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Novel Difference in IF_1 Reactivity to Zn^{2+} in Rabbit versus Rat Cardiomyocytes, Mitochondria, and Submitochondrial Particles[1]

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Zn^{2+} has a paradoxical effect on IF_1-ATPase interaction in cardiac muscle mitochondria in so-called slow heart-rate mammalian species like rabbit. In such slow heart-rate mammalian species, it completely prevents IF_1-mediated ATPase inhibition regardless of pH while concomitantly causing full IF_1 binding to the ATPase, again, regardless of pH (Rouslin *et al.* (1993) *J. Bioenerget. Biomembr.* **25,** 297–306). While our earlier study suggested that there are two kinds of IF_1-ATPase interaction, a docking interaction and an ATPase inhibitory interaction with Zn^{2+} promoting docking and interfering with inhibition, it did not yield information on whether Zn^{2+} interacted primarily with IF_1, with the ATPase, or with both. In the present study we show that, in contrast to its effects in rabbit cardiomyocytes, mitochondria, and SMP in which Zn^{2+} fully blocked IF_1-mediated ATPase inhibition, Zn^{2+} actually enhanced ATPase inhibition in rat cardiomyocytes, although the extent of this effect was limited by the low level of IF_1 in rat cardiomyocytes. Moreover, Zn^{2+} had no effect on IF_1-mediated ATPase inhibition in rat heart mitochondria and, as suggested by inter- and intra-species IF_1 binding to SMP, the different effects of Zn^{2+} in rabbit versus those in rat appear to be mediated primarily through the different reactivities of rabbit and rat IF_1 to Zn^{2+}.

It has been known for many years that, among divalent cations, Mg^{2+} and Mn^{2+} can provide effective support for IF_1-mediated mitochondrial ATPase inhibition, while Zn^{2+} and Co^{2+} do not (1). Later work showed that Zn^{2+} and Cd^{2+} were particularly effective in interfering with IF_1-mediated ATPase inhibition (2) and it was proposed that Zn^{2+} may bind primarily to the deprotonated or inactive form of IF_1 in the IF_1-ATPase complex rendering the IF_1 in the complex noninhibitory (3). While this mechanism remains to be demonstrated experimentally, it is not inconsistent with our earlier work in this area (4).

In this earlier study (4) we demonstrated that, in rabbit heart mitochondria, Zn^{2+} had the paradoxical effect of preventing MgATP-dependent, IF_1-mediated ATPase inhibition regardless of pH while, at the same time, its presence caused full IF_1 binding to the membranes. While we had demonstrated earlier that Ca^{2+} had a similar effect on IF_1-ATPase interaction, although it was only a fractional effect (5), the much more dramatic effect of Zn^{2+} allowed us to postulate that there appear to be two distinct kinds (or sites) of IF_1-ATPase interaction, IF_1 docking and IF_1 inhibitory binding. Thus Zn^{2+} promotes IF_1 docking, but interferes with IF_1 inhibitory binding to the ATPase (4).

The present study was begun because of the unexpected finding that, while Zn^{2+} produced maximal ATPase activity in intact rabbit cardiomyocytes, in contrast, it appeared to actually enhance somewhat the inhibition of the mitochondrial ATPase in rat cardiomyocytes. Thus there was clearly something different about the interaction of Zn^{2+} with either or both the IF_1 and the ATPase in the rat heart cell system. Further analysis suggested that it was rat heart

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IF_1, itself, that was primarily responsible for the different effect of Zn^{2+} in this species. Implications of these findings are discussed.

MATERIALS AND METHODS

Preparation and incubation of rabbit and rat cardiomyocytes. Rabbit and rat cardiomyocytes were prepared using a modification of the method of Silver et al. (6). One-and-one-half kg male New Zealand White rabbits were heparinized i.v. and then anesthetized with pentobarbital sodium i.v. to effect. Three hundred g male Sprague-Dawley rate were heparinized i.v. and then anesthetized with pentobarbital sodium i.p. to effect. In each experiments, the heart was then removed and rinsed in Sanquinetti buffer containing 132 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 5 mM glucose, 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES)-NaOH, pH 7.4, plus one unit/ml heparin at 37°C. While the heart remained immersed in the above buffer, the aorta was cannulated. The heart, no longer immersed in buffer, was then perfused retrogradely at 10 to 12 ml/min using a peristaltic pump with Ca^{2+}-free minimal-essential-medium (MEM) (Joklik modified) bubbled with 95% O_2, 5% CO_2 containing, 10 mM HEPES-NaOH, pH 7.4, one unit/ml heparin and 30 μM ethylene glycol-bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) for 4 to 5 min at 37°C. The heart was then perfused with MEM (Joklik modified) containing 10 mM HEPES-NaOH, pH 7.4, 0.1 % fatty acid-free BSA, 1.6% donor calf serum and 100 units/ml of each of collagenase 1 and 2 (Worthington) for 2 min. Thereafter the final concentration of Ca^{2+} was increased by 50 μM and the buffer to was filtered and recirculated. After approximately an additional 25 min of perfusion with digestion, the heart was removed from the cannula, trimmed of the atria, great vessels and fat and the remaining tissue sliced into strips. The tissue strips were then aggitated in MEM (Joklik's modified) containing 0.1 % bovine serum albumin (BSA), 10 mM HEPES-NaOH, pH 7.4, and 80 μM Ca^{2+} without additional collagenase at 37°C. Remaining undigested tissue fragments were removed and placed back into the collagenase-containing buffer for 5 to 10 min after which the collagenase was diluted approximately four-fold with MEM (Joklik's modified) containing 0.1 % BSA, 10 mM HEPES-NaOH, pH 7.4, and 80 μM Ca^{2+}. The process was repeated twice on remaining undigested tissue fragments. The three aliquots of cells produced were pooled, resuspended in the above buffer and allowed to settle out into a loose pellet. The supernatant was then removed and the cells resuspended in 35 ml of the same buffer but now containing 4% BSA. The cells were then filtered through a 292 μm polyethylene mesh and allowed to settle through the above buffer. The cell settling procedure was repeated three times. Those cells that didn't settle out in approximately 5 min in each settling step were discarded. The cells were then transferred to Sanquinetti buffer containing 80 μM Ca^{2+} which had been saturated with 100% O_2 and allowed to settle out at room temperature. This latter step was then repeated twice.

In vitro incubations of rabbit and rat cardiomyocytes. The cells were divided into an appropriate number of equal aliquots for a given experimental design and allowed to settle out. The supernatant was removed and the cells then resuspended at approximately 10 mg of cell protein per 0.4 ml of buffer. The cells were then incubated with gentle shaking at 37°C in Sanquinetti buffer with 10 mM 2-(N-morpholino)ethane-sulfonic acid (MES)-NaOH) at pH pH 6.4 in the presence of 2.5 mM NaCN minus or plus 5 mM $ZnSO_4$ (Fig. 1) or the amount of Zn^{2+} indicated (Fig. 2). At the conclusion of the incubation, the cell aliquots were suspended in 4 ml of ice-cold 0.25 M sucrose, 10 mM 3-(N-morpholino)ethanesulfonic acid (MOPS)-KOH, pH 7.2 and sonicated 10s.

Preparation and incubation of rabbit and rat heart mitochondria and submitochondrial particles and preparation of pure bovine IF_1 and of rabbit and rat IF_1-containing extracts. Rabbit and rat heart mitochondria were prepared by Polytron homogenization as described previously (7-10). For the experiments on isolated mitochondria presented in Fig. 3, mitochondria were incubated at approximately one mg/ml for 20 min at 37°C in 0.25 M sucrose, 4 mM Pi, 20 mM MES-KOH, pH 6.4 at the Zn^{2+} concentrations indicated. At the conclusion of the incubations, the mitochondria were centrifuged at 17,400 $\times$ g for 10 min and resuspended in ice-cold 0.25 M sucrose, 10 mM MOPS-KOH, pH 7.2 and sonicated using three 10s bursts.

IF_1-depleted rabbit heart particles used in the experiments depicted in Table I were prepared essentially by the same method used to prepare ''regular'' submitochondrial particles except that sonication was carried out at pH 9.0 in the presence of 1.0 mM MgATP and they were centrifuged after sonication for 60 min at 226,000$\times$g. The low speed centrifugation step normally employed for the removal of large membrane fragments was eliminated from the procedure. The procedure served to strip away at least 90% of the endogenous IF_1 present on the particles. Rat heart SMP used in the experiments depicted in Table I were prepared as described previously (9-11). Briefly, rat heart SMP were prepared in large batches from 50 to 100 rat hearts by sonication of rat heart mitochondria in 0.25 M sucrose, 1 mM EGTA, 20 mM MOPS-KOH, pH 7.2 using two 10 s bursts. The sonicated particles were centrifuged for 15 min at 32,000 $\times$ g at 20°C and the pellets discarded. The supernatants were then centrifuged for 60 min at 226,000 $\times$ g at 20°C and the pellets resuspended at 20°C in 0.25 M sucrose, 5 mM MOPS-KOH, pH 7.2 at 1.0 mg/ml. The particles were frozen in small aliquots in liquid nitrogen.

Homogeneously pure bovine heart IF_1 was prepared by the method of Pullman (12) and frozen in several small aliquots. A single standard pure bovine heart IF_1 preparation was used for the entire study. Rabbit and rat heart IF_1-containing extracts used for rebinding to IF_1-depleted rabbit heart particles (Table I) were prepared by alkaline extraction of intact mitochondria as described earlier (10,11,13,14).

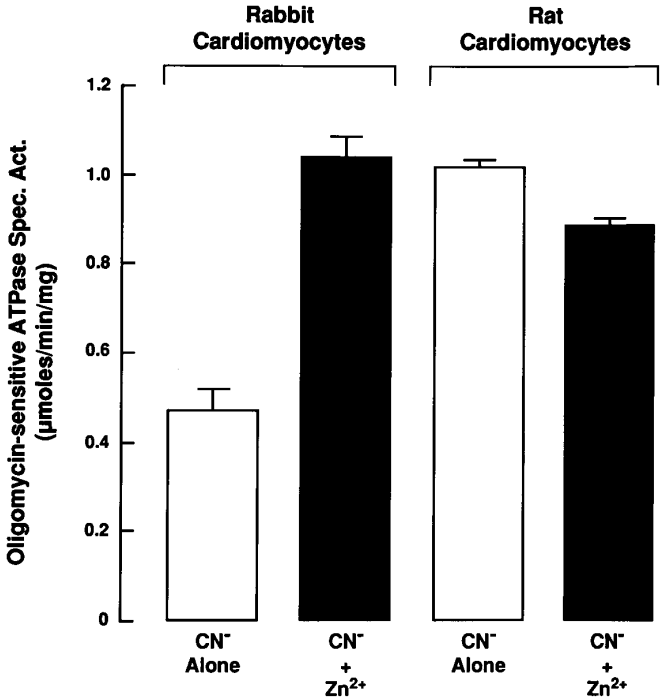

FIG. 1. Rabbit and rat cardiomyocyte mitochondrial ATPase activities. The cells were incubated for 40 min at 37°C at pH 6.4 at 25 mg/ml in media containing 2.5 mM NaCN, or NaCN plus 5 mM ZnSO4 and then sonicated for assay of oligomycin-sensitive ATPase activity. Experimental conditions are described in the text. The data are averages ± SEM of four separate determinations.

In the experiments presented in Table I, rabbit and rat particles were incubated with rabbit and rat IF_1-containing extracts for 20 min at 37°C in 0.25M sucrose, 0.5 mM MgATP, 20 mM MES-KOH, pH 6.4 minus and plus 50 μM $ZnSO_4$. The Zn^{2+}-containing samples were additionally preincubated for 5 min at room temperature at pH 7.2 to allow the Zn^{2+} to interact with both the IF_1 and the ATPase before IF_1-ATPase complex formation. In these experiments approximately 70% of the species-endogenous activity of rabbit heart mitochondrial IF_1 was combined with 100% of the species-endogenous activity of rabbit IF_1-depleted SMP ATPase or with an equal activity of rat heart SMP ATPase, and an activity of rat heart IF_1 equal to the activity of the rabbit heart IF_1 used was combined with the same activities of either rabbit or rat heart SMP ATPase used for the experiments with rabbit heart IF_1. However, while total IF_1 and particle ATPase activities were used to design these experiments, the results are expressed as specific activities.

Assay of oligomycin-sensitive ATPase activity and and protein. Mitochondrial ATPase activity was measured in sonicated rabbit and rat cardiomyocytes or in sonicated mitochondria and SMP using a modification of the method of Tzagoloff (15) as described by us previously (7-10). Briefly, the 1.0 ml reaction mixture contained approximately 45 μmol tris(hydroxymethyl)aminoethane (Tris)-SO_4, pH 7.8, 10 μmol $MgCl_2$ and 50 μl of sonicated mitochondria at 0.5 mg/ml or 100 μl of the RHMP incubation mixture described above. The reaction was started by the addition of 10 μmol ATP-Tris, run for 5 min at 30°C and stopped by the addition of 1 ml of 10% trichloroacetic acid followed by the assay of the Pi produced by the reaction. Specific activities were calculated as μmoles Pi/mg/min. Where present, oligomycin was used in the assay mixture at 2 μM. Cell and mitochondrial protein were estimated using the Lowry procedure (16).

RESULTS AND DISCUSSION

Figure 1 shows the effects of 40 min of acidotic (pH 6.4) chemical ischemia (2.5 mM CN^-) in the absence and presence of 5 mM Zn^{2+} on the activity of the mitochondrial ATPase in intact rabbit and rat cardiomyocytes. We had shown previously, in isolated rabbit heart mitochondria, that Zn^{2+} had the paradoxical effect of fully preventing MgATP-dependent, IF_1-mediated ATPase inhibition regardless of pH while, at the same time, its

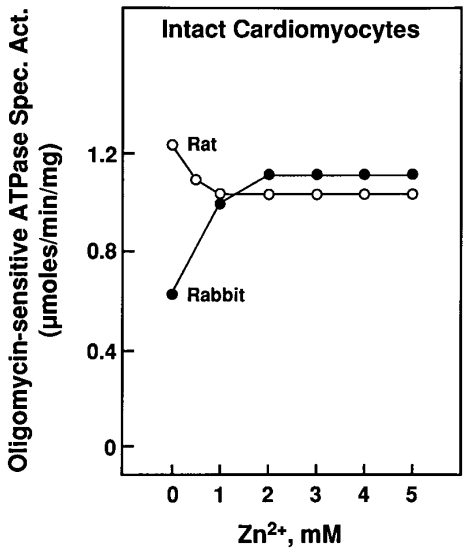

FIG. 2. Rabbit and rat cardiomyocyte mitochondrial ATPase activities. The cells were incubated for 40 min at 37°C at pH 6.4 at 25 mg/ml in media containing 2.5 mM NaCN and the concentration or NaCN indicated and then sonicated for assay of oligomycin-sensitive ATPase activity. Experimental conditions are described in the text.

presence caused full IF_1 binding to the membranes (4). In the present study Zn^{2+} had a similar effect in intact rabbit cardiomyocytes in that it fully prevented IF_1-mediated mitochondrial ATPase inhibition. In doing so it produced maximally uninhibited mitochondrial ATPase activities useful as a benchmark against which to compare the effects of other treatments on mitochondrial ATPase activity in rabbit heart cells. Thus, relative to the mitochondrial ATPase activity of Zn^{2+}-treated rabbit heart cells, rabbit cardiomyocytes made chemically ischemic and acidotic in the absence of Zn^{2+} showed nearly a 60% IF_1-mediated mitochondrial ATPase inhibition (Fig. 1), a degree of inhibition similar to that observed by us earlier in totally ischemic rabbit hearts (4,9,17-18).

While chemical ischemia with acidosis produced by incubating rabbit cardiomyocytes for 40 min at 37°C with 2.5 mM CN^- at pH 6.4 produced approximately a 60% inhibition of the mitochondrial ATPase in rabbit heart cells, it produced no ATPase inhibition in rat cardiomyocytes. This result is consistent with the relative lack of mitochondrial ATPase inhibition reported by us earlier in totally ischemic rat hearts (9,18). However, of particular interest within the context of the present study is the finding that Zn^{2+} had apparent opposite effects on ATPase inhibition in rabbit and rat heart cells. Thus, while incubation in 5 mM Zn^{2+} fully prevented IF_1-mediated mitochondrial ATPase inhibition in rabbit cardiomyocytes, incubation of rat cardiomyocytes in 5 mM Zn^{2+} resulted in a moderate degree of mitochondrial ATPase inhibition (Fig. 1). The data presented in Fig. 2 confirm these results and show further that Zn^{2+} concentrations of approximately 1 to 2 mM were required to produce these opposite effects in intact cardiomyocytes of the two species. The effect of Zn^{2+} in rat cardiomyocytes is fairly small primarily because of the low level of IF_1 present in hearts of this species (9,18,19).

As mentioned above, we had reported earlier that, in isolated rabbit heart mitochondria, Zn^{2+} had the paradoxical effect of fully preventing MgATP-dependent, IF_1-mediated ATPase inhibition regardless of pH while, at the same time, its presence caused full IF_1 binding to the membranes (4). Figure 3 once again reports the effects of varying Zn^{2+} concentration on the ATPase activity of isolated rabbit heart mitochondria incubated at

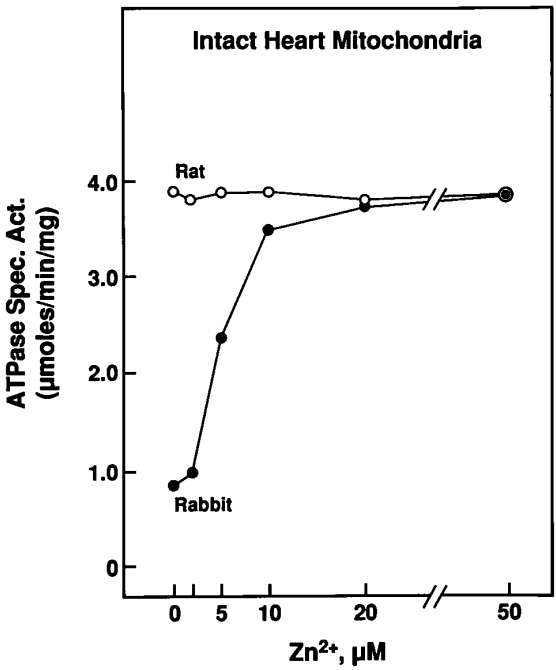

FIG. 3. ATPase activities of rabbit and rat heart mitochondria. The mitochondria were incubated at approximately 1 mg/ml for 20 min at 37°C in 0.25 M sucrose, 4 mM Pi, 20 mM MES-KOH, pH 6.4 at the Zn^{2+} concentrations indicated. At the conclusion of the incubations, the mitochondria were centrifuged at 17,400 × g for 10 min and resuspended in ice-cold 0.25 M sucrose, 10 mM MOPS-KOH, pH 7.2 and sonicated using three 10s bursts. They were then assay for ATPase activity as described in the text.

pH 6.4. It also shows that there was no effect of Zn^{2+} on the ATPase activity of rat heart mitochondria treated identically. Once again, the reason for the lack of an effect of Zn^{2+} on the ATPase activity of rat heart mitochondria is the low level of IF_1 present in heart mitrochondria of this species (9,17,18). However, in that there is approximately 20% as much IF_1 present in rat heart mitochondria as in rabbit heart mitochondria, one would have expected to see a fractional effect of Zn^{2+} in the rat. A comparison of the Zn^{2+} concentrations required to produce maximal effects on mitochondrial ATPase activity in whole cells (Fig. 2) versus intact mitochondria (Fig. 3) shows that approximately 100-fold less Zn^{2+} is required in isolated mitochondria. The difference is presumably due to the adsorption of most of the Zn^{2+} by cell surface membranes in whole cells.

Table I presents the results of species-homologous and species-heterologous combinations of rabbit and rat heart IF_1 and ATPase in the absence and presence of 50 μM Zn^{2+}. As described under Materials and Methods, these experiments were carefully constructed to normalize the activities of the two species of IF_1 and of particle ATPase to one another. In addition the same experiment was conducted using purified bovine heart IF_1 on rabbit heart particles. As may be seen, Zn^{2+} interfered with IF_1-mediated ATPase inhibition by rabbit heart IF_1 to a considerably greater extent than it did with ATPase inhibition by the rat heart inhibitor. Moreover, the different level of interference of IF_1-mediated ATPase inhibition by Zn^{2+} was clearly related to the species source of the IF_1, not to the species source of the ATPase. Finally, Zn^{2+} interfered with IF_1-mediated ATPase inhibition by pure bovine heart IF_1 to essentially the same extent as it did with rabbit IF_1. In the presence of a level of pure bovine IF_1 normalized to the level of rabbit and rat IF_1 used in the experiments presented in Table I, 50 μM Zn^{2+} increased the

TABLE I
Representative Species-Homologous and Species-Heterologous Combinations of Rabbit and Rat FoF_1-ATPase and IF_1 in the Absence and Presence of Zn^{2+}

Rabbit ATPase alone	6.51 (1.00)	Rat ATPase alone	3.94 (1.00)
+ 1× Rabbit IF_1	0.84 (0.13)	+ 1× Rabbit IF_1	1.05 (0.27)
+ 1× Rabbit IF_1 + Zn^{2+}	4.59 (0.71)	+ 1× Rabbit IF_1 + Zn^{2+}	2.78 (0.71)
Rabbit ATPase alone	6.51 (1.00)	Rat ATPase alone	3.94 (1.00)
+ 1× Rat IF_1	1.03 (0.16)	+ 1× Rat IF_1	0.95 (0.24)
+ 1× Rat IF_1 + Zn^{2+}	2.49 (0.38)	+ 1× Rat IF_1 + Zn^{2+}	1.44 (0.37)

Note. ATPase activities expressed as μmoles/min/mg. 1× rabbit IF_1 equals approximately 70% of the species-endogenous level of rabbit IF_1. 1× rat IF_1 equals approximately the same inhibitory activity used for rabbit IF_1.

ATPase specific activity to 80% of that of the rabbit particles in the absence of added IF_1. Thus, the bovine and rabbit inhibitors behaved much alike in this respect.

An analysis of the data presented in Table I suggests that the difference in the reactivities of the rabbit and rat inhibitors to Zn^{2+} were a matter of degree. Thus, on rabbit particles, the net reversal of ATPase inhibition by Zn^{2+} was approximately 2.5-fold greater for rabbit IF_1 than for rat IF_1.

Our earlier work with Zn^{2+} allowed us to postulate that there appeared to be two distinct kinds of IF_1-ATPase interaction, namely, IF_1 docking and IF_1 inhibitory binding. Thus, Zn^{2+} promoted IF_1 docking, while interfering with IF_1 inhibitory binding to the ATPase in rabbit heart mitochondria (4). As a variation on and extension of these earlier results, the present study suggests that Zn^{2+} interferes significantly less with the inhibitory binding of rat heart IF_1 than it does with the inhibitory binding of the rabbit heart inhibitor regardless of the species source of the ATPase and that the bovine inhibitor behaves much like the rabbit inhibitor. While the present results are not inconsistent with an enhancement of rat IF_1 docking by Zn^{2+}, they say nothing directly about the effects of Zn^{2+} on rat IF_1 docking.

The primary structure of rabbit IF_1 is not published at the time of this writing, but in that the rabbit and bovine inhibitors behave alike with respect to their reactivity to Zn^{2+}, it may be instructive to compare the published sequences of the bovine (20) and rat (21) inhibitors, particularly with respect to the histidine residues each contains in relation to the above mentioned idea that Zn^{2+} may bind primarily to the deprotonated form of IF_1 within the IF_1-ATPase complex (3). The distribution of histidines in the bovine and rat inhibitors is virtually the same, i.e., both contain two pairs of histidines at positions 48,49 and 55,56 and a single histidine at position 70. The rat inhibitor additionally possesses a single histidine residue at position 82. While the histidines in the two species of inhibitor may conceivably have different reactivities to Zn^{2+} due perhaps to different exposures within the inhibitor-enzyme complex, based upon IF_1 primary structures alone, this seems unlikely. Thus, while the data in Table I suggest that Zn^{2+} may react directly with IF_1, the different reactivities of the rabbit (bovine) and rat inhibitors to Zn^{2+} remain difficult to explain simply in terms of primary structural differences between the bovine (rabbit) and rat inhibitors.

REFERENCES

1. Horstman, L. L., and Racker, E. (1970) *J. Biol. Chem.* **245,** 1336–1344.
2. Chernyak, B. V., Khodjaev, E. Y., and Kozlov, I. A. (1985) *FEBS Lett.* **187,** 253–256.
3. Khodjaev, E. Y., Komarnitsky, F. B., Capozza, G., Dukhovich, V. F., Chernyak, B. V., and Papa, S. (1990) *FEBS Lett.* **272,** 145–148.
4. Rouslin, W., Broge, C. W., and Chernyak, B. V. (1993) *J. Bioenerget. Biomemb.* **25,** 297–306.

5. Rouslin, W., and Broge, C. W. (1989) *Arch. Biochem. Biophys.* **275,** 385–394.
6. Silver, L. H., Hemwall, E. L., Marino, T. A., and Houser, S. R. (1983) *Am. J. Physiol.* **245,** H891–H896.
7. Rouslin, W. (1983) *J. Biol. Chem.* **258,** 9657–9661.
8. Rouslin, W. (1983) *Am. J. Physiol.* **244,** H743–H748.
9. Rouslin, W. (1987) *Am. J. Physiol.* **252,** H622–H627.
10. Rouslin, W. (1987) *J. Biol. Chem.* **262,** 3472–3476.
11. Rouslin, W., and Pullman, M. E. (1987) *J. Mol. Cell. Cardiol.* **19,** 661–668.
12. Pullman, M. E. (1986) *in* Methods in Enzymology (Fleischer, S., and Fleischer, B., Eds.), Vol. 125, pp. 460–470, Academic Press, Orlando, FL.
13. Pullman, M. E., and Monroy, G. C. (1963) *J. Biol. Chem.* **238,** 3762–3769.
14. Frangione, B., Rosenwasser, R., Penefsky, H. S., and Pullman, M. E. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7403–7407.
15. Tzagoloff, A., Byington, K. H., and MacLennan, D. H. (1968) *J. Biol. Chem.* **243,** 2405–2412.
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
17. Rouslin, W., and Broge, C. W. (1990) *Arch. Biochem. Biophys.* **280,** 103–111.
18. Rouslin, W., Frank, G. D., and Broge, C. W. (1995) *J. Bioenerget. Biomembr.* **27,** 117–125.
19. Rouslin, W., and Broge, C. W. (1993) *Arch. Biochem. Biophys.* **303,** 443–450.
20. Frangione, B., Rosenwasser, R., Penefsky, H. S., and Pullman, M. E. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7403–7407.
21. Lebowitz, M. S., and Pedersen, P. L. (1993) *Arch. Biochem. Biophys.* **301,** 64–70.