Regulation of the mitochondrial ATP synthase in intact rat cardiomyocytes

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The ATP synthase capacity of rat heart myocytes can be measured in sonicated cell suspensions and in sonicated preparations of cultured cardiomyocytes. This procedure allows the rapid measurement of mitochondrial function in response to changes in the metabolic status of the cell. In cultured myocytes, transitions in ATP synthase capacity (with no detectable change in cellular ATP concentration) accompany a change to anoxia or electrically stimulated contraction (rise of 70%). These changes are reversed on returning to the original conditions. Exposure of myocytes to low pH has little effect on basal ATP synthase capacity (down to values less than pH 6), but markedly affects cellular ATP levels and the response of the cells to anoxia and reoxygenation, possibly mimicking changes seen in ischaemic heart. Similar effects are seen in suspensions of freshly prepared myocytes, but these preparations are less stable and more pH-sensitive than are cells in culture. It is proposed that mitochondria *in vivo* are directly regulated at the level of the ATP synthase, and that a regulator protein, the naturally occurring inhibitor protein from mitochondria, may be responsible for this regulation.

INTRODUCTION

The mitochondrial ATP synthase (F_1F_0 -ATPase) (for a review see Senior, 1988) is responsible for most of the ATP synthesis in (aerobic) heart tissue. As energy demand changes, the flux through this enzyme must also change so that ATP synthesis matches ATP utilization. In heart, 5–10-fold increases in flux are not uncommon.

These changes in flux might, in principle, be brought about by the ATP synthase responding to ADP levels directly. The ATP synthase, on this model, has a high, fixed, capacity relative to its normal turnover rate, and, as ADP levels rise, it achieves a higher degree of saturation (and thus a faster turnover). A phenomenon explicable in these terms has been demonstrated, in highly reduced mitochondria *in vitro*, and has been termed 'respiratory control'.

Recently, however, several intramitochondrial enzymes also thought to respond simply to substrate levels have been shown instead to be controlled *in vivo* by specific regulatory elements. Pyruvate dehydrogenase, for many years thought to respond passively to substrate/product concentration ratios (NADH/NAD⁺, acetyl-CoA/CoA), was instead shown to be controlled by a (Ca²⁺-dependent) protein kinase. Isocitrate dehydrogenase and 2-oxoglutarate are regulated, allosterically, by intramitochondrial Ca²⁺ (for a review, see McCormack & Denton, 1986). It is our contention that the ATP synthase, too, is subject to independent regulation, at least in the rat heart system.

In the work below, we describe a system suitable for monitoring the capacity of the mitochondrial ATP synthase in cardiac cells, using rat cardiomyocytes in

suspension or primary culture. This allows us to demonstrate that modulation of ATP synthase activity in mitochondria does occur in a manner leading to (active or inactive) states stable to membrane breakage and dilution. The ATP synthase exhibits a minimal capacity if the cells are uncoupled or anoxic, and rises, through quiescence to beating cells (high capacity). This is at variance with the simple 'substrate control' model outlined above. Possible mechanisms for such modulation are discussed.

METHODS

Cell preparations

Male Wistar rats (250–300 g), fed *ad libitum*, were anaesthetized by diethyl ether and their hearts quickly removed into ice-cold saline (0.9% NaCl). The hearts were mounted on a glass cannula and perfused in a retrograde direction, through the aorta. Ca²⁺-tolerant cardiomyocytes were obtained by collagenase perfusion, by the method of Powell *et al.* (1980). To increase yields, two hearts were perfused in parallel.

Freshly isolated myocytes were cultured by the rapid cell-attachment procedure of Piper *et al.* (1982). Briefly, polystyrene culture dishes (Falcon, type 3002) were preincubated overnight at 37 °C with 4% (v/v) fetal-calf serum in medium 199. Isolated cells, from the above procedure, were diluted in 4% fetal-calf serum/medium 199 to about 1×10^5 cells/ml, and a 3 ml portion was added to the precoated culture dishes and incubated for 4 h at 37 °C. Under these conditions, live cells attached to the plates and dead cells could be removed by aspiration.

Abbreviations used: F_1 , soluble portion of the mitochondrial ATP synthase complex; IF₁, naturally occurring inhibitor protein of F_1 ; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine.

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The isolation procedure of Powell *et al.* (1980) yielded a suspension of cells, 70 % of which were rod-shaped (i.e. intact) cardiomyocytes. Fresh myocytes had cellular ATP levels of 30.9 nmol/mg of protein (adjusted to 100 % rod-shaped cells), in agreement with published values (Haworth *et al.*, 1981; Piper *et al.*, 1982; Cheung *et al.*, 1985). After 2 h, the number of rod-shaped cells began to decline by 10–15 %/h. The mitochondrial ATPase activity, as measured below, also declined with time, but rather more slowly (results not shown).

After attachment to the culture plates, the proportion of rod-shaped cells increased to >95 % and cellular ATP to 37.9 nmol/mg of protein, again in agreement with published values (Piper *et al.*, 1982). Cells in culture were stable for several days. Cultured cells, obtained after an attachment period of 4 h, were used in most of the experiments described below.

For experimentation, freshly isolated myocytes in suspension were diluted in Buffer I (25 mM-Hepes, 110 mM-NaCl, 2.6 mM-KCl, 1.2 mM-KH₂PO₄, 1.2 mM-MgSO₄, 1 mM-CaCl₂, adjusted to pH 7.4 with NaOH) to a concentration of about 1×10^5 cells/ml. Portions (3 ml) of this suspension were shaken at 37 °C at 20 strokes/min. For the cultured cells, Petri dishes with attached myocytes (typically 1×10^5 cells per dish) were washed twice with Buffer I, and 2 ml of Buffer I was added. The dishes were placed in specially designed glass water baths, each with an airtight lid containing a gas inlet and a port for buffer change.

Assay procedures

Cell suspensions were diluted 1:5 with Buffer II (20 mM-Hepes, 1 mM-MgCl₂, 2 mM-EGTA, adjusted to pH 7.0 with NaOH), and immediately sonicated for 3×10 s at 25 °C with a MSE probe sonicator (150 W) at 10 μ m amplitude. This neutral low-salt buffer was chosen for cell disintegration as IF₁ is displaced from F₁ at high salt or high pH. With cultured cells, Buffer I was replaced by 2 ml of Buffer II (pre-gassed with O₂ or N₂, as necessary) by aspiration and sonication performed as above.

ATPase activity was assayed by the continuous spectrophotometric assay of Rosing *et al.* (1975), except that 2 mM-EGTA replaced EDTA in the reaction medium. NADH oxidase activity was assayed in the same medium but without the coupling enzymes lactate dehydrogenase and pyruvate kinase, and with the omission of KCN from the medium.

ATP was extracted from cells with HClO₄, and the deproteinized mixture was neutralized as described by Harris & Slater (1975). EDTA was present in the neutralizing base to give a final concentration of 3 mM in the extract; this complexes Mg²⁺ and prevents the activity of traces of creatine kinase and adenylate kinase which have survived the HClO₄ treatment. ATP was measured in the neutralized extracts with firefly luciferin/luciferase as supplied in a test kit from LKB Produkta.

Protein was measured by the method of Bensadoun & Weinstein (1976).

Materials

Collagenase (Worthington type 1, *Clostridium histolyticum*) was obtained from Lorne Laboratories (Twyford, Berks., U.K.). For cell isolations, purified bovine serum albumin (Behringwerke, Marburg, Germany) or Pentex bovine albumin, fraction V (Miles

Laboratories, Naperville, IL, U.S.A.) was used. For cell cultures, bovine albumin fraction V (essentially fatty-acid-free) and Hepes were obtained from Sigma (St. Louis, MO, U.S.A.), and medium 199 and fetal-calf serum were from Boehringer (Mannheim, Germany). FCCP was from Fluka (Buchs, Switzerland), and other chemicals, of the highest purity available, were from BDH (Poole, Dorset, U.K.).

RESULTS

Measurement of ATP synthase capacity of myocyte mitochondria

The H⁺-ATP synthase is an enzyme of the mitochondrial inner membrane. When uncoupled, it will hydrolyse (rather than synthesize) ATP. The fraction of ATP synthase molecules active in the myocyte can thus be estimated by breaking open the cell and mitochondria (to allow access of ATP to the enzyme) and measuring mitochondrial ATPase activity at saturating ATP (ATPase 'capacity').

A variety of procedures to break open myocytes and their mitochondria were investigated, in both cell suspensions and cell cultures (prepared as in the Methods section). Detergents (deoxycholate, cholate or Triton X-100) would not completely expose the mitochondrial ATPase, except at concentrations so high as to yield an unstable ATPase preparation. Freeze-thawing suspensions of myocytes also led to incomplete mitochondrial breakage (results not shown). Brief ultrasonic treatment (3×10 s), however, completely exposed the mitochondrial ATPase and, in the low-salt buffer used, yielded the ATPase preparation stable for up to 2 h at 25 °C. Further sonication showed no increase in ATPase activity. Ultrasonic disintegration was thus used routinely for rapid breakage of myocyte preparations.

The assay buffer used contained 2 mM-EGTA, to minimize Ca²⁺-dependent ATPase activity (such as from actomyosin), and less than 5 mM-Na⁺, to minimize Na⁺/K⁺-dependent ATPase activity. Table 1 shows that, under these conditions, over 80 % of the measured ATPase activity from sonicated cell suspensions, and over 90 % of that from sonicated cell cultures, was mitochondrial in origin, as judged by sensitivity to oligomycin. ATPase activity was also inhibited by azide and aurovertin, both of which inhibit F₁ directly (results not shown). The activity was insensitive to uncoupler and carboxyatractylate, showing that the mitochondria were broken and the ATP synthase was accessible. Less than 5 % of the ATPase activity was sensitive to vanadate or ouabain, showing only minor contributions from plasma-membrane and reticular ATPases.

Rates of ATP hydrolysis by sonicated cell preparations were typically 4.8 μ mol/min per mg of protein for the cell suspensions and 3.7 μ mol/min per mg of protein for the cultured cells. These are of the same order (allowing for differences in assay temperature) as those observed in broken mitochondria from rat heart by Rouslin (1987), implying that a significant proportion of cell protein in myocytes must be mitochondrial. Values from the literature indicate that this proportion is indeed high, mitochondria comprising around 40 % of total cell protein (Idell-Wenger *et al.*, 1978).

Transients in mitochondrial ATP synthase capacity

Table 2 shows that both anoxia and electrical stimu-

Table 1. Sensitivity to inhibitors of the ATPase activity of sonicated rat heart myocytes

Fresh cardiomyocytes in suspension, and cultured myocytes, were prepared and incubated in oxygenated Buffer I as described in the Methods section. They were then diluted into Buffer II, immediately sonicated, and their ATPase activity was measured in the assay buffer described, except that inhibitors were added to the assay buffer as indicated. Results are given as means $\pm$ s.d. ($n = 3$).

Cells	ATPase activity, no addition ($\mu\text{mol}/\text{min}$ per mg)	Inhibition (%) by:			
		Oligomycin (8 $\mu\text{g}/\text{ml}$)	Ouabain (10 μM)	Vanadate (2 μM)	Carboxyatractylate (10 μM)
In suspension	4.8 ± 0.3 ($n = 25$)	79 ± 4	5.0 ± 0.4	1.0 ± 0.2	1.6 ± 0.4
In culture	3.8 ± 0.2 ($n = 40$)	94 ± 4	5.2 ± 0.4	3.5 ± 0.5	1.2 ± 0.2

lation altered the ATP synthase capacity of cultured myocytes. Under anoxia (when oxidative ATP synthesis ceases), ATPase activity in the extract declined from 100% ($3.8 \mu\text{mol}/\text{min}$ per mg) to 63% within the time resolution of the method (1–2 min). Under electrical stimulation (when oxidative ATP synthesis increases) the ATPase capacity rose by 71%. Both of these transients were rapidly reversible: the ATPase activity reverted to the control value within 2 min of reoxygenation or cessation of stimulation respectively (Table 2).

Oligomycin-sensitivity was constant at $94 \pm 4\%$ (see Table 1), irrespective of the absolute values of the ATPase activity measured in these and all further studies on cultured cells (results not shown). This demonstrates that the transients observed were indeed due to changes in mitochondrial ATPase capacity, and not to other cellular hydrolases.

It is possible that the transients might reflect changes in mitochondrial recovery under the various conditions, rather than changes in mitochondrial activity. This possibility was ruled out by monitoring, in addition to ATPase activity, the NADH oxidase activity of each preparation as a mitochondrial marker. NADH oxidase activity was constant at $0.1 \text{ nmol}/\text{min}$ per mg of protein (s.d. = 0.02; $n = 14$), irrespective of which treatment of the cells was involved. It was concluded that mitochondrial recovery was constant, but that the capacity of the ATP synthase did indeed vary with the metabolic condition imposed on the cells.

Cell suspensions were more difficult to manipulate in this way, not least because they beat spontaneously in the shaking suspensions used. A slow (5–10 min) increase in mitochondrial ATPase activity of about 2-fold was observed on diluting the (anoxic) cells from a concentrated non-agitated suspension into a mechanically agitated incubation buffer, in keeping with the above results; but, owing to the instability of these cell preparations, reversible transients could not be demonstrated. Further work was continued with cultured cell preparations only.

Properties of the mechanism for ATP synthase modulation

It is demonstrated above that the ATP synthase capacity changes in myocyte mitochondria with changes in the metabolic condition of the cells. These changes cannot be due to direct modulation by cytoplasmic ATP levels, which were unaffected either by stimulation or by a 15 min period of anoxia (results not shown), in agreement with Piper *et al.* (1985a).

The effect of anoxia appears to be due not to the removal of O_2 directly, but more probably to an effect of decreasing the electrochemical gradient of protons across the mitochondrial membrane. This is shown in Table 3, where cyanide (which blocks O_2 utilization) and, in particular, FCCP (which uncouples oxidation from phosphorylation) are both shown to decrease ATP hydrolytic activity even more than does anoxia. (Controls

Table 2. ATP synthase capacity in cultured cardiomyocytes exposed to metabolic transitions

Cultured myocytes (1×10^5 cells/plate) were prepared, and incubated in 2 ml of Buffer I (see the Methods section) gassed with 100% O_2 . ATPase activity of a sonicated myocyte preparation was measured in the absence and presence of oligomycin (8 $\mu\text{g}/\text{ml}$) as in Table 1, and the difference between these values, the oligomycin-sensitive ATPase activity, was taken as a measure of mitochondrial ATPase capacity. Where indicated, O_2 was replaced with 100% N_2 for 15 min, or the cells were stimulated electrically (20 V/cm at 6 Hz, 0.5 ms per pulse) for 2 min, and the ATPase capacity was again measured. Finally, such cells were returned to the original conditions of incubation and, after 15 or 3 min, respectively, the ATPase activity was again measured. Values are means $\pm$ s.d. of > 12 duplicate readings; * indicates significant difference ($P < 0.01$).

Treatment	Oligomycin-sensitive ATPase in sonicated myocytes ($\mu\text{mol}/\text{min}$ per mg of protein)		
	Initial value	During treatment	Return to initial condition
Anoxia	3.6 ± 0.3	$2.3 \pm 0.2^*$	3.4 ± 0.3
Electrical stimulation	3.6 ± 0.3	$6.2 \pm 0.3^*$	3.6 ± 0.2

Table 3. ATP synthase capacity in cultured cardiomyocytes subjected to inhibitors or low temperatures

The ATPase capacity of cardiomyocytes exposed to O₂ or N₂ was measured as in Table 2, except that the temperature of exposure was changed from 37 °C to 25 °C or 4 °C. Assays on sonicated extracts were then performed at 37 °C (see the Methods section). Alternatively, cells exposed to O₂ at 37 °C were treated with 1 mM-NaCN or 2 µM-FCCP for 15 min before sonication. Results are expressed as oligomycin-sensitive ATPase (see Table 2), and are means ± s.d. of > 12 duplicate readings: * indicates significant difference from 37 °C (control) value ($P < 0.01$); ** indicates significant difference from value obtained with oxygenated cells at the same temperature ($P < 0.01$); n.d., not determined.

Cells exposed to	Temp. of exposure...	Mitochondrial ATPase capacity (µmol/min per mg)		
		37 °C	25 °C	4 °C
O ₂		3.6 ± 0.3	3.1 ± 0.2*	3.0 ± 0.2*
N ₂		2.3 ± 0.2**	2.1 ± 0.1**	2.6 ± 0.1**
O ₂ , cyanide		2.0 ± 0.2**	n.d.	n.d.
O ₂ , FCCP		1.4 ± 0.1**	n.d.	n.d.

showed that, over the incubation periods used, cell death, as indicated by 'rounding up' of the cells, did not occur in the presence of these agents.)

Table 3 also shows the effects of temperature on the modulation of ATP synthase capacity. Control values (i.e. ATPase activity of mitochondria from oxygenated quiescent myocytes) declined significantly if the temperature of incubation was lowered to 25 °C, but no further if the incubation temperature was 4 °C. (In all cases, the assay was performed at 37 °C; the cells were exposed to the different temperatures only before sonication.) As the incubation temperature fell, the effect of anoxia was, however, less marked; the fall on anoxia was only 16% at 4 °C, as opposed to 37% at 37 °C. These observations may represent a temperature-dependence of the control mechanism.

Mitochondrial activity in anoxia and ischaemia

The effects of low extracellular pH, in combination with anoxia, on myocyte mitochondrial ATPase capacity and cellular ATP levels were investigated, in an attempt to provide a model for the ischaemic heart. The relationship between extracellular and intracellular pH in myocytes is not known precisely; however, some influence of extracellular pH on intracellular processes is observed.

Lowering the buffer pH to 6.1 (with HCl or lactic acid) decreased cellular ATP levels by about 25% in oxygenated cells, with little further decline down to pH 5.0. (Fig. 1a). Mitochondrial ATPase capacity, in contrast, was unaffected down to pH 5.8, but declined by 25% between pH 5.8 and pH 5.0 (Fig. 1b).

Anoxic cells appeared well buffered against changes in ATP levels at pH 7.4; ATP content was unchanged, in anoxia, for up to ½ h (in agreement with Piper *et al.*, 1985b). At pH 6.1 and below, however, ATP levels fell in anoxia considerably below those observed in oxygenated cells; about half the ATP content of cells incubated at pH 6.1 was lost in anoxia, as compared with only 25% in oxygenated cells. Again, below pH 6.1, little further change was observed down to pH 5.0 (Fig. 1a).

As noted above, at pH 7.4 anoxia induced a 37% decrease in mitochondrial ATPase capacity. Remarkably, this was roughly halved at pH 6.1 and 5.8 (Fig. 1b, lowest curve). It therefore appears that at low pH myocyte mitochondria partially lose their ability to respond to anoxia; the 'control mechanism' is impaired in some

Fig. 1. Cellular ATP levels (a) and mitochondrial ATP synthase capacity (b) in cultured cardiomyocytes as functions of external pH

Cardiomyocytes were incubated with O₂ or N₂ as in Table 2, except that the buffer pH was modified by addition of appropriate amounts of lactic acid (15 mM-sodium lactate was added to the pH 7.4 buffer as a control). After 15 min, mitochondrial ATPase and cellular ATP levels were measured as described. In the cells incubated under N₂, the buffer pH was then raised to 7.4 and the solution was oxygenated. ATPase and ATP were measured after a further 15 min incubation. These results were unchanged (within experimental error) if either HCl or citric acid replaced lactic acid in lowering the buffer pH (results not shown). Values are means ± s.e.m.: $n = 6$ in (a) and 8 in (b). *, Incubated under O₂; ■, incubated under N₂; ○, incubated under N₂ and then reoxygenated at pH 7.4.

Table 4. Effects of pH on recovery of mitochondrial ATP synthase capacity from anoxia

Cultured myocytes were incubated at various pH values, under O₂ or N₂, as described in Table 2. After incubation under N₂ (anoxia) for 15 min, cells were reoxygenated either at the pH of the original incubation or at pH 7.4, again for 15 min. Oligomycin-sensitive ATPase activity was measured as in Table 2. Values are given as means of 8 readings. In all cases, S.D. < 8% of quoted value (see Table 2); * indicates significant difference from the ATPase in oxygenated cells at the respective pH ($P < 0.01$).

Incubation at pH	Mitochondrial ATPase capacity ($\mu\text{mol}/\text{min per mg}$)			
	Incubation with		Recovery from anoxia with	
	O ₂	N ₂	O ₂	O ₂ , pH 7.4
7.4	3.6	2.4*	3.5	—
6.1	3.5	2.9*	3.5	3.0*
5.0	2.7	2.1*	2.7	1.3*

way. However, even at pH 5.0, some mitochondrial response to anoxia was retained (Fig. 1b).

It is important to realize that in all these preparations, even at pH 5.0, the cells were rod-shaped (by microscopic examination) and viable. ATP levels were still within the range ($> 12 \text{ nmol}/\text{mg}$) reported for cell viability (Piper *et al.*, 1985a). Furthermore, reoxygenation of the cells in their respective buffers (within 30 min of inducing anoxia) led to an increase in mitochondrial ATPase activity to the value observed before anoxia, as was observed with cells incubated at pH 7.4 (Tables 2 and 4). Thus cells treated in this way retain the ability to recover from the period of anoxia.

This ability is lost if the reoxygenation takes place at pH 7.4, rather than at the (low) incubation pH. This treatment was much more deleterious to the myocytes. Reoxygenation at physiological pH (mimicking reperfusion of ischaemic heart) led to considerable cell death: after incubation at pH 5.0, up to 50% of cells rounded up and detached from the culture dishes. In the remaining cells, mitochondrial ATPase activity did not recover as it did on reoxygenation at the low pH; indeed,

in cells originally incubated at pH 5.0 a marked loss of activity was found (Table 4). ATP levels, too, fell further if cells were reoxygenated at pH 7.4 (Fig. 1a). These observations are consistent with the view that much of the damage caused by ischaemia occurs on reperfusion of the heart with oxygenated solutions at physiological pH.

The mitochondria of cultured myocytes, as shown above, were relatively insensitive to pH in the range 7.4–5.8. Myocytes in suspension, however, were much more sensitive to external pH; their mitochondrial ATPase was inhibited by 45% in (oxygenated) cells at pH 6.2 (Fig. 2). This may reflect a difference in permeability of the cells to protons in the two types of preparation; indeed, an increased membrane permeability has been reported for freshly isolated myocytes in suspension (Claycomb *et al.*, 1984). It is also possible that the local pH near a surface (bearing cultured myocytes) may be different from that in the bulk solution, i.e. the pH seen by the cultured myocytes may be higher than that expected from the buffer used.

DISCUSSION

A regulator of the mitochondrial ATP synthase *in vivo*

We show above that the mitochondrial ATPase capacity in rat heart cells can be simply and rapidly measured in myocyte preparations. Measurements can be made within 1–2 min of a change in metabolic state, allowing us to monitor changes in mitochondrial activity over a time scale comparable with those of the regulatory mechanisms themselves. Previous attempts to measure mitochondrial activity in heart cells have required the isolation of mitochondria from heart tissue, which may take 2–3 h (Rouslin, 1983, 1987).

The mitochondrial ATPase, under aerobic conditions, is responsible for ATP synthesis inside cells. An increase in ATPase capacity represents, *in vivo*, an increase in the activity of ATP synthase molecules available for synthesis. Thus we see that during electrical stimulation, when ATP must be made more quickly, an increase in ATPase (ATP synthase) capacity is observed (Table 2). Conversely, when O₂ is removed and the ATP synthase would hydrolyse ATP *in vivo*, the ATPase is switched off (Table 2) and ATP is conserved (Fig. 1a). These changes are rapidly reversible *in vivo*, when control conditions are re-established, as would be required of a regulatory device in mitochondria. Down-regulation of

Fig. 2. Mitochondrial ATP synthase capacity in cardiomyocytes in suspension as a function of external pH

A cardiomyocyte suspension (3 ml, 3×10^5 cells/ml) was incubated with shaking at 37 °C in oxygenated Buffer I. pH was adjusted, as in Fig. 1, with lactic acid. After incubation for 10 min, ATPase activity was measured as in the Methods section. Results are given as means $\pm$ S.E.M.

mitochondrial ATPase in ischaemic heart has been demonstrated in some animals (although not in rat) by Rouslin (1987, 1988), suggesting that such a device may operate generally.

Regulation of ATP synthesis at the level of the ATP synthase is not a widely accepted concept. Typically, it is believed that the ATP synthase is not regulated (its capacity is unchanged) with varying metabolic conditions, and that it responds to increasing ATP demand simply by responding to increasing ADP levels, the normal response of an (unsaturated) enzyme to increasing substrate. This view cannot account for the changes in ATP synthase capacity observed above; these clearly demonstrate that some direct regulation of the ATP synthase does occur within cells.

Since the regulated states of the ATP synthase are stable to dilution of broken mitochondria into a large volume of assay buffer, a rapidly reversible, allosteric, mechanism with a small-molecule regulator is ruled out. Instead, some modifier inducing a stable active/inactive transition of the ATP synthase must be present. One possibility might be phosphorylation by a protein kinase, such as occurs in the regulation of pyruvate dehydrogenase (McCormack & Denton, 1984). However, despite many attempts to demonstrate phosphorylation of F_1 (in studying the mechanism of ATP synthesis and hydrolysis), no evidence for a kinase has been forthcoming.

A more likely modifier might be a regulatory protein. Such a protein, designated IF_1 , has been demonstrated to interact with the ATP synthase in mitochondria of many species, including the rat (Cintron & Pedersen, 1979). In ox heart mitochondria, it has been shown to respond rapidly to the energy state of the mitochondrial membrane in a manner consistent with its use as a regulator (Harris *et al.*, 1979; Lippe *et al.*, 1988). In this case, the increase in ATP synthase capacity (V_{max}) observed reflects an increase in the fraction of F_1 molecules in the active state (e_o) rather than an increase in the rate of turnover (k_{cat}) of each enzyme molecule, as previously discussed (Harris *et al.*, 1979).

We thus propose that the mitochondrial ATP synthase in rat heart *in vivo* may be regulated by varying its association with a naturally occurring inhibitor protein, IF_1 . This proposal is at variance with the finding by Rouslin (1987) that only small quantities of IF_1 can be isolated from rat heart mitochondria. However, since no estimate of total yield was made during those extractions, resolution of this problem requires further experimentation.

Effects of hypoxia and acidosis

As with the intact myocardium (e.g. Kubler & Spieckermann, 1970; Piper & Das, 1986), cardiomyocytes subjected to anoxia at pH 7.4 show little change in ATP content (in agreement with Piper *et al.*, 1985a), but a marked fall in mitochondrial ATPase activity (Table 2). This might indicate that ATPase inhibition serves to preserve cellular ATP levels in anoxia. In isolated myocytes, these changes are reversible on reoxygenation (Table 2), consistent with the demonstration by Piper *et al.* (1984, 1985b) that there is no 'oxygen paradox' (cell death induced by reoxygenation) at the cellular level. Presumably the 'oxygen paradox', in heart tissue, is due to mechanical cell interactions (Ganote & Kaltenbach, 1979) or radical formation by xanthine oxidase in the underlying epithelium (Schrader, 1985).

The inactivation of mitochondrial ATPase observed in anoxia is less marked if the extracellular pH is lowered (Fig. 1b) (modelling ischaemic conditions), and ATP levels fall dramatically (Fig. 1a). This may indicate that low pH impairs the protective mechanism, which maintains ATP levels in anoxia at pH 7.4; however, it is also possible that the fall in ATP level itself impairs the action of the protective mechanism. In other words, lack of ATPase inhibition may be the cause of, or may be caused by, lowered cellular ATP levels. Further work is necessary to resolve these options.

Table 4 shows experiments in which viable cells, made anoxic at low pH, are reoxygenated at pH 7.4, an experiment analogous to the subjection of heart tissue to ischaemia followed by reperfusion. With myocytes, as in the intact tissue, this treatment leads to cell damage, and this is reflected in 'rounding up' of cells, their detachment from the plates, further decrease in ATP levels and inability of the ATPase to recover from its decreased activity, in contrast with the situation when reoxygenation occurs without a shift up in pH (above). The reasons for cell death and damage under these conditions are uncertain, but the procedures developed here will at least make their study feasible at the level of mitochondrial function.

Mild acidosis ($7.4 > \text{pH} > 6.5$) has been shown to have a protective effect on the hypoxic myocardium (Bing *et al.*, 1973; Nayler *et al.*, 1979; Cobbe & Poole-Wilson, 1980; Acosta & Li, 1980), presumably by somehow maintaining cellular ATP levels. It has been suggested that this protection reflects a lower mitochondrial ATPase activity in cells exposed to mild acidosis (Rouslin, 1988). Our results indicate that, at least in the rat, this is not the case, since in anoxia ATPase levels hardly fall in cells exposed to pH values down to 5 (Fig. 1b). A more likely explanation for this protection is therefore that impaired mechanical performance of the heart (i.e. decreased ATP utilization) is responsible for this protective effect (Nayler *et al.*, 1979).

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