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Content and Binding Characteristics of the Mitochondrial ATPase Inhibitor, IF_1, in the Tissues of Several Slow and Fast Heart-Rate Homeothermic Species and in Two Poikilotherms

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We determined the IF_1 contents of pig, rabbit, rat, mouse, guinea pig, pigeon, turtle, and frog heart mitochondria and the effects of varying ionic strength upon the IF_1-mediated inhibition of the ATPase activity of IF_1-depleted rabbit heart mitochondrial particles (RHMP) by IF_1-containing extracts from these same eight species. The IF_1 binding experiments were run at both species-endogenous IF_1 levels and at an IF_1 level normalized to that present in rabbit heart mitochondria. When species-endogenous levels of rabbit heart IF_1 or either species-endogenous or normalized levels of pig heart IF_1 were incubated with RHMP over a range of KCl concentrations, increasing the [KCl] to 150 mM had relatively little effect on IF_1-mediated ATPase inhibition. When either species-endogenous or normalized levels of guinea pig, pigeon, turtle, or frog heart IF_1 were incubated with RHMP under the same conditions, increasing [KCl] to 150 mM nearly completely blocked IF_1-mediated ATPase inhibition. While species-endogenous levels of rat and mouse heart IF_1 inhibited the ATPase activity of RHMP virtually not at all at any [KCl] examined, normalized levels of rat and mouse IF_1 inhibited the ATPase activity of RHMP to the same extents as species-endogenous levels of pig and rabbit heart IF_1, respectively, in the presence of increasing [KCl]. These experiments suggest that, while pig and rabbit heart mitochondria contain a full complement of higher-affinity IF_1, pigeon, guinea pig, turtle, and frog heart mitochondria cell contain essentially a full complement of a lower-affinity form of IF_1. In contrast, rat and mouse heart mitochondria contain only low levels of IF_1 which exhibit binding characteristics similar to those of the pig and rabbit heart inhibitor. The guinea pig is the only mammal thus far examined that contains a lower-affinity form of IF_1. In the present study we also determined the IF_1 contents and IF_1-to-F_1-ATPase activity ratios of cardiac muscle, skeletal muscle, liver, and brain mitochondria of rabbit, pigeon, and rat, species representative of the three homeothermic regulatory classes.

KEY WORDS: Mitochondrial ATPase; ATPase inhibitor protein; IF_1; myocardial ischemia; effects of ionic strength; higher and lower affinity IF_1; homeothermic and poikilothermic species; tissue distribution of IF_1; cardiac muscle; skeletal muscle; liver; brain.

INTRODUCTION

The F_1-ATPase inhibitory subunit, IF_1, was first isolated from bovine cardiac muscle mitochondria 31 years ago (Pullman and Monroy, 1963). In so-called slow heart-rate mammalian species, which include rabbits and all larger mammals thus far examined, including the human (Rouslin, 1987a), the inhibitor binds to the ATPase, one mole of inhibitor per mole of enzyme (Klein *et al.*, 1980) under nonenergizing conditions at low mitochondrial matrix pH such as occurs during anoxia or ischemia (Rouslin, 1987a,b; Rouslin and Pullman, 1987). Subsequent mitochondrial energization causes the rapid release of much of the bound IF_1 (Schwertzmann and Pedersen, 1981;

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Husain and Harris, 1983; Klein and Vignais, 1983; Rouslin, 1987b; Rouslin and Pullman, 1987).

Glycolysis-driven cell acidification (Rouslin, 1983a,b, 1988; Rouslin and Broge, 1989, 1990; Rouslin *et al.*, 1986, 1990) characterizes most systems under anoxia or ischemia. The lack of oxygen presumably also produces a gradual mitochondrial inner membrane deenergization resulting, in turn, in a relaxed, IF_1-receptive conformation of the F_0F_1-ATPase to which the inhibitor binds (Rouslin, 1987b; Rouslin and Pullman, 1987). The binding of the inhibitor during ischemia serves to diminish a wasteful hydrolysis of glycolytically produced ATP by the undriven mitochondrial ATP synthase (Rouslin, 1988, 1991; Rouslin *et al.*, 1986, 1990). Inhibitor binding thus slows net rates of tissue ATP depletion during ischemia, thereby delaying cell injury and death (Rouslin, 1988, 1991; Rouslin *et al.*, 1986, 1990).

Some years ago we demonstrated that the IF_1-mediated ATPase inhibition just described occurs only in the intact hearts or in the isolated cardiac muscle mitochondria of so-called slow heart-rate mammalian species (Rouslin, 1987a). It was not, however, evident in so-called fast heart-rate mammalian and avian species that were studied (Rouslin, 1987a). Fast heart-rate mammalian species, including the rat, hamster, and mouse, contain only approximately 20–30% as much IF_1 per unit of energized cardiac muscle mitochondrial ATPase activity or per mg of mitochondrial protein as slow heart-rate mammalian cardiac muscle mitochondria (Rouslin, 1987a, 1988), an amount which is apparently too low to produce significant ATPase down regulation in intact mitochondria under nonenergizing conditions.

In contrast to the situation in most fast heart-rate mammalian hearts, the cardiac muscle mitochondria of certain avian species, including the pigeon, do contain a full complement of IF_1 (Rouslin, 1987a; Rouslin and Broge, 1989). However, for reasons which remained obscure until relatively recently (Rouslin and Broge, 1992, 1993), the pigeon system also fails to exhibit a marked ATPase inhibition during either ischemia in intact hearts or in isolated intact mitochondria made acidotic under nonenergizing conditions (Rouslin, 1987a; Rouslin and Broge, 1989, 1990). The reason for this lack of ATPase inhibition in the pigeon system appears to be the presence of a lower-affinity form of IF_1 in that species (Rouslin and Broge, 1992, 1993).

In the present study we have extended these observations to include an additional species within each of the three regulatory classes which have been characterized and described by us thus far (Rouslin, 1987a; Rouslin and Broge, 1990, 1993). The three regulatory classes are (A) slow heart-rate mammalian species that contain approximately one mole if IF_1 per mole of F_1-ATPase in their cardiac muscle mitochondria and which exhibit a marked IF_1-mediated mitochondrial ATPase inhibition during ischemia, (B) fast heart-rate mammalian and nonmammalian (homeothermic) species that also contain a full complement of IF_1 in their cardiac muscle mitochondria but which fail to show a marked ATPase inhibition during ischemia, and (C) fast heart-rate mammalian species that contain low levels of IF_1 in their cardiac muscle mitochondria and which also exhibit little or no ATPase inhibition. The present study also extends our observations for the first time to two poikilothermic species, the turtle and frog. Moreover, the present study includes determinations of the IF_1 contents and IF_1-to-F_1-ATPase activity ratios of four different tissues in species representative of each of the three regulatory classes, A, B, and C, described above.

MATERIALS AND METHODS

Preparation of Control-Energized and Ischemic Pig, Rabbit, Guinea Pig, Pigeon, Rat, Mouse, Turtle, and Frog Heart Mitochondria; IF_1-Depleted Rabbit Heart Mitochondrial Particles; Mitochondria and Mitochondrial IF_1-Containing Extracts from Hearts of Pig, Rabbit, Guinea Pig, Pigeon, Rat, Mouse, Turtle, and Frog and From Cardiac Muscle, Skeletal Muscle, Liver, and Brain of Rabbit, Pigeon And Rat

Thirty-five to 40-lb. farm pigs, 1-kg male New Zealand White rabbits, 400-g male Hartley guinea pigs, 400-g Indian Fantail pigeons, 300-g male Sprague-Dawley rats, 25-g male Swiss Webster mice, 400-g *Pseudemys scripta* elegans turtles, and 4-inch *Rana pipiens* frogs were anesthetized with sodium pentobarbital (i.v. to effect for pigs and rabbits or i.p. to effect for the other species) and killed by removal of the heart. The hearts were placed either into ice-cold 180 mM KCl, 10 mM EGTA[2] (KE

[2] Abbreviations used: EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; RHMP, rabbit heart mitochondrial particles; SMP, submitochondrial particles.

solution) (control samples) or incubated for 20 min in sealed Ziplok plastic bags immersed in a circulating water bath set at 37°C as described earlier (Rouslin, 1983a,b, 1987a,b) (ischemic samples).

The hearts were then minced finely in ice-cold 180 mM KCl, 10 mM EGTA, 0.5% bovine serum albumin, and 10 mM MOPS[2]-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). For all experiments utilizing control-energized mitochondria, the mitochondria were energized 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 *in vitro* incubations.

IF_1-depleted rabbit heart particles (RHMP) used in the experiments depicted in Figs. 1 and 2 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 × *g*. This procedure served to strip away at least 90% of the endogenous IF_1 present on the particles. The low-speed centrifugation step normally employed for the removal of large membrane fragments was eliminated from the procedure so that the yield of RHMP protein would be effectively 100%. This made the calculation of the ratio of IF_1-depleted RHMP to IF_1 or IF_1-containing extracts to be added later a relatively simple matter.

Mitochondria from rabbit, pigeon, and rat skeletal muscle, liver, and brain samples were prepared as follows. Rabbit and rat back muscle and pigeon breast muscle and the livers and brains from all three were rapidly dissected free and placed in ice-cold homogenization buffer. The procedure used for the preparation of skeletal muscle mitochondria was the same as that used for the preparation of cardiac muscle mitochondria. Mitochondria from livers and brains were prepared as follows. The tissues were placed in ice-cold 0.25 M sucrose, 10 mM EGTA, 0.5% BSA, and 10 mM MOPS-KOH, pH 7.2, and minced in 1–2 ml of the same per gram of tissue.

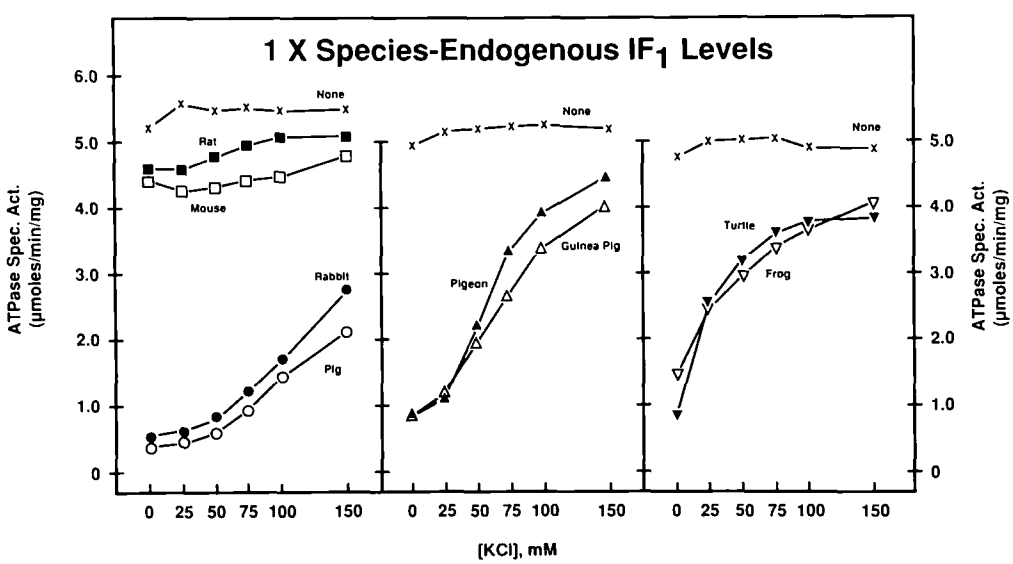

Fig. 1. Effects of ionic strength, i.e., [KCl], on IF_1-mediated ATPase inhibition of IF_1-depleted rabbit heart mitochondrial particles (RHMP) by naturally occurring (species-endogenous) levels of IF_1 from each of eight species where RHMP and IF_1-containing extracts were made from equal amounts of mitochondria. IF_1-containing extracts made from 0.5 mg of intact heart mitochondria from each species were incubated with RHMP made from 0.5 mg of rabbit heart mitochondria for 20 min at 37°C at pH 6.2 in a final volume of 2.0 ml. Nothing (×) and naturally occurring levels of IF_1-containing extracts of heart mitochondria from: pig (open circles), rabbit (solid circles), rat (solid squares), mouse (open squares), guinea pig (open triangles), pigeon (solid triangles), turtle (solid inverted triangles), and frog (open inverted triangles).

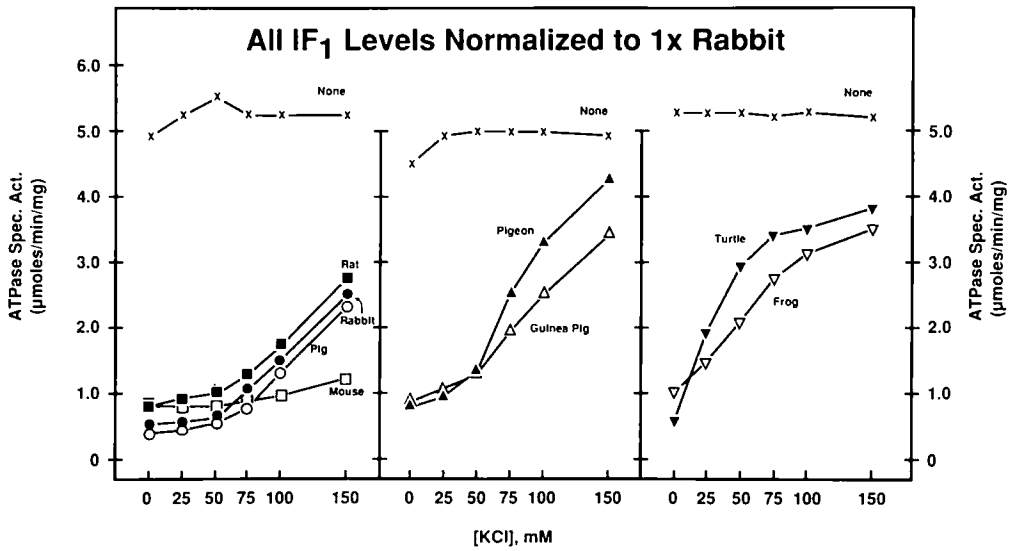

Fig. 2. Effects of ionic strength, i.e., [KCl], on IF_1-mediated ATPase inhibition of IF_1-depleted rabbit heart mitochondrial particles (RHMP) by amounts of IF_1 from each of eight species normalized to the level present in rabbit heart mitochondria. Volumes of IF_1-containing extracts made from intact heart mitochondria from each species calculated to contain ATPase inhibitory activity equal to that present in 0.5 mg of rabbit heart mitochondria, i.e., rabbit-heart-normalized levels, were incubated with RHMP made from 0.5 mg of rabbit heart mitochondria for 20 min at 37 C at pH 6.2 in a final volume of 2.0 ml. Nothing (×) and normalized levels of IF_1-containing extracts of heart mitochondria from: pig (open circles), rabbit (solid circles), rat (solid squares), mouse (open squares), guinea pig (open triangles), pigeon (solid triangles), turtle (solid inverted triangles), and frog (open inverted triangles).

The liver and brain minces were then homogenized with a glass-Teflon homogenizer using two sets of three passes each with 1 min cooling between the sets. The homogenates were then mixed with approximately 9 ml of buffer per gram of tissue and centrifuged for 15 min at approximately $600 \times g$. The supernatants were decanted through two layers of cheese cloth and centrifuged for 10 min at approximately $17{,}400 \times g$. After carefully asperating off the fat layer and the remainder of the supernatant, the loose pellets were then resuspended in four volumes of buffer and recentrifuged for 10 min at $17{,}400 \times g$. This procedure was repeated two more times.

IF_1-containing extracts used either for rebinding to IF_1-depleted RHMP as in the experiments presented in Figs. 1 and 2, or for the determination of IF_1 content, as in the experiments presented in Tables III, and IV, were prepared by alkaline extraction of intact mitochondria as described earlier (Pullman and Monroy, 1963; Frangione *et al.*, 1981; Rouslin, 1987b, 1988; Rouslin and Pullman, 1987).

In vitro Incubation Conditions and Other Procedures

The *in vitro* incubations of inhibitor-containing extracts with IF_1-depleted RHMP for the experiments presented in Figs. 1 and 2 were carried out for 20 min at 37 C at pH 6.2 in a final volume of 2.0 ml. The incubation medium contained 0.25 M sucrose, 0.1 mM MgATP, and 20 mM MES^2-KOH, pH 6.2, in the presence of the KCl concentrations indicated. Aliquots of RHMP containing the equivalent of 0.5 mg of rabbit heart mitochondria were added from a pooled suspension of RHMP made from a known amount of mitochondria. Aliquots of mitochondrial extracts from each species which contained either the amount of IF_1 present in 0.5 mg mitochondria (naturally occurring or species-endogenous level experiments in Fig. 1) or a volume calculated to contain the level of IF_1 present in rabbit heart (rabbit-heart-normalized experiments in Fig. 2) were added.

The rat heart SMP^2 titration procedure described by us previously (Rouslin, 1987a,b; Rouslin and Pullman, 1987) was used for the normalization of the IF_1-containing extracts for the experiments presented in Fig. 2. Briefly, the IF_1 content of a given mitochondrial extract was estimated by titration on rat heart SMP. The amounts of the extracts were adjusted or normalized so that their inhibitory activities were equal to that present in an amount of

Table I. Conscious Heart Rate Ranges[a] and Regulatory Classes of Species Studied

Species	Conscious heart rate range	Regulatory class
Pig	60–86	Slow H.R.—Full IF_1 complement
Rabbit	205–220	Slow H.R.—Full IF_1 complement
Rat	301–656	Fast H.R.—Low IF_1 content
Mouse	402–834	Fast H.R.—Low IF_1 content
Guinea pig	230–300	Fast H.R.—Lower affinity IF_1
Pigeon	240–370	Fast H.R.—Lower affinity IF_1
Turtle	21–44	Poikilotherm (slow H.R.)—Lower affinity IF_1
Frog	37–60	Poikilotherm (slow H.R.)—Lower affinity IF_1

[a] Heart rate data from *Biology Data Handbook*, 2nd edn., Vol III, P. L. Altman and D. S. Dittmer, eds., FASEB, 1974.

a "benchmark" rabbit heart extract derived from 0.5 mg of rabbit heart mitochondria.

Mitochondrial ATPase activity was measured in sonicated mitochondria (Tables II, III, and IV) or in RHMP (Figs. 1 and 2) using a modification of the method of Tzagoloff (Tzagoloff *et al.*, 1968) as described by us previously (Rouslin, 1983a,b, 1987a,b). Briefly, the 1.0-ml reaction mixture contained approximately 45 μmol 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 μmol Pi/mg/min.

The IF_1 content determinations presented in Table II were carried out as described previously using our rat heart SMP titration procedure (Rouslin, 1987a,b; Rouslin and Pullman, 1987). As demonstrated by us earlier (Rouslin and Pullman, 1987), the rat heart SMP titration procedure employed in the present study produced results when estimating the inhibitor from dog heart which were comparable to an RIA for the dog heart inhibitor. Moreover, a recent analysis of factors affecting functional assays for IF_1 supports the quantitative validity of the assay when applied to either a single species or to different species (Rouslin and Broge, 1994). Since units of ATPase inhibitory activity, i.e., of IF_1 activity, are the same as units of ATPase activity, i.e., μmol/min/mg, ratios of these two activities such as are presented in Tables III and IV were calculated directly from the activities of each. Protein was estimated by the Lowry procedure (Lowry *et al.*, 1951).

RESULTS

Table I lists the conscious heart rate ranges and regulatory classes of the pig, rabbit, rat, mouse, guinea pig, pigeon, turtle, and frog, i.e., the eight species included in the present study. Table II

Table II. Control and 20-min Ischemic Mitochondrial ATPase Specific Activities and Ratios of the Two in Bovine, Rabbit, Mouse, Rat, Guinea Pig, Pigeon, Turtle, and Frog Heart Mitochondria[a]

Species	Control ATPase spec. act.[b]	20-min ischemic ATPase spec. act.	Ischemic to control activity ratio
Pig	3.08 ± 0.07	1.33 ± 0.15	0.44 ± 0.06
Rabbit	3.84 ± 0.20	1.32 ± 0.18	0.34 ± 0.03
Rat	3.45 ± 0.05	3.27 ± 0.11	0.95 ± 0.02
Mouse	3.23 ± 0.05	3.31 ± 0.07	1.02 ± 0.01
Guinea pig	2.79 ± 0.10	3.04 ± 0.06	1.09 ± 0.02
Pigeon	3.69 ± 0.29	2.99 ± 0.12	0.82 ± 0.03
Turtle	3.06 ± 0.14	2.72 ± 0.09	0.90 ± 0.06
Frog	3.19 ± 0.08	2.94 ± 0.02	0.92 ± 0.02

[a] All data are averages ± SEM of four separate determinations.
[b] ATPase specific activity is expressed as μmol/min/mg.

Table III. ATPase Inhibitor Content, Maximal (Energized) Oligomycin-Sensitive ATPase Specific Activity, and Ratios of the Two in Bovine, Rabbit, Rat, Mouse, Guinea Pig, Pigeon, Turtle, and Frog Heart Mitochondria[a]

Species	IF_1 content (I.U.[b]/mg)	ATPase spec. act.[c]	IF_1/F_1 Ratio[d]
Pig	4.16 ± 0.14	2.76 ± 0.02	1.51 ± 0.06
Rabbit	3.70 ± 0.17	3.32 ± 0.24	1.12 ± 0.04
Rat	0.56 ± 0.04	2.78 ± 0.48	0.20 ± 0.03
Mouse	0.99 ± 0.02	3.25 ± 0.05	0.30 ± 0.01
Guinea pig	3.20 ± 0.05	2.93 ± 0.02	1.09 ± 0.02
Pigeon	3.29 ± 0.11	3.10 ± 0.04	1.06 ± 0.04
Turtle	2.29 ± 0.10	2.04 ± 0.13	1.14 ± 0.12
Frog	3.14 ± 0.18	2.50 ± 0.04	1.26 ± 0.08

[a] All data are averages ± SEM of four separate determinations.
[b] One I.U. (inhibitory unit) is that amount of inhibitor which fully inhibits one international unit of enzyme activity by 100%, i.e., 1 μmol ATP hydrolyzed/min.
[c] Oligomycin-sensitive ATPase specific activity is expressed as μmol/min/mg.
[d] The activities of the IF_1 and of ATPase are expressed in identical units.

presents the control-energized and 20-min ischemic ATPase specific activities and ischemic-to-control activity ratios of pig, rabbit, rat, mouse, guinea pig, pigeon, turtle, and frog heart mitochondria. The data are all averages ± SEM of four separate experiments. While the two slow heart-rate species, pig and rabbit, exhibited substantial ATPase inhibition due to ischemia *in situ*, the two fast heart-rate species containing nearly full complements of IF_1, guinea pig and pigeon, the two fast heart-rate species containing low levels of IF_1, rat and mouse, and the two poikilothermic species, turtle and frog, exhibited either relatively little ATPase inhibition or no inhibition at all.

The IF_1 contents of pig, rabbit, rat, mouse, guinea pig, pigeon, turtle, and frog heart mitochondria are given in Table III. In so far as there is overlap, these IF_1 content values and the degrees of ATPase inhibition due to ischemia *in situ* reported in Table II agree closely with data reported by us

Table IV. ATPase Inhibitor Content, Maximal (Energized) Oligomycin-Sensitive ATPase Specific Activity, and Ratios of the Two in Rabbit, Pigeon, and Rat Cardiac Muscle, Skeletal Muscle, Liver, and Brain Mitochondria[a]

Species (tissue)	IF_1 content (I.U.[b] mg)	ATPase spec. act.[c]	IF_1/F_1 ratio[d]
Rabbit			
Cardiac muscle	3.70 ± 0.17	3.32 ± 0.24	1.12 ± 0.04
Skeletal muscle	2.27 ± 0.30	1.00 ± 0.16	2.32 ± 0.15
Liver	0.65 ± 0.04	0.58 ± 0.03	1.13 ± 0.07
Brain	1.28 ± 0.03	0.80 ± 0.02	1.60 ± 0.02
Pigeon			
Cardiac muscle	3.29 ± 0.11	3.10 ± 0.04	1.06 ± 0.04
Skeletal muscle	2.39 ± 0.09	2.70 ± 0.11	0.88 ± 0.03
Liver	1.22 ± 0.04	0.90 ± 0.06	1.36 ± 0.06
Brain	1.51 ± 0.10	1.05 ± 0.08	1.44 ± 0.06
Rat			
Cardiac muscle	0.56 ± 0.04	2.78 ± 0.48	0.20 ± 0.03
Skeletal muscle	0.35 ± 0.03	1.83 ± 0.19	0.19 ± 0.02
Liver	0.41 ± 0.02	0.63 ± 0.04	0.65 ± 0.05
Brain	0.62 ± 0.13	0.52 ± 0.04	1.22 ± 0.06

[a] All data are averages ± SEM of four separate determinations.
[b] One I.U. (inhibitory unit) is that amount of inhibitor which fully inhibits one international unit of enzyme activity by 100%, i.e., 1 μmol ATP hydrolyzed/min.
[c] Oligomycin-sensitive ATPase specific activity is expressed as μmol/min/mg.
[d] The activities of the IF_1 and of ATPase are expressed in identical units.

previously (Rouslin, 1987a; Rouslin and Broge, 1990, 1993).

The experiments presented in Fig. 1 show the effects of increasing ionic strength on IF_1-mediated inhibition of the ATPase activity of RHMP by naturally occurring or species-endogenous levels of IF_1 from pig, rabbit, rat, mouse, guinea pig, pigeon, turtle, and frog heart mitochondria. Increasing the ionic strength up to approximately 100 mM KCl had relatively little effect upon IF_1-mediated ATPase inhibition of RHMP by species-endogenous levels of pig and rabbit IF_1, but had a relatively large effect upon IF_1-mediated ATPase inhibition of RHMP by species-endogenous levels of guinea pig, pigeon, turtle, and frog IF_1. Species-endogenous levels of rat and mouse heart IF_1 inhibited the ATPase activity of RHMP virtually not at all at any KCl concentration examined. The experiments presented in Fig. 2 show the effects of increasing ionic strength on IF_1-mediated inhibition of the ATPase activity of RHMP by amounts of IF_1 from pig, rabbit, rat, mouse, guinea pig, pigeon, turtle, and frog heart mitochondria that were normalized to match the IF_1 level present in rabbit heart mitochondria. Increasing ionic strength up to approximately 100 mM KCl had relatively little effect upon IF_1-mediated inhibition of the ATPase of RHMP by normalized levels of rabbit, beef, rat, and mouse IF_1, but markedly interfered with IF_1-mediated ATPase inhibition by normalized levels of pigeon and guinea pig heart inhibitor. Thus, while the pigeon and guinea pig are an avian and a mammalian species, respectively, they, nevertheless, appear to be members of the same regulatory class with respect to both IF_1 content and IF_1 type.

Table IV presents the IF_1 contents, oligomycin-sensitive mitochondrial ATPase activities (maximal or control-energized), and IF_1/F_1-ATPase activity ratios of mitochondria from four different tissues: cardiac and skeletal muscle, liver, and brain, in species representative of three homeothermic regulatory classes: rabbit, pigeon, and rat. The data are all averages ± SEM of four separate experiments. Greatest variation in IF_1 content was observed between rabbit and rat heart: 3.70 and 0.56 IU/mg, respectively. Of the three species examined, rat tissues consistently contained the least IF_1. Interestingly, based upon IF_1/F_1-ATPase activity ratios, rat brain was the one tissue in the rat that contained approximately 1 IU of IF_1 per unit of ATPase activity. Thus, among the four tissues and three species examined, only brain consistently contained relatively high levels of IF_1.

DISCUSSION

In a precursor study (Rouslin and Broge, 1993), we demonstrated that varying the KCl concentration in IF_1 binding incubation mixtures was a useful technique for distinguishing between higher- and lower-affinity forms of IF_1 in different species. It should be emphasized, however, that this ionic strength paradigm reflects our first successful attempt at distinguishing between different classes of IF_1 binding behavior and, therefore, the results obtained using this procedure may be regarded as somewhat preliminary. By and large, as shown in Figs. 2 and 3, higher-affinity inhibitors all exhibit upwardly concave shaped curves while lower-affinity inhibitors all exhibit upwardly convex shaped curves. Thus, the results obtained using this experimental design are relatively unquantitative in nature.

The earlier study just referred to (Rouslin and Broge, 1993) also showed that it was the species source of the IF_1 that was responsible for the differences in IF_1-mediated ATPase inhibition observed between different species and not the species source of the F_1-ATPase. Thus, for example, increasing KCl interfered with the binding of pigeon IF_1 to either the rabbit or pigeon ATPase, but did not interfere with the binding of rabbit IF_1 with either the rabbit or pigeon enzyme. Even when the amounts of IF_1 from different species are normalized to the level present in a typical slow heart-rate mammalian species like rabbit as they were in the present study, these differences in binding behavior in the face of increasing ionic strength persist.

The present study extends a number of relationships between species that had begun to become apparent in earlier studies (Rouslin, 1987a; Rouslin and Broge, 1990, 1993). One unexpected finding of the present study was that, unlike any other mammalian species thus far examined, the guinea pig falls into the class of fast heart-rate species that, to now, included only certain avian species, i.e., species that contain a full complement of a lower-affinity form of IF_1 (Rouslin and Broge, 1990, 1993). Thus, with respect to the mechanism of IF_1-mediated ATPase inhibition present, the guinea pig is clearly different from both mammalian slow heart-rate species like pig and rabbit as well as from mammalian fast heart-rate species like rat and mouse. While the rat and mouse contain a low level of IF_1 in their cardiac muscle mitochondria, the present study suggests that the little IF_1 that is present has binding

properties which are much like those of the higher-affinity form of IF_1 present in slow heart-rate mammalian species. Thus, IF_1 from rat and mouse behaved much like those from pig and rabbit in the face of increasing ionic strength.

In that the guinea pig system clearly resembles that present in birds (Rouslin and Broge, 1990, 1993 and the present study), reptiles, and amphibians (present study only), it may be useful to regard it as an evolutionarily less modern system than that present in the other mammals that have been investigated. Indeed, work by others on molecular phylogenetic relationships between guinea pigs and rodents and between guinea pigs and other mammalian orders suggests that the old classification of guinea pigs as rodents should be abandoned (Li *et al.*, 1990). The guinea pig would appear to be a member of a separate order of mammals that branched off from the vertebrate evolutionary tree earlier than rodents but somewhat later than marsupials, birds, and reptiles (Li *et al.*, 1990).

A second unexpected finding of the present study was that poikilothermic species with slow heart rates, i.e., the turtle and frog (see Table I), possess what are apparently nonfunctional complements of IF_1. Like the guinea pig and pigeon, these two species exhibit little ATPase inhibition during ischemia *in situ* (see Table II) and contain full complements of lower-affinity inhibitor (Table III and Figs. 1 and 2). Thus, turtles and frogs are the first species examined that have slow heart rates, but which also possess nonfunctional IF_1. Thus, the relationship between heart rate and IF_1-mediated ATPase inhibition that appears to apply to all homeothermic species thus far examined appears not to apply to poikilotherms.

While the rat, mouse and, from earlier work (Rouslin, 1987a), the hamster are all fast heart-rate mammalian species containing low levels of IF_1 in their cardiac muscle mitochondria, the present study suggests that the form of IF_1 present in these species is the higher-affinity form similar to that present in all slow heart-rate mammals thus far examined. Thus, for the time being, the rat, hamster, and mouse are all regarded by us as possessing a fast kinetic variant of the possibly more recently evolved slow heart-rate mammalian ATPase regulatory system. It may be mentioned that the amino acid sequence of rat IF_1 is approximately 70% homologous to that present in beef cattle, a slow heart-rate species (Frangione *et al.*, 1981; Lebowitz and Pedersen, 1993), and also that there appears to be just one isoform of IF_1 present per species in rat (Lebowitz and Pedersen, 1993) and in beef cattle (Walker *et al.*, 1987). Similar IF_1 sequencing work remains to be done on species that contain a lower-affinity form of IF_1 like pigeon, guinea pig, turtle, and frog.

Table IV presents the IF_1 contents, oligomycin-sensitive mitochondrial ATPase activities, and IF_1/F_1-ATPase activity ratios of mitochondria from four different tissues: cardiac and skeletal muscle, liver, and brain, from three species: rabbit, pigeon, and rat. The three species are representative of the three homeothermic slow and fast heart-rate regulatory classes described by us in this and in earlier studies. A number of points become apparent upon an examination of the data. First, there is large variation in IF_1 content and ATPase specific activity per mitochondrial protein both between different tissues within the same species and between the same tissue in different species. Second, of the three species studied, rat had a substantially lower IF_1 content in all tissues examined than the other two species. Particularly evident was the very low level of IF_1 present in rat cardiac and skeletal muscle mitochondria both in absolute terms and also as a ratio of IF_1 content to the ATPase activity present. Third, the one tissue that most consistently possessed a full complement of IF_1 as indicated by IF_1-to-ATPase activity ratios of greater than one was brain. This distinction was underscored by the uniquely high IF_1-to-ATPase activity ratio of rat brain compared to the other rat tissues examined. The significance of this finding for the regulation of brain energy metabolism remains to be elucidated.

One underlying reason for the species and tissue IF_1 distribution survey undertaken in the present study was to gain further insight into the regulatory role(s) of IF_1 in energy metabolism in both normal and ischemic cells. One central conclusion of our earlier biochemical and physiological studies on canine hearts (Rouslin, 1983a,b, 1988, 1991; Rouslin *et al.*, 1986, 1990) was that the main function of the reversible mitochondrial ATPase inhibition during ischemia appears to be the mitigation of a futile cycling of ATP produced by glycolysis during ischemic intervals, by the undriven mitochondrial ATP synthase. This conclusion about the pathophysiological function of IF_1 during ischemia was supported further by studies with baker's yeast mutants (Yoshida *et al.*, 1990; Ishikawa *et al.*, 1990). Tagawa and coworkers 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*. While mutants lacking either the 9- or 15-kDa subunits and/or IF_1, itself, showed no impairment of oxidative phosphorylation under energizing conditions, a collapse of membrane potential caused ATP hydrolysis by the F_0F_1-ATPase from 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 underscore two concepts: first that IF_1 has an important regulatory function in the control of ATP hydrolysis, i.e., in the control of the reverse reaction of the mitochondrial ATP synthase under nonenergizing conditions, and second, that IF_1 is apparently not required for forward-reaction catalysis by the ATP synthase. The low levels of IF_1 in the hearts of rats, hamsters, and mice further underscore the apparent lack of a requirement for a direct participation of the inhibitor subunit in oxidative phosphorylation.

While our work on ischemic canine hearts and the work of Tagawa and coworkers on yeast mutants suggest that the function of IF_1 may be limited to the regulation of the reverse reaction rate of the ATP synthase under nonenergizing conditions, it is still possible that IF_1 may play a role in the regulation of average net rates of ATP synthesis over a range of conditions which may include normal or physiological conditions. Thus, even under energizing conditions, the physical release of the inhibitor appears to be incomplete. Indeed, several studies suggest that a substantial fraction of the inhibitor may remain bound to the enzyme during the energization of SMP or intact mitochondria *in vitro* (Husain and Harris, 1983; Beltran *et al.*, 1984; Rouslin, 1987b; Rouslin and Pullman, 1987) and this bound fraction could function to regulate average net rates of ATP synthesis by limiting the effective average reverse reaction rate. The qualifier "average" is used here because the fractional release of IF_1 may be statistical in nature in that IF_1 may remain bound to different extents in different mitochondria within a population. Such microheterogeneity of mitochondrial energization may well be the norm in the beating heart and in other tissues where there are localized gradients of both oxygen supply and work demand. Thus, the inhibitor may be intermittently active in many tissues even during normal function. This would be so particularly in species and tissues possessing higher IF_1-to-ATPase ratios. Species and tissue distribution surveys of IF_1 content such as that presented in Table IV may be utilized as a basis for choices of material for experiments in which parameters relevant to the putative pathophysiological and physiological roles for IF_1 may be assessed.

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