High and Low Energy States of Cytochromes

II. IN SUBMITOCHONDRIAL PARTICLES

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SUMMARY

Reversed electron transfer in submitochondrial particles causes the oxidation of cytochromes a3 + a and c, and reduction of cytochrome b upon addition of adenosine triphosphate, indicating a crossover point for the energy-linked reversal of electron transfer between cytochromes c and b.

The cytochrome component designated b55 is uniformly observed to show increased absorption in the low to high energy transition and vice versa. It is tentatively identified as a high energy intermediate in the energy transfer pathway.

The 555 nm compound is distinguished from cytochrome c by its presence in KCl-washed cytochrome c-deficient particles and by the lack of evidence for their interconvertibility. Its identification as a cytochrome of type b is confirmed.

In a previous paper (1) we identified a compound that is responsive to the transition between high and low energy states in mitochondria, which has been tentatively identified as cytochrome b55. In order to determine the relationship of this compound to cytochrome c, submitochondrial particles deficient in this component are examined by low temperature spectroscopy. Furthermore, the extent to which high to low energy transitions affect cytochrome spectra is examined in these particles.

EXPERIMENTAL PROCEDURE

Physical Methods—The physical methods are identical to those employed in the previous paper (1). Submitochondrial particles derived from pigeon heart mitochondria were prepared by sonic oscillation. The general procedure used was as follows. Freshly prepared pigeon heart mitochondria were diluted with medium (0.225 M mannitol, 0.075 M sucrose, 0.010 M Tris-HCl buffer, pH 7.4) to a concentration of about 15 mg per ml. A protein recovery of 25 to 30% was usually obtained.

Cytochrome c-deficient submitochondrial particles were prepared from cytochrome c-deficient pigeon heart mitochondria. The method used for releasing cytochrome c from pigeon heart mitochondria was essentially that described by Jacobs and Sanadi for rat liver mitochondria (2). Three milliliters of freshly prepared pigeon heart mitochondria (20 mg of protein per ml) were diluted to 40 ml with 0.015 M KCl. The suspension was incubated for 3 min at 0° and then centrifuged at 8000 X g for 10 min. The sediment was then washed with 30 ml of 0.175 M KCl and subsequently suspended into 30 ml of medium and sonically treated as just described.

DPNH oxidase and succinate oxidase activity were measured with a Clark oxygen electrode in a reaction medium consisting of 0.225 M mannitol, 0.075 M sucrose, and 0.010 M Tris-HCl buffer, pH 7.4, at 23°. ATPase, succinate-linked DPN reduction, and cytochrome c oxidation were assayed essentially the same way as described previously (3).

RESULTS

General Properties of Sonically Treated Particles—Sonically treated particles derived from the tightly coupled pigeon heart mitochondria retain phosphorylative properties, as revealed by their ability to carry out ATP-induced DPN reduction and cytochrome c oxidation (Fig. 1B and Table I). Particles disrupted by sonic oscillation do not contain bound DPN, but they can readily reduce a substrate amount of added DPN, while, on the contrary, they can only oxidize endogenous cytochrome c but not the externally added carrier. Both of these properties are in contrast to those found with particles derived by digitonin treatment (4, 5). In line with the observation reported by Löw and Vallin (6), Mg++ is obligatory for both ATP-induced DPN reduction and cytochrome c oxidation (Fig. 1B and Table I). Particles disrupted by sonic oscillation do not contain bound DPN, but they can readily reduce a substrate amount of added DPN, while, on the contrary, they can only oxidize endogenous cytochrome c but not the externally added carrier. Both of these properties are in contrast to those found with particles derived by digitonin treatment (4, 5). In line with the observation reported by Löw and Vallin (6), Mg++ is obligatory for both ATP-induced DPN reduction and cytochrome c oxidation. Table I summarizes the various enzymic activities of particles disrupted by sonic oscillation.

ATP Responses in Sonically Treated Particles—Fig. 1A illustrates the oxidized-reduced spectrum for sonically treated particles derived from pigeon heart mitochondria. The electron transfer components show the α band of cytochrome a + a3 at 599 nm and the γ band as a doublet at 447 and 442 nm. The α and γ bands of cytochrome c are at 547.5 and 415 nm, respectively. Under these conditions only a small shoulder on the long wave...
FIG. 1. A, the cytochrome components of sonically treated particles prepared from pigeon heart mitochondria. The reference sample is oxidized; the measured sample (+ Sulfide) is treated with 3 mM succinate and 0.76 mM sulfide for at least 2 min. Submitochondrial fragments are suspended in medium with a protein concentration of 1.9 mg per ml in a volume of 0.3 ml. The spectrum is measured at 77° K. (Experiment 735 3II). B, effect of ATP upon the respiratory components of submitochondrial particles supplemented with succinate. The particle suspension (1.9 mg per ml) is treated with 3 mM succinate, 0.3 mM DPNH, 3.3 mM Mg++, and 0.76 mM sulfide in a volume of 0.3 ml. After 2 min, the reference sample (+ Sulfide) is removed and frozen. Then 0.67 mM ATP is added to the remainder, and after 30 sec the measured sample (+ ATP) is frozen (Experiment 735a 1 III). The absorbance calibration is p 0.005, not 0.05/8 mm, as shown on the figure.

TABLE I

<table>
<thead>
<tr>
<th>Enzymic activities of submitochondrial particles</th>
<th>Rate</th>
<th>Ratioa</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPase ..................................</td>
<td>1130</td>
<td>230</td>
</tr>
<tr>
<td>DPNH oxidase ................................</td>
<td>470</td>
<td>100</td>
</tr>
<tr>
<td>Succinate oxidase ............................</td>
<td>31</td>
<td>6.6</td>
</tr>
<tr>
<td>ATP-induced succinate-linked DPN+ reduction (+18 μM DPN+)</td>
<td>40</td>
<td>8.5</td>
</tr>
<tr>
<td>ATP-induced cytochrome c oxidation (endogenous cytochrome c)</td>
<td>2.4</td>
<td>0.5</td>
</tr>
<tr>
<td>ATP-induced cytochrome c oxidation (+1 μM cytochrome c)</td>
<td>2.4</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* The ratio among various enzymatic activities was calculated on the basis of DPNH oxidase activity of the same enzyme preparation. The DPNH oxidase activity was arbitrarily chosen as 100.

The components activated by reversed electron transfer are distinguishable by ATP addition if the reference material consists of the sulfide-treated preparation supplemented with succinate, DPNH, and magnesium, as indicated in Fig. 1B. Upon addition of ATP the oxidation of cytochrome c is observed at 547 μm, and oxidation of the cytochrome a + a3 is observed by troughs at 600 and 440 μm. The γ band of cytochrome c is obscured by the large 431 μm peak. Two components showing increased absorbance indicative of increased reduction have peaks at 556 and 562 μm; their γ bands are fused together to give a maximum at 431 μm. Other preparations may show a larger response of cytochrome a + a3, nearly equal to that of cytochrome c.

Transition from High to Low Energy States—In Fig. 2 a preparation similar to that described in Fig. 1 is supplemented with succinate, DPNH, sulfide, magnesium, and ATP. Addition of 50 μM Dicumarol causes increased reduction of cytochrome c,
indicated by the $\alpha$ and $\gamma$ peaks at 547 and 413 $\mu$m, with a barely detectable increased reduction of cytochrome $a + a_3$, indicated by the peak at 441 $\mu$m. The troughs observed at 555 and 426 $\mu$m are characteristic of the $\alpha$ and $\gamma$ bands of the $\delta_{451}$ component observed in intact mitochondria (1).

The appearance of the absorption bands of cytochrome $c$ under the same conditions as the disappearance of the 555 and 426 $\mu$m bands in Fig. 2 would, in the absence of other data, suggest a relationship between the 555 $\mu$m compound and cytochrome $c$. For this reason we have undertaken the study of the 555 $\mu$m compound in cytochrome $c$-deficient particles obtained by washing with KCl. These particles show only one-third of the cytochrome $c$ compared to the particles in Fig. 1 and are employed in the experiment shown in Fig. 3. Dicumarol treatment of the sulfide-, magnesium-, and ATP-treated particles results in a clear cut absorbance decrease with a maximum of 556 $\mu$m. There is no detectable change in wave lengths appropriate to cytochrome $c$ in this spectrum, but if one takes the irregularities of the baseline at 550 $\mu$m as indications of the maximum detectable cytochrome $c$, the ratio of the change in cytochrome $c$ to that in the 555 $\mu$m pigment is less than 1:15, assuming the latter has a molecular extinction coefficient typical of the $b$ or $c$ type.

Other evidence against the participation of cytochrome $c$ in this reaction is indicated in Fig. 4, where the high to low energy transition of submitochondrial particles is caused by phosphate. Following the addition of sulfide, the particles are energized with magnesium and ATP and then supplemented with phosphate. The disappearance of a compound similar to $\delta_{451}$ is indicated by the trough at 555 and 427 $\mu$m. There is no absorbance change that can be attributed to cytochrome $c$, thus confirming the conclusions derived from the previous figure. The reduction of oxidized cytochrome $a$ is indicated by a $\gamma$ band at 447 $\mu$m and a peak at 555 $\mu$m. The trough at 602 $\mu$m also shows that cytochrome oxidase is affected by the high to low energy transition.

A similar and clearer response is observed with ADP and phosphate. These data are discussed in Paper III (7).

REFERENCES
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