Stimulation of the Respiratory Chain of Rat Liver Mitochondria between Cytochrome c_1 and Cytochrome c by Glucagon Treatment of Rats

By ANDREW P. HALESTRAP

Department of Biochemistry, Medical School, University of Bristol, Bristol BS8 1TD, U.K.

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Mitochondria from glucagon-treated rats oxidize succinate, but not ascorbate plus tetramethylphenylenediamine, faster in the uncoupled state than do control mitochondria. The rate of O_2 uptake in the presence of both substrates is equal to the sum of the rates of the O_2 uptake in the presence of either substrate alone. It is concluded that the mitochondrial respiratory chain is limited at some point between cytochromes b and c and that this step is regulated by glucagon. Measurement of the cytochrome spectra under uncoupled conditions in the presence of succinate and rotenone demonstrates a crossover between cytochromes c and c_1 when control mitochondria are compared with those from glucagon-treated rats, cytochrome c being more oxidized and cytochrome c_1 more reduced in control mitochondria. Under conditions where pyruvate metabolism is studied the control mitochondria are generally more oxidized than those from glucagon-treated rats, the redox state of cytochrome b -566 correlating with the rate of pyruvate metabolism in sucrose medium. However, when the redox state of the mitochondria is taken into account, a crossover between cytochromes c and c_1 is again apparent. The spectra of the b cytochromes are complex, but cytochrome b -562 appears to become more reduced relative to cytochrome b -566 in mitochondria from glucagon-treated rats than in control mitochondria. This can be explained by the existence of a more alkaline matrix in glucagon-treated rats, the redox potential for cytochrome b being pH-sensitive. It is concluded that glucagon stimulates electron flow between cytochromes c_1 and c . The physiological significance of these findings is discussed.

Mitochondria isolated from the liver of rats treated with glucagon show a variety of differences from those of control rats. They show enhanced rates of pyruvate metabolism (Adam & Haynes, 1969, Garrison & Haynes, 1975), enhanced rates of coupled and uncoupled respiration with NADH- and flavo-protein-linked substrates (Yamazaki, 1975), higher mitochondrial concentrations of Mg^{2+} , K^+ and ATP (Haynes, 1976; Halestrap, 1978*b*), higher rates of citrulline synthesis and valinomycin-induced K^+ flux (Yamazaki & Graetz, 1977; Yamazaki *et al.*, 1977), and a more alkaline matrix pH and greater membrane potential under metabolizing conditions (Halestrap, 1978*b*). Yamazaki & Graetz (1977) have suggested that some of these effects may be due to the enhanced respiratory activity of mitochondria from glucagon-treated rats (Yamazaki, 1975) and in the preceding paper (Halestrap, 1978*b*) this suggestion was confirmed. Glucagon appears to exert its effects on mitochondria through the mediation of an increase in intracellular 3':5'-cyclic AMP (Garrison & Haynes, 1975). The pertinent question in the present paper is how a rise in intracellular cyclic AMP concentration can activate the respiratory chain.

Yamazaki (1975) demonstrated that only the oxidation of substrates entering the respiratory chain before cytochrome b is enhanced after glucagon treatment. There is no effect of glucagon on the oxidation of ascorbate in the presence of *NNN'*-tetramethylphenylenediamine; this has been confirmed by Titheradge & Coore (1976) and Halestrap (1978*b*). The present paper demonstrates that the rate-limiting step in the respiratory chain is between cytochrome b and cytochrome c . Spectral analysis of the cytochromes under uncoupled and metabolizing conditions shows that glucagon causes a cross-over between cytochrome c_1 and cytochrome c . This implies an activation of the oxidation of complex 3. The significance of this direct control of the respiratory chain for metabolic regulation is discussed.

Experimental

Materials

The source of all chemicals and biochemicals was the same as that given in the preceding paper (Halestrap, 1978*b*). Mitochondria were prepared from control and glucagon-treated rats as described (Halestrap, 1978*b*).

Methods

Measurements of pyruvate metabolism and mitochondrial respiration were performed as described by Halestrap (1978b).

Measurements of cytochrome spectra. Measurements of the absorbance of mitochondrial respiratory components at fixed wavelengths were made with a conventional split-beam or double-beam spectrophotometer designed and built in this department, which permitted full-scale chart-recorder deflections of 0.0125 with less than 5% noise. For scanning the cytochrome spectra, one of three techniques was used. For preliminary experiments, the split-beam spectrophotometer described above was used. In later experiments the spectrophotometer was refined to measure spectra at liquid-N₂ temperatures. Samples (0.7 ml) of mitochondria (approx. 12 mg of protein/ml) under the appropriate conditions were transferred to 0.2 cm-light-path Perspex cuvettes inserted in a metal cooling fin, and the assembly was rapidly cooled in liquid N₂. Control and sample cuvettes were present in the same assembly, which could be mounted in a conventional split-beam spectrophotometer, such that the cooling fin dipped into liquid N₂ contained in a Dewar flask. The spectra were then scanned in the conventional manner.

To obtain accurate spectra rapidly under metabolizing conditions, a computer-linked single-beam spectrophotometer was used as described by Cogdell (1973) using the principles of Norris & Butler (1961). This machine allowed a scanning rate of 6.4 nm/s and the data to be stored (512 data points stored per scan). Baselines were determined by scanning spectra of aerobic mitochondria in the presence of uncoupler and rotenone. The spectra under the relevant conditions were obtained by scanning the same mitochondrial suspension and then subtracting the baseline. Spectra could be displayed on a cathode-ray oscilloscope or X-Y chart-recorder at a variety of sensitivities. Difference spectra between two batches of mitochondria incubated under the same conditions were readily calculated by the computer. Spectra could also be 'normalized' to take into account differences in protein concentration between different incubations. To accentuate the position of the cytochrome peaks, for example to discriminate between cytochromes *c* and *c*₁, spectra could be differentiated four times as described by Butler & Hopkins (1970). Details of the exact conditions used are given in the legends to the Figures.

Results and Discussion

Effect of glucagon on mitochondrial respiration

Fig. 1 shows the rates of O₂ uptake by uncoupled mitochondria in the presence of succinate and ascorbate + *NNN'*-tetramethylphenylenediamine.

Fig. 1. Effect of glucagon treatment on mitochondrial respiration

Liver mitochondria (approx. 1 mg/ml) were incubated in medium (125 mM-KCl/2.5 mM-potassium phosphate/5 mM-MgCl₂/20 mM-Tris/HCl/10 μ M-rotenone, pH 7.4) in a Clark oxygen electrode at 25°C. Additions were made as indicated: A, 1 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; B, 10 mM-potassium succinate; C, 10 mM-sodium ascorbate + 0.3 mM-*NNN'*-tetramethyl-*p*-phenylenediamine; D, 10 μ M-antimycin. The broken lines represent incubations where antimycin was added at C. Rates of O₂ uptake are given as ng-atoms of O/min per mg of mitochondrial protein.

The enhancement by glucagon treatment of rats of the rate of succinate oxidation is apparent as reported previously (Yamazaki, 1975; Titheradge & Coore, 1976; Halestrap, 1978b), whereas no effect on ascorbate oxidation is seen. The rate of oxidation of succinate and ascorbate + *NNN'*-tetramethylphenylenediamine is equal to the sum of the rates of O₂ uptake of each substrate on its own. This implies that there is a rate-limiting step in the respiratory chain before cytochrome *c*. Further, since the oxidation of succinate and all NADH-linked substrates tested is enhanced after glucagon treatment, it appears that the rate-limiting step is between ubiquinone and cytochrome *c* (i.e. in complex 3), and that this is the step that is activated in mitochondria from glucagon-treated rats.

Effects of glucagon on the spectra of the cytochromes during respiration

To confirm this and to establish the exact location of the stimulation, the spectra of the cytochromes

Fig. 2. *Effect of glucagon on the cytochrome spectra of uncoupled liver mitochondria oxidizing succinate* Mitochondria (approx. 10 mg of protein/ml) were incubated at 25°C in KCl medium containing succinate and uncoupler as described in Fig. 1. Spectra were measured at 25°C in a computer-linked single-beam spectrophotometer (a) or with a split-beam spectrophotometer at -170°C (b) as described under 'Methods'. Exact protein concentrations are given in parentheses. Data from the same batches of mitochondria are shown in (a) and (b). The rate of succinate-dependent O₂ uptake was 105 ng-atoms/min per mg for the control mitochondria and 172 ng-atoms/min per mg for the mitochondria from glucagon-treated animals. In (a) the spectrum of the fully reduced control mitochondria is shown. This was obtained by allowing the incubation to become anaerobic. Details of the fourth-derivative procedure are given under 'Methods'. Spectra are derived from mitochondria from either control or glucagon-treated rats as shown below and at the protein concentrations given in parentheses. In (a) curves (i) and (ii) control (10.6 mg/ml), (iii) glucagon (10.6 mg/ml), (iv) control-glucagon difference spectrum; in (b) curve (i) glucagon (11.3 mg/ml), (ii) control (10.7 mg/ml).

were studied in uncoupled mitochondria oxidizing succinate. The spectra from control and glucagon-treated mitochondria measured both at room temperature (20°C) and liquid-N₂ temperature are shown in Figs. 2(a) and 2(b). Cytochrome *c* is more reduced and cytochromes *c*₁ and *b* are more oxidized in mitochondria from glucagon-treated rats. The difference spectrum measured on the computer-linked spectrophotometer (Fig. 2a, spectrum iv) shows a clear cross-over between cytochromes *c* and *c*₁. The fourth derivatives of the spectra are also shown to accentuate the peak positions (see under 'Methods'). This procedure highlights two problems. Firstly because the α -band of cytochrome *c* (550 nm) is very close to that of cytochrome *c*₁ (554 nm), separation of the two peaks is difficult and was not achieved in the aerobic mitochondria oxidizing succinate. It could, however, be picked up in the fourth derivative of the fully reduced spectra (i). Secondly, cytochrome *b* shows a complex α -peak. It is now fairly well established that there are two pools of cytochrome *b* in the mitochondrial membrane, cytochrome *b*_K (α -peak at 562) existing predominantly on the inside of the membrane and cytochrome *b*_T (α -peak at 566 nm) existing predominantly on the outside of the membrane. There may also be an energized form of cytochrome *b*-566 (α -peak at 558 nm) (see Lemberg & Barrett, 1973; Hagihara *et al.*, 1975; Papa, 1976, for reviews). The fourth-derivative spectra (ii, iii) suggest that, in uncoupled mitochondria oxidizing succinate, the major forms of cytochrome *b* are cytochromes *b*-558 and *b*-562. In uncoupled anaerobic mitochondria cytochrome *b*-558 is not apparent as expected (spectra i). Despite these complexities, the existence of a cross over between cytochromes *c*₁ and *c* is immediately apparent (spectrum iv) and is confirmed by the spectra measured at liquid-N₂ temperature where resolution of the cytochromes *c* and *c*₁ peaks is possible (Fig. 2b). Thus glucagon causes an enhancement of electron flow between cytochromes *c*₁ and *c*, leading to reduction of cytochrome *c* and oxidation of cytochromes *c*₁ and *b*.

Effects of glucagon on the cytochrome spectra of metabolizing mitochondria

It was argued (Halestrap, 1978b) that the enhanced mitochondrial pyruvate metabolism induced by glucagon treatment of rats was due to a more alkaline matrix produced by stimulated respiratory-chain activity. If this is the case, it should be possible to demonstrate a crossover in the respiratory chain during metabolism when mitochondria from glucagon-treated rats are compared with controls. The experiments recorded in Fig. 3 demonstrate that this is indeed the case. Fig. 3 shows the cytochrome spectra of mitochondria metabolizing pyruvate in sucrose and KCl media. It is apparent that in both

media, but notably in sucrose media, the control mitochondria are more oxidized than mitochondria from glucagon-treated rats. Indeed Fig. 4 shows that the oxidation state of cytochrome *b*-566 correlates very well with the rate of pyruvate metabolism, cytochrome *b*-566 being more reduced under conditions in which metabolism is enhanced. This may be explained if it is remembered that the only source of reducing power under these conditions comes from the oxidation of pyruvate, since no other substrate is available. Pyruvate entry is enhanced in mitochondria from glucagon-treated rats (Halestrap, 1978b) and thus a greater supply of NADH is available for the respiratory chain. Furthermore the ATP concentrations within mitochondria are increased in glucagon-treated mitochondria under these conditions (Halestrap, 1978b), and it has been demonstrated that the redox state of the respiratory chain on the substrate side of cytochrome *c* oxidase is in equilibrium with the ATP/ADP ratio (Davis & Blair, 1977; Wilson *et al.* 1977). Thus an increased reduction of the cytochromes would be expected in glucagon-treated mitochondria. Taking the difference of the redox state of the mitochondria into account, it is apparent that glucagon causes a relative increase in reduction of cytochrome *c* compared with cytochrome *b*.

A more detailed analysis of this change is made in Fig. 3(c), where the spectrum of the mitochondria from glucagon-treated animals was normalized to bring its redox state to a value similar to that of the control mitochondria. This normalization factor was calculated as the ratio of the area of the cytochrome α -peaks in the control mitochondria (Fig. 3b, spectrum i) to those in the glucagon-treated mitochondria (Fig. 3b, spectrum ii). After normalization it is clear that cytochrome *c* is more reduced and cytochrome *b*-566 more oxidized in mitochondria from glucagon-treated rats. Cytochrome *c*₁ is not readily resolvable, but the fourth derivative does suggest that cytochrome *c*₁, like cytochrome *b*-566, is more oxidized in mitochondria from glucagon-treated animals. Thus under metabolizing conditions there is again evidence for a crossover between cytochromes *c* and *c*₁. The fourth derivative of the difference spectra (iii) would also suggest a crossover between cytochromes *b*-562 and *b*-566, cytochrome *b*-562 becoming more reduced and *b*-566 more oxidized in mitochondria from glucagon-treated rats. Assuming there is stimulation of electron flow between cytochromes *c*₁ and *c*, it would be expected that all the cytochromes *b* would become more oxidized. However, cytochrome *b*-562 is on the inner surface of the membrane and sensitive to the matrix pH (Papa, 1976; Papa *et al.*, 1975). The redox potential of cytochrome *b* is pH-dependent between pH 6.2 and 8.1 (see Hagihara *et al.*, 1975), such that at higher pH cytochrome *b* becomes more reduced at the same redox potential. Since the

Fig. 3. Effect of glucagon treatment on the cytochrome spectra of liver mitochondria metabolizing pyruvate Mitochondria (approx. 10mg/ml) were incubated at 37°C in medium containing 10mM-KHCO₃, 2.5mM-potassium phosphate, 5mM-MgCl₂, 20mM-Tris/HCl, and 4mM-sodium pyruvate and either 250mM-sucrose (a) or 125mM-KCl (b). The pH was 7.4. After incubation for 2min with shaking, samples were taken for measuring spectra either at room temperature with a computer-linked single-beam spectrophotometer, or at -170°C on a split-beam spectrophotometer, as described under 'Methods'. Exact protein concentrations are given in parentheses. The rates of pyruvate metabolism, measured as described in Halestrap (1978b), were (results expressed as nmol/min per mg of protein) 11.9 and 26.4 for control mitochondria in sucrose and KCl media respectively and 21.6 and 51.5 respectively for mitochondria from glucagon-treated rats. In (c) the data are the same as that for KCl medium given in (b) except that the spectrum of the glucagon-treated mitochondria was normalized by multiplying by 0.65 as described under 'Methods'. Spectra are derived from mitochondria from either control or glucagon-treated rats as shown below and at the protein concentrations given in parentheses. In (a), (i), glucagon (10.4mg/ml), (ii) control (9.4mg/ml), (iii) glucagon-control difference spectrum, (iv) control (5.9mg/ml), (v) glucagon (5.0mg/ml); in (b) (i) glucagon (9.7mg/ml), (ii) control (9.7mg/ml), (iii) glucagon-control difference spectrum, (iv) control (7.2mg/ml), (v) glucagon (7.7mg/ml); in (c) spectra are the same as those in (b) (i)-(iii) but normalized.

mitochondrial matrix becomes more alkaline in the mitochondria from glucagon-treated rats during metabolism, it is not unexpected that cytochrome

b-562 should become more reduced relative to cytochrome *b*-566. This would be more likely if cytochrome *b*-562 were on the substrate side of

Fig. 4. Correlation between the redox state of liver mitochondria as judged by the cytochrome *b*-566 oxidation state and the rate of pyruvate metabolism

Mitochondria (approx. 6mg/ml) were incubated at 37°C in the sucrose-based medium described in Fig. 2. The absorbance at 566–575nm was measured in a double-beam spectrophotometer as described under 'Methods'. The fully oxidized absorbance was obtained by adding uncoupler and rotenone, and the fully reduced absorbance by adding succinate and allowing the incubation to become anaerobic. Pyruvate metabolism was measured as described by Halestrap (1978*b*). Control mitochondria (■) and mitochondria from glucagon-treated rats (●) were both used. The correlation coefficient is 0.93, the slope 0.044 ± 0.004 and the intercept -0.16 ± 0.10 .

cytochrome *b*-566, since the tendency of cytochrome *b*-562 to become more oxidized in response to the stimulation of electron flow between cytochromes *c*₁ and *c* would not mask the reducing effect of elevating the matrix pH. Thus the data would favour the model for electron transport of Papa (1976) rather than that of Mitchell (1972). However, the ubiquinone cycle proposed by Mitchell (1975) could accommodate the findings reported in the present paper.

General conclusions

The present paper identifies a site of action of glucagon on the respiratory chain between cytochromes *c*₁ and *c*. It is probable that glucagon exerts its effects on mitochondrial metabolism through a rise in intracellular 3':5'-cyclic AMP concentration (Garrison & Haynes, 1975) and subsequent protein phosphorylation. Phosphorylation of liver inner mitochondrial membranes has been shown to occur *in vivo* and this may be enhanced by glucagon treatment (Zahlten *et al.*, 1972). Preliminary work in this laboratory with isolated hepatocytes confirms these observations (W. A. Hughes & R. M. Denton, unpublished work). A protein kinase has been found on the inner mitochondrial membrane, but this does

not seem to be sensitive to cyclic AMP (Vardanis, 1977). However, cytochrome *c*₁ is on the outer surface of the inner mitochondrial membrane (see Racker, 1970; Papa, 1976) and might be available for phosphorylation by a cyclic AMP-dependent protein kinase between the inner and outer mitochondrial membranes.

It has been generally assumed (see Newsholme & Start, 1973) that the rate of the respiratory chain is controlled purely by the availability of reduced substrate and the ratio of ATP/ADP, the latter usually being considered the normal regulator of the respiratory chain. However, it would seem from the present data that there is also hormonal control of the respiratory chain. The situation can be likened to a hydrodynamic analogy where the control of flow through a pipe can be mediated either by the pressure differential (equivalent to NADH and ATP concentrations) or the setting of a control valve in the pipe (equivalent to the hormonal effect on the respiratory chain). To have two such control mechanisms regulating an enzyme is not uncommon, an example being the regulation of glycogen phosphorylase by either AMP and Ca²⁺ (internal signals) or hormones, such as glucagon and adrenaline (external signals) (see Cohen, 1976). Similarly pyruvate dehydrogenase may be controlled either by feedback inhibition through acetyl-CoA and NADH or through insulin action (see Denton *et al.*, 1975). The significance of a stimulation of the respiratory chain by glucagon is not hard to see. Glucagon causes a stimulation of gluconeogenesis and nitrogen metabolism in liver (see Wieland *et al.*, 1969; Parilla *et al.*, 1975) both of which require ATP. Stimulation of the respiratory chain will allow the rate of production of ATP to be increased without requiring either a rise in NADH or drop in ATP. The ability to have an enhanced rate of ATP production without lowering ATP concentrations will allow an enhanced rate of ATP utilization by the gluconeogenic pathway and the urea cycle. Yamazaki & Graetz (1977) have shown that the rate of citrulline synthesis is enhanced after glucagon treatment and is regulated by the supply of ATP. The stimulation of pyruvate metabolism in mitochondria from glucagon-treated rats would appear to be through a rise in the matrix pH caused by an increased respiratory-chain activity leading to enhanced pyruvate transport (Halestrap, 1978*a,b*). However, the ability of the respiratory chain to supply ATP at an enhanced rate is also essential for gluconeogenesis. Indeed where an alternative oxidizable substrate to pyruvate is available it may be that the mitochondrial ATP concentration rather than the rate of pyruvate transport controls pyruvate metabolism (Walter & Stucki, 1970; Stucki *et al.*, 1972). Thus stimulation of the respiratory chain is likely to be of central importance in the regulation of liver metabolism by glucagon.

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