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Cytochrome Oxidase and Cytochromes a and a_3 in Crab Mitochondria

By D. H. BURRIN AND R. B. BEECHEY

Department of Physiology and Biochemistry, The University, Southampton

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Using a highly-purified mammalian cytochrome-oxidase preparation, Yonetani (1960) unequivocally demonstrated the spectrophotometric separation of cytochromes a and a_3 . In the present paper we give evidence for the presence of cytochromes a and a_3 in phosphorylating mitochondria obtained from the hepatopancreas of *Carcinus maenas* (Beechey, 1961*a*, *b*). Little detailed biochemical information is available for crustaceans.

Beechey (1961*a*) presented kinetic evidence for the presence of cytochromes a and a_3 in these mitochondria. In the present paper the responses of cytochromes a and a_3 and of cytochrome-oxidase activity to cyanide and carbon monoxide are reported and discussed. A preliminary report has been published (Burrin & Beechey, 1962).

METHODS

Animals. Specimens of *Carcinus maenas* were obtained from Southampton Water. They were maintained in the Laboratory in 75% artificial sea water (Lyman & Fleming, 1940). This water was continuously circulated through the aquarium at 13°.

Preparation of hepatopancreas mitochondria. The mitochondria were isolated in 0.25M-sucrose from the hepatopancreas of *Carcinus maenas* by the method of Beechey (1961*a*). The final pellet was suspended in 0.25M-sucrose or in one of the media shown in Table 1.

Respiratory activity. The consumption of O_2 was measured at 25° in Warburg manometers. Normally CO_2 was absorbed in 20% (w/v) KOH in the centre well. In those experiments where HCN was present CO_2 was absorbed in

a suspension of 10% (w/v) $Ca(OH)_2$, containing $Ca(CN)_2$, in the centre well (Robbie, 1948). Solutions of HCN were prepared before use by mixing and diluting equal volumes of 0.2M-KCN and 0.2N-HCl to give a neutral solution of HCN and KCl (0.1M-HCN-KCl). Rates of respiration are expressed as q_{O_2} (μ l. of O_2 consumed/mg. of protein/hr.).

Protein determinations. These were made by the biuret procedure of Cleland & Slater (1953). The biuret reagent was calibrated with crystallized bovine plasma albumin (Armour Pharmaceutical Co. Ltd.).

Gas mixtures for manometric purposes. These were freshly prepared before each experiment by displacement of water from 5 l. or 10 l. aspirators. The N_2 , CO and O_2 used in these mixtures were taken directly from cylinders. The required atmosphere in the manometric flask was obtained by the method of Umbreit, Burris & Stauffer (1957).

Difference spectra. These were obtained with a Zeiss spectrophotometer PMQ11 fitted with a double monochromator that had glass prisms. These gave half band-widths of 0.7, 1.2 and 1.7 $m\mu$ at 400, 445 and 500 $m\mu$ respectively. Samples were contained in cuvettes with a 1 cm. light-path. The constancy of a preparation during an experiment was checked by repeated reference to the extinction value of the preparation at 445 $m\mu$. If the value changed significantly the experiment was terminated. 445 $m\mu$ was chosen as the reference point since the extinction coefficient of the difference spectrum of cytochromes a plus a_3 is maximal at this wavelength. All experiments were performed at 4°. A thin film of detergent was used on the optical faces of the cuvettes to prevent condensation of atmospheric moisture. Cuvettes containing mitochondria treated with HCN or CO were sealed with a greased cap. At some stage in the experiment when the contents of both the experimental and test cuvettes had been treated identically the difference spectrum of the cuvettes was measured. The resulting

Table 1. *Composition of reaction media*

Reaction media ...	Concn. of component (mm)	
	'No substrate A' 'Succinate B' 'Fumarate A'	'No substrate B' 'Succinate D' 'Ascorbate A and B'
Sodium, potassium phosphate buffer, pH 7.4	5	25.5
Magnesium chloride	5	17
ATP (trisodium salt)	1	6
Sucrose	200	73
Substrate (sodium salt)	5	8.5 ('Succinate D') 25 ('Ascorbate A') 50 ('Ascorbate B')

curve, the base line, which reveals any slight differences in the cuvettes themselves, in their contents and in their detergent films, was used to correct the difference spectra under study. This is basically the technique of Chance (1953).

Reversals. A reversal is defined as the changes in extinction that occur with time as a suspension of mitochondria passes from the aerobic to the anaerobic state. These were carried out as described by Holton (1955).

Nomenclature. Cytochromes *a*^{'''} and *a*₃^{'''}, and cytochromes *a*^{''} and *a*₃^{''}, refer respectively to the oxidized and reduced forms of cytochromes *a* and *a*₃. Cytochrome *a*₃^{'''}-CN and cytochrome *a*₃^{''}-CN refer to the oxidized and reduced states respectively of the complex formed between cytochrome *a*₃ and cyanide. Similarly cytochrome *a*₃^{'''}-CO refers to the complex formed by cytochrome *a*₃ and CO.

RESULTS

Experiments with hydrogen cyanide

Effect of hydrogen cyanide on the respiration of the hepatopaneas mitochondria. Fig. 1 shows the effect of hydrogen cyanide on the oxidation of fumarate, succinate and cytochrome *c* reduced by ascorbate. Half-maximum respiration was obtained in the presence of 5 μM-hydrogen cyanide and the respiration was 93% inhibited at concentrations greater than 50 μM-hydrogen cyanide.

Difference spectrum of the substrate-reduced mitochondria minus the substrate-reduced mitochondria treated with cyanide. The main feature of this difference spectrum is the large absorption band at 445 mμ (Fig. 2). At wavelengths higher than 445 mμ the extinction falls rapidly, forming a trough at about 470 mμ, but there are no further variations until the 600 mμ region is reached. Below 445 mμ the line of the difference spectrum invariably forms a broad trough lying between 410 and 430 mμ, but the relationship of this minimum absorption to that at 470 mμ is variable. It may lie at the level indicated in Fig. 2 or it may be higher. The causes of these variations are unknown but they have been reported by other workers; Yonetani (1960) described a decrease in the *E*₄₄₅ (red.):*E*₄₂₄ (ox.) ratio after repeated freezing

Fig. 1. Effects of hydrogen cyanide on the respiration of hepatopaneas mitochondria. The mitochondria, containing 1-4 mg. of protein, were suspended in 'Succinate B' (○), 'Fumarate A' (●) or 'Ascorbate B' containing cytochrome *c* (0.1 mM) (▽).

and thawing of a preparation of purified mammalian cytochromes *a* plus *a*₃. The presence of cytochrome *a*₃ in these mitochondria may be deduced since the results of Fig. 2 are best interpreted by supposing that the cytochrome *a*₃^{'''}-CN complex, which absorbs less light at 445 mμ than does cytochrome *a*₃^{''} (Keilin & Hartree, 1939; Yonetani, 1960), has been formed. All the respiratory pigments in the experimental cuvette and all save cytochrome *a*₃ in the reference cuvette would be reduced by substrate. Hence the spectrum in Fig. 2 would then be equivalent to the difference spectrum of cytochrome *a*₃^{''} minus cytochrome *a*₃^{'''}-CN.

*Relative contributions of cytochromes *a* and *a*₃ to the absorption of light at 445 mμ.* The fact that

cytochrome a_3^m and cytochrome a_3^m -CN have almost identical absorption spectra (Yonetani, 1960) was used to determine the relative contributions of cytochromes a and a_3 to the absorption of light at $445\text{ m}\mu$. The difference between the extinctions at $445\text{ m}\mu$ and a reference wavelength, either $455\text{ m}\mu$ or $460\text{ m}\mu$, was obtained from the difference spectrum of cytochrome a_3^m minus cytochrome a_3^m -CN as in Fig. 2. This value was then expressed as a percentage of the change in

Fig. 2. Difference spectrum of the substrate-reduced mitochondria minus the substrate-reduced mitochondria treated with cyanide. Samples (2.7 ml.) of mitochondria, containing 5–10 mg. of protein, respiring in 'Succinate B' or 'Fumarate A', were added to each cuvette. Then 0.3 ml. of 10 mM-HCN-KCl was added to the reference cuvette and 0.3 ml. of 15 mM-KCl to the test cuvette.

extinction measured during a reversal at $445\text{ m}\mu$, by using the contents of the test cuvette. The calculations (Table 2) show that cytochrome a_3 is responsible for 65% of the absorption of light at $445\text{ m}\mu$ when $460\text{ m}\mu$ is used as a reference wavelength. However, when $455\text{ m}\mu$ is used as the reference wavelength the contribution of cytochrome a_3 is 51% of the total. The more reliable reference wavelength for this preparation is probably $455\text{ m}\mu$, since it is an isosbestic point for the difference spectrum of the reduced minus the oxidized mitochondria.

Absorption of light at $445\text{ m}\mu$ by cytochrome a . Spectrum A (Fig. 3) represents the results of an experiment in which all of the respiratory pigments of the mitochondria in the reference cuvette were reduced, with the exception of cytochrome a_3 which was in the form of cytochrome a_3^m -CN. Since cytochrome a_3^m and cytochrome a_3^m -CN have similar absorption spectra (Yonetani, 1960), this difference spectrum is that of the reduced mitochondria minus the oxidized mitochondria (see spectrum B, Fig. 3) save that the contribution of cytochrome a_3 is absent. The absorption band at $445\text{ m}\mu$ in spectrum A is due to cytochrome a . It is separated clearly from the band at $430\text{ m}\mu$ due to cytochrome b .

The contribution of cytochrome a_3 to the absorption at $445\text{ m}\mu$ was calculated by using $455\text{ m}\mu$ as a reference wavelength. The value was 47% if the height of the reversals at $445\text{ m}\mu$ was taken as 100%. This value agrees with the data listed in Table 2.

Experiments with carbon monoxide

Effect of carbon monoxide on the respiration of the hepatopancreas mitochondria. The rate of oxidation of succinate and fumarate by the mitochondria was

Table 2. Relative contributions of cytochromes a and a_3 to the absorption of light at $445\text{ m}\mu$

The height of the reversal at $445\text{ m}\mu$ is the mean of at least three values. The differences in extinction were measured from the difference spectrum of substrate-reduced mitochondria minus the substrate-reduced mitochondria treated with cyanide (see Fig. 2).

Reversal height (E_{445})	Extinction difference ($E_{445} - E_{455}$)	Contribution of cytochrome a_3 ($\frac{E_{445} - E_{455}}{E_{445}} \times 100$)	Extinction difference ($E_{445} - E_{460}$)	Contribution of cytochrome a_3 ($\frac{E_{445} - E_{460}}{E_{445}} \times 100$)
0.039	0.023	60	0.032	82
0.023	0.007	35	0.009	39
0.039	0.023	60	0.032	82
0.082	0.030	37	0.038	46
0.071	0.045	64	0.056	79
0.071	0.043	60	0.058	78
0.071	0.040	56	—	—
0.076	0.026	34	0.040	50
0.048	0.027	56	0.037	77
0.027	—	—	0.022	81
0.077	—	—	0.040	52
0.077	—	—	0.050	65
Mean $\pm$ S.D.		51 ± 12		65 ± 13

measured in an atmosphere of carbon monoxide plus oxygen (95:5). The effect of alternate light and darkness on these rates of oxidation was also studied, usually in the sequence light, dark, light. Table 3 shows the effect of carbon monoxide on the oxidation of succinate (six experiments) and fumarate (three experiments). There were no obvious differences between the effects of carbon monoxide on the oxidation of succinate and fumarate and the results are grouped together. In the dark there is a 20–30% inhibition of the uptake of oxygen, which is reversed by light. This type of inhibition is a characteristic property of cytochrome oxidase (Warburg, 1926).

Difference spectrum of the substrate-reduced mitochondria minus the substrate-reduced mitochondria treated with carbon monoxide. The main features of this difference spectrum are the absorption band at 445 m μ and the trough at 430 m μ (see Fig. 4). The

absorption band is that of cytochrome *a*₃⁺, which is present in the substrate-reduced mitochondria and absent in the substrate-reduced mitochondria treated with carbon monoxide. The trough at 430 m μ is due to cytochrome *a*₃⁺-CO in the carbon monoxide-treated mitochondria in accordance with the known properties of mammalian cytochrome *a*₃ (Keilin & Hartree, 1939; Chance, 1953).

Cytochrome-oxidase activity

Table 4 shows that the hepatopancreas mitochondria oxidize ascorbate in the presence of catalytic concentrations of horse-heart cytochrome *c*. The mean respiratory activity for the oxidation

Table 3. *Photosensitive inhibition of respiration by carbon monoxide*

The atmosphere of the experimental flasks was CO + O₂ (95:5) and that of the control flasks N₂ + O₂ (95:5). Samples (1 ml.) of mitochondria, containing 2–5 mg. of protein, suspended in 'Succinate D' or 'Succinate B' or 'Fumarate A' were placed in manometer flasks; 0.1 ml. of 10% (w/v) KOH was in the centre well. The rates of respiration were measured during alternate 20 min. periods of light and dark. The results are presented as means $\pm$ S.D.

Inhibition of respiration (%)				No. of experiments
Dark	Light	Dark	Light	
21 $\pm$ 7	3 $\pm$ 5	12 $\pm$ 6	—	7
—	5 $\pm$ 7	30 $\pm$ 5	16 $\pm$ 2	2

Fig. 3. Difference spectra of substrate-reduced mitochondria treated with cyanide minus oxidized mitochondria (A), and of substrate-reduced mitochondria minus oxidized mitochondria (B). Each cuvette contained 1 ml. of mitochondria suspended in 0.25M-sucrose, containing 5–10 mg. of protein; other additions were: reference cuvette, 2.4 ml. of 'No substrate A' and 0.3 ml. of 15 mM-KCl; test cuvette for spectrum A, 2.4 ml. of 'Succinate B' and 0.3 ml. of 10 mM-HCN-KCl; test cuvette for spectrum B, 2.4 ml. of 'Succinate B' and 0.3 ml. of 15 mM-KCl. Air was bubbled through the reference cuvette. The contents of the test cuvettes were allowed to become anaerobic.

Fig. 4. Difference spectrum of the substrate-reduced mitochondria minus the substrate-reduced mitochondria treated with carbon monoxide. A sample (3.0 ml.) of a suspension of mitochondria in 'Succinate B', containing 15–30 mg. of protein, was placed in each cuvette. Carbon monoxide was gently bubbled through the reference cuvette for 2 min. When the contents of the test cuvette had become anaerobic the difference spectrum was measured.

of ascorbate in the presence of cytochrome *c* is 77 % of that for the oxidation of succinate. The composition of 'Succinate D' is optimum for the oxidation of succinate (B. D. Thompson, personal communication), whereas no attempt has been made to establish the optimum conditions for the oxidation of ascorbate. Thus it appears that the cytochrome-oxidase system has the capacity to carry most of the electron flux associated with the oxidation of succinate.

Table 4 also shows that the mitochondria will not oxidize ascorbate in the absence of horse-heart cytochrome *c* and that the presence of cytochrome *c* had no effect on the endogenous respiration rate. In other experiments it has been shown that the presence of cytochrome *c* has no effect on the oxidation of succinate by the hepatopancreas mitochondria. Thus any loss of endogenous cytochrome *c* during the preparation of the mitochondria which is required for the oxidation of succinate cannot be replaced by horse-heart cytochrome *c*. The addition of 20 μ g. of antimycin A/g. of mitochondrial protein has been shown to inhibit 94 % of the oxygen consumption due to the oxidation of succinate and

fumarate. Difference spectra in the presence of this concentration of antimycin A have shown that the site of inhibition lies between cytochrome *b* and cytochromes *c* plus *c*₁, as in mammalian respiratory chains. Thus the exogenous cytochrome *c* must reduce some component of the respiratory chain on the oxygen side of cytochrome *b*.

DISCUSSION

These results show that information that had been obtained for cytochromes *a* and *a*₃ with non-phosphorylating preparations also applies to the respiratory system in phosphorylating mitochondria from crustaceans. Table 5 shows that the crab and mammalian cytochromes *a* and *a*₃ and cytochrome oxidase are similar.

The response of the absorption spectrum of the hepatopancreas mitochondria towards cyanide and carbon monoxide confirms the previous identification of cytochrome *a*₃ in these particles (Beechey, 1961*a*). Also the cytochrome-oxidase activity of these particles is highly sensitive to the presence of these compounds. Finally the simple nature of the

Table 4. *Relative ability of the hepatopancreas mitochondria to oxidize succinate and ascorbate*

The mitochondria were suspended in 'No substrate B' medium. The final composition of the suspension medium was achieved by diluting the mitochondrial suspension with equal volumes of media containing twice the final concentration of substrates and horse-heart cytochrome *c*. Samples (1 ml.) of the final mitochondrial suspension, containing 2–5 mg. of protein, were placed in manometer flasks; 0.1 ml. of 10 % (w/v) KOH was in the centre well. The respiration rates were then measured. The results of four experiments are shown.

Suspension medium ...	Respiration rate (μ l. of O ₂ /hr./mg. of protein)				
	'No substrate B'		'Ascorbate B'		'Succinate D'
	0.0	0.1	0.0	0.1	0.0
Concn. of cytochrome <i>c</i> (mM)					
	5	5	4	46	56
	—	—	4	54	64
	5	6	—	48	59
	5	8	—	54	79

Table 5. *Comparison of the properties of the terminal respiratory systems in hepatopancreas mitochondria and mammalian preparations*

	Phosphorylating hepatopancreas mitochondria	Purified mammalian cytochromes <i>a</i> plus <i>a</i> ₃ preparations	Particulate heart-muscle preparations
Percentage contribution of cytochrome <i>a</i> ₃ to absorption at 445 m μ	51 (455 m μ)	51 (460 m μ)*	60–65 (460 m μ)*
Peak absorption of cytochrome <i>a</i> ₃ –CO	65 (460 m μ)	—	—
Absorption peak of difference spectra of: Cytochrome <i>a</i> ₃ minus cytochrome <i>a</i> ₃ –CN	430 m μ	430 m μ *	430 m μ †
Cytochrome <i>a</i> ₃ –CN minus cytochrome <i>a</i> ₃ –CN	445 m μ	444 m μ *	—
CO inhibition of respiration	445 m μ	444 m μ *	—
Inhibition of cytochrome-oxidase activity by: HCN	Light-reversible	—	Light-reversible‡
Antimycin A	93 % at 50 μ M-HCN Insensitive	100 % at 10 μ M-HCN§ Insensitive§	90 % at 500 μ M-HCN

* Yonetani (1960).

§ Griffiths & Wharton (1961).

† Chance (1953).

|| Wainio & Greenlees (1961).

‡ Keilin (1930).

difference spectra in Figs. 2 and 4 indicate that there is only a single pigment on the oxygen side of the points inhibited by cyanide and carbon monoxide. Thus the cytochrome oxidase and the cytochrome a_3 of these mitochondria are identical, as suggested originally by Keilin & Hartree (1939) for heart-muscle preparations.

Keilin & Hartree (1939), using a heart-muscle preparation, and Wainio (1955), using a purified cytochrome-oxidase preparation, have presented evidence that the spectra of cytochrome a_3 -CN and cytochrome a_3'' differ significantly. However, by reducing cytochrome a_3 -CN with dithionite Yonetani (1960) showed that cytochrome a_3 -CN and cytochrome a_3'' have almost identical absorption spectra in a highly purified mammalian cytochrome-oxidase preparation. Though our results obtained with phosphorylating mitochondria agree with those of Yonetani (1960), the mitochondria used bear more resemblance to the heart-muscle preparation of Keilin & Hartree (1939) than to the purified cytochrome-oxidase preparation used by Yonetani (1960). It would appear that, unlike carbon monoxide and nitric oxide (Wainio, 1955; Sekuzu, Takemori, Yonetani & Okunuki, 1959), hydrogen cyanide does not profoundly modify the spectra of haems a_3'' and a_3''' , nor does it prevent their interconversion. These anomalies suggest that the block of electron flow is not due to the formation of a haem a_3 -cyanide complex but is due to a cyanide block between haems a and a_3 .

The high degree of the cyanide inhibition of the oxygen uptake suggests that most of the oxidative capacity of these mitochondria is mediated via cytochrome a_3 . But the role of these mitochondria in the metabolism of the intact hepatopancreas remains unsettled. Preliminary experiments suggest that 60–70 % of the respiration of homogenates of the whole hepatopancreas is cyanide-sensitive.

SUMMARY

1. A study has been made of the cytochrome oxidase and cytochromes a and a_3 in phosphorylat-

ing mitochondria isolated from the hepatopancreas of *Carcinus maenas*.

2. 50 μ M-Hydrogen cyanide inhibits 93 % of the respiration due to the oxidation of succinate, fumarate and reduced cytochrome c .

3. In the dark 95 % carbon monoxide causes a 20–30 % inhibition of respiration. This inhibition is completely reversed by light. Carbon monoxide combines with reduced cytochrome a_3 to form a complex which absorbs light maximally at 430 m μ .

4. About 51–65 % of the absorption of light at 445 m μ in the difference spectrum may be ascribed to cytochrome a_3 .

5. It is concluded that cytochrome a_3 is the cytochrome oxidase of these mitochondria.

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