BBA 45589

THE LOW-TEMPERATURE SPECTRAL PROPERTIES OF MAMMALIAN CYTOCHROME OXIDASE

II. THE ENZYME ISOLATED FROM BEEF-HEART MITOCHONDRIA

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(Received April 4th, 1967)

SUMMARY

A series of low-temperature spectra of isolated cytochrome oxidase are presented.

1. A technique for the selective reduction of cytochrome *a* with dithionite is given.

2. The reduced *minus* oxidized difference spectrum of cytochrome *a* shows a 601 mμ α-peak and a split Soret band with maxima at 447 and 442 mμ for ferrous *a* and a Soret absorption band at 426 mμ for ferric *a*.

3. The cytochrome a_3 difference spectrum is quite different. Its ferrous α-peak is found at 604 mμ and its Soret band has a single maximum at 444 mμ. The ferric γ-band is at 412 mμ.

4. Sulfide, like cyanide, was found to react with ferricytochrome a_3.

5. Azide was found to react with ferrocytochrome *a*. The 601 mμ peak of ferrous *a* was shifted to 597 mμ and the split Soret band to 447 and 440 mμ.

6. Under anaerobic conditions the azide effect was reversed, indicating that ferrocytochrome *a* can exist in two forms differing in reactivity toward azide. The form which reacts with azide is labile and in the absence of active turnover is converted to the form which does not react with azide.

7. 'Oxygenated' cytochrome oxidase showed no Soret band splitting at low temperature.

INTRODUCTION

Few spectral studies of isolated cytochrome oxidase (cytochrome $c:O_2$ oxidoreductase, EC 1.9.3.1) at low temperatures have been reported and these are incomplete compared with those which are available at room temperatures[1–3]. The use of low-temperature techniques is of particular value for studying the complex spectral behavior of oxidase. YONETANI[2] used low temperatures to show that cytochrome a_3 cannot easily be reduced in the presence of cyanide and later[4] to show the photodissociation spectrum of a_3–CO compound where the photodissociation was done at

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77 °K. The absorption contribution of reduced *a* and a_3 at 445 mμ appeared to be 50:50 from this spectrum.

Extensive low-temperature studies have been done with mitochondria. This technique has been used to trap aerobic steady states of mitochondria inhibited with CO, cyanide, sulfide and azide[5–8]. The findings with azide are of particular interest. The azide inhibited aerobic steady state of mitochondria (State 3) has a unique oxidase difference spectrum. The α-peak appears at 596 mμ instead of 602 mμ found for the normal reduced peak and the Soret absorption band is split having maxima at 438 and 447 mμ. This splitting of the Soret band also occurs in the presence of CO, cyanide and sulfide but the maxima are at 441 and 448 mμ. The azide effect is reversed by anaerobiosis, uncouplers of oxidative phosphorylation, and the classical inhibitors CO and cyanide. WILSON and co-workers have interpreted this as evidence that active turnover of the electron-transport system causes cytochrome *a* to cycle through a labile reduced form which is capable of reacting with azide. It was further suggested that this labile form of reduced cytochrome *a* may be a common intermediate of the electron transfer and energy conservation reactions.

The present communication presents a complete series of low-temperature spectra of isolated cytochrome oxidase. The low-temperature technique was used to stop reduction of the oxidase at mid-point in order to obtain individual spectra of cytochromes *a* and a_3. Advantage was taken of the increased resolution at low temperatures to examine closely the spectrum of the 'oxygenated' form of cytochrome oxidase. Studies of the trapped aerobic steady state of the isolated enzyme in the presence of inhibitors which parallel those by WILSON AND GILMOUR[8] of the mitochondrial oxidase are included. Here, as with the mitochondrial study, particular emphasis is given to the effect of azide.

EXPERIMENTAL

Isolated cytochrome oxidase was prepared according to YONETANI[2] as modified by MANSLEY, STANBURY AND LEMBERG[9] except that the oxidase was finally dissolved in 0.5 % Tween-80 (Sigma Chemical) instead of Emasol. For all assays cytochrome oxidase was diluted with 0.1 M phosphate buffer (pH 7.4) containing 0.5 % Tween-80. The cytochrome oxidase concentration is expressed as μM heme *a* on the basis of $\varepsilon_{mM}^{605\ m\mu} = 23$ for ferrous oxidase.

The technique of spectroscopy at liquid N_2 temperature is described by CHANCE and co-workers[10,11] and is a modification of the method of ESTABROOK[12,13] and BONNER[14].

In order to reduce cytochrome *a* selectively advantage was taken of the different rates of reduction of cytochromes *a* and a_3 with dithionite. To a solution of the ferric enzyme in an open test tube an appropriate amount of sodium dithionite was added. The contents were mixed by inversion then frozen at liquid N_2 temperature 20 sec after the dithionite addition. The dependence of the spectral changes of cytochrome oxidase as a function of time after dithionite addition is shown in Fig. 1. The absorbance change at 605 mμ is 73 % complete in 20 sec while the absorbance change at 445 mμ is only 30 % complete. The corresponding percentage reduction of cytochrome *a* can be calculated by using the value 81–85 % (refs. 15, 16) for the relative contribution of cytochrome *a* to the 605 mμ peak. In 20 sec 85–90 % of cytochrome *a*

is reduced. Cytochrome a_3 reduction does not significantly begin until after this time and requires minutes to complete. The uncertainty[2,15,17] concerning the relative contribution of cytochromes *a* and a_3 to the 445 mμ peak makes the use of this absorbance unsatisfactory for this calculation. The dependence of the reduction rate on dithionite concentration is less than proportional (even less than a square root dependence) for cytochrome *a* and nearly independent for cytochrome a_3.

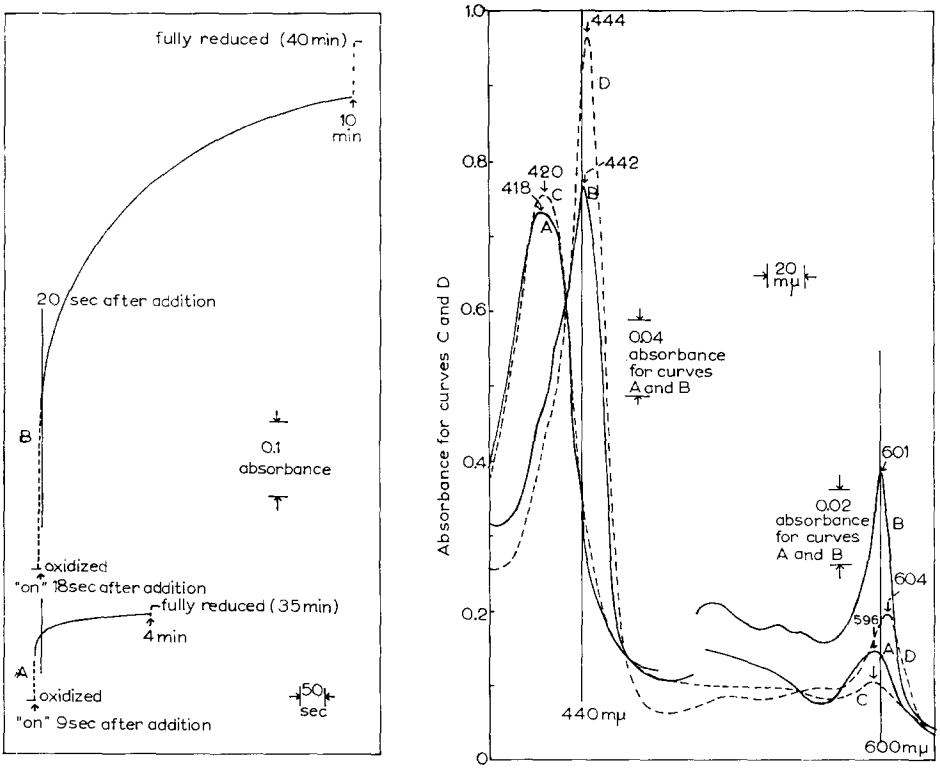

Fig. 1. Sodium dithionite reduction of cytochrome oxidase as a function of time observed at 605 and 445 mμ. Reduction was done in an evacuated Thunberg cell. Curve A, reduction observed at 605 mμ. 0.09 mg of dithionite (diluted 1:100 with sucrose for accurate weighing) was mixed with 2.1 ml of an 9.8 μM oxidase solution. Curve B, reduction observed at 445 mμ. 0.09 mg of dithionite was mixed with 2.4 ml of an 8.6 μM oxidase solution.

Fig. 2. Absolute spectra of oxidized and reduced cytochrome oxidase at room and liquid N_2 temperatures. 8.1 μM oxidase was used for all spectra. ———, Curve A, oxidized oxidase at 77 °K. A 2-mm light path was used at low temperature; ———, Curve B, oxidase reduced with approx. 5 mM dithionite then brought to 77 °K for recording of the spectrum; -------, Curve C, oxidized oxidase at 23°. The normal 10-mm light path was used at room temperature; -------, Curve D, reduced oxidase at 23°.

Where reduction was carried out under anaerobic conditions a Thunberg cell was used. O_2 was removed from the system by evacuating to less than 5 mm of Hg before the reductant which was in the hollow stopper was mixed with the enzyme solution. Excess reductant was added to remove any residual O_2 remaining after evacuation. For formation of the oxygenated compound 30 min was allowed for

complete reduction of the oxidase in the Thunberg cell; the cell was then opened and immediately mixed with O_2 either as air or O_2 gas.

Other reagents used were the same as those of WILSON AND GILMOUR[8].

RESULTS

The absolute spectra of cytochrome oxidase

Oxidized and reduced absolute spectra of cytochrome oxidase at both room and low temperatures are presented for comparison in Fig. 2. In the Soret region of the spectrum the ferric peak of this particular enzyme preparation appears at 420 mμ at room temperature and 418 mμ at liquid N_2 temperature; the ferrous peak is at 444 mμ and 442 mμ, respectively. The α-peak of the ferric enzyme at 596 mμ is unaltered by low temperature, whereas the ferrous peak appears at 601 mμ at low temperature compared with 604 mμ at room temperature. The accuracy of the peak position is ± 1.0 mμ.

The spectral properies of oxygenated cytochrome oxidase

Where reduced cytochrome oxidase is mixed with O_2 the oxygenated form of the enzyme is produced[18–21]. This form is characterized by a Soret maximum at 428 mμ at room temperatures and an α-maximum at 600 mμ. The symmetry of the 428 mμ peak suggests that this form is unique and does not represent a mixture of ferrous and ferric components. We extended this investigation by looking for band splitting at low temperature. The absolute spectrum of the oxygenated compound at liquid N_2 temperature is given as Curve A of Fig. 3. The Soret band appears at 425 mμ and the α-band at 598 mμ. There is no splitting of the Soret band, thus, this method reveals no inhomogeneity in the compound. Curve B is the difference spectrum of oxygenated compound *minus* fully reduced oxidase. The 427 mμ Soret maximum represents the oxygenated form.

The reduced minus oxidized difference spectra of cytochromes a and a_3

The reduced *minus* oxidized difference spectrum is represented by Curve C in Fig. 4. The spectrum shows the Soret peak at 444 mμ with a broad trough at 414 mμ and the α-peak at 600 mμ. Although cytochromes *a* and a_3 are not physically separable entities it is possible to obtain their individual difference spectra. Curve A of Fig. 4 shows the cytochrome *a* spectrum. Cytochrome *a* was selectively reduced by the reaction of dithionite for 20 sec, and this partially reduced enzyme ($a^{2+} + a_3{}^{3+}$) *minus* the oxidized enzyme ($a^{3+} + a_3{}^{3+}$) gives the difference spectrum ($a^{2+} - a^{3+}$). The α-peak at 601 mμ is similar but of slightly decreased absorbance to that of the totally reduced enzyme, whereas, the Soret region is quite different; the trough occurs at 426 mμ and the γ-peak has a double maximum at 447 mμ and 442 mμ. This Soret band split has been reported also by BENDALL AND BONNER[22] from work of these authors with LEMBERG AND GILMOUR. The observed band split is of particular interest with regard to the original finding by WILSON[5] and WILSON AND CHANCE[6] that azide inhibition results in a split Soret band with slightly different maxima.

The cytochrome a_3 spectrum is represented by Curve B of Fig. 4. This spectrum was obtained by taking the fully reduced ($a^{2+} + a_3{}^{2+}$) *minus* partially reduced ($a^{2+} + a_3{}^{3+}$) difference spectrum. It shows the α-peak at 604 mμ, 3 mμ to the red

of the cytochrome *a* spectrum. The height of this peak is more than would be expected for a_3 alone[16] indicating a small cytochrome *a* contribution. It is experimentally difficult to get an uncontaminated a_3 spectrum. The partially reduced sample is not actually ($a^{2+} + a_3{}^{3+}$) but (a^{2+} (85–90 %) + a^{3+} (10–15 %) + $a_3{}^{3+}$) which leaves some (a^{2+}) not cancelled in the fully reduced sample. The Soret band is a single peak at 444 mμ and the trough is at 412 mμ.

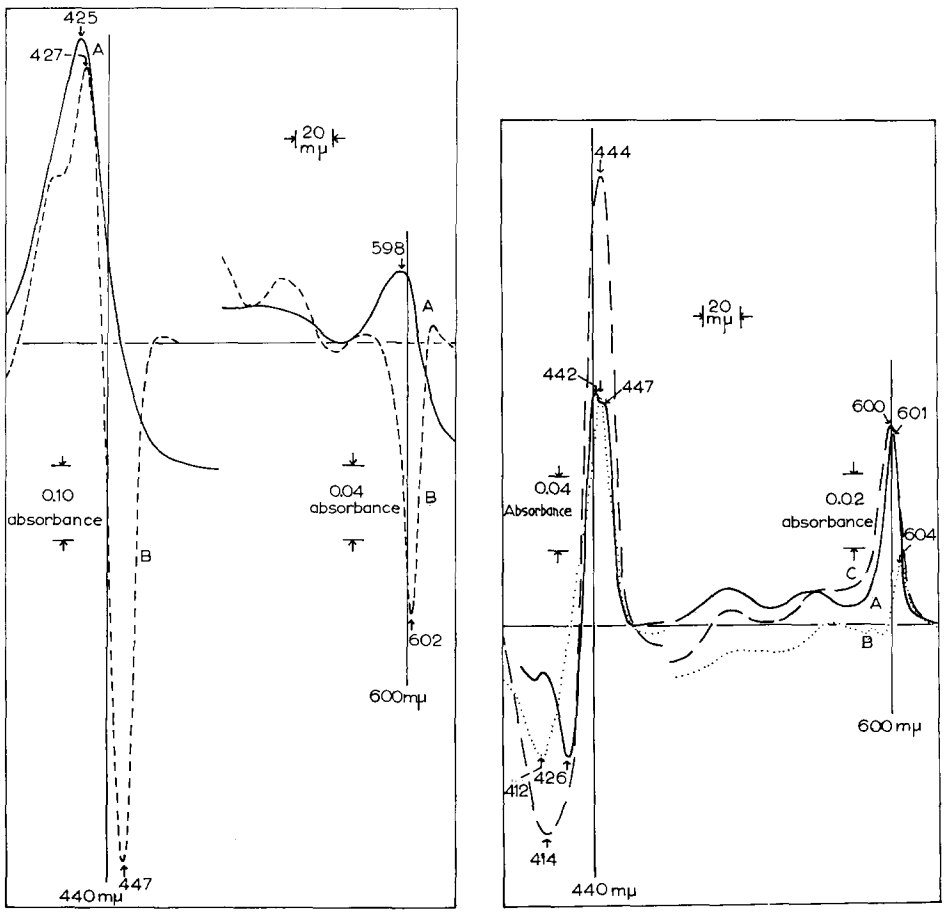

Fig. 3. Liquid N_2 temperature; absolute and difference spectra of oxygenated cytochrome oxidase. ———, Curve A, absolute spectrum of 16.8 μM oxygenated oxidase; -------, Curve B, oxygenated *minus* fully reduced difference spectrum. 16.3 μM enzyme solution was used.

Fig. 4. Liquid N_2 temperature individual difference spectra of cytochromes *a* and a_3. 8.1 μM oxidase solution was used. ———, Curve A, cytochrome *a* difference spectrum. Partially reduced (20 sec with approx. 5 mM dithionite) *minus* oxidized oxidase; ·····, Curve B, cytochrome a_3 difference spectrum. Reduced (30 min with approx. 5 mM dithionite) *minus* partially reduced oxidase; – – – –, Curve C, difference spectrum of the total enzyme. Reduced *minus* oxidized oxidase.

Rough quantitative consideration of the spectra shows that at 444 mμ cytochromes *a* and a_3 have equal absorption and their sum equals that for the total enzyme. The trough representing the oxidized enzyme is quite broad for the total

enzyme with a minimum at 414 mμ whereas that of the cytochrome *a* spectrum is found at 426 mμ, that of the a_3 spectrum at 412 mμ. Summation of the *a* and a_3 troughs equals that of the total enzyme with a 414 mμ minimum. The relative order of the γ/α ratios for the difference spectra of cytochromes *a* and a_3 and the total enzyme agree with those at room temperature reported by LEMBERG *et al.*[3].

The CO effect is clearly and uniquely shown in the difference spectra presented in Fig. 5. The spectrum of the reduced enzyme *plus* CO *minus* the reduced enzyme (Curve A) shows the CO transition from 597 to 585 mμ in the α-region and from 445 to 426 mμ in the Soret region. In Curve B the reduced cytochrome *a* contribution has been almost eliminated from the CO spectrum by placing the partially reduced

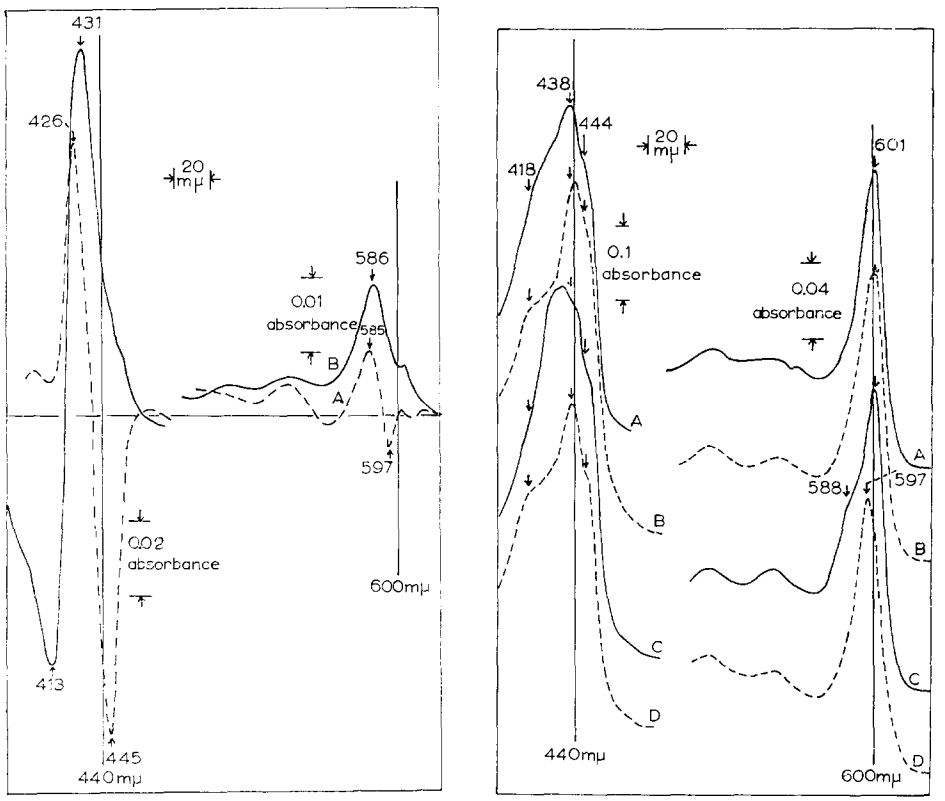

Fig. 5. Liquid N_2 temperature difference spectra of CO–cytochrome oxidase. 8.1 μM oxidase solution was used. -------, Curve A, reduced (30 min with approx. 5 mM dithionite) *plus* CO *minus* reduced oxidase spectrum; ———, Curve B, reduced *plus* CO *minus* partially reduced (20 sec with approx. 5 mM dithionite) oxidase spectrum.

Fig. 6. Absolute spectra of cytochrome oxidase in the aerobic steady state in the presence of various inhibitors. To 16 μM oxidase was added 30 mM ascorbate, 200 μM tetramethyl-*p*-phenylenediamine and then an inhibitor. This solution was then aerated for several min before being frozen at liquid N_2 temperature. The reference solution was 0.5% Tween-80 in 0.1 M phosphate buffer (pH 7.4) *plus* 30 mM ascorbate and 200 μM tetramethyl-*p*-phenylenediamine. ———, Curve A, *plus* 1 mM cyanide; -------, Curve B, *plus* 1 mM sulfide; ———, Curve C, the oxidase solution was bubbled with CO gas before and after the addition of the reductant. It was then allowed to stand without aeration for several minutes before being frozen; -------, Curve D, *plus* 1 mM azide.

enzyme in the reference cuvette. This gives essentially the $(a^{2+} + CO\text{–}a_3^{2+}) - (a^{2+} + a_3^{3+})$ difference spectrum. The α- and γ-peaks are found at 586 and 431 mμ, respectively with a trough at 413 mμ representing oxidized a_3.

The absolute spectra of the inhibited aerobic steady state

A series of absolute spectra of cytochrome oxidase in the aerobic steady state in the presence of various inhibitors is presented in Fig. 6. The system was kept aerobic by continual inversion of the reaction vessel. Curve A shows the cyanide-inhibited spectrum. In the α-region there is a normal ferrocytochrome *a* peak at 601 mμ. In the Soret region the maximum is found at 438 mμ with a shoulder on either side of the peak, one at about 444 mμ and the other on the shorter wavelength side at about 426 mμ. The 426 mμ shoulder represents the small $CN\text{–}a_3^{3+}$ shift[1,2]. The 438 mμ peak and 444 mμ shoulder represent the split Soret band of reduced *a*. In the cyanide-inhibited aerobic steady state, therefore, cytochrome a_3 is present as $CN\text{–}a_3^{3+}$ and cytochrome *a* as the normal reduced component.

The effect of sulfide is shown in Curve B. The spectrum is similar to that with cyanide. The α-peak is at 600 mμ and the Soret maximum is at 440 mμ with 2 shoulders, one on either side of the peak. However, the reduced *a* band split is not as marked and the oxidized shoulder on the shorter wavelength side of the Soret peak occurs at 418 mμ. Sulfide which reacts with oxidized a_3 does not shift the oxidized γ-band as does cyanide.

CO reacts with ferrocytochrome a_3 altering the spectrum in both the α- and Soret regions (see Curve C). The 588 mμ shoulder and the 433 mμ peak represent the $CO\text{–}a_3^{2+}$ complex. Cytochrome *a* shows the typical reduced spectrum with a 600 mμ α-peak and a split Soret band represented by the 440 and 444 mμ double shoulder. The absorbance contributed by ferrocytochrome *a* to the spectrum is of the same magnitude when CO reacts with ferrous a_3 as when sulfide reacts with ferric a_3 (Curve B).

The aerobic steady-state azide spectrum is given by Curve D. Unlike the previous inhibitors azide causes a marked shift of the α-peak. The peak is found at 597 mμ instead of 601 mμ. It is symmetrical and is about the same height as that of normal ferrocytochrome *a*. In the Soret region the peak lies at 437 mμ with a shoulder at 444 mμ representing the split band of reduced *a*. Azide causes a more pronounced split than do the other inhibitors. The Soret peak also has a shoulder on the shorter wavelength side at 418 mμ which represents normal oxidized a_3. In the presence of azide, therefore, cytochrome *a* gives a reduced and modified spectrum; a_3 gives a normal oxidized spectrum. Spectrally, there is no indication that cytochrome a_3 is reduced to any extent or modified in any way.

The difference spectra for the reduced minus the inhibited aerobic steady state

Cyanide, sulfide, CO and azide effects are compared further in the reduced *minus* inhibited aerobic steady-state spectral series presented in Fig. 7. The cyanide difference spectrum (Curve A) representing $(a^{2+} + a_3^{2+}) - (a^{2+} + CN\text{–}a_3^{3+})$, shows a small alteration in the α-region with a maximum at 607 mμ and a minimum at 603 mμ. In the Soret region the maximum is at 445 mμ representing reduced a_3 and there is a minimum at 424 mμ displaced 12 mμ from the normal oxidized minimum at 412 mμ (see Fig. 4, Curve B) and representing $CN\text{–}a_3^{3+}$. The 445 mμ peak has a higher absorption than would be expected for a normal $(a_3^{2+} - a_3^{3+})$ spectrum.

The difference spectrum with sulfide (Curve B) although it shows minor differences is similar to that with cyanide. It has an ill-defined maximum at about 606 mμ with a 590 mμ minimum. The γ-peak is at 445 mμ and the trough of the S^{2-}–a_3^{3+} complex is at 408 mμ and is asymmetric and shallower than that of the normal a_3 difference spectrum.

The typical CO behavior is shown by Curve C. The α- and γ-minimum at 587 and 428 mμ, respectively represent formation of the CO–a_3^{2+} complex, and the 445 mμ maximum represents (a_3^{2+} — CO–a_3^{2+}).

The unique effect of azide is clearly seen in Curve D. There is a shift from the 603 mμ α-peak of the reduced sample to 595 mμ in the azide-inhibited sample. This represents the formation of a ferrocytochrome *a*–azide complex. In the Soret region the maximum at 445 mμ is typical of (a_3^{2+} — a_3^{3+}). There is a small double minimum at 425 and 407 mμ.

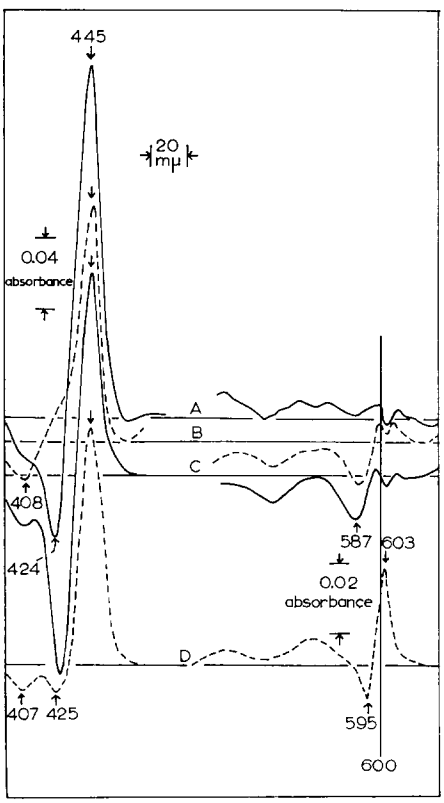

Fig. 7. Difference spectra of reduced cytochrome oxidase *minus* cytochrome oxidase in the aerobic steady state in the presence of various inhibitors. The sample cuvette for each curve contained 16 μM oxidase, 30 mM ascorbate, 200 μM tetramethyl-*p*-phenylenediamine, and approx. 5 mM dithionite. 15 min was allowed for reduction before freezing the solution at liquid N_2 temperature. Each reference solution consisted of 16 μM oxidase, 30 mM ascorbate, 200 μM tetramethyl-*p*-phenylenediamine and an inhibitor added last. This solution was continually aerated for several minutes before being frozen. ———, Curve A, *plus* 1 mM cyanide; -------, Curve B, *plus* 1 mM sulfide; ———, Curve C, the reference solution was bubbled with CO gas before and after the addition of the reductant. It was then allowed to stand without aeration for several minutes before being frozen; -------, Curve D, *plus* 1 mM azide.

Further observations on the effect of azide on the spectral properties of cytochrome oxidase

Additional studies of the effect of azide on isolated cytochrome oxidase are presented in Fig. 8. Curve A is the spectrum of the aerobic steady state with azide *minus* the oxidized sample. The α-peak is found at 597 mμ, 4 mμ to the blue compared

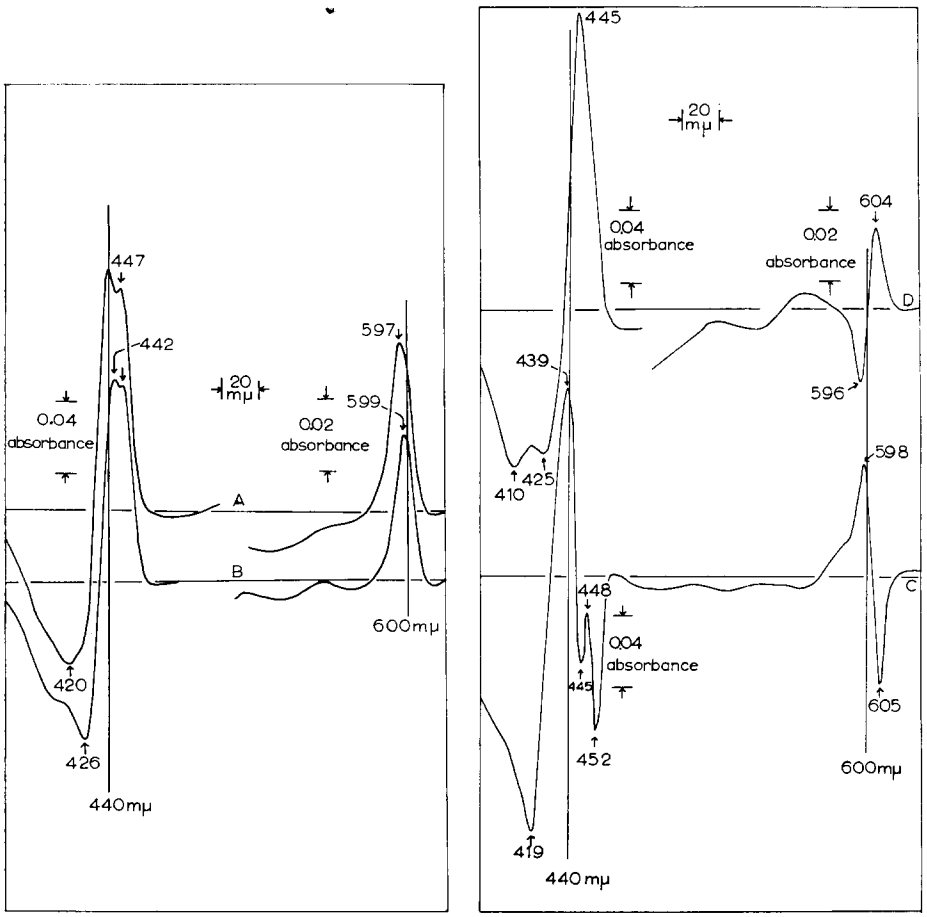

Fig. 8. The effect of azide on partially reduced cytochrome oxidase and the reversal of the azide effect on anaerobiosis. Curve A, the azide-inhibited aerobic steady state *minus* oxidized cytochrome oxidase difference spectrum. The sample cuvette contained 8.1 μM oxidase, 15 mM ascorbate, 200 μM tetramethyl-*p*-phenylenediamine, and 1 mM azide added last. The solution was then aerated for about 1 min before being frozen at liquid N_2 temperature. The reference cuvette contained 8.1 μM oxidase and 15 mM ascorbate and was frozen immediately. Curve B, the partially reduced *plus* azide *minus* oxidized spectrum. The sample cuvette contained 8.1 μM oxidase *plus* 1 mM azide to which approx. 5 mM dithionite was added and the solution then frozen after 20 sec at liquid N_2 temperature. The reference cuvette contained 8.1 μM oxidase. Curve C, the aerobic steady state with azide *minus* that without azide. Both sample and reference cuvettes contained 16.2 μM oxidase, 15 mM ascorbate, and 100 μM tetramethyl-*p*-phenylenediamine. The sample cuvette contained in addition 1 mM azide. Both were aerated for about 1 min before being frozen at liquid N_2 temperature. Curve D, the aerobic steady state with azide *minus* the anaerobic oxidase with azide. Both sample and reference cuvettes contained 9.2 μM oxidase, 15 mM ascorbate, 150 μM tetramethyl-*p*-phenylenediamine, and 2 mM azide added last. The reference solution was aerated for about 1 min before being frozen at liquid N_2 temperature. 5 mM dithionite was added to the sample cuvette and the solution allowed to stand 15 min for formation of complete anaerobiosis before being frozen.

with the cytochrome *a* difference spectrum (Curve A, Fig. 4). The Soret band split with maxima at 447 and 440 mμ is slightly more pronounced than that for cytochrome *a* alone. When the oxidase in the presence of azide was only partially reduced with dithionite, *i.e.* when only cytochrome *a* was reduced, Curve B was obtained. The α-peak is found at 599 mμ but the split Soret band is the same as that of the non-inhibited system. Azide does not appear to react readily, therefore, with reduced cytochrome *a* of the partially reduced oxidase.

The aerobic steady state with azide *minus* the aerobic steady state without azide spectrum (Curve C) is that of $(a_3^{3+} + N_3^- - a^{2+}) - (a_3^{3+} + (x)\ a^{2+} + (1-x)\ a^{3+})$. The proportion of (a^{2+}) and (a^{3+}) in the aerobic steady state is unknown. The effect of azide on ferrocytochrome *a* is shown by the shift from 605 to 598 mμ. In the somewhat complicated Soret region there are 2 minima at 445 and 452 mμ and 2 maxima at 439 and 448 mμ which show the more pronounced split and slight shift of the double peak due to azide. The presence of oxidized cytochrome *a* in the sample without azide is shown by the trough at 419 mμ.

When the azide-inhibited sample is allowed to become anaerobic the spectrum (Curve D) of fully reduced oxidase with azide *minus* the aerobic steady state with azide is obtained. The α-peak of cytochrome *a* has moved to longer wavelengths on anaerobiosis as indicated by the trough at 596 mμ and the peak at 604 mμ. Cytochrome a_3 has become reduced as indicated by the maximum in the Soret region at 445 mμ and the minimum at 410 mμ. The cytochrome *a* transition shows that the azide-induced spectral shift is reversed when the sample is made anaerobic even though the azide is still present. A similar reversal effect is obtained if cyanide (2 mM) instead of dithionite is added to the sample cuvette which is kept aerobic. The spectrum of the aerobic steady state with azide *plus* cyanide *minus* that without cyanide showed a trough at 596 mμ and a peak at 604 mμ.

DISCUSSION

There are several notable differences between the individual low-temperature difference spectra of ferrocytochromes *a* and a_3. The α-peak of cytochrome *a* is at 601 mμ whereas the a_3 peak is found further towards the red at 604 mμ. About 85 % of the reduced α-peak absorbance of the total enzyme is contributed by cytochrome *a* (*cf.* ref. 16) and it, therefore, has the same maximum as *a* at 601 mμ. This is similar to the finding of HORIE AND MORRISON[23] at room temperature. With the aid of cyanide inhibition and an elaborate difference spectral set-up these authors obtained the spectra of $(a^{2+} + a_3^{3+})$ with a 603 mμ peak, the same as the totally reduced enzyme, and $(a^{3+} + a_3^{2+})$ with a peak 2 mμ to the red at 605 mμ. HORIE[24] also making use of cyanide-inhibited spectra calculated the individual absolute spectra of cytochromes *a* and a_3 from available room temperature spectra of the total enzyme. The ferrocytochrome *a* peak occurred at 602 mμ with that of a_3 6 mμ to the red at 608 mμ. These calculations were based on the assumption that the molar ratio of *a* to a_3 is 5:1. However, VANNESTE[17] assuming a 1:1 molar ratio calculated the spectra of cytochromes *a* and a_3 from absolute room temperature spectra of the various forms (oxidized, reduced, inhibited) of cytochrome oxidase and found quite different results in the α-region; the ferrocytochrome *a* peak occurred at 605 mμ and that of a_3 was 2 mμ to the blue at 603 mμ.

The second a, a_3 difference at low temperature is the split Soret band of ferrocytochrome a with maxima at 447 and 442 mμ compared with a single maximum for ferrous a_3 at 444 mμ. Because the a_3 maximum is midway between the two a maxima, the peak for the total enzyme does not show the a, a_3 differences but appears as a single maximum. YONETANI[2] has presented individual difference spectra of ferrocytochromes a and a_3 at room temperature and found essentially no difference between the peak positions of a and a_3 in either the α- or Soret region. However, a close examination of YONETANI's CO spectra at low temperature[25] shows a shoulder on the red side of the Soret peak representing the reduced a split. The discrepancy may be explained by the increased resolution of spectra at low temperatures. Neither HORIE AND MORRISON's[23] difference spectra at room temperature nor HORIE's[24] and VANNESTE's[17] calculated spectra of ferrocytochrome a show a split Soret band. In agreement with YONETANI our low-temperature difference spectra show that approx. one-half of the 445 mμ absorption of the total reduced enzyme is contributed by a and one-half by a_3. However, this is a very rough approximation.

The split Soret band of cytochrome a was first observed by WILSON AND CHANCE[5,6] in azide-inhibited mitochondria and subsequently by WILSON AND GILMOUR[8] in cyanide-, sulfide-, and CO-inhibited mitochondria. The split is not a result of inhibition but is inherent in the normal enzyme as shown by the ferrocytochrome a difference spectrum of Fig. 4, Curve A. Here the split is obtained with the isolated enzyme by the partially reduced *minus* oxidized spectrum. It appears, therefore, that cytochrome a has an inhomogeneity which has not been detected by other methods.

Another a, a_3 difference of considerable interest is that the minima of the a and a_3 difference spectra which represent the ferric forms are found at different wavelengths (426 and 412 mμ, respectively). Thus ferricytochromes a and a_3 have different absorption spectra. This confirms the earlier room-temperature studies of HORIE AND MORRISON[23], HORIE[24] and VANNESTE[17]. HORIE AND MORRISON reported an oxidized Soret peak at 424 mμ for cytochrome a and a 421 mμ shoulder for cytochrome a_3 from their spectra of $(a^{3+} + a_3{}^{2+})$ and $(a^{2+} + a_3{}^{3+})$, respectively. HORIE reported for the calculated absolute spectra peaks at 426 mμ for ferric a and 413 mμ for ferric a_3, and VANNESTE reported 426 and 414 mμ, respectively. In addition VANNESTE's computed oxidized minima for the difference spectra gave values at 425 mμ for cytochrome a and 411 mμ for cytochrome a_3. Examination of the ferric absolute spectrum of the total enzyme shows that the Soret peak is broad and not completely symmetrical, the longer wavelength arm of the peak having a greater absorbance. However, the low-temperature spectrum reveals no band splitting.

Our findings of the effects of inhibitors on isolated cytochrome oxidase with emphasis on azide and sulfide action are very similar to those of WILSON AND GILMOUR[8] for oxidase in intact mitochondria. The action of the classical inhibitors cyanide and CO is well defined[1–3] and our spectra at low temperature showed no deviations. The effects observed with sulfide confirm the suggestion originally made by KEILIN AND HARTREE[26] and supported by the findings of WILSON AND GILMOUR[8] that sulfide inhibits by binding oxidized cytochrome a_3 to form an inactive complex. Thus the action of sulfide is similar to cyanide except there is no indication that, like cyanide, sulfide can also bind reduced a_3. In addition, unlike cyanide, there is no spectral alteration of the ferric complex with sulfide. In Fig. 6 the oxidized a_3 shoulder

remains at 418 mμ and the trough in Fig. 7 is at 408 mμ as opposed to those of the cyanide spectrum at 426 and 424 mμ, respectively.

In the presence of cyanide, sulfide, and CO which react with cytochrome a_3, cytochrome *a* is in the reduced form and neither its absorbance nor its peak position is altered, *i.e.* its 601 mμ peak remains unchanged even by the 590 mμ CO rise and its characteristic split Soret band is unaltered. Changes in the cytochrome a_3 spectrum whether they be to the ferric or the ferrous form do not, therefore, obscure the normal reduced *a* spectrum. Azide is markedly different from the other inhibitors in that it alters the reduced *a* spectrum. The α-peak is shifted to 597 mμ and the Soret band split magnified and slightly shifted. We wish to emphasize here that the whole of the α-peak has shifted, *i.e.* there is no absorption remaining at 601 mμ which could be attributed to normal reduced *a*. Reduced *a* is, therefore, the site whereby azide reacts. We see no alternate explanation of the results and the generally accepted concept that cytochrome *a* is unable to bind ligands must be altered.

MUIJSERS, SLATER AND VAN GELDER[27] recently observed that azide on prolonged standing with ferricytochrome oxidase caused spectral alterations. They conclude that such alterations are due to combination of azide with ferricytochrome a_3. Because the azide effect on ferric oxidase is slow, being incomplete even after 19 h incubation, it is unlikely that this effect is relevant to our findings with the inhibited aerobic state of oxidase which are complete within 1 min.

A comparison of the azide-inhibited spectrum of isolated oxidase with that in intact mitochondria shows that azide apparently has a more marked effect on the intact enzyme. This can be readily seen from the aerobic steady state *plus* azide *minus* oxidized difference spectrum (represented by Curve A of Fig. 7 for the isolated enzyme and by Fig. 2 of WILSON AND GILMOUR[8] for the intact enzyme). The intact enzyme shows a 6 mμ shift to the blue from 602 to 596 mμ whereas the isolated enzyme shift is only 4 mμ from 601 to 597 mμ. In the Soret region the split band of the intact enzyme has maxima at 438 and 447 mμ compared with 440 and 447 mμ for the isolated enzyme.

WILSON[7] using the intact enzyme observed that the azide effect could be reversed merely by allowing the system to become anaerobic which concomitantly allows cytochrome a_3 to become reduced. This same phenomenon occurs with isolated cytochrome oxidase. An explanation of how the absence of O_2 and/or the reduction of cytochrome a_3 releases the azide effect remains elusive. WILSON showed that uncouplers of oxidative phosphorylation also release the azide-induced spectral shift. Furthermore, WILSON AND GILMOUR[8] found that the azide effect did not appear to depend upon the redox state of cytochrome a_3. Azide had no effect when cytochrome a_3 was trapped in the oxidized state by cyanide or sulfide, or when it was trapped in the reduced state by CO. Here there is the possibility that the inhibitors themselves may interfere with the action of azide. Our experiment in which azide was reacted with partially reduced oxidase where cytochrome *a* was selectively reduced, a_3 remaining oxidized, is inconclusive since only a partial shift was obtained. Evidently azide can react with this oxidase system but not readily.

The characteristics of the azide inhibition have led WILSON AND GILMOUR[8] to propose that reduced cytochrome *a* is able to exist in two forms differing in chemical reactivity. The reduced cytochrome *a* of the anaerobic or fully reduced oxidase which is not involved in active turnover is insensitive to azide. Active enzymatic turnover

of the cytochrome oxidase gives rise to an intermediate labile reduced form of cytochrome *a* and it is this form with which azide reacts to give either a ligand change or a ligand modification causing inhibition of electron transport. Our studies with the isolated enzyme give similar results with azide and thus support this proposal.

In coupled mitochondrial electron transport uncouplers of oxidative phosphorylation greatly effect the kinetics of the azide inhibition of respiration[5,6]. It has, therefore, been suggested that the labile form of cytochrome *a* is also an intermediate in the energy conservation reactions. If this interpretation is correct then isolated cytochrome oxidase has retained at least part of the energy conservation mechanism.

ACKNOWLEDGEMENTS

It is a pleasure for the authors to acknowledge the continued support and guidance of Prof. B. CHANCE.

The investigation was supported by grants from the Muscular Dystrophy Associations of America, Inc., U.S. Public Health Service grants GM 12202, 2-F2-GM-18 154 and HE 07827 04, and in part by the National Health and Medical Research Council of Australia.

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