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MINI-REVIEW

Regulation of the Mitochondrial ATPase *In Situ* in Cardiac Muscle: Role of the Inhibitor Subunit

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Abstract

The mitochondrial F_1-ATPase inhibitor protein, IF_1, binds to β subunits of the F_1-ATPase both *in vitro* and *in situ* under nonenergizing conditions, i.e., under conditions that allow a net hydrolysis of ATP by the mitochondrial ATPase to take place. This reversible IF_1 binding occurs in a wide variety of cell types including (anaerobic) baker's yeast cells and (ischemic) mammalian cardiomyocytes under conditions that limit oxidative phosphorylation. The binding of inhibitor results in a marked slowing of ATP hydrolysis by the undriven mitochondrial ATP synthase. An apparent main function of this reversible IF_1 binding, at least in cells that undergo aerobic–anaerobic switching, is the mitigation of a wasteful hydrolysis of ATP produced by glycolysis during anoxic or ischemic intervals, by the mitochondrial ATPase. While this apparent main function is probably of considerable importance in cells that normally either can or must undergo aerobic–anaerobic switching such as baker's yeast cells and skeletal myocytes, one wonders why a full complement of IF_1 has been retained in certain cells that normally do not undergo such aerobic–anaerobic switching, cells such as adult mammalian cardiomyocytes of many species. While some mammalian species have, indeed, not retained a functional complement of IF_1 in their cardiomyocytes, those that have can benefit significantly from its presence during intervals of myocardial ischemia.

Key Words: Oxidative phosphorylation; F_1-ATPase; IF_1; ATPase inhibitor protein; ATPase regulation *in situ*; cardiac muscle; myocardial ischemia; cell acidosis; mitochondrial matrix pH; glycolysis.

Introduction: A Selective Retrospective

The F_1-ATPase inhibitory subunit, now referred to as IF_1, was first isolated

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from bovine cardiac muscle mitochondria nearly 30 years ago (Pullman and Monroy, 1963). When complexed with soluble F_1-ATPase, the inhibitory factor was found to markedly inhibit ATP hydrolysis by the enzyme in a pH-dependent manner but did not interfere with F_1-dependent oxidative phosphorylation (Pullman and Monroy, 1963). However, these pioneering investigators were understandably not yet able to formulate a general mode of action for the inhibitor or to demonstrate a regulatory role for it either in oxidative phosphorylation coupling or respiration.

Seven years later, in 1970, Ernster and coworkers provided a pivotal and, for me, a powerfully heuristic insight into the mode of action of IF_1 (Asami *et al.*, 1970). Working with bovine heart material, they were the first to suggest that the inhibitor might be unidirectional in its action, controlling only the reverse reaction rate of the ATP synthase, i.e., the rate of ATP hydrolysis and thus the backflow of energy from ATP to mitochondrial energy-linked functions. Put another way, these workers provided the first evidence that IF_1 could slow ATP hydrolysis by the mitochondrial ATPase under nonenergizing conditions without intefering with coupled oxidative phosphorylation occurring upon the resumption of respiratory activity. Much later, in 1980, it was shown that the inhibitor binds to one or more β subunit-localized sites on the F_1-ATPase (Klein *et al.*, 1980), probably including a region near the C-terminus of the β subunit (Jackson and Harris, 1988), and that its binding appears to decrease the proton conductivity of the F_0F_1-ATPase (Guerrieri *et al.*, 1987a).

In 1973, three years after the pivotal paper by Ernster and coworkers cited above, Van de Stadt *et al.* (1973) provided the first evidence, though still of a preliminary nature, that the inhibitor becomes displaced from the enzyme when energy is generated by substrate oxidation. They showed that the low endogenous ATPase activity of bovine heart SMP became activated severalfold when the SMP were incubated with NADH. While they correctly inferred that their activity measurements reflected a release of inhibitor from the enzyme, a demonstration of this phenomenon which included direct measurements of inhibitor being released from SMP during energization did not occur for another eight years (Schwertzmann and Pedersen, 1981). These latter workers demonstrated parallel time courses of ATPase activity activation and inhibitor protein release into the supernatant during the incubation of rat liver IF_1-supplemented rat liver SMP with NADH. Both the ATPase activity activation and inhibitor release were completely blocked when FCCP was present in the incubation medium. As discussed later on, rat liver, like rat cardiac muscle, contains fairly low levels of IF_1. It was this fact that may have made it necessary for these workers to employ rat liver SMP supplemented with an excess of purified rat liver inhibitor in their energization time course experiments (Schwertzmann and Pedersen, 1981). Thus,

while rat liver may not have been the best possible choice for the demonstration of a large, IF_1-mediated ATPase activity modulation reflecting changes in endogenous IF_1 binding, the conclusions drawn from work with IF_1-supplemented rat liver SMP are undoubtedly quite valid.

In 1983, two years after the report by Schwertzmann and Pedersen, we presented the first preliminary evidence for the operation of a reversible, IF_1-mediated mitochondrial ATPase inhibition *in situ* in an intact vertebrate organ, the canine heart (Rouslin, 1983), although two reports had appeared previously, one on IF_1-mediated changes in mitochondrial ATPase activity in response to catabolite repression in the fission yeast, *S. pombe* (Lloyd and Edwards, 1976), and a second on a reversible inactivation of azide-sensitive ATPase activity in ischemic and reperfused rabbit hearts (Godin *et al.*, 1980). The latter study included an examination of a variety of enzyme activities including the Na^+, K^+-ATPase, and the authors postulated that the inhibition and reactivation of the azide-sensitive mitochondrial ATPase activity might be due to changes in the level of some metabolite such as long-chain fatty acyl-CoA esters during ischemia and postischemic reperfusion (Godin *et al.*, 1980).

In our 1983 report (Rouslin, 1983), we showed that a reversible inhibition and reactivation of the mitochondrial ATPase occurred during canine myocardial ischemia and reperfusion, respectively, and, on the basis of a variety of lines of evidence, we proposed that the observed ATPase inhibition was due to the reversible binding of IF_1 to the enzyme *in situ* (Rouslin, 1983). Another four years elapsed, however, before we confirmed these preliminary studies with direct measurements of the IF_1 contents of SMP derived from control and ischemic canine cardiac muscle (Rouslin and Pullman, 1987). Such comparative IF_1 content measurements performed by ourselves and others were possible because the degrees of ATPase inhibition and IF_1 binding achieved during perturbations produced either *in vitro* or *in situ* are effectively preserved during subsequent SMP isolation and ATPase and IF_1 content assays, provided that the particles are neither reenergized nor exposed to other factors that might promote either further inhibitor binding or loss into the supernatant during the interval between the initial perturbation and the eventual assay.

Our studies on reversible changes in IF_1 binding *in situ* during canine myocardial ischemia were both accompanied (Rouslin *et al.*, 1986) and followed (Rouslin, 1988; Rouslin *et al.*, 1990) by *in situ* physiological studies which attempted to assess the quantitative contribution made by the reversible IF_1-mediated inhibition of the mitochondrial ATPase to the conservation of tissue ATP levels *in situ* in ischemic heart muscle. We found that, in totally ischemic canine hearts, IF_1-mediated mitochondrial ATPase inhibition contributed greatly to a slowing of the net rate of depletion of the largely

glycolytically generated tissue ATP pool (Rouslin *et al.*, 1986; Rouslin, 1988; Rouslin *et al.*, 1990). One central conclusion of both our biochemical and physiological studies on canine hearts was that the main function of the reversible mitochondrial ATPase inhibition appeared to be the mitigation of a wasteful hydrolysis of ATP produced by glycolysis during ischemic intervals, by the undriven mitochondrial ATP synthase. Our results with ischemic dog hearts have been confirmed by Jennings and coworkers although their data have not appeared at the time of this writing (Jennings and Reimer, 1991).

Our central conclusion about the physiological function of IF_1 has since been compellingly corroborated by studies with baker's yeast mutants (Yoshida *et al.*, 1990; Ichikawa *et al.*, 1990). Tagawa and coworkers have demonstrated that 9- and 15-kDa regulatory subunits appear to be required for the stable binding of IF_1 to the F_1-ATPase in *S. cerevisiae* (see Hashimoto *et al.*, 1990 for an overview of the work of Tagawa and coworkers in this area). While mutants lacking either the 9- or 15 k-Da subunits and/or IF_1, itself, showed no impairment of oxidative phosphorylation under energizing conditions, a collapse of membrane potential-induced ATP hydrolysis by the F_0F_1-ATPase of the mutants, but not by the ATPase of wild-type cells (Yoshida *et al.*, 1990; Ishikawa *et al.*, 1990). These pivotal findings of Tagawa and coworkers further underscore two concepts: first that IF_1 has an important regulatory function in the control of ATP hydrolysis by the mitochondrial ATPase under nonenergizing conditions, and second, that IF_1 is apparently neither a required factor for, nor, in all likelihood, a regulator of coupled oxidative phosphorylation.

For additional references on historical aspects and on other details of the field, the reader is referred to one or more earlier, and, in some cases, more comprehensive, reviews of the area (see, e.g., Cross, 1981; Pedersen *et al.*, 1981; Chernyak and Kozlov, 1986; Schwertzmann and Pedersen, 1986; Hashimoto *et al.*, 1990).

Factors Affecting Cardiac Muscle IF_1 Binding and Release *In Vitro* and *In Situ*

Bovine cardiac muscle IF_1 is a water-soluble, heat-stable polypeptide (Pullman and Monroy, 1963) containing 84 amino acid residues whose combined residue molecular weights add up to a polypeptide molecular weight of 9578 (Frangione *et al.*, 1981). The bovine heart inhibitor contains five histidine residues, four of which are arranged in two pairs (Frangione *et al.*, 1981), at least one of the five of which may function as a pH sensor. Glycolysis-driven cell acidification (Rouslin, 1988; Rouslin and Broge, 1989b; Rouslin and Broge, 1990; Rouslin *et al.*, 1990) characterizes most

systems under hypoxia or anoxia. The lack of oxygen produces a concomitant mitochondrial inner membrane deenergization resulting, in turn, in a relaxed, IF_1-receptive conformation of the F_0F_1-ATPase. The glycolysis-driven cell acidification is rapidly transmitted to the mitochondrial matrix compartment mediated primarily by the Pi^-/H^+ symporter activity of the mitochondrial phosphate carrier, at least in so-called slow heart-rate hearts (Rouslin and Broge, 1989b). This matrix space acidification presumably results, in turn, in a protonation of at least one of the five histidines of the beef heart inhibitor (Frangione *et al.*, 1981; Panchenko and Vinogradov, 1985), and, presumably, the one histidine (His 39) of the baker's yeast inhibitor (Matsubara *et al.*, 1981; Fujii *et al.*, 1983). Histidine protonation may thus be a component of, and possibly the initiating event in, the pH-induced conformational transition that accompanies the activation of both the beef heart and baker's yeast inhibitors (Fujii *et al.*, 1983; Panchenko and Vinogradov, 1985). Treatment of purified beef heart inhibitor with the histidine-modifying agent diethylpyrocarbonate blocks histidine protonation thereby inactivating its ATPase inhibitory function (Panchenko and Vinogradov, 1985; Guerrieri *et al.*, 1987b). However, the blocking of up to three of the five histidine residues of the beef inhibitor with diethylpyrocarbonate appears not to have affected its activity (Harris, 1985).

Complex formation between equal numbers of inhibitor molecules and F_0F_1-ATPase assemblies (Gomez-Fernandez and Harris, 1978) can occur under nonenergizing conditions under the control of a few critical matrix milieu conditions. While the main, rapidly changing, regulatory parameters appear to be inner membrane energization state ($\Delta\mu H^+$) and matrix space pH, other conditions that have been studied that may conceivably affect IF_1–enzyme interaction *in situ* include the concentrations of Mg^{2+} and ATP (Pullman and Monroy, 1963; Horstman and Racker, 1970; Galante *et al.*, 1981; Rouslin and Broge, 1989a), Ca^{2+} concentration (Yamada *et al.*, 1980; Yamada and Huzel, 1989; Rouslin and Broge, 1989a), and ionic strength (Van de Stadt and Van Dam, 1974).

During normal heart function, the mitochondrial ATP synthase is driven in the direction of net ATP synthesis, supplying in excess of 95% of all of the ATP utilized by the heart, the remaining small balance being provided by a low rate of aerobic glycolysis. In normally functioning heart muscle, cardiomyocyte cytosolic pH values are typically above neutrality between 7.0 and 7.4 and matrix pH values of actively respiring cardiac muscle mitochondria *in situ* are significantly more alkaline to the extent the ΔpH across the inner membrane is built up. Thus, while neither the energization state of the ATPase nor the prevailing matrix pH are conducive to IF_1 binding in normally functioning hearts, matrix Mg^{2+} (LaNoue *et al.*, 1981; Jung *et al.*, 1990) and matrix ATP (LaNoue *et al.*, 1981; Asimakis and Conti,

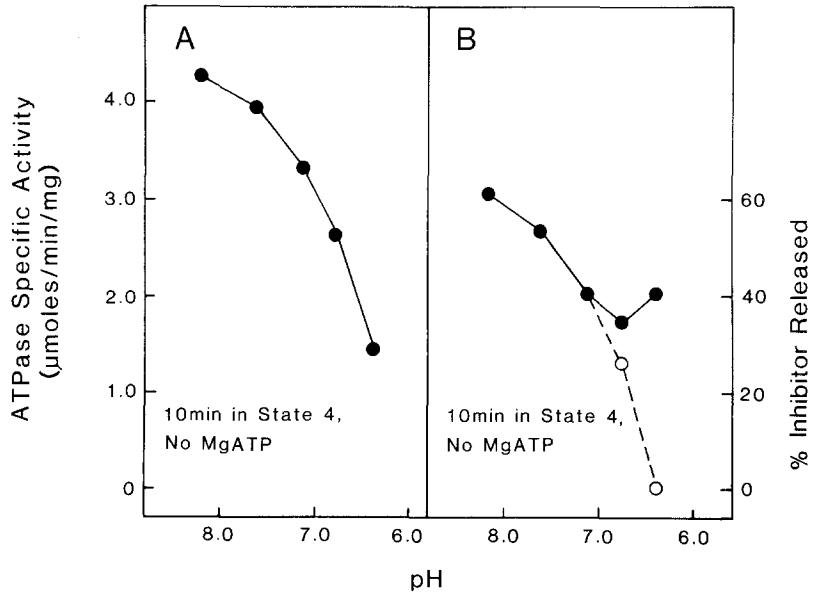

Fig. 1. (A) Effect of pH during sonication on the reactivation of the mitochondrial ATPase. Intact mitochondrial samples from 20-min ischemic canine heart muscle were reenergized for 10 min. At the conclusion of reenergization the samples were sonicated in 0.25 M sucrose and either 20 mM TAPS-KOH, pH 8.2, 20 mM MOPS-KOH, pH 7.6, 7.2 or 6.8 or 20 mM MES-KOH, pH 6.4. The reference control sample was at pH 6.4 and also contained 2 mM MgATP and 2 μM FCCP. SMP were then prepared from the sonicated samples and assayed for ATPase activity. (B) Effect of pH during sonication on the release of inhibitor protein. The mitochondrial samples were the same ones used for the experiment presented in (A). SMP were prepared from these mitochondria, and inhibitor-containing extracts were prepared from the SMP. These extracts were then assayed for their inhibitor content using our rat heart SMP titration procedure. The data points shown as open circles were calculated from the corresponding ATPase activity points presented in (A). Reproduced from Rouslin and Broge (1989a) with permission.

1984) levels are more than adequate to support IF_1 binding to the ATPase. Typical responses of cardiac mitochondrial ATPase activity and IF_1-ATPase interaction to pH and MgATP concentration pre presented in Figs. 1 and 2, respectively (from Rouslin and Broge, 1989a). As shown by a number of earlier studies (e.g., Horstman and Racker, 1970; Galante *et al.*, 1981), IF_1-mediated ATPase inhibition is promoted by decreasing pH between approximately 8.0 and 6.0 and requires the presence of submillimolar concentrations of Mg^{2+} plus ATP.

Physiological ionic strengths may not be particularly conducive to the binding of IF_1 to the ATPase, at least under conditions that have been used in a number of *in vitro* IF_1 binding studies (e.g., Van de Stadt and Van Dam, 1974), but the ionic strengths prevailing in the matrix milieu obviously do not

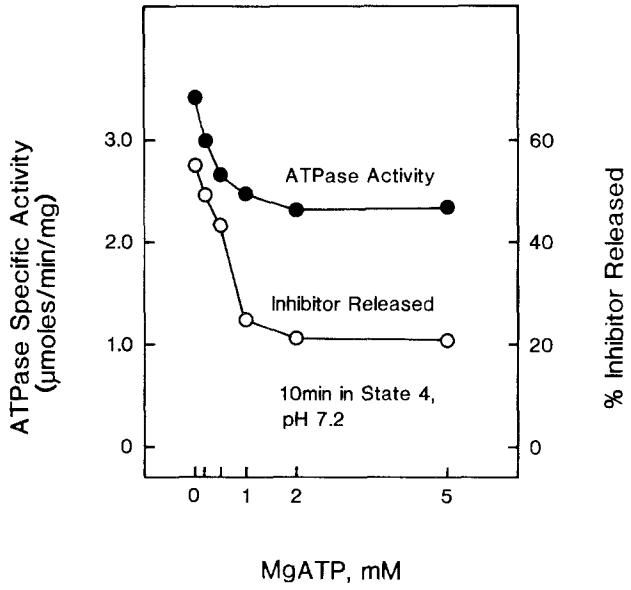

Fig. 2. Effect of MgATP concentration during sonication on the reactivation of the mitochondrial ATPase and on the release of inhibitor protein. Intact mitochondrial samples from 20-min ischemic canine heart muscle were reenergized for 10 min. At the conclusion of reenergization the samples were sonicated in 0.25 M sucrose and 20 mM MOPS-KOH, pH 7.2, at the MgATP concentrations indicated. The reference control sample also contained 5.0 mM MgATP and 2 μM FCCP. SMP were then prepared from the sonicated samples and assayed for ATPase activity (solid circles) and for inhibitor content (open circles) using our rat heart SMP titration procedure. Reproduced from Rouslin and Broge (1989a) with permission.

prevent IF_1 binding *in situ* in, for example, ischemic slow heart-rate mammalian hearts (Rouslin, 1983; Rouslin and Pullman, 1987).

In general, Ca^{2+}, in the submicromolar to micromolar range, appears to be an activator of mitochondrial ATPase activity and this activating effect is, in general, consistent with the observed Ca^{2+}-orchestrated activation of cardiac muscle contractility and mitochondrial dehydrogenase activities (see, e.g., Hansford, 1985). Exposure of mitochondria to *supra*-physiological Ca^{2+} concentrations, however, results in a suppression of oxidative phosphorylation in favor of the pumping of Ca^{2+} from the medium into the mitochondrial matrix (Rossi and Lehninger, 1964; Varcesi *et al.*, 1978). While a potentially massive accumulation of Ca^{2+} into the mitochondrial matrix may suppress the activity of the mitochondrial ATPase, possibly by an IF_1-mediated mechanism (Tuena de Gomez-Puyou *et al.*, 1980; Hillered *et al.*, 1983), caused either directly by an effect of increased matrix Ca^{2+} on IF_1 binding, or indirectly through an effect upon ATPase energization state, the effect of Ca^{2+} concentration over the physiological range on IF_1-mediated

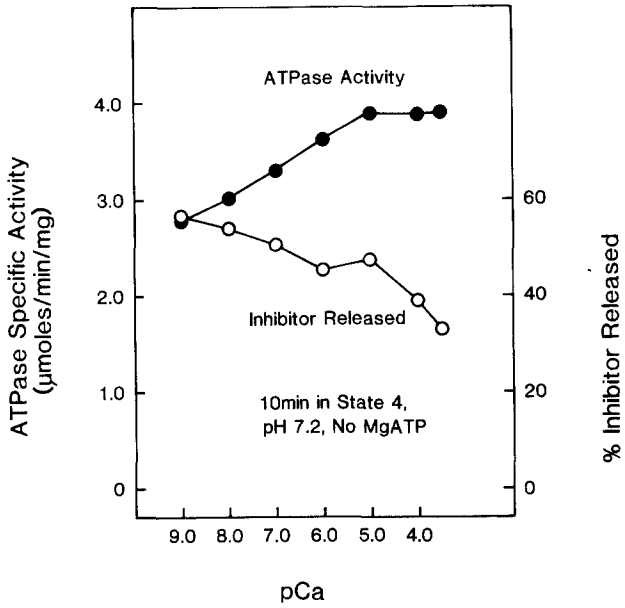

Fig. 3. Effect of the pCa of the sonication medium on the reactivation of the mitochondrial ATPase and on the release of inhibitor protein. Intact mitochondrial samples from 20-min ischemic canine heart muscle were reenergized for 10 min. At the conclusion of reenergization the samples were washed once in 0.25 M sucrose, 10.0 mM EGTA, and 20 mM MOPS-KOH, pH 7.2. The mitochondrial samples were then suspended in the same solution plus sufficient $CaCl_2$ to produce the pCa values indicated. The reference control sample was at pCa 3.5 and also contained 2 mM MgATP and 2 μM FCCP. SMP were then prepared from the sonicated samples and assayed for ATPase activity (solid circles) and for inhibitor content (open circles) using our rat heart SMP titration procedure. Reproduced from Rouslin and Broge (1989a) with permission.

modulations in the activity of the mitochondrial ATPase may not be particularly large or very significant from a regulatory point of view, at least at Ca^{2+} concentrations that prevail in the cytosol of beating cardiomyocytes and, therefore, in the matrix milieu of cardiac muscle mitochondria (see, e.g., Robertson *et al.*, 1982). That this may be the case is suggested by the relatively modest responses of cardiac mitochondrial ATPase activity, on the one hand, and IF_1 release, on the other, to changes in pCa in the submicromolar to micromolar range presented in Fig. 3 (from Rouslin and Broge, 1989a). In these studies free Ca^{2+} concentration was controlled using Ca-EGTA buffers. The paradoxical effect that increasing Ca^{2+} had upon ATPase activity activation, on the one hand, and IF_1 binding, on the other, will be discussed below.

The results presented in Fig. 3 are undeniably very different from those

reported by Yamada and coworkers on the effects of varying Ca^{2+} concentration on the activity of a rat skeletal muscle mitochondrial ATPase inhibitor protein (Yamada *et al.*, 1980). These workers incubated inhibitor-depleted "A" particles prepared from rat skeletal muscle mitochondria with a rat skeletal muscle ATPase inhibitor protein at Ca^{2+} concentrations ranging from 10^{-9} to 10^{-3} molar. They observed a sharp peak of ATPase activation at 10^{-6} M Ca^{2+} and approximately 60 and 75% ATPase activity inhibitions occurring at 10^{-9} M and 10^{-3} M Ca^{2+}, respectively. While the rat skeletal muscle system studied by Yamada and coworkers may, in fact, respond differently from the canine cardiac system to changes in Ca^{2+} concentration both below and above the micromolar level, there remain two questions: did Yamada and coworkers control free Ca^{2+} concentrations in their experiments below the micromolar level, i.e., between 10^{-9} and 10^{-6} M Ca^{2+}, in that they apparently did not employ a Ca^{2+} buffering system (Yamada *et al.*, 1980), and second, were the apparent differences in IF_1–ATPase interaction observed in response to changes in Ca^{2+} concentration due, either additionally or instead, to the different identities of the rat skeletal muscle and canine cardiac ATPase inhibitor proteins studied by Yamada and coworkers and by ourselves, respectively, i.e., the rat skeletal muscle inhibitor studied by Yamada and coworkers may have been a distinct protein from the inhibitor of Pullman and Monroy, a question left unresolved in their initial report (Yamada *et al.*, 1980).

In more recent work, Yamada and Huzel reported essentially the same Ca^{2+}-concentration-dependent behavior for a calcium-binding ATPase inhibitor protein from bovine heart mitochondria, here demonstrated to be a distinct protein from the inhibitor of Pullman and Monroy (Yamada and Huzel, 1988, 1989). While this more recent work by Yamada and coworkers appears to suffer from the same lack of control of Ca^{2+} concentrations below the micromolar level due to the continued practice of these workers not to employ a Ca^{2+} buffering system, one is, nonetheless, very impressed with the degree to which the ATPase activity of bovine heart SMP can be inhibited by increases in Ca^{2+} concentation *above the micromolar level* when SMP are supplemented with the Ca^{2+} binding inhibitor protein (Yamada and Huzel, 1989). While this review is primarily about IF_1-mediated ATPase regulation, a few words about Yamada's Ca^{2+}-dependent ATPase inhibitor would not be out of place.

The provocative 1988 and 1989 reports by Yamada and coworkers (Yamada and Huzel, 1988, 1989) present data which suggest that a distinct Ca^{2+}-dependent ATPase inhibitor protein is present in bovine heart SMP in similar amounts to IF_1, but that, unlike IF_1, the Ca^{2+} dependent protein is capable of alternatively either stimulating the ATP synthase activity or

inhibiting the ATPase activity of bovine heart SMP, both in a Ca^{2+}-concentration-dependent manner. Thus, in the presence of the Ca^{2+}-dependent inhibitor, micromolar Ca^{2+} both maximally stimulated ATPase activity and maximally inhibited ATP synthase activity. Increasing the Ca^{2+} concentration from the micromolar to the millimolar level progressively inhibited ATPase activity, as it had in their earlier rat skeletal muscle study (Yamada *et al.*, 1980), and progressively activated ATP synthase activity. While both the ATP synthase and ATPase activities of Ca^{2+}-dependent inhibitor-protein-depleted SMP were relatively unresponsive to changes in Ca^{2+} concentration, these workers proposed that the actions of the Ca^{2+} dependent protein suggest a role for it in the previously documented Ca^{2+}-mediated suppression of ATP synthesis in favor of Ca^{2+} pumping (Rossi and Lehninger, 1964; Varcesi *et al.*, 1978). This is an extremely interesting set of observations which one would like to see reproduced with appropriate Ca^{2+} buffering.

As stated earlier, Van de Stadt *et al.* (1973) provided the first evidence that the inhibitor becomes displaced from the enzyme when energy is generated by substrate oxidation. Since then, a number of workers have investigated the process of energization-induced, IF_1-mediated ATPase activation while also performing independent, direct measurements of changes in the IF_1 content of the energized particles (e.g., Schwertzmann and Pedersen, 1981; Husain and Harris, 1983; Klein and Vignais, 1983; Rouslin, 1987a; Rouslin and Pullman, 1987; Lippe *et al.*, 1988a, b). The effects of energization on IF_1-enzyme interaction are presumably mediated primarily through energization-induced conformational changes in the ATPase, and probably only secondarily through independent changes in the inhibitor protein itself. Thus, for example, matrix pH may increase during the energization process, independently initiating the relatively slow, pH-induced conformational changes in the inhibitor protein discussed above. $\Delta\mu H^+$-induced changes in IF_1–enzyme interaction can be rapid and are manifested as rapid changes in enzyme activity. The physical release of IF_1 into the supernatant is usually incomplete or fractional (Husain and Harris, 1983; Beltran *et al.*, 1984; Rouslin, 1987a; Rouslin and Pullman, 1987) and, under certain conditions, may be considerably slower than changes in ATPase activity (Van de Stadt *et al.*, 1973; Dreyfus *et al.*, 1981; Rouslin, 1987a). Thus, at least early in the time course of energization, there may exist complexes of enzyme with nonfunctionally bound inhibitor where the inhibitor no longer inhibits enzyme activity but has not yet physically dissociated from the enzyme. Along these lines, our experiments in which Ca^{2+} concentration was varied (Fig. 3) produced what may be the clearest demonstration thus far of the independence of IF_1-mediated ATPase activity regulation and IF_1 binding (Rouslin and Broge, 1989a). In these experiments, increasing Ca^{2+} concentration was accompanied by increases in both enzyme activity and IF_1

binding. Increasing Ca^{2+} concentrations would thus appear to promote the stabilization of noninhibitory IF_1-ATPase complexes. Alternatively, it may be argued that IF_1 is a relatively loosely bound regulatory subunit of the F_1-ATPase complex that normally never fully dissociates from the enzyme *in situ*, although our studies with ischemic (deenergized) and control (energized) dog hearts suggest that a fractional displacement of IF_1 into the soluble phase of the matrix milieu may actually take place *in situ* (Rouslin, 1987a; Rouslin and Pullman, 1987). Notwithstanding these observations, the incremental or fractional nature of the displacement of inhibitor observed both *in vitro* and *in situ* remains somewhat of a puzzle which might possibly be explained by heterogeneity of membrane structural integrity within populations of SMP or mitochondria being studied.

While there is general agreement that $\Delta\mu H^+$ provides the impetus for a rapid displacement of IF_1 from an activity regulatory site if not from all IF_1-interaction sites on the ATPase, there remains the possibly trivial question as to which component of $\Delta\mu H^+$, $\Delta\psi$ or ΔpH, is the more important *in situ* in cells and tissues. When beef heart SMP were energized, elimination of the electrical component of $\Delta\mu H^+$ effectively prevented both ATPase activation and IF_1 displacement from the SMP, whereas elemination of the proton gradient abolished only about 50% of the response if an electrical gradient was still present in the SMP (Husain and Harris, 1983). In contrast, during the *in vitro* energization of intact mitochondrial isolated from 20-min ischemic canine hearts, both ATPase reactivation and IF_1 release appeared to be more sensitive to the elimination of the pH gradient than to a loss of $\Delta\psi$, although the importance of the $\Delta\psi$ component gradually increased to near that of the ΔpH component as the KCl concentration approached 150 mM (Rouslin, 1987a). These results suggest that, possibly because of conditions prevailing within the matrix milieu of intact mitochondria, ΔpH and possibly pH, *per se*, may be more important than $\Delta\psi$ in overcoming the IF_1-mediated ATPase inhibition produced *in situ* during ischemia.

Relative Importance of IF_1 Binding as an ATP Sparing Mechanism in Totally Ischemic Cardiac Muscle

As stated above, a primary pathophysiological role of IF_1 appears to be the sparing of ATP generated primarily by glycolysis during anaerobic intervals. Consistent with this idea, it may be stated unequivocally that the greatly enhanced glycolytic flux ongoing in ischemic cardiac muscle constitutes the single most important mechanism for the maintenance of ATP levels during ischemia in both slow and fast heart-rate hearts (Rouslin *et al.*, 1990).

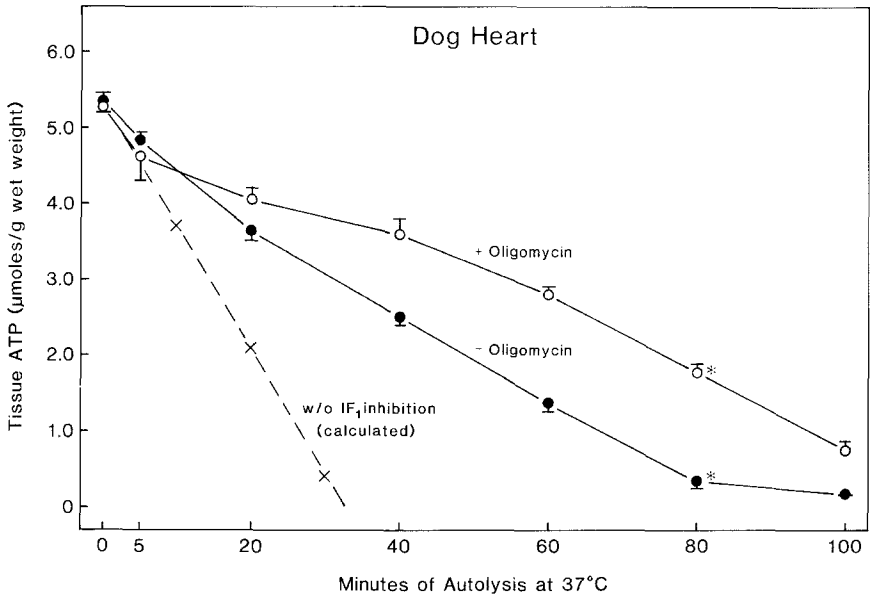

Fig. 4. Time courses of tissue ATP depletion in dog hearts during zero-flow ischemia (autolysis) at 37°C. Dog hearts were perfused in the Langendorff mode in the absence and presence of 10^{-5} M oligomycin. During subsequent ischemic incubations, tissue samples weighing 1 g or less were frozen in liquid nitrogen at the times indicated. The frozen samples were then lyophilized, finely powdered, and the powders extracted with 6% TCA. Aliquots of the extracts were then analyzed enzymatically for ATP content. The calculated ATP depletion time course shows the rate of ATP depletion based upon the additional mitochondrial ATPase activity that would be expressed in the absence of IF_1-mediated inhibition. All data are averages $\pm$ SEM of six separate determinations. $^*p < 0.0001$. Reproduced from Rouslin *et al.* (1990) with permission.

The *in situ* IF_1-mediated inhibition of the mitochondrial ATPase places a fairly distant second in terms of its relative importance as an ATP sparing mechanism, and, this, in ischemic slow heart-rate hearts only. Thus, in totally ischemic dog heart, a slow heart-rate heart, enhanced glycolytic flux produces approximately 0.5 μmol of ATP/min/g wet weight of tissue. In contrast, the approximately 50% IF_1-mediated inhibition of the dog heart mitochondrial ATPase *in situ* accounts for the sparing of a net of only approximately 0.1 μmol of ATP/min/g wet weight of tissue or approximately five times less ATP than can be produced glycolytically in the same totally ischemic dog hearts (Rouslin *et al.*, 1990). Nevertheless, as is evident from the time course data presented in Fig. 4, the relatively small net contribution made by IF_1-mediated ATPase inhibition in ischemic dog heart produces a very large delaying effect in the time course of tissue ATP depletion (Rouslin *et al.*, 1990). In contrast, as is evident from the data presented in Fig. 5, the contribution to ATP maintenance made by the oligomycin inhibition of

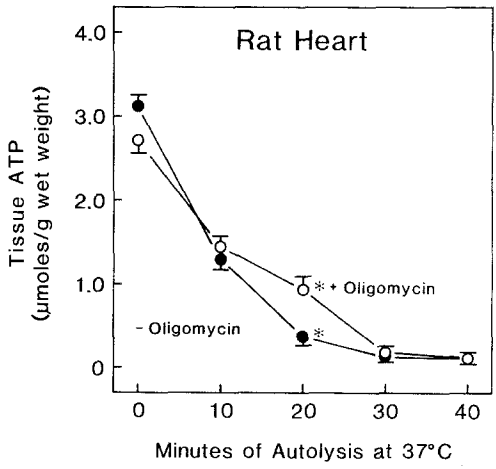

Fig. 5. Time courses of tissue ATP depletion in rat hearts during zero-flow ischemia (autolysis) at 37°C. Rat hearts were perfused in the Langendorff mode in the absence and presence of 10^{-5} M oligomycin. During subsequent ischemic incubations, tissue samples were frozen in liquid nitrogen at the times indicated. The frozen samples were then lyophilized, finely powdered, and the powders extracted with 6% TCA. Aliquots of the extracts were then analyzed enzymatically for ATP content. All data are averages ± SEM of six separate determinations. $*p < 0.0001$. Reproduced from Rouslin *et al.* (1990) with permission.

the mitochondrial ATPase *in situ* in totally ischemic rat heart, a fast heart-rate containing only low levels of IF_1, is negligible (Rouslin *et al.*, 1990).

Species, Tissue-Specific, and Possible Regulatory or Developmental Determinants of Intracellular IF_1 Level and *In Situ* Function

The amount of IF_1 present in mitochondria from the same tissue from different species can vary considerably. Thus, for example, cardiac muscle mitochondria from all so-called slow heart-rate mammals which include rabbits and all larger mammals thus far examined including the human (Rouslin, 1987b), appear to contain approximately one unit of IF_1 inhibitory activity per unit of fully energized mitochondrial ATPase activity (Rouslin, 1987b) where one unit of IF_1 inhibitory activity is the amount required to inhibit one international unit of ATPase activity by 100%, i.e., 1 μmol of ATP hydrolyzed/min (Rouslin, 1987a; Rouslin and Pullman, 1987). On the other hand, cardiac muscle mitochondria from so-called fast heart-rate mammals, i.e., rats, hamsters, and mice, contain only approximately 0.2 to 0.3 unit of IF_1 inhibitory activity per unit of fully energized ATPase activity (Rouslin, 1987b). Moreover, the low level of IF_1 present in these so-called fast

heart-rate hearts appears to produce a fractional ATPase inhibition during ischemia *in situ* that is even lower than would be expected from the low level of IF_1 present in these species (Rouslin, 1987b; Rouslin and Broge, 1990).

When one compares the ATPase activities and IF_1 contents of, for example, cardiac muscle and liver mitochondria, both within the same species and between species, one finds some additional interesting differences. Thus, the energized ATPase specific activities of rabbit liver mitochondria (a slow heart-rate species) and rat liver mitochondria (a fast heart-rate species) based upon total mitochondrial protein are both somewhat less than 20% of the corresponding energized cardiac muscle mitochondrial ATPase specific activities from the same two species. Moreover, the IF_1 inhibitory specific activities present in rabbit and rat liver mitochondria are both approximately 50 to 60% as great as the respective energized ATPase specific activities in the liver mitochondria of these same two species (unpublished data). Thus, based upon the fairly preliminary data in hand at the time of this writing, the differences in IF_1 content between, for example, rabbit and rat heart mitochondria and, by extension, between slow and fast heart-rate cardiac muscle mitochondria in general, appear to be tissue specific between different species and *not* species specific affecting all tissues within a given species. That is, both IF_1 content and IF_1 function *in situ* appear to differ between cardiac muscle from slow versus fast heart-rate species, but not necessarily between other tissues and organs from slow versus fast heart-rate species. We are currently expanding our data base to include additional tissues from species already examined (Rouslin, 1987b) and also to include a variety of additional non-mammalian vertebrate species.

Certain tumor cells appear to contain significantly increased levels of IF_1 compared to normal cells like those from which the tumor cells had been derived (Luciakova and Kuzela, 1984; Chernyak *et al.*, 1987). While the specific pathophysiological role of IF_1 may differ somewhat in liver from that, for example, in muscle and yeast cells, the increased levels of IF_1 in hepatoma mitochondria are consistent with the higher rates of aerobic glycolysis observed in hepatoma cells compared to normal liver cells.

Phylogenetic, ontogenetic, and regulatory studies on both the content and *in situ* function of IF_1 have scarcely begun. For example, one might expect the level of IF_1 to change in certain tissues just before, during, or following the transition from the pre- to the postnatal environment, particularly in tissues where there may be a shift from primarily glycolytic to primarily aerobic energy metabolism. Similarly, one might expect to find different levels (or types) of IF_1 in tissues of animals that live in intermittent or permanent hypoxic environments versus those that live under stable normoxic conditions. Such studies will almost certainly afford a greater understanding of the pathophysiological role(s) of IF_1.

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