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PROPERTIES OF THREE CYTOCHROME *b*-LIKE SPECIES IN MITOCHONDRIA AND SUBMITOCHONDRIAL PARTICLES

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SUMMARY

1. Oxidoreduction of cytochrome *b* in rat-liver mitochondria and sonicated particles from beef-heart mitochondria was studied with emphasis on the influence of red/ox and energy conditions.

2. Difference spectra revealed three cytochrome *b*-like species, b_{558}, b_{562} and b_{566}, designated provisionally after the position of their absorption maxima at room temperature. b_{558} and b_{566} appeared to be low-potential species in comparison with b_{562} (classical cytochrome *b*).

3. Energy-linked reduction of b_{558} and b_{566} was described, driven either by respiration with (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) + ascorbate or succinate, or anaerobically by ATP.

4. Complete reduction of all three cytochrome *b*-like species was also accomplished anaerobically without ATP, and in the presence of uncoupler, if substrate couples of low red/ox potential or dithionite were added. The spectra of b_{558}, b_{562} and b_{566} thus obtained were identical with the spectra where reduction was facilitated by ATP.

5. It was concluded that the three species may represent three cytochromes *b*, or one cytochrome in three different environments. No evidence in favour of interconversion between the species was found, and no evidence in favour of 'high-energy' derivatives of cytochrome *b*. The function of two, or all three species as an entity was proposed to be essential for energy conservation at coupling site 2.

INTRODUCTION

In 1958 CHANCE[1] suggested that three cytochromes *b* may be present in Keilin–Hartree particles. A long wavelength species was originally thought to become reduced by substrate only in the presence of antimycin[1,2], and a short wavelength species in the presence of dithionite[1]. The identity of the latter with a *b*-type cytochrome was, however, not generally accepted[2]. The third cytochrome *b* is the classical

Abbreviations: FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; PMS, phenazine methosulphate; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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species described by KEILIN[3] already in 1925. The presence of three cytochromes *b* in plant mitochondria is well established[4–6]. WAINIO *et al.*[7] suggested distinction between three cytochromes *b* in beef-heart mitochondria on the basis of studies with inhibitors. This was recently confirmed by DUTTON *et al.*[8], who identified potentiometrically three species in beef-heart mitochondria. WIKSTRÖM[9] reported that three cytochrome *b*-like components could be distinguished spectrophotometrically in rat-liver mitochondria without requirement for antimycin. The potentiometric technique of DUTTON *et al.*[8] differentiates only between two species in rat-liver[8,10] and pigeon-heart[8,11] mitochondria.

The concept of 'high-energy' derivatives of cytochrome *b*, predicted by the chemical hypothesis of oxidative phosphorylation[12,13], was put forward on an experimental basis by CHANCE and co-workers[14,15], who discovered the species b_{555} (λ_{max} = 555 nm at 77°K) in pigeon-heart mitochondria and submitochondrial particles supplemented with ATP. Energy-linked reduction of a long-wavelength cytochrome *b* was first reported by SLATER *et al.*[16,17] in beef-heart submitochondrial particles, by WIKSTRÖM[9] in rat-liver, by WEGDAM *et al.*[18] in rat-heart, and by BONNER AND SLATER[19] in potato mitochondria. Energy-linked reduction of a short wavelength cytochrome *b* simultaneously with the long wavelength form was shown by WIKSTRÖM[9] in rat-liver mitochondria, and similar findings have recently been made in sonicated particles from beef-heart mitochondria, and in rat-heart mitochondria by SLATER AND LEE[20]. These authors also concluded that the short wavelength species (λ_{max} = 558 nm at room temperature) may be identical with b_{555} of CHANCE and co-workers[14,15].

WILSON AND DUTTON[10] and CHANCE *et al.*[11] showed a considerable change in the measured midpoint potential of part of cytochrome *b* in rat-liver[10] and pigeon-heart[11] mitochondria. This led them to propose a mechanism of Site 2 phosphorylation[11] involving the 'high-energy' species cytochrome $b_T \sim I$, differing almost 300 mV in midpoint potential from the corresponding 'low-energy' form, cytochrome b_T. A second cytochrome *b* component was proposed to function in electron transport only.

The mechanism proposed by SLATER *et al.*[17] (see also ref. 20) includes the 'high-energy' forms of two cytochromes *b* (*b* and b_i), one in the reduced ($b^{2+} \sim X$) and the other in the oxidized form ($b_i^{3+} \sim X$).

In the present paper the evidence in favour of 'high-energy' derivatives of cytochromes *b* will be tested and discussed. Some of the presented data have been reported preliminarily[9].

METHODS AND MATERIALS

Preparation of mitochondria and sub-mitochondrial particles

Rat-liver mitochondria were isolated as described previously[21]. Mg^{2+}–ATP particles were prepared from beef-heart mitochondria as described by LÖW AND VALLIN[22].

Oxidoreduction of cytochrome b

The red/ox state of cytochrome *b* was followed with the Aminco–Chance dual wavelength spectrophotometer. The cuvettes contained 3 ml of reaction mixture and the light path was 1 cm. The slit width was routinely 0.2 mm.

Difference spectra

Difference spectra were scanned point by point with the Aminco–Chance instrument by either of two procedures. In one method two cuvettes (sample and reference) were used. The instrument was balanced at one particular wavelength couple with the reference cuvette, after which the sample cuvette was inserted and the difference in transmission read. This procedure was repeated throughout the desired spectral range (with a constant reference wavelength), appropriate control measures being taken to assure that no time-dependent changes took place during the scan.

In the other method, which was applied *e.g.* when time-dependent changes made the first procedure uncertain, the wavelength scanning was performed point by point with a separate incubation for each wavelength couple. With this method care was taken to perform a sufficient number of experiments to minimize pipetting errors. This method was found accurate enough, since deviations from the arithmetic mean of measurements at one particular wavelength couple never exceeded 5 %.

The wavelength scale of the Aminco–Chance instrument was calibrated using the known mercury lines. With a slit width of 0.2 mm the accuracy was found safe within at least $\pm$ 1 nm.

Reaction mixture and chemicals

The standard reaction mixture consisted of 0.2 M mannitol–0.07 M sucrose–0.02 M KCl–0.03 M Tris–HCl buffer, (pH 7.4). For detailed description of experimental conditions, see figure legends. The reaction temperature was 23°.

Most chemicals were commercial products of analytical grade. The uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) was kindly donated by Dr. P. G. Heytler.

RESULTS

Energy-linked oxidoreduction of cytochrome b

Figs. 1A and 1B show the red/ox state of cytochrome *b* as measured at 566 *minus* 575 nm during respiration in State 4 (see ref. 23 for nomenclature) with succi-

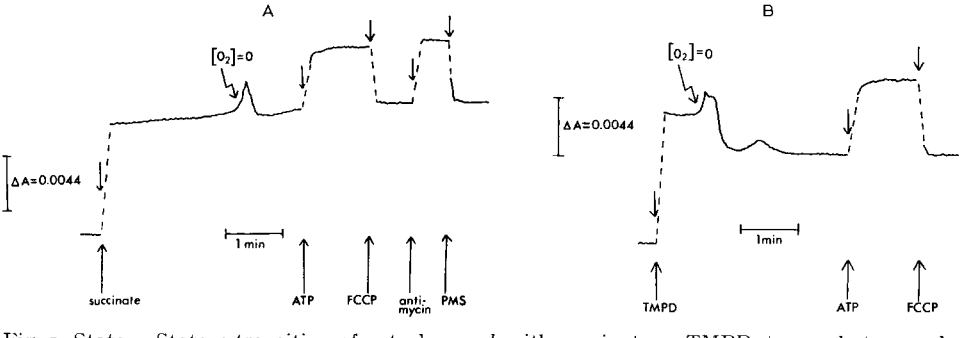

Fig. 1. State 4–State 5 transition of cytochrome *b* with succinate or TMPD + ascorbate as substrate; the effects of ATP and antimycin in State 5. The Aminco–Chance instrument was set at 566 *minus* 575 nm. An upward deflection of the trace reflects an increase in the absorption difference. Rat-liver mitochondria (2.7 mg protein/ml) were suspended in standard reaction mixture (see METHODS AND MATERIALS) in the presence of 5 μM rotenone. In B, 10 mM ascorbate was also present initially. After 3 min preincubation the following additions were made, indicated in the figures: 6.7 mM Tris–succinate (A), 0.33 mM TMPD (B), 1.33 mM ATP (A, B), 1 μM FCCP (A, B), 0.4 μg/ml antimycin (A) and 12 μM PMS (A).

nate and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) + ascorbate, respectively. It should be noted that the measuring wavelength was set a few nm more to the red than is customary for measuring cytochrome *b*. Both traces show the transition from State 4 ('the resting state') to the anaerobic State 5. This transition is biphasic (*cf.* ref. 24). Initial reduction is apparently followed by a secondary oxidation phase. With TMPD + ascorbate (Fig. 1B) there is moreover a small reduction–oxidation cycle subsequent to the large oxidation phase. This cycle will be referred to as the 'oscillation effect'.

Addition of ATP in State 5 (Figs. 1A and 1B) caused a considerable increase in the absorption difference indicating reduction of part of cytochrome *b*. This effect was reversed (or prevented) by uncoupling agents (Figs. 1A and 1B) and, more slowly, by oligomycin. Addition of antimycin after the uncoupler in the presence of succinate (Fig. 1A) caused an increase in the absorption difference to a similar level as after ATP. This effect was reversed (or prevented) by phenazine methosulphate (PMS).

Fig. 2A shows the State 3–State 5 transition (transition from the active phosphorylating to the anaerobic state) with TMPD + ascorbate as substrate. The anaerobic state reached from State 3 differs from that reached from State 4 in that the concentration of ATP is considerably higher in the former state as compared to the latter, where ATP has been formed only from endogenous ADP and P_i. The State 3–State 5 transition (Fig. 2A) is monophasic lacking the secondary oxidation phase, which was characteristic for the State 4–State 5 transition (see Fig. 1B). Thus a highly reduced State 5 level is reached, similar to that after the addition of ATP in Fig. 1B. Both uncouplers and oligomycin (Fig. 2A) induced oxidation to a similar level as after the complete State 4–State 5 transition (*cf.* Fig 2C).

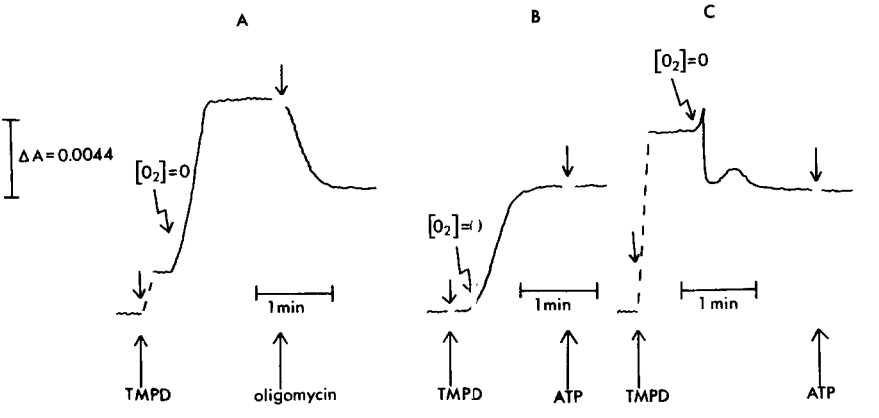

Fig. 2. Aerobic–anaerobic transition of cytochrome *b* with TMPD + ascorbate as substrate, in the presence of ADP + P_i (A), uncoupler (B), or oligomycin (C). Dual-wavelength spectrophotometry at 566 *minus* 575 nm. An upward deflection connotes increase in the absorption difference. The initial conditions were the same as in Fig. 1B, except that 0.67 mM ADP and 10 mM KH_2PO_4 were further present in A, 1 μM FCCP in B, and 3 μg/ml oligomycin in C. The additions indicated in the figures were: 0.33 mM TMPD (A, B, C), 3 μg/ml oligomycin (A), and 1.33 mM ATP (B, C).

Fig. 2B shows the transition from the uncoupled aerobic state (TMPD + ascorbate) to State 5. Cytochrome *b* was completely oxidized in the aerobic steady-state, and monophasic reduction was seen at anaerobiosis. The final level of reduction is,

however, low and similar to the final state after the complete State 4–State 5 transition (*cf.* Figs. 1B, 2A and 2C). Subsequent addition of ATP had no effect.

In the presence of oligomycin (Fig. 2C) cytochrome *b* was highly reduced in the aerobic state (*cf.* Fig. 1B), apparently by energy-linked reversal of electron flow driven by the coupled respiration with TMPD + ascorbate[25] (*cf.* Fig. 2B). The transition to State 5 was qualitatively similar to the 'normal' State 4–State 5 transition with secondary oxidation followed by the 'oscillation effect'. In the presence of oligomycin, however, the oxidation phase was very fast, lasting only 5–7 sec. Further addition of ATP had no effect as expected.

The following conclusions may be drawn from these data: Anaerobically part of cytochrome *b* was reduced only in the presence of an energy source. Aerobically with TMPD + ascorbate as substrate, the entire reduction of cytochrome *b* was dependent on the supply of energy. Thus the energy required for reduction could be supplied either by ATP, this pathway being effectively blocked by oligomycin, or by coupled respiration, in that case without oligomycin sensitivity. In both cases uncouplers of oxidative phosphorylation were completely inhibitory.

The secondary oxidation phase observed at anaerobiosis (Figs. 1A, 1B and 2C) is due to 'de-energization' of the mitochondria at the stop of energy conservation coupled to oxygen consumption (*cf.* ref. 24). 'De-energization' may be slow due to the presence of endogenous ATP (Figs. 1A and 1B), fast in the presence of oligmycin (Fig. 2C), or may be absent at high concentrations of ATP (Fig. 2A). In the presence of oligomycin the time of oxidation may thus be a measure of the maximal 'life-time' of the energized state (5–7 sec, *cf.* ref. 26).

Although reduction of cytochrome *b* in the aerobic steady state with TMPD + ascorbate was entirely energy-dependent, a considerable part of cytochrome *b* was reduced without energy dependency in the anaerobic state (Fig. 2B), probably by endogenous substrates entering the respiratory chain above the site of rotenone inhibition. This part of cytochrome *b* hence appears to have a more positive mid-point red/ox potential in the uncoupled state than the part which was reduced in the anaerobic state only if an energy source was present. Antimycin appeared, according to the experiment of Fig. 1A, to simulate the effect of energization. This effect was reversed by the red/ox mediator PMS. The effect of antimycin will be dealt with in detail in a subsequent publication.

Spectral identification of the part of cytochrome b responsible for the anaerobic energy-linked behaviour

Fig. 3 shows the wavelength dependence of the oxidation phase after anaerobiosis (*cf.* Fig. 1B), the effect of ATP added in State 5 (*cf.* Fig. 1B), and the 'oscillation effect' (*cf.* Figs. 1B and 2C). Note that all effects are plotted in the same direction in the figure for comparison, although some are due to increase and some to decrease in absorption.

The oxidation phase after anaerobiosis is obviously due to a component absorbing maximally at 565–566 nm, but a clear shoulder at 557–558 nm suggests contribution from a second species (*cf.* ref. 9). The effect of ATP added to State 5 is clearly reduction of the 566- and 558-nm components. Scanning of the effect of uncoupler added after the ATP (see Fig. 1B) revealed oxidation of the 566- and 558-nm components as expected (not shown). From these data it is clear that the 'energy-linked' part

of cytochrome *b* in the anaerobic state is spectrally different from classical cytochrome *b* (see below).

Both the reduction and the oxidation phases of the 'oscillation effect' (*cf.* Figs. 1B and 2C) are plotted in Fig. 3. The spectra suggest that the effect is a reduction–oxidation cycle of the 566-nm component without significant contribution from the 558-nm species. This indicates that the 566-nm peak and the 558-nm shoulder do not belong to the same species. It is concluded that the 'energy-linked' part of cytochrome *b* under the above conditions appears to be the sum of two species, provisionally designated b_{558} and b_{566}, respectively.

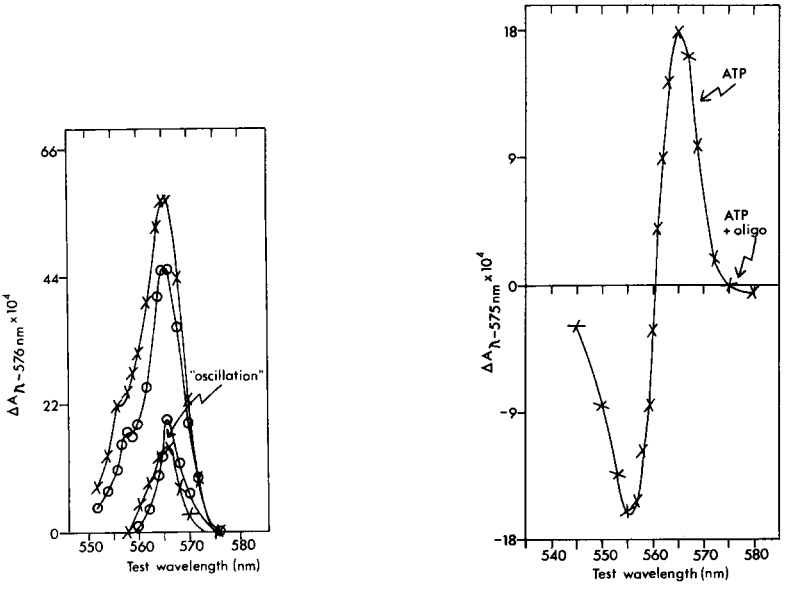

Fig. 3. Wavelength dependence of the oxidation phase at anaerobiosis, the effect of ATP in State 5, and the 'oscillation' effect. The experimental conditions were the same as in Fig. 1B. The wavelength scanning was performed as described in METHODS AND MATERIALS with a separate incubation for each wavelength pair. ×—×, the effect of ATP addition to State 5 (increase in absorption); ○—○, the oxidation phase at anaerobiosis (decrease in absorption). The two lower peaks designated 'oscillation' describe the wavelength dependence of the reduction (×—×) and oxidation (○—○) phases of the 'oscillation effect' (see text).

Fig. 4. Wavelength dependence of the effect of ATP addition in State 5 with succinate as substrate. A suspension of standard reaction mixture, 5 μM rotenone, and rat-liver mitochondria (2 mg protein/ml) was divided into two cuvettes (sample and reference). After 3 min preincubation 6.7 mM Tris–succinate was added to both cuvettes, and incubation was continued until anaerobiosis. A control scan was run to assure that both cuvettes were identical, after which 1.33 mM ATP and 10 μl ethanol were added to the sample cuvette and 1.33 mM ATP and 2 μg/ml oligomycin to the reference. The difference spectrum, sample *minus* reference, was immediately scanned as described in METHODS AND MATERIALS.

With succinate as substrate (see Fig. 1A) similar reduction of b_{566} occurred under the influence of ATP as was described with TMPD + ascorbate above. With succinate, however, a deep trough was seen in the difference spectrum at approx. 555 nm (Fig. 4). This was previously interpreted as due to oxidation of the b_{558} component[9], but since the corresponding trough in the Soret region occurs immediately below 420 nm, the effect is more probably due to oxidation of cytochrome c_1 by reversal of electron trans-

fer. This conclusion is substantiated by the finding that the 555-nm trough disappears if the conditions are made unfavourable for reversal of electron transfer, *e.g.* by application of a high succinate/fumarate ratio (Fig. 5). Under such conditions, and also if TMPD + ascorbate are added in addition to the succinate to keep cytochromes $c + c_1$ fully reduced, reduction of both b_{558} and b_{566} is again observed under the influence of ATP (Fig. 5).

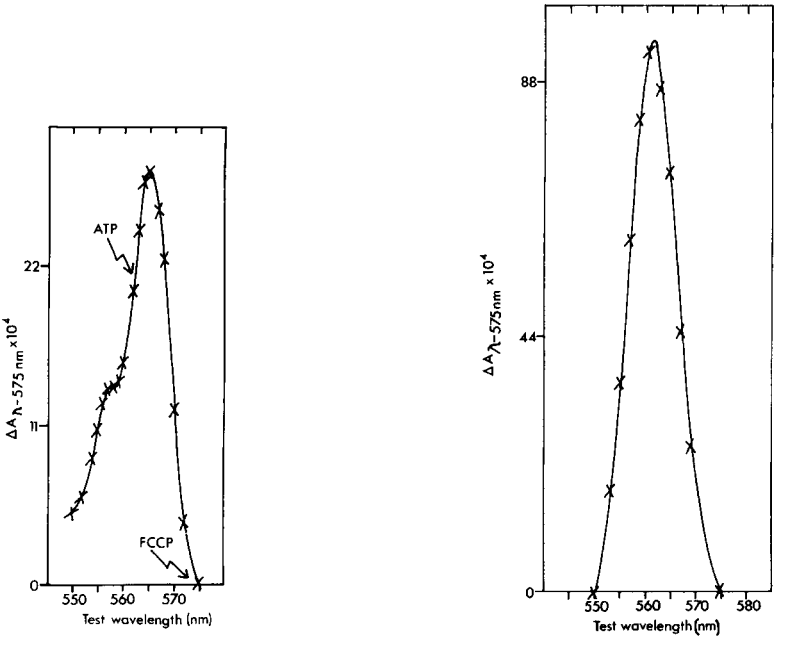

Fig. 5. Wavelength dependence of the effect of ATP addition to State 5 at a high succinate/fumarate ratio. The basic experimental conditions were the same as described in the legend to Fig. 4, except that the concentration of mitochondrial protein was 2.7 mg/ml. Both the sample and reference cuvette were bubbled with N_2 for 3 min to minimize the oxygen concentration. Both cuvettes were supplemented with 20 mM Tris–succinate after which anaerobiosis occurred immediately. The aqueous layer was covered with mineral oil to minimize oxygen diffusion. A control scan showed no significant spectral difference between the cuvettes in the 550–570-nm region. 1.33 mM ATP was added to the sample cuvette and 1 μM FCCP to the reference. The difference spectrum, sample *minus* reference, was scanned as outlined in METHODS AND MATERIALS.

Fig. 6. Classical cytochrome *b* (b_{562}). Two cuvettes were supplemented with standard reaction mixture, 5 μM rotenone, 1 μM FCCP and rat-liver mitochondria (2.4 mg protein/ml). The sample cuvette was further supplemented with 10 mM ascorbate and 0.17 mM TMPD after which anaerobiosis occurred rapidly. To the reference 10 mM ascorbate and 1 mM KCN were first added, followed by 0.17 mM TMPD after 2 min incubation. The difference spectrum, sample *minus* reference, is shown in the figure.

Classical cytochrome b

A considerable fraction of cytochrome *b* was reduced in the anaerobic state with TMPD + ascorbate in the presence of rotenone and uncoupler (Fig. 2B), probably due to endogenous substrates. Spectral identification of this part of cytochrome *b* was complicated by the simultaneous appearance of the large peak of ferrocytochromes $c + c_1$ in the difference spectra. Aerobically in the presence of 1 mM KCN, uncoupler and TMPD + ascorbate, cytochrome $c + c_1$ were practically completely reduced, but

only very little reduction occurred at 562 *minus* 575 nm (cytochrome *b*)*, possibly due to a leak through the cyanide-inhibited site. In any case, advantage was taken of this by recording the difference spectrum anaerobic *minus* aerobic + cyanide, in the presence of uncoupler and TMPD + ascorbate (Fig. 6). In this way it was possible to identify spectrally the cytochrome *b* species which became reduced anaerobically without energy dependence. The spectrum shows a sharp and symmetrical peak with maximum at 561.5 nm, clearly due to classical cytochrome *b* (ref. 3), which we here prefer to call b_{562}.

Reduction of b_{558} and b_{566} without energy dependence

Fig. 7 shows that both b_{558} and b_{566} became reduced anaerobically also in the presence of an uncoupler, at a sufficiently high succinate/fumarate ratio. The anaerobic equilibration of b_{558} and b_{566} with the succinate/fumarate and β-hydroxybutyrate/acetoacetate couples is extremely slow in the non-energized state with rat-liver mito-

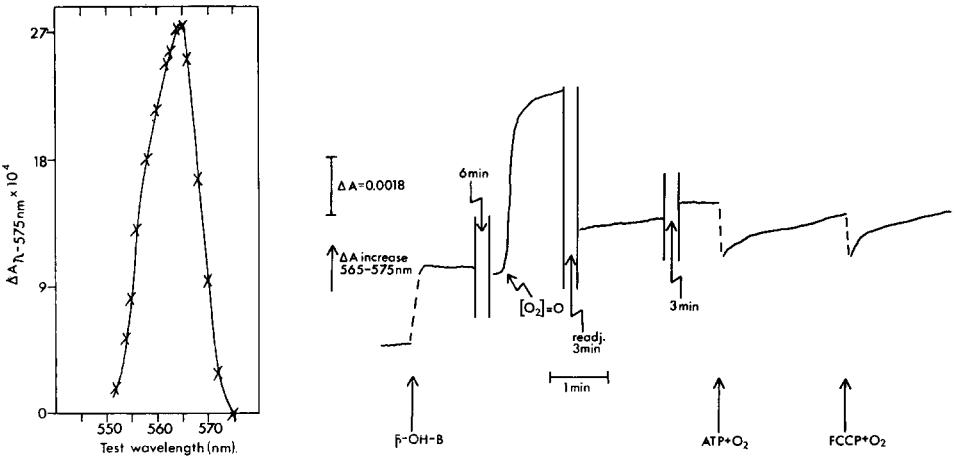

Fig. 7. The effect of two different succinate/fumarate ratios on the red/ox state of cytochrome *b* in uncoupled State 5. The cuvettes were supplemented with standard reaction mixture, 5 μM rotenone, 1 μM FCCP and rat-liver mitochondria (2.7 mg protein/ml). The sample cuvette was bubbled with N_2 for 3 min, and then 20 mM Tris–succinate was added, immediately followed by anaerobiosis. The surface of the mixture was covered by mineral oil. The reference cuvette (no N_2 treatment) was supplemented with 1.7 mM succinate and incubated until anaerobiosis occurred. Both cuvettes were incubated in the anaerobic state until no significant change in absorption occurred in the cytochrome *b* region. The difference spectrum, sample *minus* reference, is shown in the figure.

Fig. 8. Reduction of cytochrome *b* by β-hydroxybutyrate in anaerobic Mg^{2+}–ATP particles. The standard reaction mixture was supplemented with 3.3 mM $MgCl_2$, Mg^{2+}–ATP particles (1.0 mg protein/ml) and 0.33 mM NAD^+. Further additions indicated in the figure were: 13.3 mM β-hydroxybutyrate (Tris salt), 1.33 mM ATP and 1 μM FCCP. Slight stirring (addition of O_2) had the same effect as ATP and FCCP shown in the figure.

* The behaviour of b_{562} (classical cytochrome *b*) is also dependent on the energy-state of the mitochondrion[9,20]. Addition of ATP to cyanide-inhibited rat-liver mitochondria (no added substrates) causes reduction of b_{562} (in addition to b_{558} and b_{566}, and oxidation of cytochrome c_1 (unpublished observation), which is uncoupler-sensitive. Addition of TMPD + ascorbate to cyanide-inhibited mitochondria causes reduction of b_{562} alone, this effect being insensitive to oligomycin but rapidly reversed by uncouplers (unpublished observation). The latter effect, which might be due to a small leak through the cyanide-inhibited site, is presently under investigation.

chondria. Complete reduction of the two species was not achieved without ATP in a reasonable time. A slow but steady rate of reduction of these species indicated that the equilibrium had not been reached. With freshly prepared beef-heart mitochondria, however, complete reduction of b_{558} and b_{566} was achieved with β-hydroxybutyrate anaerobically, without energy-supply, and within a reasonable time (approx. 25 min). Thus no further reduction occurred on addition of ATP (*cf.* Fig. 8). Under less reducing conditions, similar findings were made with beef-heart mitochondria as with rat-liver mitochondria (Figs. 3 and 5).

With Mg^{2+}–ATP particles reduction of b_{558} and b_{566} was observed upon addition of ATP in the anaerobic state (*cf.* ref. 20) with succinate as substrate in a similar fashion as shown above with rat-liver mitochondria. Fig. 8 shows the anaerobic equilibration of the cytochrome *b* complex with the β-hydroxybutyrate/acetoacetate couple in Mg^{2+}–ATP particles. A situation very close to equilibrium is reached in 10 min. After this, further additions of ATP and FCCP had no effect (Fig. 8) other than a slight oxidation, which was due to simultaneous addition of oxygen. Fig. 9 shows the difference spectrum anaerobic + β-hydroxybutyrate *minus* anaerobic + succinate in the presence of uncoupler. The peaks of b_{558} and b_{566} are clearly visible showing the requirement of low-potential conditions for their reduction in the absence of an ener-

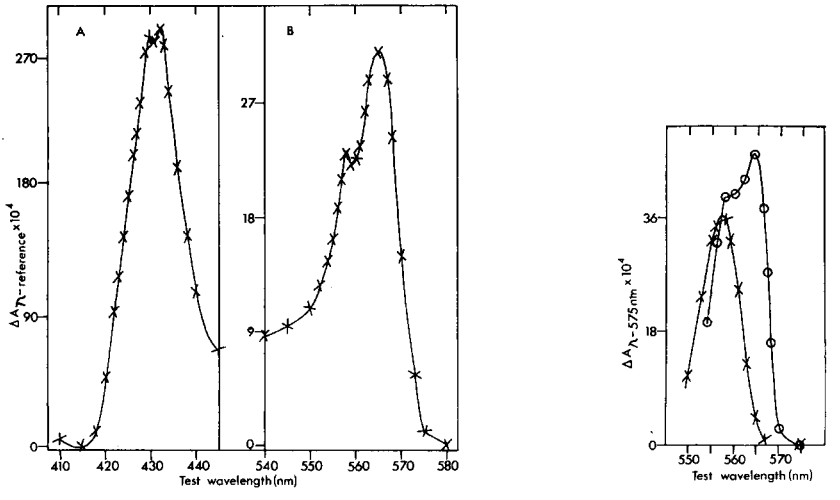

Fig. 9. Reduction of the b_{558} and b_{566} species without energy dependence in Mg^{2+}–ATP particles. Standard reaction mixture, 3.3 mM $MgCl_2$, 1 μM FCCP and Mg^{2+}–ATP particles (1.0 mg protein/ml) were added to two cuvettes (sample and reference). To the sample cuvette was further added 0.33 mM NAD^+ and 13.3 mM β-hydroxybutyrate (Tris-salt), and after anaerobiosis the liquid-surface was covered with mineral oil. The cuvette was incubated for 10 min after which no significant change in cytochrome *b* absorption took place (see Fig. 8). The reference cuvette was supplemented with 5 μM rotenone and 6.7 mM succinate. After anaerobiosis this cuvette was handled as described above for the sample. The difference spectrum, sample *minus* reference, is shown in the figure. The reference wavelength is 415 nm in A, and 580 nm in B.

Fig. 10. Comparison between the wavelength dependence of cytochrome *b* reduced by dithionite- or under the influence of ATP. The sample cuvette was supplemented with standard reaction mixture, 1 μM FCCP, rat-liver mitochondria (2.6 mg protein/ml) and a few grains of solid dithionite (×—× and ○—○). In ×—× the reference cuvette contained standard reaction mixture, 5 μM rotenone, 16.7 mM Tris–succinate and rat-liver mitochondria (2.6 mg protein/ml). After anaerobiosis 1.33 mM ATP was further added. In ○—○ the reference cuvette was further supplemented with 1 μM FCCP. The two difference spectra, sample *minus* reference, are shown in the figure.

gy source. The spectral properties of the reduced b_{558} and b_{566} components are, moreover, exactly the same irrespective of whether reduction was facilitated by ATP, or by substrate alone.

Complete reduction of the three cytochrome *b*-like components was also accomplished by dithionite, which also caused reduction of cytochrome b_5 in rat-liver mitochondria. The spectrum of dithionite-reduced mitochondria differed from that of anaerobic mitochondria supplemented with ATP, only with respect to cytochrome b_5, the peak of which appeared in the difference spectrum (Fig. 10). If the ATP-supplemented anaerobic cuvette (reference cuvette in Fig. 10) was further supplemented with an uncoupler, reversing the energy-linked reduction of b_{558} and b_{566}, the peaks of these species appeared in the difference spectrum (Fig. 10) in addition to the peak of cytochrome b_5.

Although cytochrome b_5 has spectral properties very similar to b_{558} in the α-region, the two species may be distinguished by their different absorption properties in the Soret region (Fig. 9A). Moreover, the addition of rotenone in the presence of β-hydroxybutyrate, NADH and Mg^{2+}–ATP particles (*cf.* Fig. 9) completely inhibited the reduction of b_{558} (and b_{566}), whereas cytochrome b_5 would have been expected to remain reduced due to the rotenone-insensitive pathway[27]. The use of an inner-membrane preparation such as the Mg^{2+}–ATP particles[22] naturally makes the identity of b_{558} with cytochrome b_5 even more improbable[28]. Rat-liver mitochondria are more uncertain in this respect, but the clear energy-dependent behaviour of the b_{558} species (Figs. 1–3 and 5) makes its identity with cytochrome b_5 highly improbable.

The results presented in this section show clearly that the reduced forms of the species b_{558} and b_{566} are not specific for the energized state of mitochondria or submitochondrial particles, although this conclusion might have been drawn on the basis of the initial experiments (Figs. 1–5). Thus although b_{558} and b_{566} appeared 'energy-dependent' under certain conditions, there is no spectral evidence whatsoever favouring the conclusion that the short and long wavelength peaks reflect 'high-energy' forms of cytochrome *b* (contrast refs. 14–18 and 20). An obvious alternative explanation for the energy-requirement of reduction of b_{558} and b_{566} is simply that these species may be low-potential components in relation to the potential of the substrate couple. An exactly equivalent phenomenon would then be the well known energy requirement for reduction of NAD^+ by succinate (see *e.g.* ref. 22).

The relative amount of b_{558}, b_{562} and b_{566} in rat-liver mitochondria

The relatively sharp absorption characteristics of the three cytochrome *b*-like species, the position of their α-and Soret maxima (see also ref. 9), and the Soret/α-absorption ratio (approx. 6) indicate that they may indeed be cytochromes of *b*-type. Their response towards changes in red/ox and energy conditions in mitochondria justifies the conclusion that they are members of the respiratory chain. The fact that there are conditions where absorption spectra of b_{562} and b_{566} may be recorded without notable interference from each other, or from b_{558}, makes it possible to approximate their relative amount. Such calculations are of course based on the assumption that the molar absorptivities are the same, or nearly the same, for the three species. With this assumption and rounding off for the closest integer, the ratio $b_{566}:b_{562}$ is about 1:2. The relative amount of b_{558} is much more difficult to approximate. However, if it is assumed that both b_{558} and b_{566} are fully oxidized in the uncoupled State 5 with

TMPD + ascorbate, and fully reduced in State 5 with succinate and ATP, the ratio $b_{558}:b_{566}$ is about 1:2. Thus the total ratio $b_{558}:b_{562}:b_{566}$ may be approximately 1:4:2.

In rat-liver mitochondria b_{562} contributes approx. 72 % to the total reduced *minus* oxidized spectrum at 562 *minus* 575 nm, while the rest, 28 %, is due to b_{558} + b_{566}. At 566 *minus* 575 nm the contribution of b_{562} and b_{566} is about equal with negligible interference from b_{558}. One implication from these data is that none of the three species alone could possibly be identical with the 'energy-dependent' cytochrome b_T of WILSON AND DUTTON[10], since this component was reported to account for 50 % of the cytochrome *b* absorption at 430 *minus* 410 nm in rat-liver mitochondria[8,10] (see DISCUSSION).

DISCUSSION

Three cytochromes b in the respiratory chain

At present there is spectrophotometrical evidence of three cytochrome *b*-like absorption maxima in the α-region in submitochondrial particles from beef-heart[1,20] (Fig. 9) and in rat-liver mitochondria[9] (Figs. 3, 5, and 6). In pigeon-heart mitochondria the situation may be the same, since both a short wavelength[14,15] and a long wavelength[11] species has been reported. Potentiometrical evidence[8] and studies with inhibitors[7] have also indicated the presence of three cytochromes *b* in beef-heart mitochondria (see INTRODUCTION). In view of the data accumulated so far, the occurence of three cytochrome *b*-like components in the respiratory chain may well be a common feature of mitochondria from several, widely different, organisms.

Since the long and short wavelength peaks appear under very similar conditions[9,20] (Figs. 3, 5 and 9) the possibility arises that the two absorption maxima might belong to the same component. The ratio $\Delta A_{558\text{ nm}}/\Delta A_{566\text{ nm}}$ is, however, rather variable. Moreover, b_{558} did not participate in the 'oscillation effect' of b_{566} (Fig. 3), indicating that the two absorption maxima may indeed belong to two different entities. SLATER AND LEE[20] have arrived at the same conclusion on the basis of different pH dependencies of the two absorption peaks.

Different spectral properties of the same chemical cytochrome *b* molecule may well arise from differences in the environment within the membrane (*cf.* cytochromes *a* and a_3, reviewed by LEMBERG[29]). Therefore it may not be correct to consider three cytochromes *b*, but perhaps one cytochrome in three different environments. It is known from the work of GOLDBERGER *et al.*[30] that the environment, or binding, of cytochrome *b* has a profound effect on its red/ox properties.

'High-energy' derivatives of cytochrome b

The chemical hypothesis of oxidative phosphorylation[12] requires a red/ox carrier of the respiratory chain forming the primary 'high-energy' intermediate in the process. In the mechanism proposed by SLATER *et al.*[17] (see also SLATER AND LEE[20]), the long wavelength absorption (565–566 nm) is due to a 'high-energy' derivative ($b^{2+}\sim X$) of classical cytochrome *b* in the absence of antimycin*. According to this view it is difficult to understand how the long-wavelength species could possibly persist in the un-

* According to SLATER AND LEE[20], cytochrome b_i^{2+} antimycin has its peak absorption at long wavelength (565 nm), b_i^{2+} at short wavelength (559 nm), b^{2+} (classical *b*, ref. 3) at 561 nm, and $b^{2+}\sim X$ at 565 nm.

coupled state (Figs. 7 and 9) without reverting to the corresponding 'low-energy' form b^{2+} (λ_{max} = 561–562 nm). The proposal of SLATER *et al.*[16,17] that cytochrome b_i, the short-wavelength (λ_{max} = 558 nm) species[20], accounts for half of total cytochrome *b* is also very hard to reconcile with our data if it is not assumed that the molar absorbance of b_{558} is much lower than of classical cytochrome *b*.

The mechanism proposed by CHANCE *et al.*[11] is more flexible, since the 'high-energy' derivative $b_T^{2+} \sim I$ is thought to have identical spectral properties with the 'low-energy' form b_T^{2+}. This mechanism fails to explain, however, the significance of the short-wavelength species b_{558} (see NOTE ADDED IN PROOF).

From the present data it is concluded that there are no spectral effects specific for the 'energized' state in the α-region of cytochrome *b*, and no evidence in favour of interconversion of two spectrally different species (contrast ref. 20). The data may simply be interpreted in terms of one high-potential cytochrome *b* component (b_{562}) and two low-potential components *in situ* (b_{558} and b_{566}). Energy, *e.g.*, in the form of ATP, is required for reduction of anyone of these three species* if the red/ox potential of the substrate couple is too high (positive) to permit spontaneous reduction. It is obvious that the red/ox state of all three cytochrome *b* components is dependent upon the energy-transducing device and the phosphate potential of the mitochondria, but we wish to emphasize that there is no spectral evidence indicating that this dependence is a direct one, *i.e.*, the primary intermediate of oxidative phosphorylation at Site 2 being a 'high-energy' form of cytochrome *b*.

The 'energy-transducing' cytochrome *b* (b_T) accounted for approx. 50% of total cytochrome *b* in rat-liver mitochondria[8,10]. From the present data it is clear that neither b_{558} nor b_{566} alone could possibly account for such a high proportion. Neither could b_{562} alone be identical with cytochrome b_T, since classical cytochrome *b* does not have a midpoint potential of −55 mV in the uncoupled state[10,31] (see also Figs. 2B and 6). At the wavelength couple 430 *minus* 410 nm, where the absorption peaks of the three cytochrome *b* species are quite close (Fig. 9A), the combination $b_{558} + b_{566}$ could maximally account for 43% of total cytochrome *b*, but at 562 *minus* 575 nm the contribution is maximally 30% in rat-liver mitochondria (contrast 40% b_T in pigeon-heart mitochondria at this wavelength couple[11]). Thus it is concluded that the entity cytochrome b_T must be a mixture of at least two (b_{558} and b_{566}), possibly even all three cytochrome *b* species *in situ*. The kinetically derived spectrum of cytochrome $b_T^{2+} \sim I$ (ref. 11) was indeed very broad, suggesting the involvement of more than one component. The homogeneous potentiometrical behaviour of cytochrome b_T, together with the present data, suggests that concomitant functioning of at least two components (b_{558} and b_{566}) may be essential for conservation of energy. HOMMES[32] has indeed shown that second-order oxidoreduction of cytochrome *b* is characteristic for coupled mitochondria, while first order kinetics are found in the uncoupled state.

The change in midpoint potential of part of cytochrome *b* upon 'energization' (refs. 8, 10 and 11) does not necessarily imply formation of a 'high-energy' derivative of cytochrome *b*. The question of whether the red/ox mediators used in the potentiometrical technique[8] interact directly with the cytochrome *b* complex rather than, *e.g.*, *via* cytochromes $c + c_1$ (see ref. 33) is not satisfactorily answered. Moreover, the possibility that cytochrome *b* *in situ* may formally be a hydrogen carrier[34,35] which

* See footnote on p. 339.

is buried within a lipid membrane phase, could give the changes in midpoint potential a completely different meaning. If the protons which may be released upon oxidation become localized in the membrane[36] furnishing the driving force for ATP synthesis[36,37], the midpoint potential of cytochrome *b* may follow the phosphate potential simply through changes in the activity of the protons[38]. This possibility will be approached experimentally in a subsequent paper[39].

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In a paper which appeared after submission of this manuscript, SATO *et al.*[40] concluded that the short wavelength shoulder (λ_{max} 558 nm at room temperature and 555 nm at liquid nitrogen) and the long wavelength peak (λ_{max} 565 nm at room temperature and 562.5 nm at liquid nitrogen) belong to the same species, cytochrome b_T. This is in disagreement with the present data as well as with those of CHANCE AND SCHOENER[14]. The latter authors (see their Figs. 2 and 3) showed that the short wavelength component can be detected in pigeon-heart mitochondria at 77°K without appearance of the other *b*-species, its Soret band being centered at 424–425 nm in contrast to the 430 nm maximum reported by SATO *et al.*[40].

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