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ENERGY-DEPENDENT ACCUMULATION OF IRON BY ISOLATED RAT LIVER MITOCHONDRIA

IV. RELATIONSHIP TO THE ENERGY STATE OF THE MITOCHONDRIA

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SUMMARY

1. The energy-dependent accumulation of iron by isolated rat liver mitochondria, respiring on endogenous substrates, is strongly dependent on the efficiency of energy coupling in the respiratory chain as measured by respiratory control with ADP and the endogenous energy dissipation. The accumulation reached a saturation level at respiratory control with ADP values (with succinate as the substrate) of approx. 4.0.

2. In the presence of exogenous substrate, the energy-dependent accumulation of iron was markedly reduced, primarily due to binding of iron as carboxylate complexes having less favourable dissociation constants than the iron(III)-sucrose complex(es).

3. The effect of added ATP was at least 2-fold, i.e. that of providing energy and that of chelating iron. When the mitochondria respired on endogenous substrate, the energy-dependent accumulation of iron increased at low concentrations of ATP, whereas higher concentrations ($> 50\ \mu M$) gradually inhibited the uptake.

4. Energization of the mitochondria by the generation of an artificial K^+ gradient across the inner membrane with valinomycin in a K^+-free medium increased the energy-dependent accumulation of iron.

INTRODUCTION

In previous studies [1–3], rat liver mitochondria were shown to accumulate iron by an energy-dependent as well as by an energy-independent mechanism. The energy-dependent accumulation, probably an inner membrane and matrix loading with iron [3], appeared to be very similar to the high-affinity binding of Ca^{2+} [2]. Furthermore, in the presence of P_i iron induced a stimulation of State 4 as well as State 3 respiration together with an increase in the rate of endogenous energy dis-

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

sipation notably at higher concentrations of the cation [4–6]. Thus, the ability of mitochondria to carry out energy-dependent accumulation of iron appeared to be very similar to their capacity for energy-dependent accumulation of other cations (for review, see ref. 7). On the other hand, whereas cation uptake in general can be increased several fold by the addition of substrates or ATP [8–11], the energy-dependent accumulation of iron was markedly inhibited under these conditions [1]. As already pointed out, these peculiarities are probably related to the strong tendency of iron ions to form complexes of high stability [1]. Thus, so far all our studies on the accumulation of iron have been performed on mitochondria respiring on endogenous substrates in the absence of P_i.

In the present study the relationship between the iron accumulation and the mitochondrial energy state has been studied in more detail. From these studies it is concluded that the energy-dependent accumulation of iron is closely linked to the energy state of the inner membrane. Thus the amount of iron accumulated in State 1 is positively correlated to the degree of energy coupling and negatively correlated to the rate of endogenous energy dissipation. Furthermore, the energy-dependent accumulation is increased by energization of the mitochondria either by valinomycin in a K^+-free medium or by low concentrations of ATP as recently reported for Ca^{2+} [12].

MATERIALS AND METHODS

Animals and preparation of mitochondria

Rat liver mitochondria were prepared essentially as previously described [2]. In the valinomycin experiments, the mitochondria were prepared and stored in a K^+-free medium. The functional integrity of the mitochondria was tested as described [13]. Increasing degree of loose-coupling was obtained by incubating the mitochondria in a water bath at 10 °C and removing samples at timed intervals.

Accumulation of iron and calcium

The iron accumulation experiments were performed as described [2]. Alterations and additions are indicated in the legends to figures and table.

In the valinomycin experiments K^+-free media were used, and the osmolarity was adjusted to the same final value by increasing the concentration of sucrose. The ionophore and iron were added simultaneously.

Accumulation of calcium was measured by incubating the mitochondria as described for iron except that the reaction time was 60 s. Aliquots of 1 ml were withdrawn and transferred to 5 ml of ice-cooled incubation medium for centrifugation. The pellet was rinsed with isotonic sucrose and taken up in Unisolve and counted in a Mark I Liquid Scintillation Counter (Nuclear Chicago Corp.) to an accuracy of 1 %.

All experiments were performed in duplicate. Energy-dependent accumulation of calcium was taken as the difference between the accumulation in the absence and presence of 17 μM CCCP [1–3].

Spectrophotometry and other analytical methods

The measurement of oxidation-reduction levels of mitochondrial *b*-type cytochromes was performed as previously described [14]. The K^+ content of the

media and the mitochondria was determined by flame photometry on an Eel photometer. Protein was determined using the Folin-Ciocalteau reagent [15].

Chemicals

ADP, ATP, oligomycin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were from the Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Atractyloside (A grade), valinomycin and HEPES (A grade) were purchased from Calbiochem (Lucerne, Switzerland), and $^{59}FeCl_3$ and $^{45}CaCl_2$ from Institutt for Atomenergi, Kjeller, Norway.

Other chemicals were of highest purity commercially available. Double quartz-distilled and deionized water was used throughout.

RESULTS

Effect of protein concentration on the iron accumulation

The net accumulation of iron varied with the amount of mitochondria (Fig. 1) as well as with the concentration of iron [1]. Moreover, as shown in Fig. 1, the energy-dependent accumulation behaved quite differently from the energy-independent accumulation to changes in the ratio of iron/protein in the incubation medium. Thus, at 0.25 mM iron(III)-sucrose the energy-dependent accumulation reached a level at approx. 1.5 mg protein/ml (Fig. 1), whereas the energy-independent accumulation decreased progressively at protein concentrations above 1 mg/ml. The percentage of energy-dependent accumulation of iron, therefore, increased with increasing

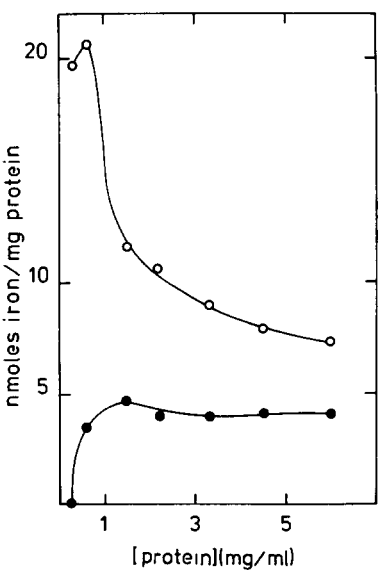

Fig. 1. Effect of mitochondrial protein concentration on the energy-dependent (●) and energy-independent (○) accumulation of iron by isolated rat liver mitochondria. The mitochondria were preincubated for 10 min at 25 °C in a medium containing in a final volume of 1.5 ml: 225 mM sucrose, 10 mM HEPES buffer, pH 7.4, 5 mM $MgCl_2$ and 10 mM KCl; 0.25 mM iron was added and the reaction followed as described, t = 30 s (For details, see Materials and Methods).

protein concentration and it represented approx. 35 % of the total amount of iron accumulated at a concentration of 6 mg protein/ml.

Effect of the degree of energy-coupling on the accumulation of iron and calcium in the absence and presence of exogenous substrate

A variation in the energy-dependent accumulation of iron from one mitochondrial preparation to the other was repeatedly observed during these studies. From the data of Fig. 1, this variation could not be due to differences in the amount of protein in the incubation mixture. As expected, however, the energy-dependent accumulation, when measured in mitochondria respiring on endogenous substrates in the absence of P_i, is highly dependent on the functional integrity of the mitochondria as measured by the respiratory control with ADP (Fig. 2A). Thus, the amount of iron accumulated increased with increasing degree of coupling until a level was reached at respiratory control with ADP values of

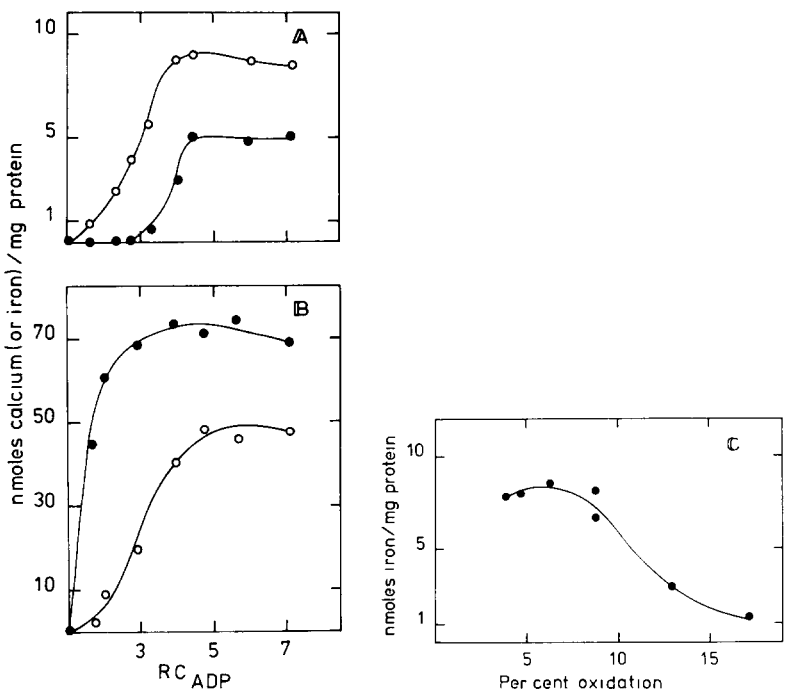

Fig. 2. (A and B) Effect of the degree of energy coupling, measured as respiratory control with ADP ($R.C._{ADP}$), on the energy-dependent accumulation of iron (A) and calcium (B) in State 1 (in the absence of P_i, ○) and State 4 (with 3.3 mM succinate as the substrate, ●). For experimental details, see Materials and Methods and legend to Fig. 1. The concentration of iron and calcium was 0.25 mM. (C) Relationship between the oligomycin-induced energy-dissipation and the energy-dependent accumulation of iron. Mitochondria, 4–5 mg of protein, were incubated in the medium of the polarographic experiments (see Materials and Methods) in the presence of 3 μM rotenone, 3.3 mM cyanide, 4.2 mM ascorbate and 90 μM TMPD. Following maximal energization by 1 mM ATP, 10 μg oligomycin was added and the rate of oxidation of the *b*-type cytochromes recorded; t = 60 s. 100 % oxidation represents the difference in transmission ΔT (%) = $\Delta(T_{564\ nm} - T_{575\ nm})$, value in the presence of ATP and 17 μM CCCP. Accumulation of iron was performed as described in Fig. 1.

3.5–4.0. On the other hand, in the presence of 3.3 mM succinate, no measurable energy-dependent accumulation of iron could be detected in mitochondria with respiratory control with ADP values below 2.8. Only in tightly coupled mitochondria was an energy-dependent accumulation observed in the presence of succinate, and the saturation level reached at respiratory control with ADP values greater than 4.0 represented only 50–60 % of the amount accumulated in the absence of succinate.

For the purpose of comparison, the results of analogous experiments with calcium are given in Fig. 2B. In this case, the accumulation in mitochondria respiring on endogenous substrate in the absence of P_i reached a saturation level at respiratory control with ADP values of 4.5–5.0, whereas in the presence of 3.3 mM succinate, a saturation level was obtained at respiratory control with ADP values of 2.5–3.0. At the highest degree of energy coupling, the accumulation in the absence of succinate amounted to approx. 70 % of that in the presence of succinate.

Relationship between the endogenous energy dissipation and iron accumulation

It has been generally found that it is impossible to isolate mitochondria without some leakage of the 'energy potential' [16]. Furthermore, by aging and loose-coupling, the endogenous energy leakage increases and simultaneously the 'energy potential' decreases, thereby reducing the efficiency of all energy-dependent processes [17, 18]. Recently Flatmark and Pedersen [19] have estimated the magnitude of the endogenous energy dissipation in brown adipose tissue and liver mitochondria maximally energized by ATP, from the rate and extent of the oligomycin-induced oxidation of the cytochrome *b* complex. Using this parameter, it is seen from Fig. 2C that the energy-

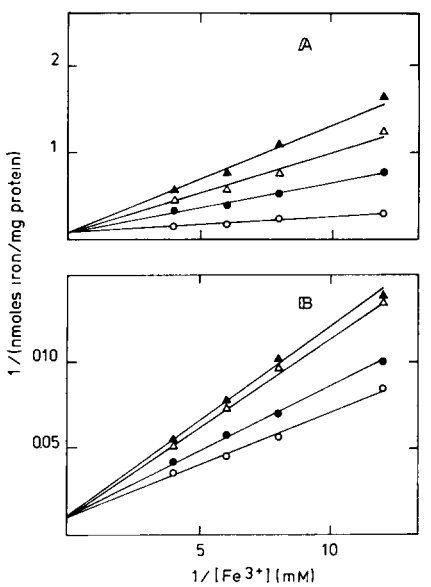

Fig. 3. Effect of substrates on energy-dependent (A) and energy-independent (B) accumulation of iron. The mitochondria, 4–5 mg of protein, were incubated as described (see Materials and Methods and legend to Fig. 1). The substrates were added 60 s before iron (0.25 mM). ○, no addition; ●, 5 mM succinate, △, 5 mM pyruvate; ▲, 5 mM malate. The results are presented as Lineweaver-Burk plots.

dependent accumulation of iron is inversely related to the rate of energy dissipation thus determined.

Effect of exogenous substrates

All the studies on mitochondrial iron accumulation have so far been performed with mitochondria respiring on endogenous substrates in the absence of P_i [1–6]. As already mentioned (Fig. 2A and ref. 1), the attempts to increase the energy-dependent iron accumulation by adding substrates were not successful.

As seen from Figs 3A and 3B, succinate, pyruvate and malate all inhibited the energy-dependent (Fig. 3A) as well as the energy-independent (Fig. 3B) accumulation of iron by increasing the K_m value of iron without changing the V; the inhibition was most pronounced with malate. Essentially similar results were obtained with β-hydroxybutyrate and α-ketoglutarate.

Effect of ATP and ADP

Figs 4A and 4B show the effect of ATP-induced energization on the iron accumulation. It is seen that the energy-independent accumulation decreased as the ATP concentration increased (Fig. 4B), whereas the energy-dependent accumulation (Fig. 4A) was stimulated at low ATP concentrations. Higher concentrations of ATP ($>50\ \mu M$), however, gave a progressive inhibition of the iron uptake. Upon inhibition

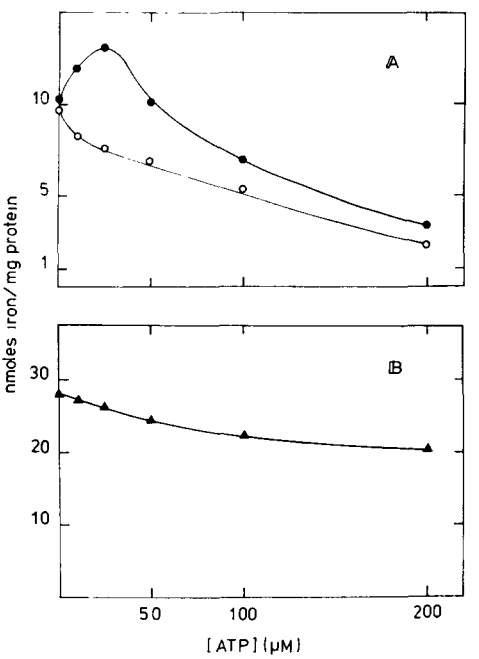

Fig. 4. Effect of ATP on the energy-dependent accumulation of iron (A) in the absence (●) and presence (○) of atractyloside, and on energy-independent accumulation of iron in the absence of atractyloside (B). The mitochondria, 4–5 mg of protein, were incubated as described (see Materials and Methods and legend to Fig. 1). ATP was added 30 s before iron (0.25 mM), and in the atractyloside experiments, the mitochondria were preincubated with the inhibitor for 3 min.

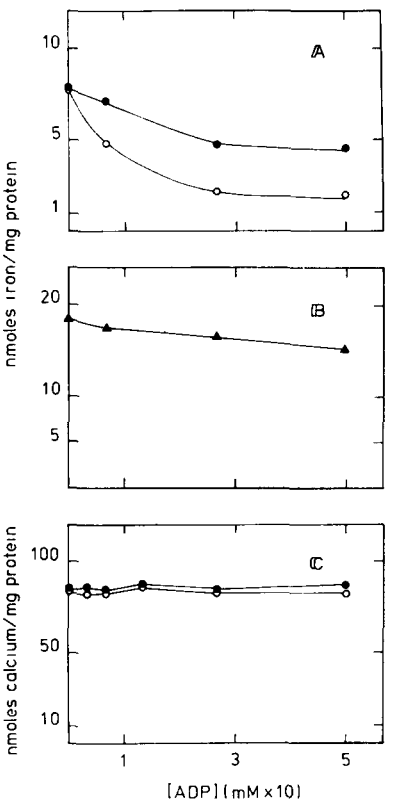

Fig. 5. Effect of ADP on the energy-dependent accumulation of iron (A) in the absence (●) and presence (○) of atractyloside, and on the energy-independent accumulation of iron in the absence of atractyloside (B). Mitochondria, 4–5 mg of protein, were incubated as described (see Materials and Methods and legend to Fig. 4). (C) Effect of ADP on substrate-supported accumulation of calcium in the absence (●) and presence (○) of atractyloside. The incubation medium was supplemented with 3.3 mM succinate, and the accumulation of calcium was performed as described (see Materials and Methods) in the absence and presence of 50 μM atractyloside. ADP was added 30 s before calcium (0.25 mM).

of the ATP translocation by atractyloside, no stimulation of the energy-dependent accumulation was observed; at all concentrations of ATP tested, the accumulation of iron was inhibited by atractyloside.

In contrast to ATP, ADP was inhibitory at all concentrations tested (Figs 5A and 5B) and the inhibition was more pronounced in the presence of atractyloside; atractyloside alone had virtually no effect on the iron accumulation.

For comparison, it is seen from Fig. 5C that ADP and atractyloside had no effect on the substrate-supported calcium accumulation.

Effect of energization by an artificial K^+ gradient

The generation of an artificial K^+ gradient across the mitochondrial inner membrane induces a high energy state [20–22] which can be utilized to drive energy-requiring processes such as ATP synthesis, reversed electron flow and cation trans-

TABLE I

EFFECT OF VALINOMYCIN-INDUCED K^+ EFFLUX ON ENERGY-DEPENDENT ACCUMULATION OF IRON

Mitochondria, approx. 4–5 mg of protein, were incubated at 25 °C in a medium containing in a final volume of 1.5 ml: 0.25 M sucrose, 10 mM HEPES buffer, adjusted to pH 7.4 with NaOH, and 5 mM $MgCl_2$. The mitochondria were preincubated for 10 min with the uncoupler (CCCP), whereas valinomycin and 0.25 mM iron(III)-sucrose were added simultaneously. Energy-dependent accumulation is defined as the amount of iron accumulated in the absence of CCCP minus the amount accumulated in the presence of 17 μM CCCP in a parallel experiment. The mean $\pm$S.D. of eight different experiments are given.

Addition	Energy-dependent accumulation of iron (nmol · mg^{-1} protein)
None	7.6$\pm$1.5
Valinomycin (1 μg)	11.3$\pm$1.7

location [23–25].

From Table I it is seen that the valinomycin-induced energization [20–22] can be utilized for energy-dependent accumulation of iron. Thus, when measured in mitochondria (containing 80–145 nmol of $K^+ \cdot mg^{-1}$ protein) respiring on endogenous substrates and suspended in a medium containing less than 1 μM K^+, valinomycin increased the energy-dependent accumulation by approx. 50 %.

Furthermore, on preincubation of the mitochondria in isoosmotic media containing different concentrations of KCl (Fig. 6A–C), an energization as well as a marked increase in the energy-dependent accumulation of iron was observed. Both effects were maximal at 20–30 mM K^+, whereas the energy-independent accumulation was only slightly stimulated.

DISCUSSION

As previously reported [3], the energy-independent accumulation of iron in rat liver mitochondria represents a low-affinity binding mainly to the outer membrane, whereas the energy-dependent accumulation represents a high-affinity inner membrane and matrix loading [1, 2]. These conclusions are further supported by the present study. Thus, from Fig. 1 it can be seen that the mitochondria possess two classes of iron binding sites with an inverse relationship between capacity and affinity to accumulate iron from the suspending medium. Furthermore, the data of Fig. 1 point to the noxious effect of iron [1] at high iron/protein ratios; at a ratio $>$375 nmol iron/mg of protein, the mitochondria tend to aggregate (unpublished observation) and concomitantly the energy-dependent accumulation approaches zero.

As expected, it was found (Fig. 2) that the efficiency of the energy coupling determined the ability of the mitochondria to carry out energy-dependent cation accumulation. To the best of my knowledge, this has not previously been emphasized, and requires a short comment. In State 1, in the absence of P_i, qualitatively the relationships between the degree of energy coupling and the accumulation of iron and calcium were very similar. On the other hand, when the accumulation was measured in the presence of 3.3 mM succinate, in contrast to calcium, the accumulation of iron

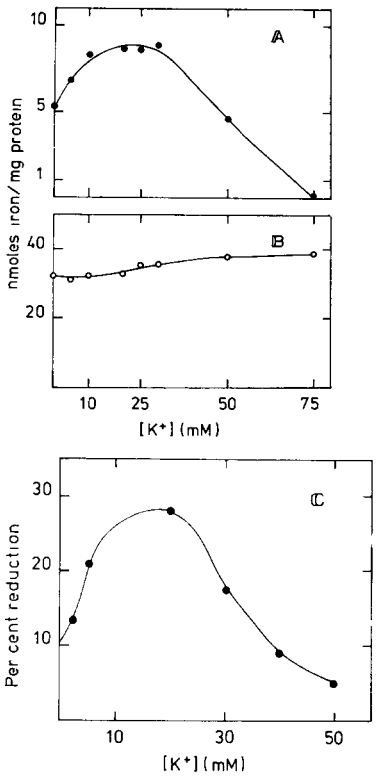

Fig. 6. Effect of KCl on energy-dependent (A) and energy-independent (B) accumulation of iron. Mitochondria, 4–5 mg of protein, prepared in K^+-free media, were incubated as described (see Materials and Methods) at increasing concentrations of KCl. The concentration of iron was 0.25 mM. (C) Effects of KCl on the reduction level of the *b*-type cytochromes. Mitochondria, 4–5 mg of protein and isolated in a K^+-free medium, were incubated as described (see Materials and Methods), in a final volume of 1 ml: 225 mM sucrose, 10 mM HEPES/NaOH buffer, pH 7.4, 5 mM $MgCl_2$, 3 μM rotenone, 3.3 mM NaCN, 4.2 mM asorbate, 90 μM TMPD and increasing concentrations of KCl. The change in transmission ΔT (%) = $\Delta(T_{564\ nm} - T_{575\ nm})$. 100 % reduction represents the difference in transmission $\Delta(T_{564\ nm} - T_{575\ nm})$ between the level obtained by adding excess dithionite and the initial ascorbate/TMPD level.

was markedly reduced (Figs 2A and 2B). This effect may be attributed primarily to reduced mitochondrial binding of iron (Figs 3A and 3B) due to the formation of iron-carboxylate complexes [26] which have less favourable stability constants than the iron(III)-sucrose complex(es), and for this reason, accumulation of iron should be studied in State 1 in the absence of P_i [1–3, 27]. Furthermore, the data of Figs 2A and 2B point to quantitative differences in the ability of rat liver mitochondria to accumulate calcium and iron, in agreement with our recent findings on the energy-dependent accumulation of iron and calcium in mitochondria from different tissues [27].

The inhibition of the energy-dependent accumulation of iron by ATP and ADP can also be explained as a chelate effect since both nucleotides form stable complexes with ferric iron [28] (e.g. at pH 7.0 the minimal value for the formation

constant of Fe(III) · ADP is 10^{29} M^{-1} [29]). The experimental evidence favouring extra-mitochondrial formation of iron-nucleotide complexes are indicated by the observations that atractyloside plus ATP markedly reduced the amounts of iron accumulated, whereas atractyloside alone had virtually no effect on the accumulation of iron (Fig. 4). At lower concentrations of ATP, however, the inhibitory effect was compensated due to the low K_m value of the nucleotide for energization of the inner membrane [14] and the excess of iron relative to ATP. This finding is in substantial agreement with that of Spencer and Bygrave [12] on the effect of increasing ATP concentrations on the accumulation of calcium by rat liver mitochondria.

As to the effect of valinomycin-induced K^+ efflux on iron accumulation, the results are in essential agreement with those reported by Scarpa et al. [24] and Vinogradov and Scarpa [30] in experiments with calcium.

Contrary to the results reported by van Dam et al. [20–22], we found an increased steady-state reduction of the *b*-type cytochromes in the presence of increasing concentrations of KCl (Fig. 6C). This discrepancy may be due to differences in experimental design. Thus, whereas van Dam et al. [20–22] added KCl and recorded the immediate changes in the redox state of the *b*-type cytochromes, in the present study, the mitochondria were preincubated for 10 min at increasing concentrations of KCl before the redox level of the cytochrome *b* complex was measured, and it is therefore unlikely that the results obtained (Fig. 6A–C) reflect the generation of a diffusion potential [20–22]. On the other hand, K^+ has been shown to evoke a series of responses in isolated mitochondria [31–35], and of particular importance to the present study is the finding that in K^+-depleted rat liver mitochondria respiration could be restored by adding 10–20 mM K^+ to the medium [35] or very close to the values reported for maximal reduction of the *b*-type cytochromes and the accumulation of iron. Thus, the effect of KCl on the redox level of the cytochrome *b* complex and the energy-dependent accumulation of iron may be mediated at least in part by reconstituting the mitochondria of their K^+ content and thereby increase the degree of energy coupling and energy conservation.

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