broge, 1989a). The addition of FCCP alone completely blocked both ATPase activation and IF_1 release. Zn^{2+} alone caused a slight slowing of ATPase activation, but the level of ATPase activation reached after 20 min was essentially identical to that observed in the "No Additions" protocol (Fig. 4A). In contrast to its effect on ATPase activation, in the presence of Zn^{2+} alone, there was an initial partial release of IF_1 followed by its rebinding to the enzyme to form non-inhibitory IF_1-ATPase complexes by 10 min of energization (Fig. 4B). This transient release of IF_1 is believed to have occurred during a brief time lag required for Zn^{2+} to reach an effective concentration in the matrix compartment in these experiments. Interestingly, the addition of FCCP plus Zn^{2+} was accompanied by very slow and limited increases in both ATPase activity and IF_1 release. The level of IF_1 release observed in the presence of FCCP plus Zn^{2+} appeared to approach that produced by Zn^{2+} alone after 20 min of energization.

Figures 5A and 5B show the effect that 50 μM Zn^{2+} had on the percent maximal membrane potential developed and held after 1 min and on the percent maximal safranin dye response, respectively, in intact rabbit heart mitochondria, both as a function of time and of energization. These experiments are identical to the 'No Additions" and Zn^{2+} alone protocols presented in Fig. 4. As can be seen, 50 μM Zn^{2+} had a substantial depressing effect on membrane potential development in these experiments.

Figure 6A shows the effect of varying Zn^{2+} concentration on ATPase activity in intact rabbit heart mitochondria essentially as shown in the pH 6.4 protocol in Fig. 2A above, and also in sonicated rabbit heart mitochondria studied under the same conditions except for the absence of added Pi and the presense of 0.5 mM MgATP. Zn^{2+} was maximally effective at approximately a 3-fold lower concentration in intact mitochondria than in sonicated mitochondria, sug-

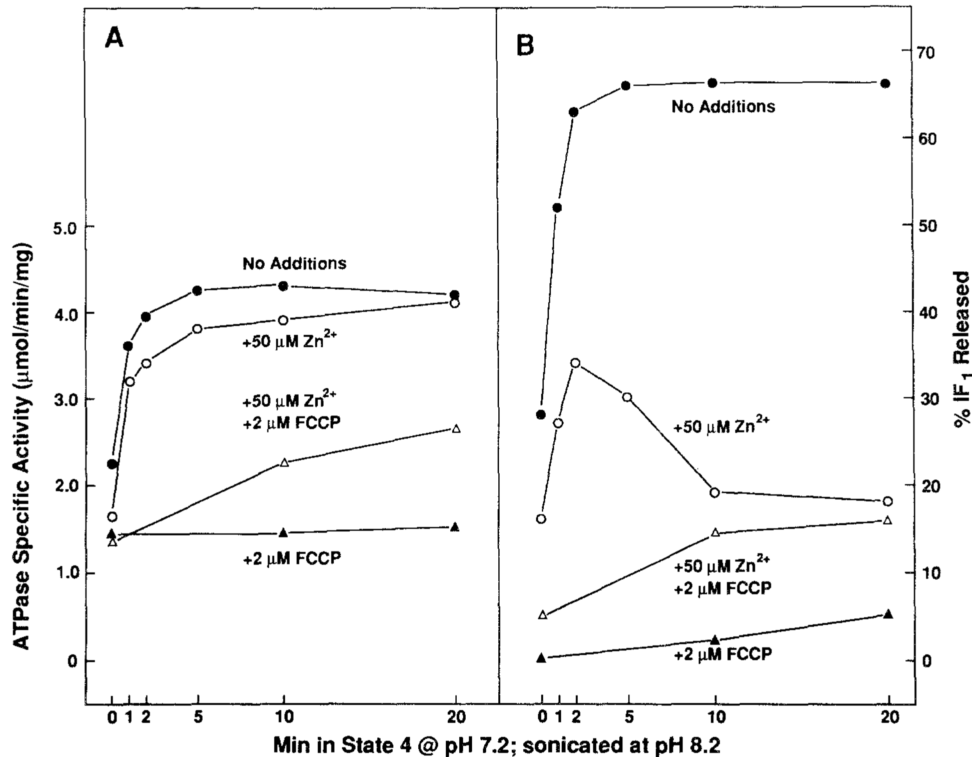

Fig. 4. The effect of 50 μM Zn^{2+} alone, 2 μM FCCP alone, or both Zn^{2+} and FCCP on the activation of the ATPase (A) and on the release of IF_1 from the enzyme (B) during the state 4 incubation (reenergization) of 20-min ischemic rabbit heart mitochondria. Intact rabbit heart mitochondria from 20-min ischemic hearts were incubated at pH 7.2 at 37°C for the times indicated in the presence of 6.25 mM glutamate, 6.25 mM malate, 2.5 mM Pi, and Zn^{2+} and FCCP where indicated. The mitochondria were sonicated at pH 8.2 and SMP prepared from each sample, and these were assayed for ATPase activity and IF_1 content.

gesting that Zn^{2+} may have become concentrated in the matrix compartment to a small extent in these experiments. The experiment presented in Fig. 6B shows that ruthenium red had no effect on the concentration dependence of the effect of Zn^{2+} on ATPase activity in intact rabbit heart mitochondria, suggesting a lack of involvement of the Ca^{2+} uniporter in the transport of Zn^{2+} into the matrix compartment under the conditions employed.

DISCUSSION

In the present study it has been shown clearly for the first time that Zn^{2+} has a paradoxical effect on IF_1-ATPase interaction. As shown in Table I and in Figs. 1 and 2, incubation of either rabbit heart IF_1-depleted SMP in the presence of added IF_1 and Zn^{2+} or of intact rabbit heart mitochondria in the presence of Zn^{2+} resulted in a fully active ATPase even at pH 6.4 while, at the same time, it caused nearly full IF_1 binding to the membranes. At pH 8.2, Zn^{2+} still caused a nearly full binding of IF_1 to the membranes without a concomitant inhibition of the ATPase. Moreover, the noninhibitory IF_1 binding which occurred in the presence of Zn^{2+} required the presence of at least trace amounts of MgATP and, in this latter respect, the Zn^{2+}-induced nonfunctional binding of inhibitor resembled the normal, inhibitory IF_1 binding which occurs in the absence of Zn^{2+}. That is, the MgATP dependence of both the inhibitory and noninhibitory binding of IF_1 in the absence and presence of Zn^{2+}, respectively, suggests that, in both cases, IF_1 became bound to the F_1-ATPase. It should be said, however, that the Zn^{2+}-induced noninhibitory IF_1 binding observed in the present study presumably never occurs under physiological conditions.

The concept of noninhibitory or nonfunctional IF_1 binding is not new. It is an idea that was first suggested by Van de Stadt *et al.* (1973) and again by Dreyfus *et al.* (1981). We reintroduced the idea as a result of observations on the time courses of ATPase

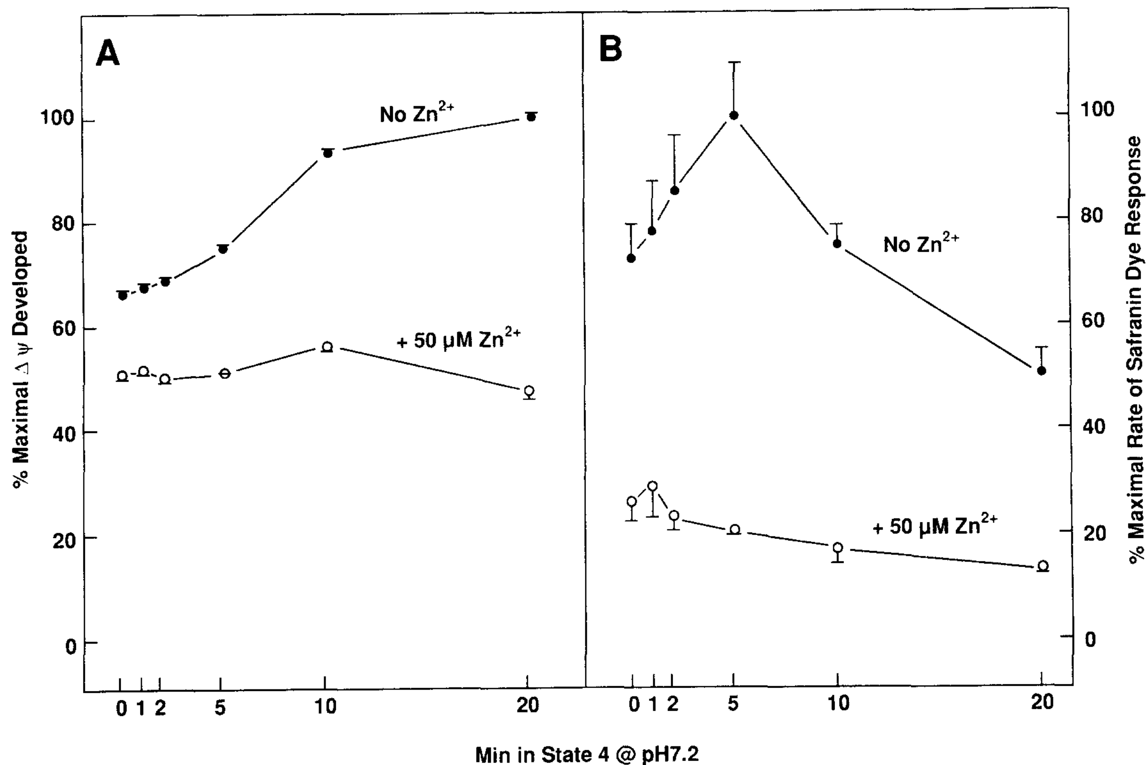

Fig. 5. The effect of 50 μM Zn^{2+} on the maximal membrane potential developed and held after 1 min (A) and on the rate of the safranin dye response (B) during the state 4 incubation (reenergization) of 20-min ischemic rabbit heart mitochondria. Intact rabbit heart mitochondria from 20-min ischemic hearts were incubated at pH 7.2 at 37°C in the presence of 6.25 mM glutamate, 6.25 mM malate, and 2.5 mM Pi. Aliquots were taken at the times indicated and membrane potential estimated using the safranin dye uptake technique.

activation versus inhibitor release during the energization of ischemic dog heart mitochondria (Rouslin, 1987a) and, again, as a result of observations on the effects of Ca^{2+} on ATPase activity versus IF_1 release during the sonication of ischemic-reenergized dog heart mitochondria (Rouslin and Broge, 1989a). In our studies on time courses of ATPase activation versus inhibitor release during the reenergization of ischemic dog heart mitochondria, we observed that, under certain specific conditions, ATPase activation appeared to be a more rapid process than the physical release of inhibitor from the membranes into the soluble phase (Rouslin, 1987a), suggesting that the release of IF_1 from noninhibitory docking sites can be a slower process than IF_1 release from activity regulatory sites. In our studies on Ca^{2+} effects, we reported that, like Zn^{2+}, Ca^{2+} also had a paradoxical effect on IF_1-ATPase interaction in that Ca^{2+} enhanced somewhat the binding of IF_1 to the ATPase while, at the same time, it produced a moderate increase in ATPase activity (Rouslin and Broge, 1989a). An important difference between the effects of Ca^{2+} reported by us earlier and those of Zn^{2+} reported here is that, while the effects of Ca^{2+} were partial or fractional, those of Zn^{2+} are fairly complete and, thus, much more convincing.

Both our former studies on Ca^{2+} effects as well as the data presented here on Zn^{2+} lead to the conclusion that ATPase activity is regulated either by the specific conformation of the IF_1 which is bound to the enzyme, or, alternatively, by the specific site on the ATPase to which the IF_1 is bound, rather than simply by the amount of IF_1 which is bound to the enzyme. Active and inactive conformations of IF_1 versus different sites of IF_1 binding may be thought of as alternative models which can explain our findings with Zn^{2+}. The currently available data do not allow one to choose between these two models. In the model involving active versus inactive forms of IF_1, one can imagine that Zn^{2+} binds only to the deprotonated or inactive form of the free inhibitor and also causes the inactive inhibitor to form noninhibitory complexes with the ATPase. Thus, Zn^{2+} may act like H^+ in its effect on IF_1 binding to the enzyme, while, at the same

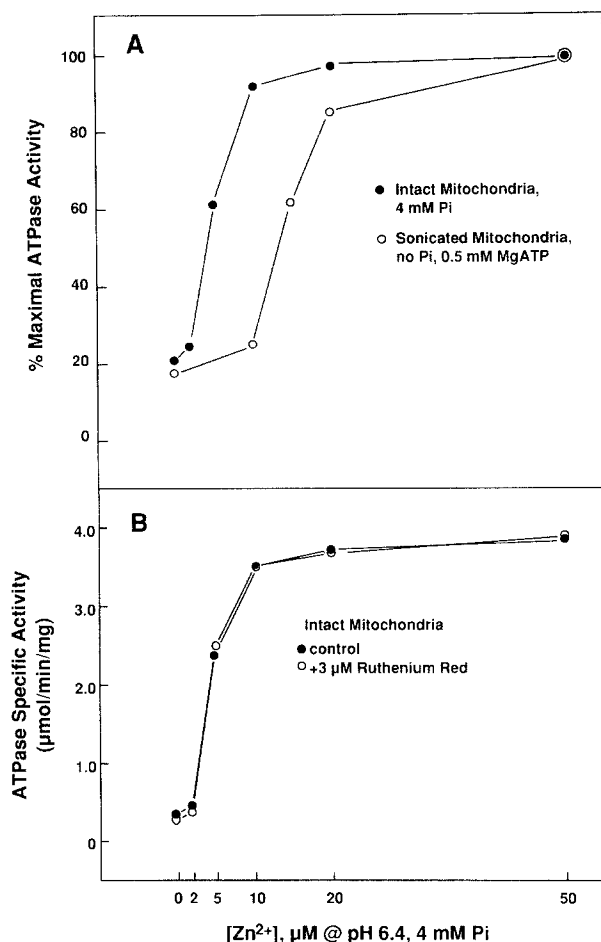

Fig. 6. (A) The effect of varying Zn^{2+} concentration on the formation of functional IF_1-ATPase complexes in intact rabbit heart mitochondria at pH 6.4 in the presence of 4 mM Pi (solid circles) and in sonicated rabbit heart mitochondria at pH 6.4 in the presence of 0.5 mM MgATP (open circles). Intact or sonicated rabbit heart mitochondria were incubated for 20 min at 37°C at the Zn^{2+} concentrations indicated and assayed for ATPase activity as sonicated mitochondria. (B) The effect of 3 μM ruthenium red upon the effect of Zn^{2+} concentration on the formation of inhibitory IF_1-ATPase complexes in intact rabbit heart mitochondria at pH 6.4. Intact rabbit heart mitochondria were preincubated for 6 min at 37°C at pH 6.4 in the presence of 4 mM Pi in the absence and presence of 3 μM ruthenium red and then further incubated for 20 min at 37°C at the Zn^{2+} concentrations indicated under the same conditions. Sonicated mitochondria were prepared from each sample and assayed for ATPase activity.

time, preventing IF_1-mediated ATPase inhibition. Indeed, it was proposed earlier that the inactivation of free IF_1 by Zn^{2+} or Cd^{2+} may involve interference with histidine protonation by these metal ions (Khodjaev *et al.*, 1990) and thus with the pH-dependent and time-dependent conformational transition involved in the activation of the inhibitor that has been reported for the IF_1 from baker's yeast (Fujii *et al.*, 1983) and for the beef heart inhibitor (Panchenko and Vinogradov, 1985). However, such a mode of action for Zn^{2+} and Cd^{2+} on the inhibitor remains to be demonstrated experimentally.

Alternatively, Zn^{2+} or Cd^{2+} and, to a lesser extent, Ca^{2+} may be thought of as enhancing the binding of IF_1 to a noninhibitory IF_1 docking site on the ATPase while preventing IF_1 interaction at a second presumably closely adjacent inhibitory site on the enzyme. Such effects could be due either to direct effects of Zn^{2+} on either the free inhibitor or on the free enzyme or possibly on some susceptible transition form of the IF_1-ATPase complex. The experiment presented in Fig. 3 in which Zn^{2+} was added after an inhibitory IF_1-ATPase complex was already established, suggests that once a functional IF_1-ATPase complex is formed, the subsequent introduction of Zn^{2+} only very slowly inactivities the IF_1 which is already functionally bound to the enzyme.

The data on the pH-induced conformational transition that IF_1 from yeast and bovine heart can undergo suggest that this transition may be a relatively slow, time-dependent process (Fujii *et al.*, 1983; Panchenko and Vinogradov, 1985). If this is the case in intact mitochondria, then this conformational transition may be too slow to be of regulatory significance. On the other hand, once anchored to the enzyme either through a noninhibitory docking site or in a noninhibitory conformation, it is quite possible that this or other conformational transitions or site interactions that the inhibitor may be able to participate in may come under the influence of other factors such as the transmembrane proton gradient and energy-dependent effects on ATPase conformation. Thus, within the IF_1-ATPase complex, the conformational transitions that IF_1 can undergo may become much more rapid, thereby assuming regulatory importance. Zn^{2+} may act by stabilizing the "docking site" interaction, transforming this initial IF_1-ATPase interaction into a pH-independent process possibly through interaction with a histidine on the free inhibitor. Zn^{2+} may additionally prevent the second or inhibitory IF_1-ATPase interaction either as a further consequence of its initial conformational effect on the inhibitor or by preventing a further conformational change in the inhibitor such as a switch-like movement on the surface of the ATPase. Alternatively, Zn^{2+} may interact directly with the inhibitory site on the enzyme, thereby blocking the inhibitory bind-

ing of IF_1 at the second site. Only high-resolution structural studies of the complex under appropriate diagnostic conditions are likely to answer these questions.

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