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REVIEW ARTICLE
Control of mitochondrial ATP synthesis in the heart

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INTRODUCTION

ATP synthesis in the heart

Most of the ATP in a beating heart is used for contraction and associated processes (Ca^{2+} and Na^+ transport). Indeed, about 2% of total cellular ATP is consumed in each heart beat in the rat [1]. Clearly, this ATP must be replenished by ATP synthesis. Under physiological conditions, the heart is fully aerobic and over 90% of its ATP is made by mitochondrial oxidative phosphorylation. Thus, to a first approximation, we can consider the heart as a system in which ATP is made by the mitochondrial ATP synthase (F_1F_0 ATPase) and consumed by contraction. The validity of this model is attested by the correlation between heart work rate and the rate of oxidative phosphorylation (as measured by oxygen uptake) [2].

A number of recent reviews have dealt with the control of oxidative phosphorylation in the heart [1,3–9]. In general, however, they have concentrated on the means employed by the cell to modulate the mitochondrial electron transfer chain; the ATP synthase was considered as essentially responding passively to changes in these other events. It is the aim of this Review to redress the balance and focus attention on the ATP synthase within heart mitochondria, and on its regulation.

Control and its terminology

The rate and force of contraction of heart muscle can be varied by external stimuli, such as nerve impulses or hormones. *In vivo*, changes in work rate (ATP utilization) may be 5–10-fold, from a low level of around 30 μmol of ATP used/min per g wet wt. (in rat heart) [10]. ATP levels, however, hardly change from a resting value of 5 μmol/g wet wt. [11]. During the short term, transient changes in the ATP level are buffered by the creatine kinase equilibrium (phosphocreatine + ADP → creatine + ATP). However, during the steady state, the rate of oxidative ATP synthesis must be closely matched to the rate of consumption. That is to say, the rate of ATP synthase turnover must also vary over the above 10-fold range.

If ATP consumption is to govern the rate of ATP synthesis, the two processes must be linked; the mitochondria will only speed up, for example, if they 'know' that more ATP is being consumed in the cytoplasm. Such communication would typically involve the movement of small 'messenger' molecules between the two compartments to signal, say, an ATP deficit in the cytoplasm.

Two types of such messenger molecule can be described. The first includes substrates for the ATP synthase. In this scenario, use of ATP in the cytoplasm produces ADP and P_i. These move into the mitochondria, increasing the rate of ATP synthase turnover purely by increasing the concentration of its substrates. The kinetic parameters of the ATP synthase remain constant; the increase in rate (v) is due to an increase in saturation with substrate (s). Since such behaviour is a passive property of all enzymes, I shall follow the nomenclature of Newsholme & Start [12], and exclude this from my definition of control. However, some other authors take a broader definition [6].

In the second case, the 'messenger' molecules interact directly or indirectly with the (regulatory) enzyme and alter its kinetic properties ($V_{max.}$ and/or K_m). One example is the allosteric activation of α-oxoglutarate dehydrogenase (in the tricarboxylic acid cycle) by Ca^{2+} moving from the cytoplasm into the mitochondrion [9]. The regulator switches the enzyme from an inactive to an active form, and the enzyme is truly controlled.

It is widely believed that the rates of ATP utilization and ATP synthesis are linked *in vivo* by a mechanism of the first type (see [1,3–6,12,13]). In short, ATP utilization is assumed to increase cellular levels of ADP (and P_i), to which the ATP synthase responds passively. Such responses can be demonstrated in isolated mitochondria, where they are designated—confusingly in terms of the above definition—'respiratory control' or 'substrate control'.

The present Review (*a*) demonstrates that, at least in heart, variations in substrate levels cannot generally account for increases in ATP synthase activity, i.e. that such models are, at best, incomplete; (*b*) shows that the kinetic properties of the ATP synthase are directly modulated *in vivo* i.e. that a true control mechanism operates on this enzyme; and (*c*) considers possible molecular mechanisms by which the ATP synthase might be controlled in heart cells.

SUBSTRATE LEVELS AND ATP SYNTHASE

What are the substrates for the ATP synthase?

The reaction catalysed by the mitochondrial ATP synthase is:

$$ADP_{in} + P_{i,in} + 3H^+_{out} \rightarrow ATP_{in} + 3H^+_{in}$$

(the suffixes representing position relative to the mitochondrial inner membrane). Concentrations of intramitochondrial ADP and P_i are difficult to measure *in vivo*. A more convenient system to consider is that shown in Fig. 1, incorporating the ATP synthase and the adenine nucleotide and phosphate transporters, yielding

$$ADP_{out} + P_{i,out} + 4H^+_{out} \rightarrow ATP_{out} + 4H^+_{in}$$

This relates the activity of the synthase to cytoplasmic concentrations of ATP, ADP and P_i, which are accessible to measurement and are, presumably, the physiological concentrations relevant to the rate of ATP synthesis *in vivo*.

In heart mitochondria, the response of ADP phosphorylation to ADP and P_i normally reflect the kinetics of the ATP synthase itself, as judged by measurements of control strength [14,15]. The translocators appear at physiological ADP/ATP ratios to be comparatively rapid in heart [14], in contrast to other tissues [16]; at very high ADP/ATP ratios, the adenine nucleotide carrier may become limiting in heart, but such conditions would be rare *in vivo*.

The remaining substrate for the ATP synthase is normally

Abbreviations used: F_1, extramembrane segment of the mitochondrial ATP synthase; IF_1, intramitochondrial protein, potential-dependent inhibitor of F_1; CaBI, intramitochondrial protein, Ca^{2+}-dependent inhibitor of F_1; J_{ATP}, net rate (flux) of ATP synthesis; e_0, concentration of active enzyme (from Michaelis–Menten equation); $\Delta\mu_{H^+}$, difference in electrochemical potential of protons across mitochondrial membrane.

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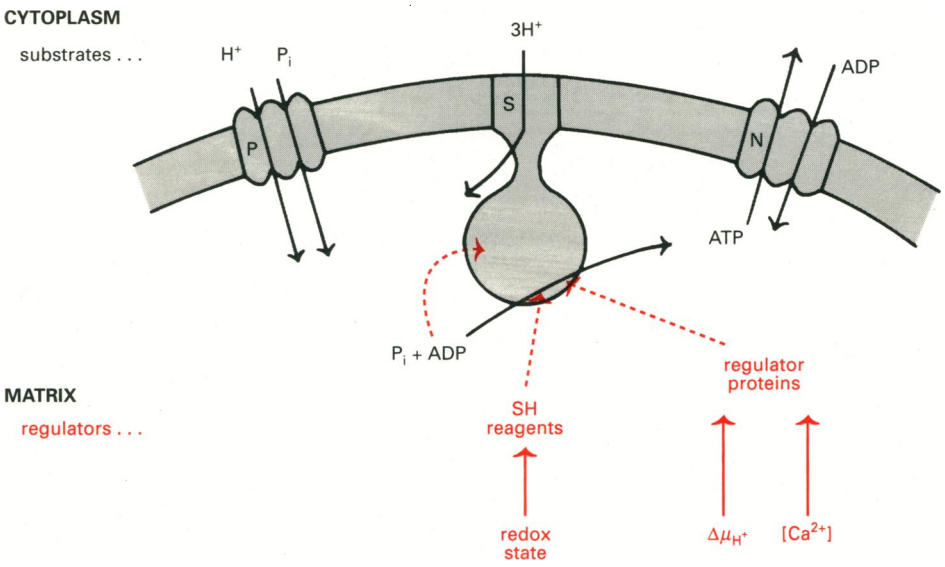


Fig. 1. Substrates and putative regulators of the system mediating ATP synthesis in mitochondria

S, ATP synthase; P, phosphate translocator; N, adenine nucleotide translocator. Regulatory elements are shown in red.

taken to be the electrochemical gradient of protons, with an effective 'concentration' of $\Delta\mu_{H^+}$, the electrochemical potential of H^+. This includes both concentration (ΔpH) and electrical ($\Delta\psi$) terms, which in principle might (*a*) vary independently, and (*b*) affect rates differently [6]. However, it is probably valid to assume that these two components vary in parallel *in vivo* [17]; thus, even if a rate is truly dependent on ΔpH, for example, the same dependence will be observed on $\Delta\mu_{H^+}$.

The substrates of the ATP synthase are not saturating *in vivo*

Measurement of cytoplasmic ADP and P_i (ADP_{out}, $P_{i,out}$, in the above equation) in intact heart, or in heart cells, is by no means trivial. The techniques most widely employed are freeze clamping and ^{31}P-n.m.r. [18,19]. Using the freeze clamp method (and enzymic determination of metabolites), the results must be corrected from total metabolite levels to free levels, which are the kinetically relevant concentrations. ^{31}P-n.m.r. detects only free metabolites; however its sensitivity is low, and [ADP], for example, must be estimated from ATP, creatine and phosphocreatine levels, assuming creatine kinase to be at equilibrium. Both methods may be subject to error if one, or more, metabolites are compartmented within the cell.

Despite these problems, consistent values for cytoplasmic ADP and P_i levels in heart cells have been obtained, at around 20–60 μM and 400–2000 μM respectively [20,21]. Importantly, these values are around the $K_{app.}$ for ADP and P_i for oxidative phosphorylation, as measured in isolated heart mitochondria [7,22]. [The value of $K_{app.}$ reflects the affinity of the ATP synthase for the relevant substrate at physiological concentrations of the other two; since the reaction has, in principle, three substrates, $K_{app.(ADP)}$ will be affected by the values of the other two, [P_i] and $\Delta\mu_{H^+}$.] Thus, the levels of ADP and P_i *in vivo* are non-saturating to the ATP synthase; the turnover of the ATP synthase would increase, passively, in response to substrate concentration in the physiological range.

The same is probably true of the third substrate, $\Delta\mu_{H^+}$. Estimates of $\Delta\mu_{H^+}$ have been made in isolated heart mitochondria [22], and in intact heart, using TPMP probe distribution methods [23,24]. The values of 130–170 mV obtained indicate that values of this parameter also fall, *in vivo*, in a range where the ATP synthase is 'unsaturated' with $\Delta\mu_{H^+}$, i.e. will respond steeply to changes in its value [25].

Dependence of ATP synthesis on ADP and P_i

The concentrations of three substrates for the ATP synthase (above) are in a range *in vivo* where substrate variation is able to influence turnover rate. If turnover were governed in this way, it should be possible to correlate varying rates of ATP synthesis with changes in concentration of the relevant substrate *in vivo*.

In attempting this correlation, attention has focused largely on ADP (and to a lesser extent P_i), for two reasons. First, they are the metabolites directly affected by cytoplasmic ATP utilization, ADP the more so since its concentration is an order of magnitude less than that of P_i. Second, if the ATP synthase works faster, $\Delta\mu_{H^+}$ would be expected to fall rather than rise. Such a fall (the basis of the respiratory control model of Chance & Williams [26]) can indeed be demonstrated in intact heart mitochondria [22] and perfused heart [23,24].

Early measurements of ATP synthesis rates, and ADP concentration, in isolated perfused heart, suggested that the two correlated well [27,28]. However, it is becoming increasingly evident that this correlation is found only under a limited range of conditions [21,29]. In recent work on the perfused rat heart, for example, isoprenaline was found to stimulate oxygen uptake 2-fold with no detectable change in steady state ADP or P_i levels [30]. Similar effects have been observed in dog heart perfused *in vivo*, paced over a 4-fold change in oxygen consumption [20,31]. Thus increased ATP synthase turnover is not irrevocably linked to increased cytoplasmic [ADP] or [P_i] in heart tissue.

These observations appear to rule out a simple model in which ATP synthesis rates vary passively in response to substrate ADP and/or P_i. This is certainly true if we consider the response of the ATP synthase to its substrate to follow Michaelis–Menten type kinetics (which is approximately true *in vitro* [32]). In this case, a 2-fold increase in rate would require at least a 2-fold increase in [ADP] or [P_i] (or an even greater change if compensation for

a drop in $\Delta\mu_{H^+}$ is considered), and such an increase would be readily detectable. Changes in [ADP] and [P_i] in the heart are thus too small to govern changes in ATP synthesis rate.

This conclusion is, however, not completely watertight. If we consider the ATP synthase to be close to equilibrium, a small percentage change in ADP (for example) could increase the net flux very greatly in the direction of ATP synthesis. This high sensitivity of flux to ADP levels in this case arises from the dynamic nature of the equilibrium, with rapid and nearly equal forward ($ADP+P_i \rightarrow ATP$) and reverse reactions. If these rates were balanced at, say, (+101) in the forward direction and (−100) in the reverse (net flux = +1 unit), a 5% increase in [ADP], leading to a 5% increase in forward flux (to +106) would actually yield a 6-fold increase in net flux (to +6 units). Thus in this near-equilibrium scenario [33], changes in ADP undetectably small by the above methods could produce large changes in ADP synthesis rate.

Again, while this scenario is reasonable in theory, it does not accord with the existing data. Measurements of flux across the ATP synthase either in perfused heart (using saturation transfer ^{31}P-n.m.r.) [10,34], and in heart mitochondria (using $^{33}P_i$ isotope exchange) [22] show a strong imbalance between forward and reverse fluxes during net ATP synthesis. In other words, the ATP synthase is not operating near equilibrium. Thus both 'kinetic' and 'thermodynamic' models which attempt to link increases in ATP synthase turnover to [ADP] and/or [P_i] concentrations can be ruled out in the functioning heart.

Dependence of ATP synthesis on $\Delta\mu_{H^+}$

Despite the steep dependence of ATP synthesis rates on $\Delta\mu_{H^+}$ in isolated mitochondria [25], little attention has been paid to the possibility of $\Delta\mu_{H^+}$ modulating ATP synthase turnover as its third substrate. $\Delta\mu_{H^+}$ is a 'product' of electron transfer to oxygen; thus for $\Delta\mu_{H^+}$ to rise *in vivo*, electron transfer must be speeded up relative to ATP synthesis (a $\Delta\mu_{H^+}$ 'consumer').

One proposed mechanism by which electron flow might be increased as ATP is consumed employs ATP as an inhibitor of electron transfer between cytochrome *c* and cytochrome oxidase [33]. According to this model, as ATP is used, its cytoplasmic levels fall, and electron flow accelerates. Inhibition of electron transfer from cytochrome *c* to cytochrome oxidase by ATP has been demonstrated [35]; however, the relative constancy of nucleotide levels with varying metabolic states (above) argue against the operation of this mechanism *in vivo*.

A more likely mechanism for increasing $\Delta\mu_{H^+}$ *in vivo* is the stimulation of the dehydrogenases of the tricarboxylic acid cycle by [Ca^{2+}]. It has been shown by a number of workers that, as the work rate of the heart increases, cytoplasmic [Ca^{2+}] levels rise, Ca^{2+} enters the mitochondria, and pyruvate, isocitrate and α-oxoglutarate dehydrogenases are all activated [9] (for a review, see [36]). It was postulated (*a*) that this activation causes the level of mitochondrial [NADH] to rise [37], (*b*) that NADH dehydrogenase (which is not saturated with substrate) thus works faster, and (*c*) that the consequent rise in $\Delta\mu_{H^+}$ stimulates ATP synthesis [38].

However this model, while self-consistent, cannot account *quantitatively* for observed changes in ATP synthesis rates. In experiments on rat heart mitochondria *in vitro*, Moreno-Sanchez *et al.* [39] showed that dehydrogenase activation did *not* necessarily lead to a sufficient rise in NADH levels to account for the observed increase in the rate of ATP synthesis. They concluded that some control on the utilization of $\Delta\mu_{H^+}$, probably at the level of the ATP synthase, must occur to match $\Delta\mu_{H^+}$ production and utilization. Recent work by LaNoue *et al.* [24], on perfused hearts, has confirmed that NADH levels do not generally rise in parallel with rates of ATP synthesis in the intact organ, leading to a similar conclusion.

Finally, direct measurements of $\Delta\mu_{H^+}$ in perfused, beating heart indicate that this parameter falls, rather than rises, as work rate increases [23,24]. Thus, on several counts, it seems unlikely that changes in $\Delta\mu_{H^+}$ can govern the changes in ATP synthase turnover observed *in vivo*. In summary, attempts to correlate the rate of ATP synthesis with the levels of [ADP], [P_i] or $\Delta\mu_{H^+}$ in heart mitochondria or heart tissue have failed, indicating that this enzyme cannot simply be responding to changes in levels of its substrates. Other factors must be involved.

ACTIVITY STATES OF THE ATP SYNTHASE

Experiments on isolated mitochondria or membranes

ATP synthesis. The evidence above suggests that ATP synthesis rates can vary in the heart without significant changes in the concentrations of ATP synthase substrates (ADP, P_i, ATP or $\Delta\mu_{H^+}$). By our definition above, this implies that the ATP synthase must itself be regulated. More direct evidence for regulation arises from the observation of kinetic abnormalities in the turnover of this enzyme. A number of such observations are summarized in Table 1.

The kinetic observations that most unambiguously suggest ATP synthase regulation relate to changes occurring during a transition in ATP synthesis from one steady state to another. Submitochondrial vesicles from (ox) heart, after a rapid transition from quiescent to energized, show a lag in the induction of a maximal rate of phosphorylation of 5–30 s, regardless of whether energization occurs through electron transfer [40,41] or via an artificially imposed H^+ gradient [42,43]. This lag is abolished by pretreatment of the vesicles by oxidizable substrate [41–43] and can be explained only by the operation of a controlling element on the ATP synthase during this lag period. A similar lag in the ability of the ATP synthase to use a H^+ gradient has been observed in (rat) liver submitochondrial vesicles [44], and intact liver mitochondria [45] and interpreted in terms of control of the ATP synthase [44,46].

Less direct indications of control arise from analysing variations in steady state ATP synthesis rates. If no regulatory system operates (i.e. with a fixed assembly of ATP synthase molecules), it is expected that, at constant [ADP] and [P_i], ATP synthesis rates should be a unique function of $\Delta\mu_{H^+}$, however this parameter may be varied. At least under some conditions, this expected relationship does not hold [47–49], and this can be most simply explained by regulation of the ATP synthase [6,49].

Similarly, if $\Delta\mu_{H^+}$, [ADP] and [P_i] are held constant, inhibition of some molecules of ATP synthase should reduce ATP synthesis in parallel. However, ATP synthesis rates in ox heart submitochondrial vesicles appear resistant to significant levels of synthase inhibition [50]. Again, this can be explained on the basis of ATP synthase regulation, if we consider ATP synthesis rates to be maintained, in this situation, by replacement of inhibited molecules with others that have been up-regulated.

Finally, a number of studies have indicated departures of ATP synthesis from a Michaelian dependence on substrate concentrations [54,55]. Even when anomalies due to (*a*) energy limitation (uncontrolled $\Delta\mu_{H^+}$), (*b*) translocase limitation (see above) and (*c*) enzyme co-operativity are eliminated (for a review see [56]), some abnormalities remain. Again, a regulatory device would readily explain all the observed abnormalities.

As with all kinetic data, abnormalities in the steady state kinetics of ATP synthesis are open to several interpretations. The participation of localized protons [57] or intramembrane collisions [58] have been suggested to account for parts of the above data. However, it is doubtful whether anything other than

Table 1. Observed kinetic anomalies in mitochondrial ATP synthesis

Observation	Explanation proposed	Explicable by ATP synthase regulation?	Reference
No excess capacity in ATP synthase at suboptimal phosphorylation rates (double inhibitor titration)	Localized proton domains*	Yes	[53]
	Collisional energy transfer†	Yes	[51,52]
Lag in ATP synthesis after shift of $\Delta\mu_{H^+}$	ATP synthase regulation	Yes	[42]
	ATP synthase regulation by IF_1	Yes	[40,41]
Rate of ATP synthesis varies with electron transfer rate at constant $\Delta\mu_{H^+}$	Localized proton domains or ATP synthase regulation	Yes	[47,48]
K_m (ADP) and K_m (P_i) vary differently as $\Delta\mu_{H^+}$ is varied by uncoupling or electron transfer inhibition	Localized proton domains	Yes	[54]
Rate of ATP synthesis not an invariant function of $\Delta\mu_{H^+}$	Collisional energy transfer or ATP synthase regulation	Yes	[49]
Inhibition of ATP synthase does not give parallel inhibition of ATP synthesis	Variation in synthase capacity	Yes	[50]
ATP is a competitive or non-competitive inhibitor of phosphorylation depending on $\Delta\mu_{H^+}$	Collisional energy transfer	Yes	[55]

* Reviewed in [56].
† Reviewed in [58].

ATP synthase regulation can account for all observations including the observations of pre-steady state 'lags'. Furthermore, in most cases the *only* evidence for the proposed 'non-chemiosmotic' models lies on such kinetic analyses, whereas independent evidence exists for a regulatory mechanism at the ATP synthase (see below).

ATP hydrolysis. It is concluded from above that the ATP synthase in mitochondria from heart (and other tissues) may exist in an inactive form (low rate of ATP synthesis) and an active form. To assess the proportions of the two forms by measuring synthesis rates, however, is difficult because (*a*) under conditions of ATP synthesis, the two forms convert within 5–30 s [41,44], and (*b*) it is difficult, reproducibly, to manipulate levels of $\Delta\mu_{H^+}$ (which affects J_{ATP}), especially in leaky membrane preparations.

More conveniently, ATP synthase activity can be measured in the direction of ATP hydrolysis. This occurs by a simple reversal of the mechanism of synthesis [59]. In a suitable assay system (saturating [ATP], zero $\Delta\mu_{H^+}$, absence of translocase limitation), the rate of ATP hydrolysis by mitochondrial membranes reflects the capacity of the ATP synthase for turnover ($V_{max.}$) at the time of sampling. Hydrolytic activity can thus be used to monitor changes in synthase capacity during activation/inactivation.

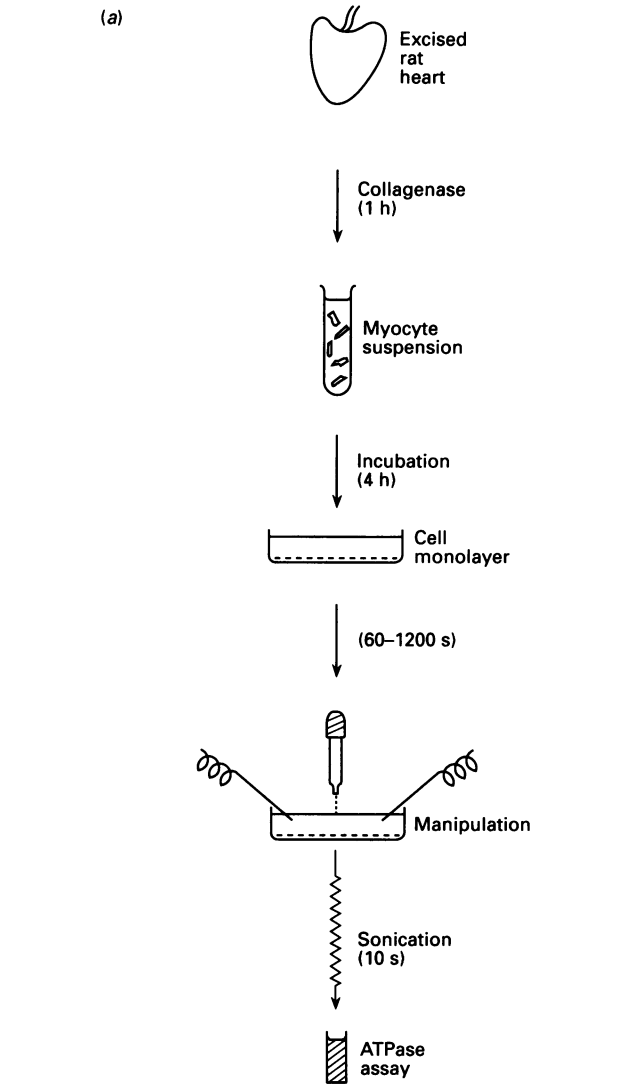
Activity transitions of the heart mitochondrial ATP synthase were demonstrated first by this method in ox heart submitochondrial vesicles [60]. They were subsequently shown to occur in intact mitochondria if the organelles were pretreated (e.g. by allowing or preventing electron flow) and the ATPase subsequently measured in sonicated [61] or detergent-permeabilized [62] membranes. Increases in ATP hydrolytic activity in heart submitochondrial vesicles could be correlated with increase in the initial rate of ATP synthesis by these vesicles [41,43], confirming that forward and reverse processes are activated in the same process (although this conclusion has been questioned in the case of liver mitochondria [44,63]).

Besides demonstrating the convenience of ATP hydrolysis measurements in following regulation of the ATP synthase, this work also demonstrated that the active and inactive enzyme forms are stable to membrane disruption and dilution into an assay medium. (Indeed, the F_1-ATPase fragment can itself be isolated from the membrane in latent [64] and active forms.) These observations rule out a rapidly reversible, allosteric regulator of the ATP synthase, and suggest that the (in)activator must produce a stable (but reversible) complex with the F_1 component of the synthase enzyme. Thus the population of membrane bound synthase is regulated by varying the proportion of active and inactive molecules; $V_{max.}$ ($= k_{cat.}e_0$) is altered by varying e_0 rather than $k_{cat.}$, the rate of turnover of any one molecule.

Experiments on tissues

Ischaemic heart muscle. Rouslin and co-workers have demonstrated a down-regulation of the ATP synthase in ischaemic heart [65,66]. In their experiments, isolated sections of heart muscle were made ischaemic by incubation for 20 min in sealed bags, and mitochondria subsequently isolated from them. ATP synthase capacity (as ATPase activity) was measured on submitochondrial vesicles prepared by sonication of the isolated mitochondria. The time between treating the tissue and ATPase assay was 2–3 h.

ATPase activities in vesicles from ischaemic muscle were typically only 30–50% of those from control muscle, and this was taken to reflect a down-regulation of the ATP synthase in the original ischaemic muscle [65]. This was not due to a general inactivation of mitochondrial activities [67]; in particular, addition of substrate and oxygen to the mitochondria isolated from



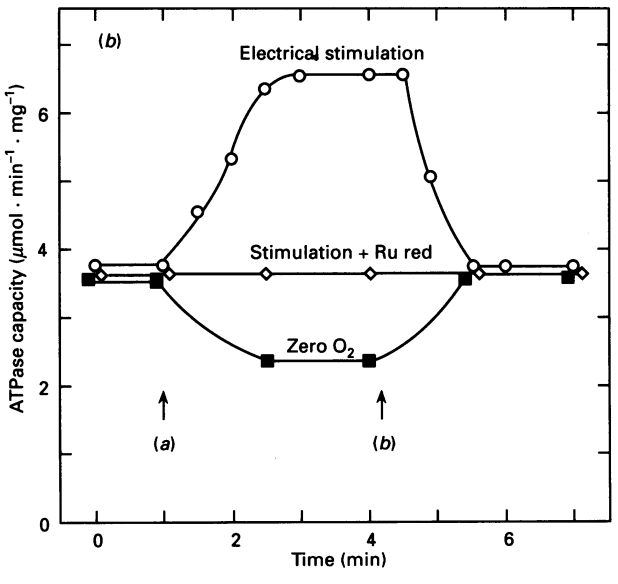


Fig. 2. Modulation of ATP synthase capacity in cultured rat cardiomyocytes

(*a*) Outline of the method for determining the ATP synthase capacity. (*b*) Cultured cardiomyocytes (10^5 cells per plate) were prepared as in [73], and incubated in 25 mM-Hepes, 110 mM-NaCl, 10 mM-glucose, 2.6 mM-KCl, 1.2 mM-KH_2PO_4, 1.2 mM-$MgSO_4$, 1 mM-$CaCl_2$, 1 mM-isoascorbate, pH 7.4 (with NaOH), at 37 °C, under an atmosphere of 100% O_2. In the uppermost curve, electrical stimulation was (*a*) initiated at 3 Hz, 20 V/cm and (*b*) terminated, as indicated. Cells were then sonicated, and ATP synthase capacity measured. In the central curve, treatment was as above, except that the cells were pretreated with ruthenium red (4 μg/ml) for 5 min before stimulation. In the lowest curve, the atmosphere was (*a*) replaced by 100% N_2, and then (*b*) returned to 100% O_2. The cellular content of ATP was 38–40 nmol/mg of protein throughout these treatments.

ischaemic muscle reactivated their ATPase to control levels [68,69]. Studies on the isolated mitochondria implicated pH values below 6.5 (such as are found in ischaemic heart) in promoting the inhibition [68,70].

This down-regulation could be observed in a variety of large mammal species (e.g. dog, rabbit, human) but not in smaller mammals, notably rat and guinea pig, or in birds. This was attributed to a fundamental difference in ATP synthase regulation between 'slow heart rate' (1–4 Hz) and 'fast heart rate' (4–10 Hz) animals [66]. However, a similar down-regulation of the mitochondrial ATP synthase was recently observed in rat heart myocytes [71]. It may thus be that the regulatory mechanism is general, but that the inhibited state of the synthase is, in some species, unstable to the prolonged period required to isolate submitochondrial vesicles from the ischaemic tissue in Rouslin's procedure.

Isolated rat cardiomyocytes. Both up-regulation and down-regulation of the ATP synthase have been demonstrated in rat heart cells [71–73]. These workers utilized stable, primary cultures of cardiomyocytes from mature rats, attached to a solid substratum [74]. These cells were > 95% viable, contained normal levels of ATP, and were able to contract on electrical stimulation [73]. Using sonication to break open cells and mitochondria simultaneously, and an ATPase assay medium which suppressed other cellular ATPases [71], ATP synthase capacity could be measured within 1 min of cell disruption. The procedure used is summarized in Fig. 2(*a*).

When cultured cardiomyocytes were made anoxic, or their mitochondrial electron flow inhibited with cyanide, their mitochondrial ATPase capacity fell to about 60% of the oxygenated control. This loss in activity was potentiated by low external pH [71]. These observations are reminiscent of those on ischaemic dog heart muscle (above). Since an uncoupler, FCCP, also caused this effect, a loss of mitochondrial membrane potential (or $\Delta\mu_{H^+}$) was implicated in causing this down-regulation.

Using this system, it was also possible to investigate the effects on ATP synthase capacity of cellular work rate. Increasing energy utilization led to an increase in myocyte ATP synthase. From a value of 100% (3.8 μmol/min per mg of protein) in quiescent cells, synthase capacity rose to 175% under electrical stimulation and further to 225% in the presence of isoproterenol, a positive inotropic agent [72,73]. Thus up-regulation of the ATP synthase, as well as down-regulation, appears to occur *in vivo*. Both up- and down-regulation were shown to be rapid ($t_{\frac{1}{2}} \approx$ 30–40 s) and reversible; control values were rapidly restored on reoxygenation of anoxic cells, or cessation of stimulation of contracting cells [72,73] (Fig. 2*b*).

Experiments with calcium channel blockers (verapamil or ruthenium red) implicated intramitochondrial [Ca^{2+}] as the primary effector in up-regulation of the ATP synthase [73]. ATP levels and intracellular pH were unchanged over the period of the experiments [71,75] and so cannot be regulators. The effects of intramitochondrial [Ca^{2+}] on stimulating ATP synthase capacity in these experiments are reminiscent of its stimulatory effects on

Table 2. ATP synthase regulation in chronic heart overload

Cardiomyocytes were prepared as in Fig. 2 from normal (WKY) rats (control I), hypertensive (SHR) rats and from hypertensive rats treated with captopril (400 mg/l of drinking water, 12 weeks), or from Wistar rats, sham-injected (control II), from Wistar rats injected with thyroxine (350 μg/kg body wt. per day for 7 days) and from Wistar rats injected with thyroxine as (*d*), and then allowed to recover for 17 days without thyroxine. Myocytes were tested for ATP synthase capacity as in Fig. 2, after the following treatments: (*a*) none (quiescent cells), (*b*) electrically stimulated (10 Hz, 20 V/cm, 2 min) or (*c*) isoproterenol (10 μM) for 5 min, and electrical stimulation as (*b*). Cellular ATP was measured, in perfused hearts by ^{31}P-n.m.r. as in [10] ([a]), or in myocyte cultures as in [71] ([b]). For full experimental details, and 100% values, see [77,78]. * Significantly different from the respective control rats; † significantly different from the respective quiescent values ($P < 0.02$).

Rats	ATP synthase capacity (% of untreated controls)			[ATP] after low-to-high activity transition (% of untreated controls)
	Untreated cells	Electrically stimulated cells	Electrically stimulated cells + isoproterenol	
WKY	100%	145%†	191%†	96%[a]
SHR	127%*	127%	131%	71%*[a]
SHR + captopril	100%	132%†	154%†	Not tested
Wistar, sham-injected	100%	135%†	197%†	102%[b]
Wistar + thyroxine	135%*	136%	132%	83%*[b]
Wistar + thyroxine, recovered	96%	118%†	175%†	Not tested

pyruvate dehydrogenase and the Krebs cycle dehydrogenases, as reported by various workers [36,76]. However, it should be noted that the effect of $[Ca^{2+}]$ on the ATP synthase cannot be a direct (reversible) allosteric effect because activation survives dilution into the ATPase assay and other buffers.

This control mechanism was also investigated in pathological conditions, notably hypertension and hyperthyroidism [77,78]. Chronic exposure to either of these conditions predisposes the heart to failure. In both cases, (*a*) the ATP synthase capacity of quiescent cardiomyocytes was raised; (*b*) down-regulation of the ATP synthase (in anoxia) was normal; and (*c*) ability to up-regulate the synthase with energy demand was lost. Thus pathological defects in the heart are associated with defects in this regulatory system. Treating the pathological defect (with antihypertensive drugs, or by reducing thyroxine levels, respectively) led to the re-establishment of normal regulation (Table 2).

The relationship of defects in ATP synthase regulation to the aetiology of these pathological states is not yet clear. Nonetheless, demonstration of such a clear cut defect in both conditions is intriguing. One possibility is that inability to up-regulate the ATP synthase *in vivo* may lead to an inability to match ATP supply to demand in the heart, and thence to heart failure. Indeed, ATP levels, which show a remarkable constancy with energy demand in control myocytes (above), have been shown to fall significantly in cardiomyocytes from hyperthyroid animals after 2 min of electrical stimulation [78], and in perfused hearts from hypertensive rats within 1 min of increased pacing (Table 2). This observation emphasizes the importance of regulating the mitochondrial ATP synthase in the maintenance of energy balance in heart cells.

MECHANISM OF ATP SYNTHASE REGULATION

Possible control mechanisms

Variations in the rate of oxidative ATP synthesis in heart cannot be explained by variations in substrate levels for the ATP synthase *in vivo*. This implies that the ATP synthase enzyme must be regulated in some way. Investigations on mitochondrial membranes and, more recently, on heart cells and tissue show that ATP synthase capacity *in vivo* is not constant, but varies with ATP demand. Thus regulation is demonstrated to occur *in vivo*. Finally, inability to up-regulate ATP synthase in pathological states (Table 2), or due to ruthenium red perfusion [29], can lead to a shortfall in ATP production in heart cells. This indicates that ATP synthase regulation is important for function *in vivo*.

What, then, is the mechanism of this regulation? The observation that the different activity states of the ATP synthase are stable to isolation and dilution [60,61,65,72] sets limits on our models for a regulatory mechanism. Thus, although intramitochondrial pH [68], Ca^{2+} [73] or $\Delta\mu_{H^+}$ [79,80] have been proposed as regulators, they are unlikely to interact allosterically with the ATP synthase or the effect would be lost on dilution.

One possible mechanism might be chemical modification of the ATP synthase, yielding separate stably modified and unmodified activity states. By analogy with mitochondrial pyruvate dehydrogenase [81], the ATP synthase might be regulated by a protein kinase system. However, despite extensive investigations (in the search for the mythical 'phosphorylated intermediate' of ATP synthesis), no phosphorylated form of the ATP synthase has been identified.

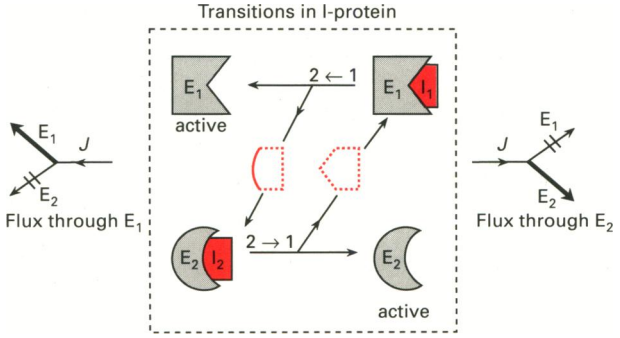


Fig. 3. Hypothetical model of bistable branched chain regulation by a single inhibitor protein

The inhibitor is proposed to exist in two forms, I_1 and I_2, which inhibit the two enzymes E_1 and E_2 acting after a branch in a metabolic pathway. Conversion of the inhibitor from I_1 to I_2 will divert the flux, J, from the E_2 branch to the E_1 branch.

Other possibilities have been indicated in Fig. 1. One intriguing possibility is regulation by modulation of redox state of the ATP synthase. A well-documented system exists in chloroplasts by which the ATP synthase is switched on by a thioredoxin mediated reduction of its γ subunit [82,83]. This idea has been applied by analogy to the regulation of mitochondrial ATP synthase [80]. However, (*a*) documented effects of oxidation/reduction on mitochondrial ATP synthase activity are secondary and/or small [84] and (*b*) no large changes in mitochondrial redox state, which could drive this oxidation/reduction, accompany changes in ATP synthase activity [reviewed in 6,39]. It is more likely that regulation by oxidation/reduction is a specialized adaptation of chloroplasts, where enzymes other than the ATP synthase are also regulated in this way.

A third possibility is regulation by ADP. This is surprising, because we would expect ADP to be a simple competitive or allosteric effector of the ATP synthase, whose effects are abolished by dilution. However, 'hysteretic' effects of ADP—in which an inhibitory effect of ADP preincubation persists after dilution into an assay medium—have been well documented for the heart mitochondrial ATPase [85,86]. Inhibition following ADP preincubation probably occurs by occlusion of ADP at one of the active sites of the synthase [87].

Again the relevance of these observations to the situation *in vivo* is doubtful. While inhibition persists for several minutes in the isolated F_1 subfragment of the synthase [85], it is very short-lived on the membrane bound enzyme [88]. Furthermore, inhibition is reversed by physiological levels of ATP and this, together with the inconvenience of a model which has the ATP synthase inhibited at low energy levels (high ADP), makes ADP an unlikely regulator of the ATP synthase *in vivo*.

Regulator proteins

The most promising candidates for regulation of the ATP synthase *in vivo* are two regulator proteins that have been isolated from heart mitochondria. Both are small, heat stable proteins (< 20 kDa) which inhibit the membrane bound ATPase. Inhibition by the first, IF_1, is reversed by membrane potential [79]; inhibition by the second (CaBI) is reversed by Ca^{2+} [89].

The use of inhibitory proteins in regulating intermediary metabolism is unusual, but not unknown. Heat-stable proteins inhibiting protein phosphatase I (of the cyclic AMP cascade) [90], and the branched chain α-oxoacid dehydrogenase phosphatase [91] for example, have been described.

Production of stoichiometric amounts of an inhibitory protein is an energy-intensive way of controlling an enzyme, and it is not clear why this method is used. One possibility is that the size of a protein allows it to interact at several sites with the regulated enzyme, producing a very highly specific, high-affinity system. However, K_d for IF_1 binding to the ATP synthase is in the nM range [85], not much higher in affinity than that expected for small molecule regulators. A more interesting speculation is that the regulator, as a protein, may have sites of interaction with several proteins. In a simple model, enzyme 1 (E_1) is inhibited by inhibitor form I_1: conversion of I_1 to I_2 will release the inhibition of E_1, and lead to the inhibition of a second enzyme, E_2 (Fig. 3). If, say, E_1 and E_2 catalyse critical steps in competing pathways, a highly sensitive switch is achieved. A similar mechanism operates in the protein phosphatase system [92].

Two stable conformations of both IF_1 and CaBI are known to occur [93,94]. However, although a *binding* site for IF_1 on a second (i.e. non-F_1) mitochondrial protein has been proposed (see below), no second site of *action* for either IF_1 or CaBI has been identified in mitochondria, and further work is necessary to identify interactions (if any) with an enzyme other than F_1.

THE POTENTIAL-DEPENDENT ATPase INHIBITOR, IF_1

Structure, and interaction with the ATP synthase

IF_1 has been isolated from mitochondria of various eukaryotic species, including mammals [66,95–97], birds [66], trypanosomes [98], yeasts [99,100] and plants [101]. No homologous protein has been detected in bacteria or chloroplasts, although in these

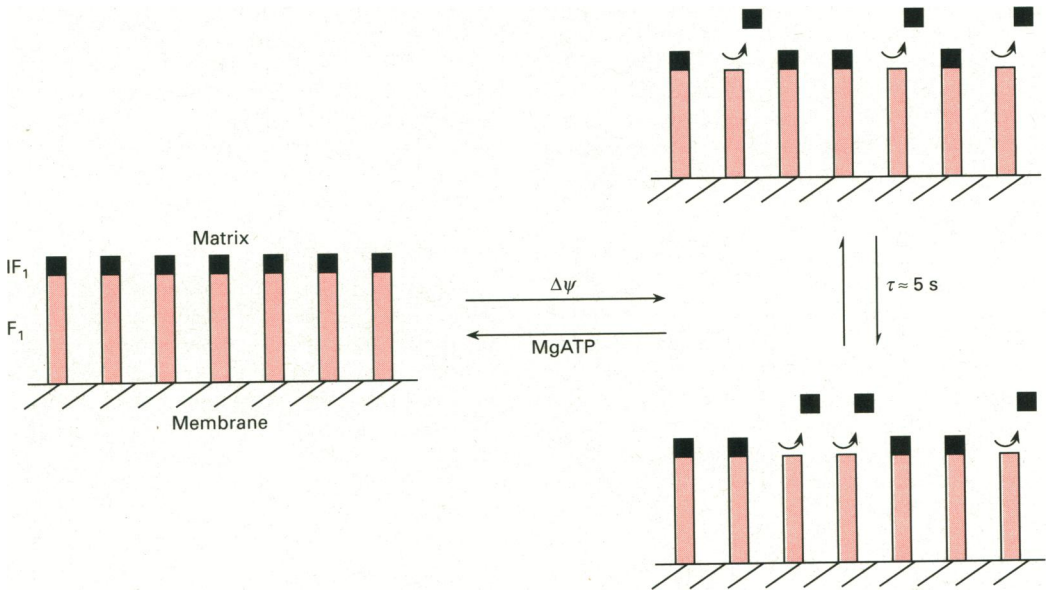


Fig. 4. Dynamic equilibrium between the F_1–IF_1 complex (inhibited) and active F_1 on the mitochondrial membrane

In the 'resting state', all F_1 molecules are inhibited. IF_1 is displaced under the influence of membrane potential, and its rebinding is promoted by MgATP. Only those F_1 molecules free of IF_1 can carry out ATP synthesis. The steady state is established with a time constant of about 5 s [41]. Due to the constant release and rebinding of IF_1, all F_1 molecules participate in the steady state, with only a fraction active at any instant [123]. Thus, decrease in $\Delta\psi$ rapidly leads to inactivation of the F_1 population. Other factors such as Ca^{2+} may perturb the steady state, through accessory proteins (see the text).

species the smallest (ϵ) subunit of F_1 often exhibits inhibitory properties [102,103].

Sequences of IF_1 from ox heart [104,105], *Candida utilis* [106] and *Saccharomyces cerevisiae* [107] are known, and show considerable sequence similarity [108]. The protein varies considerably in size between species, from 7.5 kDa in yeast [99,107] to 12.5 kDa in rat [96]; however, it is believed that the core of the protein is conserved, as an amphiphilic α-helical rod [109,110] whose hydrophobic side is responsible for interaction with F_1. Antibodies to IF_1 which inhibit [111,112] or do not affect [113] its binding to F_1 have been isolated.

It is not known which amino acid residues are essential either for inhibition by, or the energy-dependent responses of, IF_1. In ox heart IF_1, residues 1–9 can be removed proteolytically without affecting inhibitory activity, but further removal is deleterious [114]. Also in the ox heart protein, two pairs of histidine residues are potentially interesting, especially since IF_1 binds to F_1 much less strongly after treatment with diethylpyrocarbonate [115]. However, these residues are absent from the homologous yeast proteins which, like all other reported IF_1 species, cross-react with ox-heart F_1.

IF_1 inhibits the ATPase activity of F_1 at a 1:1 molar ratio, with $K_d \approx 10^{-8}$ M [85]. Binding appears to involve the *C*-terminal region of one catalytic (β) subunit of F_1 [116] and not the active site (in the central region of the β subunit [117]) directly. Indeed, binding of IF_1 apparently traps a substrate (nucleotide) molecule at an active site [87]. Nonetheless, its effect is to inactivate all three catalytic sites of F_1; IF_1 inhibits both unisite catalysis (turnover not involving intersubunit co-operativity) and multisite catalysis (in which intersubunit co-operativity is involved) [118,119]. This is in contrast to inhibitors such as azide [119] and the ϵ subunit in bacteria and chloroplasts [120,121] which prevent co-operativity in F_1 and thus inhibit only multisite catalysis.

Energy-dependent responses of IF_1

Effects on ATP hydrolysis. As outlined above, imposition of a membrane potential ($\Delta\psi$) across the mitochondrial membrane (e.g. by initiating electron flow) leads to an activation of hydrolytic capacity by the ATP synthase, both in submitochondrial vesicles and mitochondria. This is due to displacement of IF_1 from its inhibitory site on the ATP synthase [60,63,122]. Under conditions where non-specific interactions are suppressed (+50 mM-KCl, +cytochrome *c*), IF_1 is released from the membrane into free solution [44,123]. The time course and extent of release correlates directly with the increase in hydrolytic capacity observed [44,79,113,123].

If salt and/or cytochrome *c* are omitted from these experiments, some IF_1 is found associated with the mitochondrial membranes but not inhibiting the ATPase [60,96,111]. Such binding is even more marked in preparations containing detergents [62,124,149]. This indicates the presence of non-inhibitory binding sites for IF_1 on mitochondrial membranes. It is attractive to consider such sites as functional receptors for IF_1, particularly in the light of the 'two-site' model for inhibitor protein action (Fig. 3). However, more work is needed to establish whether this binding (which may be as low as 5% of total IF_1 content [113]) represents a *specific* association of IF_1 with a membrane protein.

Rebinding of IF_1 to F_1 occurs in membrane suspensions, and is stimulated by MgATP [85,96]. Thus, in energized membranes, levels of F_1–IF_1 interaction reach a steady state whose position is governed by the factors promoting release ($\Delta\psi$) and rebinding (MgATP) [109,126]. A dynamic equilibrium, in which binding and release occur simultaneously involving the entire population of synthase molecules, has been demonstrated in ox heart submitochondrial vesicles (Fig. 4) [123].

Effects on ATP synthesis. More controversial is the relationship between IF_1 content (or ATPase capacity) and the activity of the synthase in the direction of ATP synthesis. If, as one would expect, ATP synthesis and hydrolysis involve the same sites on the synthase, IF_1 should inhibit both equally. IF_1 clearly does suppress the initial rate of ATP synthesis when induced either by electron flow [41,44] or via an artificial pH gradient [43]. However, while Lippe *et al.* [79] claim a close correlation between the kinetics of activation of ATP hydrolysis and ATP synthesis capacities in heart submitochondrial vesicles, other workers [127] claim that hydrolytic capacity (and IF_1 release) rise more slowly ($t_{\frac{1}{2}} = 3$ min) than synthetic capacity ($t_{\frac{1}{2}} = 5$ s). This discrepancy in kinetics has been suggested to indicate that ATP synthesis and hydrolysis occur at different sites on the synthase enzyme [63,127], with synthesis being activated by some unknown process more rapid than IF_1 release.

Husain *et al.* [113] prefer to resolve this paradox in terms of kinetic anomalies of the assay systems used. They observed that the time courses of ATPase activation and IF_1 release were biphasic, with the rapid phase corresponding to the rate of induction of synthesis. The slow phase was interpreted in terms of IF_1 loss from the dynamic equilibrium (e.g. by aggregation or by adsorption onto the vessel walls), and was thus non-physiological in nature. This second phase would increase ATPase capacity (measured under uncoupled conditions) since increasing amounts of IF_1 were lost from the membrane; however, it would not increase synthase activity which, after sufficient synthase molecules are switched on, would be limited by energy supply. Thus it is not necessary to invoke separate ATP hydrolytic and synthetic sites on the synthase to explain the kinetics of its activation.

Mechanism of IF_1 release. The mechanism of IF_1 displacement from the ATP synthase is not known. It is not clear, for example, how IF_1 (on one side of the membrane) can sense a transmembrane charge (or pH) gradient. Experiments to implicate surface charge [41] or phospholipid head groups [128] as mediators have been inconclusive. However, one interesting possibility follows from the observation that ox heart IF_1 can exist in two conformational forms which are only slowly interchangeable in solution [93,129]. The protonated form is active (binds strongly to the ATP synthase), and the transition between protonated and deprotonated forms has a pK_a of 6.8 in free solution (shifting to pH 8.8 in the IF_1 synthase complex) [130]). Thus changes in local proton activity as a consequence of transmembrane potential may lead to deprotonation of IF_1 and, hence, dissociation of the complex.

Physiological role of IF_1

IF_1 has been shown to be responsible for down-regulation of ATP synthase capacity in ischaemic dog and rabbit hearts. In these organisms, IF_1 is present at a 1:1 molar ratio with the synthase [66,123,131] and loss of ATPase activity during ischaemia, measured in submitochondrial vesicles isolated from ischaemic muscle, correlates with increased IF_1 content of the membranes [132].

Down-regulation of ATP synthase is also observed in anoxic rat cardiomyocytes [71,72]. The role of IF_1 in this process has not yet been established, since IF_1 contents were not measured. It has been suggested that the IF_1 content of rat heart mitochondria is too low (approx. 0.25 mol/mol of synthase) for this protein to cause down-regulation of ATP synthase [66]. However, as these estimates were made on preparations which showed no down-regulation in ischaemia, the observed low ratio may reflect instability of the preparation rather than ruling out this protein as a regulator of the rat synthase.

Studies on ox-heart submitochondrial vesicles indicate that

IF_1 release is sensitive to membrane potential ($\Delta\psi$) in the range 120 mV (low release) to 160 mV (maximal release) [126]. Estimates of $\Delta\psi$ for heart mitochondria *in vivo* are equivocal; however, values of around 150 mV seem likely [22–24,133]. Thus, in ischaemia, as $\Delta\psi$ falls back to 120 mV or below, IF_1 could indeed inhibit the ATP synthase. A drop in intramitochondrial pH would also promote IF_1 binding [70,93,132].

The estimated potentials, above, suggest that IF_1 might also be involved in up-regulation of the ATP synthase in the physiological range of $\Delta\psi$. Up-regulation of the synthase has been demonstrated in rat cardiomyocytes [72,73]. However, there is no evidence to implicate IF_1 in this regulation. First, no estimates of IF_1 content of 'quiescent' or 'activated' membranes are available. Second, in hypertension/hyperthyroidism in rats, down-regulation of the ATP synthase (presumably a property of IF_1) is retained while up-regulation is lost [77,78], possibly suggesting two different mechanisms. And thirdly, the messenger in up-regulation seems to be Ca^{2+} [72,73], in the range 0.5–5 μM [36], which does not alter the IF_1–synthase interaction in isolated mitochondrial membranes.

IF_1 would therefore seem unlikely to be solely responsible for up-regulation of the ATP synthase in heart. However, its participation as part of a more complex system is not ruled out. It may be, for example, that the IF_1–synthase interaction responds to Ca^{2+} *in vivo*, but that sensitivity is lost in isolated membranes. Two possibilities emerge from the literature, each involving additional proteins to confer Ca^{2+} sensitivity. Pedersen and coworkers have reported that the positive, amphiphilic helix of IF_1 (from rat liver) binds to calmodulin, a negative amphiphilic helix, in a Ca^{2+}-dependent manner [134,135], although this has not been confirmed for heart. As intramitochondrial Ca^{2+} rises, therefore, IF_1 would bind preferentially to Ca^{2+}–calmodulin, and the synthase would be activated (see Fig. 2).

This specific model founders on the lack of convincing evidence for sufficient calmodulin inside mitochondria [136,137]. However, other Ca^{2+}-binding proteins might be involved. In this regard, the existence of accessory proteins that influence the IF_1–synthase interaction should be noted. Two such proteins (function still unknown) have been shown to occur in yeast mitochondria [138–140, reviewed in 108]. If such proteins also occur in mammals, they might confer Ca^{2+} sensitivity on the system *in vivo*, but be lost during preparation of vesicles for studies *in vitro*.

Another factor that may be relevant is tissue variation of ATP synthase regulation. IF_1 itself shows no isoforms; IF_1 proteins from ox heart, liver and kidney are identical [141], and only a single gene for IF_1 seems to be present [142]. If, say, Ca^{2+} sensitivity were preferentially a property of heart and skeletal muscle ATP synthase, the presence of differing accessory proteins would allow variation in regulation between tissues.

In conclusion, it is likely that IF_1 can switch off the heart ATP synthase *in vivo* under conditions of energy deficiency (ischaemia, anoxia etc.), probably in response to decreased transmembrane potential ($\Delta\psi$) and/or intramitochondrial pH (see Fig. 4). The mechanism for up-regulating the ATP synthase *in vivo* is less clear; it may involve IF_1 plus additional protein factors which confer Ca^{2+} sensitivity. Alternatively, Ca^{2+}-dependence of the synthase may be mediated by a second, Ca^{2+}-binding inhibitor protein, CaBI, totally separate from the IF_1 system. This is discussed further below.

THE Ca^{2+}-DEPENDENT INHIBITOR PROTEIN (CaBI)

Structure and interaction with the ATP synthase

Treatment of certain preparations of submitochondrial vesicles from ox [97] or dog [69] hearts, (or from rat skeletal muscle [143]) with Ca^{2+} at around 1 μM leads to an increase in their ATPase activity without loss of IF_1. This activation is accompanied by release from the membrane of a small, heat-stable protein (6.3 kDa) termed **Ca**lcium **B**inding **I**nhibitor (CaBI), which is distinct from IF_1 both antigenically and in amino acid composition [89,97]. The purified protein inhibits the ATPase activity of submitochondrial vesicles in the absence of Ca^{2+}. Little is known of the structure of this protein, or its affinity for, or site of interaction with, the membrane-bound ATP synthase. However, it appears to be inhibitory as a 12.5 kDa dimer, with Ca^{2+} preventing inhibition by dissociating the protein into monomers [94].

The effects of CaBI on ATP synthesis have been investigated in ox heart submitochondrial vesicles (preincubated to eliminate effects of IF_1). Vesicles with a high CaBI content showed enhanced ATP synthesis while ATP hydrolysis was suppressed; for those with a low CaBI content, synthesis was low and hydrolysis high [144]. Thus surprisingly, the effect of CaBI on ATP synthesis seemed to be opposite to its effect on hydrolysis (cf. IF_1, above, which inhibits both). However, in these investigations, an ATP-trapping system was not present when synthesis was measured, and ATP levels were assayed after 4 min; it is possible that the observed levels of ATP reflect a balance between synthesis by some vesicles and a high rate of hydrolysis by other (leaky?) vesicles, rather than initial rates of ATP synthesis by the synthase. A similar phenomenon has been observed in assays conducted with submitochondrial vesicles depleted of IF_1 [145].

Elucidation of the precise role of CaBI in ATP synthase regulation *in vivo* requires considerable further experimentation. However, heart mitochondria contain considerable amounts of this protein—5–10 mol/mol of synthase [97,143]—and it is an interesting candidate for conferring Ca^{2+} sensitivity on ATP synthase regulation in the heart.

PHYSIOLOGICAL IMPORTANCE OF ATP SYNTHASE REGULATION

Up-regulation of the ATP synthase

From above, it appears that the mitochondrial ATP synthase in heart, at least in the rat, is switched on in response to intracellular [Ca^{2+}] levels *in vivo*. Thus ATP synthase capacity arises with (*a*) increased contraction, or (*b*) positive inotropic agents, both of which raise cytoplasmic [Ca^{2+}], which in turn raises intramitochondrial [Ca^{2+}] (see [146]). Indeed, Ca^{2+} ions are the only secondary messenger at present known to enter mitochondria.

Ca^{2+} entry allows coordinate activation of (mitochondrial) pyruvate dehydrogenase, the Krebs cycle dehydrogenases [36,76] and the ATP synthase, and links increased electron flux with increased ATP synthesis (Fig. 5). Interestingly, the mechanism of response to [Ca^{2+}] of all these systems varies; pyruvate dehydrogenase is activated via a phosphatase (kinase) system, the oxoglutarate and isocitrate dehydrogenases via direct allosteric interaction, and the ATP synthase via regulator protein(s). In heart, which generates its energy mainly by fatty acid oxidation, the regulation of pyruvate dehydrogenase *in vivo*, although well characterized, is probably the least important of these three systems (see [76]).

ATP synthase regulation is important to ATP homeostasis in heart, particularly under stress conditions. If regulation is absent, either due to ruthenium red treatment [29,30] or pathological changes ([78], Table 2), ATP levels fall in working heart cells, but not in resting cells. It is, however, not obvious why such regulation is necessary: why should the mitochondria not simply exhibit a fixed, high-capacity ATP synthase as was originally believed?

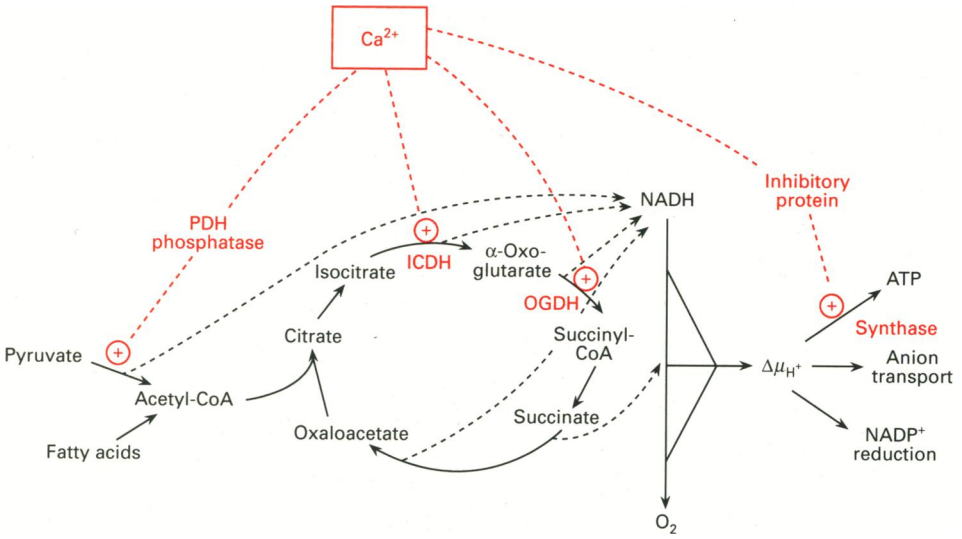


Fig. 5. Co-ordinate activation of intramitochondrial dehydrogenases and the ATP synthase by Ca^{2+}

ICDH, isocitrate dehydrogenase; PDH, pyruvate dehydrogenase; OGDH, α-oxoglutarate dehydrogenase.

Classically, the answer to this question would be that the ATP synthase follows a branch point at which $\Delta\mu_{H^+}$ can be used by several processes, not only ATP synthesis but also ion transport, $NADP^+$ reduction, etc. [147]. Control of the ATP synthase could thus help to partition $\Delta\mu_{H^+}$ between the different pathways (Figs. 3 and 5). Quantitative assessment of energy demand of these various pathways *in vivo* is required to judge the validity of this explanation.

A second answer may lie in a cellular requirement to maintain [ATP] *and* [ADP]+[P_i] levels constant within the cell; perhaps because they are cofactors in many processes, and perhaps because of the deleterious effects to cellular energetics if the free energy change for ATP hydrolysis (ΔG_P) were to change. If [ADP] and [P_i] levels are to remain constant *in vivo*, increased ATP synthesis must require activation of the synthase.

A third possibility lies in the nature of the ATP synthase reaction itself. The membrane sector of the synthase, F_0, provides a pathway from protons across the mitochondrial membrane. If F_1 were 'activated', but not turning over, protons may leak through F_0, and efficiency of energy conservation will decrease. This idea is supported by the observation that IF_1 will decrease the permeability of submitochondrial vesicles to protons *in vitro* [148]. It would therefore be energetically efficient to match ATP synthase capacity closely to any desired turnover rate. In other words, the system will operate best with a small number of ATP synthase molecules turning over at near maximal rates rather than with a large number of molecules turning over slowly. This appears to approximate to the situation occurring in phosphorylating submitochondrial vesicles under some conditions [50].

Down-regulation of the ATP synthase

It is established above that the mitochondrial ATP synthase in heart is switched off in the absence of oxidative metabolism. The synthase is unusual in that its operation in reverse, uncontrolled ATP hydrolysis, would be rapidly deleterious to the cell. Thus, when oxidative phosphorylation is inoperative, down-regulation of the ATP synthase might reduce ATP loss by hydrolysis [40,63,127]. In the heart, for example, down-regulation of the ATP synthase may be a last-ditch attempt to conserve cellular ATP levels in pathological situations such as ischaemia [65–70]. This view suggests that down-regulation of the synthase has a role very different from that of up-regulation; this may indeed be the case if two separate regulatory systems are involved.

This model is superficially attractive, since down-regulation (e.g. by IF_1) can clearly prevent ATP hydrolysis *in vitro*. To demonstrate its application *in vivo*, however, is more difficult. It is necessary to show (*a*) that ATP hydrolysis in ischaemic heart is largely due to the mitochondrial ATP synthase, and (*b*) that mitochondria become uncoupled in ischaemia, since otherwise ATP hydrolysis will be self-limiting due to the build up of $\Delta\mu_{H^+}$ across the mitochondrial membrane. The importance of the second point has been demonstrated in a mutant yeast strain lacking IF_1. This strain can grow normally both aerobically and anaerobically, showing that IF_1 is not essential for cellular function; however, it loses ATP rapidly (compared to the normal strain) if challenged with the uncoupler CCCP [150].

Unfortunately, evidence from studies *in vivo* is equivocal. In ischaemic dog heart muscle, over 90% of ATP hydrolysis has been attributed to the ATP synthase [151], while in rat cardiomyocytes, very little ATP hydrolysis is so attributable [152]. (Both these studies used oligomycin as a specific inhibitor of hydrolysis by the ATP synthase). Submitochondrial vesicles isolated from ischaemic dog heart show a lowered ATP hydrolytic capacity [65], while mitochondria from ischaemic rat heart show raised hydrolysis [153]. Finally, mitochondria from ischaemic heart (both isolated and *in situ*) have been variously reported as coupled [67,154] or uncoupled [153,155]. These variations may reflect species differences [66], difference in length of the ischaemic period [153,154] or other unknown experimental factors. In addition, care should be taken in interpreting experiments using oligomycin in whole cells. Typically this agent does not completely inhibit the ATP synthase in cells, and thus some ATP hydrolysis persists, maintaining a significant mitochondrial membrane potential even in anoxic, oligomycin-treated cells.

Few data are available from mitochondria after reperfusion of ischaemic tissue, which is when major damage to cell viability occurs. Interestingly, however, a pore has been identified in isolated heart mitochondria which opens at unphysiologically high [Ca^{2+}] levels (such as might occur in reperfusion after ischaemia) [156], and which, when open, will uncouple the mitochondria. Furthermore, blockage of this pore (e.g. by cyclosporin) decreases injury in cardiomyocytes after simulated ischaemia [157]. If this pore is indeed active *in vivo*, the protective

role of a Ca^{2+}-independent inhibitor protein like IF_1 becomes more likely.

In conclusion, therefore, the long-held view that down-regulation of the ATP synthase is an ATP sparing mechanism in ischaemia (and other pathological states) may be a valid one, but without more experimental evidence for its protective role *in vivo*, it cannot yet be regarded as proven. At the very least, this model needs reassessing in the light of recent demonstrations of up-regulation of the ATP synthase in heart cells [72,73]. It might be that down-regulation and up-regulation of the synthase represent totally separate regulatory systems, as the above model implies—but it is still possible that they represent opposite poles of a single mechanism, the details of which remain to be established.

SUMMARY AND FUTURE PROSPECTS

It has been established above that the mitochondrial ATP synthase in heart is subject to direct regulation *in vivo*. This enzyme catalyses a non-equilibrium step in the cell, and variations in flux through it cannot be explained by variations in levels of its substrates (ADP, P_i, $\Delta\mu_{H^+}$) or its product (ATP). Other kinetic anomalies in rates of ATP synthesis, such as a delay in reaching a steady state after a de-energized/energized transition, also suggest regulation of this enzyme.

Direct demonstrations of ATP synthase regulation *in vivo* come from measurements of synthase (hydrolytic) capacity in ischaemic heart muscle (down-regulation) and cardiomyocytes (both up- and down-regulation). Down-regulation has been linked to the operation of a regulatory protein, IF_1, which inhibits the synthase, and whose properties have been extensively studied. Down-regulation may be a mechanism for conserving ATP in heart tissue in the absence of oxidative metabolism, when the synthase is capable of acting as an ATP hydrolase.

The mechanism of up-regulation is unknown. The major effector promoting activation of the synthase is intramitochondrial Ca^{2+}, which reflects changes in cytoplasmic Ca^{2+} on increased contractile work or after treatment with positive inotropic agents. This allows the rapid integration of ATP synthase activity with the activities of other regulatory enzymes such as pyruvate dehydrogenase and isocitrate dehydrogenase. However, Ca^{2+} does not act directly on the ATP synthase, or on the regulator IF_1. It is possible that a second regulatory protein, CaBI, may be involved in up-regulation of the ATP synthase, but relatively little is known about this protein compared to IF_1.

Up-regulation of the ATP synthase allows the cell to match ATP synthase capacity closely to the desired flux through it. It is not clear why the synthase cannot operate at a high fixed capacity, without direct control; however, up-regulation does appear to be necessary for cell homeostasis since, in its absence, intracellular ATP levels fall. Since up-regulation is lost in the chronically overloaded rat heart (whether overload is due to hypertension or induced by thyroxine), it is interesting to speculate as to the role of ATP synthase regulation in the aetiology of heart failure in such conditions.

The above discussion leads to the conclusion that the rate of ATP synthase turnover is modulated *in vivo* by three separate mechanisms: variation in ADP and/or P_i concentrations, variation in $\Delta\mu_{H^+}$ (via dehydrogenase activation), and variation in enzyme capacity via a regulatory element. In the normal heart, the last two (and particularly the last) are apparently of greatest importance, while in pathological states one or both of these may be absent. In order to assess these elements quantitatively, however, a mathematical model needs to be developed incorporating all three, and this should be backed up by improved methods for obtaining free intracellular concentrations of the small molecules involved. Only then will we be able to predict, for example, under what conditions loss of a control mechanism may be tolerated by the heart, or when irreversible energy depletion may occur—for example in heart failure.

Further developments are also necessary at the molecular level. In particular the protein(s) involved in the Ca^{2+}-dependent regulatory system ('up-regulation') need to be positively identified, and the mechanism for their interaction with Ca^{2+} established. It may be that such proteins are related to one of the families of Ca^{2+}-regulated systems whose structure is well-defined; if so, considerable insights into their structure may be readily accessible after identification of their primary structure. In addition, studies on the expression of these proteins during growth or during induction of hypertrophy would be useful for assessing changes in control mechanisms during normal, or abnormal, development.

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