BBA 46129

BIOCHEMICAL AND BIOPHYSICAL STUDIES ON CYTOCHROME aa_3

II. CONFORMATIONS OF OXIDIZED CYTOCHROME aa_3

A. O. MUIJSERS, R. H. TIESJEMA AND B. F. VAN GELDER

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Amsterdam (The Netherlands)*

(Received December 24th, 1970)

SUMMARY

1. The spectral properties of 'oxygenated' cytochrome *c* oxidase, prepared by passing air through the dithionite-reduced enzyme solution, were compared with those of the ferric enzyme.

2. The circular-dichroism spectra of reduced and oxidized cytochrome aa_3 in the γ-band region were in agreement with the results of URRY AND VAN GELDER and in the α-band region with those of YONG AND KING. The spectrum of the oxygenated compound was similar to but not identical with that of the oxidized enzyme.

3. The EPR spectra of oxidized and oxygenated preparations were the same.

4. The effects of azide on the spectra of oxidized and oxygenated cytochrome aa_3 were similar, suggesting different sites for azide binding and 'oxygenation'.

5. A reversible slow spectral change following evacuation of the oxidized enzyme preparation is interpreted as evidence for a conformational change of the protein, one of the conformations being stabilized by molecular oxygen.

6. The position of the γ-band of the oxidized cytochrome aa_3 is strongly dependent on the type of detergent present and the incubation time.

7. At least three different conformations of oxidized cytochrome *c* oxidase have to be distinguished, possibly even four. One of these is the classical 'oxygenated compound'.

INTRODUCTION

As discovered by OKUNUKI *et al.*[1], aeration of dithionite-reduced cytochrome *c* oxidase (EC 1.9.3.1) produces a compound known as oxygenated cytochrome *c* oxidase, which can be distinguished by its γ-band at 428 nm. It was first studied by the groups of OKUNUKI[2–4], MINNAERT[5] and WAINIO[6,7] and later on extensively investigated by LEMBERG and coworkers[8–10]. As a result of later work, the original idea of an enzyme–oxygen complex had to be dropped but the designation 'oxygenated' has persisted and is also adopted throughout this paper.

A characteristic of the oxygenated oxidase is its spontaneous decomposition

Abbreviations: CD, circular dichroism; EPR, electron paramagnetic resonance.

* Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

with formation of the ferric enzyme. This property was investigated and has been described in a preliminary form[11].

A role for the oxygenated form in the enzymic activity, as advocated by LEMBERG and co-workers[12,13], was disputed by GIBSON AND WHARTON[14] and is still controversial. In this context it is essential to agree about the spectral properties of the oxidized enzyme but these are by no means clear. In this paper the existence of several conformations of ferric cytochrome *c* oxidase, depending upon the presence of molecular oxygen, is suggested.

EXPERIMENTAL

Enzyme

Cytochrome *c* oxidase was prepared by the method of FOWLER *et al.*[15] followed by the fractionation of MACLENNAN AND TZAGOLOFF[16]. Several steps were simplified to allow the large-scale preparation of a high-purity enzyme in a short time. The starting material was a modified KEILIN AND HARTREE preparation from beef heart. The procedure from fresh hearts to green residue was accomplished on the first day and the purification on the second day. The green residue and the purified enzyme were stored in liquid N_2. The final preparation contained 8–10 μmoles haem *a* per g protein. The reducibility parameters were: γ-band (reduced)/γ-band (oxidized), 1.30–1.37; $A_{444\text{ nm}}$ (reduced)/$A_{424\text{ nm}}$ (reduced) = 2.20–2.36.

In a few experiments a YONETANI[17] type of preparation was used containing 7 μmoles haem *a* per g protein and having a ratio of γ-band (reduced)/γ-band (oxidized) of about 1.25. Enzyme concentration is expressed as haem *a* and calculated with the millimolar absorbance coefficient (reduced *minus* oxidized) at 605 nm of 12.0 $mM^{-1} \cdot cm^{-1}$ (ref. 18).

Spectra

For spectra and kinetic measurements Cary 14, Cary 15 and Zeiss M4Q spectrophotometers were used. Circular dichroism (CD) spectra were measured with a Cary 60 instrument with a 6002 CD attachment. EPR spectra were measured with a Varian E3 instrument.

Oxygenation procedure

The standard method consisted of evacuation of the cooled sample for 5 min followed by anaerobic addition of a 10-fold excess $Na_2S_2O_4$. After 20 min a stream of air was passed through the solution for 15 min by means of a capillary syringe.

Chemicals

Cholic and deoxycholic acid from British Drug Houses were recrystallized from aq. ethanol and used as neutralized colourless solutions.

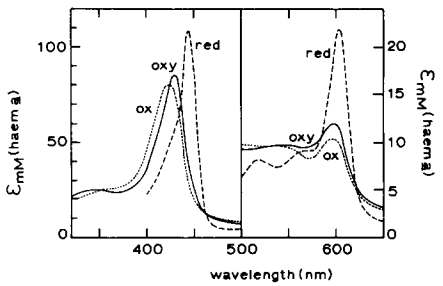
Tween 80 was from Sigma, the other chemicals from British Drug Houses Analar grade.

RESULTS

Spectra

The spectrum of the oxygenated cytochrome aa_3, as prepared by passing air through the dithionite-reduced enzyme solution, is shown in Fig. 1. The Soret peak

is at 427.5–428.5 nm, compared with that of the oxidized enzyme at 422–424 nm. The absorption of the oxygenated form shows a 7 % increase in intensity of the γ-band as compared with the oxidized enzyme. In the α-band a shift of the peak from 597 to 600 nm is accompanied by an absorbance increase of 15–20 %. The shoulder at 340 nm in the spectrum of the oxidized enzyme is a more pronounced small peak in the oxygenated form. The difference spectrum oxygenated *minus* oxidized gives a peak at 435 nm and a trough at 410 nm. The 830-nm band is 3 % higher in the oxygenated form as compared with the oxidized cytochrome aa_3 (Fig. 2).



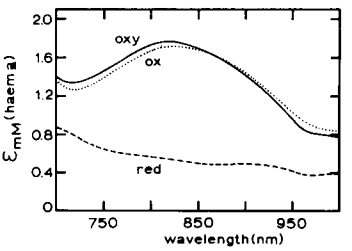
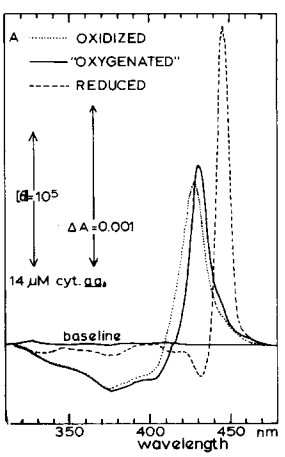


Fig. 1. Absorption spectra of oxidized (ox), reduced (red) and oxygenated (oxy) cytochrome aa_3 in 50 mM potassium phosphate (pH 7.2) and 1 % cholate (w/v). Reduction with a slight excess of $Na_2S_2O_4$ added to an evacuated enzyme solution. For oxygenation a stream of water-saturated air from a capillary tube was passed through the reduced enzyme for 10 min.

Fig. 2. Absorption spectra of oxidized, reduced and oxygenated cytochrome aa_3 in the near infrared. Conditions as in Fig. 1.

The shift to the red of the γ-band on oxygenation is also observed in the CD spectrum (Fig. 3A), as is the slightly higher intensity of the band of the oxygenated form. In the α-band region (Fig. 3B) the curves for the reduced and oxidized samples closely followed those reported by YONG AND KING[19] although our oxidized cytochrome aa_3 showed a small negative extremum at 620 nm in addition to the 570-nm



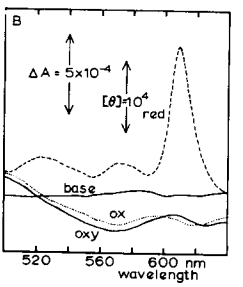


Fig. 3. CD spectra of oxidized, reduced and oxygenated cytochrome aa_3. Enzyme containing 28 (A) or 141 (B) μM haem *a* in 50 mM potassium phosphate (pH 7.2) and 1 % cholate. Light path, 1 cm. Temperature, 27°. The γ-peak of the oxygenated sample was traced 4 min after oxygenation so that no more than about 10 % of the oxygenated compound had decomposed to give the oxidized form.

negative peak already observed by YONG AND KING[19]. The spectrum of the oxygenated cytochrome closely resembles that of the oxidized enzyme but it is not identical. The CD spectrum underlines once more that the oxygenated form is not a mixture of the oxidized and reduced forms of the enzyme. This is especially clear at 436–439 nm since the ellipticity of the reduced form in this wavelength region is smaller and the ellipticity of the oxygenated form is more positive than that of the oxidized form. Concerning the more conspicuous difference at 430 nm some caution should be exerted in view of the detergent-dependent variations observed at this wavelength by MYER AND KING[20] (see DISCUSSION).

No appreciable difference is observed in the EPR spectra (Fig. 4) of the oxygenated and the oxidized forms. However, immediate freezing of the prepared oxygenated sample is necessary, not only to avoid the decomposition of the oxygenated form but also because a large $g = 6$ signal appears on standing. This phenomenon, that has also been observed by H. BEINERT (personal communication), is a secondary effect and not an essential feature of oxygenated cytochrome aa_3.

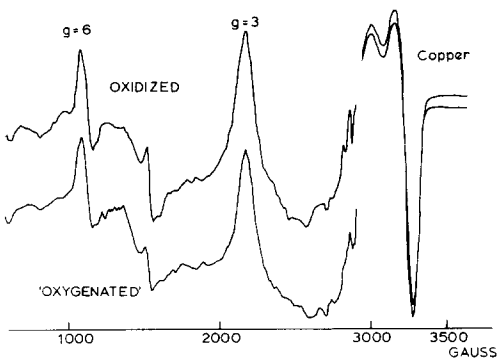


Fig. 4. EPR spectra of oxidized and oxygenated cytochrome aa_3. The oxygenated form was prepared by reducing the enzyme (700 μM haem) with a small excess of $Na_2S_2O_4$, followed by passing O_2 through the solution. The conditions of EPR spectroscopy were: microwave power 160 mW, modulation amplitude 16 gauss, microwave frequency 9131 Gc, scanning rate 313 gauss/min, time constant 0.3 sec. The gain for the low-field part of the spectra was 20 times that for the high-field part. Temperature 78° K.

Alternative methods of preparation

The oxygenation procedure has also been performed with cytochrome aa_3 preparations purified according to the method of YONETANI[17]. In our hands his method gives rise to cytochrome aa_3 with a Soret band at 421–422 nm which on oxygenation shifts to the same value of 427–428 nm as found with the preparation according to FOWLER *et al.*[15]. This is in agreement with the observations of WHARTON AND GIBSON[21] who found that the rather wide scattering in the position of the γ-bands of individual oxidized preparations was not seen with the corresponding oxygenated samples.

It has been demonstrated that in the oxygenation procedure dithionite can be replaced as a reducing agent by ferrocytochrome *c*, ferrocyanide or formamidine sulphinic acid[10]. We have obtained the same result when NADH *plus* a trace of phenazine methosulphate were used under anaerobic conditions. We could confirm the observation of WILLIAMS *et al.*[22] that the bubbling of air through the reduced preparation could be replaced by passing the reduced enzyme through a short Sephadex G-25 column.

Effect of azide

The addition of 400 μM azide to oxidized cytochrome aa_3 results in a number of small spectral changes[23], the most prominent being a shift of the Soret peak from 424 to 421–422 nm. The difference spectrum (oxidized + azide *minus* oxidized) has small peaks at 415, 558 and 678 nm and troughs at 432 and 590 nm. The difference spectrum oxygenated + azide *minus* oxygenated is similar, the γ-band of the oxygenated enzyme being shifted to about 424 nm by reaction with azide. As might be expected the spectra of the oxygenated form with azide and of the oxidized form with azide become indistinguishable from each other on standing for a number of hours. The possibility that only the small percentage of oxidized aa_3 present reacts with azide could be excluded and the decomposition of the oxygenated compound was not accelerated by azide. Thus the oxygenated enzyme itself is able to react with azide in a similar way as the oxidized form. The same conclusion can be drawn from analogous experiments where fluoride (100 mM) was used instead of azide.

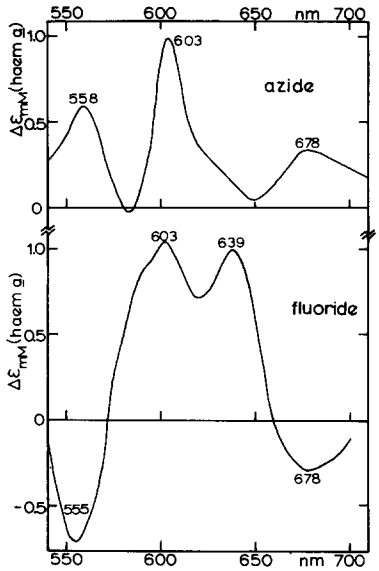
The addition of 100 mM azide or 500 mM fluoride to oxidized cytochrome aa_3 gives rise to additional spectral changes, not observed with lower concentrations of these chelators. In the region of the α-band (Fig. 5) the difference spectra in the case of azide and in the case of fluoride are almost the counterpart of each other. However, both show a peak at 603 nm and in the case of azide also one at 445 nm. As the peaks disappear largely on addition of ferricyanide and have a γ/α ratio of 3, their origin must be a partial reduction of cytochrome *a*. This can be interpreted as a slow electron transport from endogenous donors to the haem groups, which in the absence of chelators are rapidly reoxidized by O_2. In the presence of azide the electron pathway is inhibited somewhere between cytochrome *a* and O_2. The slow O_2 uptake predicted by this hypothesis was confirmed experimentally by incubating the concentrated enzyme (0.2 μmole cytochrome aa_3) without added substrate in a Warburg vessel at 25°. The O_2 consumption was about 1.5 μmoles in 4 h and was inhibited for more than 50 % by 90 mM azide or 100 mM fluoride. However, since reduction of the enzyme without added substrate or chelators is not observed under anaerobic conditions, the behaviour of oxidized cytochrome aa_3 under aerobic and anaerobic conditions appears to be different.

The effect of anaerobiosis on the spectrum of oxidized cytochrome aa_3

Under anaerobic conditions the α-band shifts 2 nm to the red without change in intensity, in contrast to the Soret peak which moves to shorter wavelength and has a slightly lowered intensity. The process becomes already visible within a few minutes after removal of the O_2 and is fully reversible as illustrated in Fig. 6. An oxidized sample was made anaerobic in a Thunberg cuvette and then partly transferred to a second evacuated cell, connected to the first. After opening and aeration of one of the cells the difference spectrum (anaerobic *minus* aerobic) was recorded at different intervals. The millimolar extinction coefficient for the trough at 430 nm is about 5, but the decrease in the 422–424 nm γ-peak region is much smaller. The inset of Fig. 6 depicts the slight shift in peak position from 424 to 421 nm on anaerobiosis and its reversal upon subsequent aeration. Qualitatively the observations are quite independent of the batch of enzyme preparation and the detergent used in the experiment but the magnitude of the spectral changes increases when Tween 80 is used instead of cholate. The spectral changes described also take place when the anaerobic

cuvette is filled with O_2-free N_2, Ar or H_2, instead of being kept under vacuum. The decisive factor appears to be only the absence of O_2.

These experiments lead to the concept of two different forms of the oxidized cytochrome aa_3. The one with a γ-band at 423–424 nm is stabilized by O_2; the other having a peak at lower wavelength is the more stable conformation in the absence of O_2.



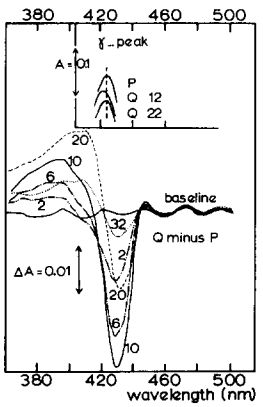


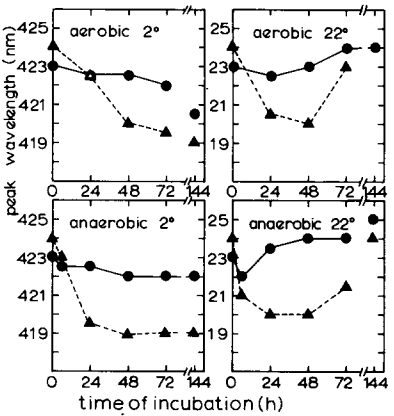
Fig. 5. Difference spectra showing the effect of high concentrations of azide and fluoride on oxidized cytochrome aa_3 in the α-band region. Enzyme in 60 mM potassium phosphate (pH 7.3) containing 0.7% (v/v) Tween 80. Upper curve, cytochrome aa_3 + 100 mM azide (after 13 min at 22°) *minus* cytochrome aa_3; lower curve, cytochrome aa_3 + 500 mM fluoride (after 30 min at 22°) *minus* cytochrome aa_3.

Fig. 6. Reversible shift of the γ-band of oxidized cytochrome aa_3 under anaerobic conditions. The enzyme solution containing 10.6 μM haem in 50 mM potassium phosphate (pH 7.3) and 1% Tween 80 was made anaerobic in a Thunberg cuvette (P) by repeated evacuation and flushing with O_2-free N_2 and finally left evacuated. Some of the solution was transferred to a second evacuated Thunberg cuvette (Q), connected to the first with a ground-glass joint and stopcock. In this way two anaerobic samples of exactly equal concentration were obtained. A baseline was traced, Q *minus* P. At zero time cell P was opened and aerated. Difference spectra were traced of the still anaerobic Q *minus* the aerobic P. The numbers in the figure give the time in min (2, 6 and 10) after the opening of cell P. The temperature was 20°. The absolute peak positions of the spectra were recorded at 12 (Q) and 14 (P) min (inset). After 16 min the second cell Q was also opened and aerated. A difference spectrum was taken at 20 min. At 22 min the absolute spectrum of Q was recorded (see inset) followed by a difference spectrum at 32 min.

The effect of detergents and ageing on the γ-band position

Ageing of preparations, even at 2°, also causes shifts of the γ-band. The nature of the shift is dependent on the temperature, the type of detergent present, and the absence or presence of O_2. The results presented in Fig. 7 were obtained with good enzyme preparations and the shifts of the γ-peak shown in the four plots are less than those found with enzyme preparations of lower quality. Generally the peak of the freshly prepared oxidized enzyme is at higher wavelength with Tween 80 than with cholate as detergent, but the peak slowly shifts 4–5 nm to lower wavelength

(see Fig. 7). At 22° this blue shift is followed after 2 days by a movement back towards longer wavelengths. With cholate as detergent, the initial blue shift is less marked. When both Tween and cholate are present the spectral changes follow the pattern given by Tween alone. The enzyme as prepared always contains traces of cholate because the last fractionation with $(NH_4)_2SO_4$ is carried out in the presence of cholate[16].



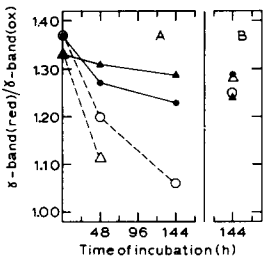


Fig. 7. The effect of ageing under varying conditions on the position of the γ-band of oxidized cytochrome aa_3. Enzyme containing 8 μM haem in 80 mM potassium phosphate (pH 7.4) containing either 0.8% cholate (●—●) or 0.8% Tween 80 (▲—▲). The two upper pictures show the effect of ageing under aerobic conditions, the lower in evacuated Thunberg cuvettes. The left-hand plots show ageing at 2°, the right-hand ones at 22°. All 2° samples were equilibrated 5 min at 22° prior to tracing spectra. The peak position of a sample aged aerobically for 144 h at 22° in Tween could not be measured, because of aggregation.

Fig. 8. The effect of ageing under varying conditions on the reducibility of cytochrome aa_3 (expressed as ratio γ-band (red)/γ-band (ox)). Enzyme containing 8 μM haem in 80 mM potassium phosphate (pH 7.4) containing either 0.8% cholate or 0.8% Tween 80. The left-hand part (A) gives the results of ageing under aerobic conditions and the right-hand part (B) of anaerobic conditions. ●—●, cholate at 2°; ○---○, cholate at 22°; ▲—▲, Tween at 2°; △---△, Tween at 22°. The samples incubated at 2° were equilibrated 5 min at 22° before recording of spectra. The absorption of reduced samples was determined 30 min after addition of a few grains of solid $Na_2S_2O_4$. After 144 h incubation at 22° in the presence of Tween under aerobic conditions, the turbidity of the sample made accurate measurements impossible.

With some preparations the initial peak wavelength in the presence of Tween was lower than observed with cholate. These preparations may be considered already partially aged because their initial behaviour parallels that of other preparations after a short period of incubation.

The changes proceed faster under anaerobic conditions. Since the spectra were first measured 6 h after anaerobiosis, the changes recorded in Fig. 7 include the rapid changes shown in Fig. 6.

The reducibility of the preparations after incubation has been determined, expressed as the ratio of the absorbances of the reduced and oxidized preparations at their respective maxima in the Soret region (Fig. 8). There is a general decline of the values on standing without much difference between cholate and Tween. However, with enzyme preparations of lower quality (in terms of activity, spectral purity and reducibility) better reduction values are always obtained when Tween is used as detergent.

The spectral shifts on ageing could be due to conformational changes in the protein or to the formation of aggregates of enzyme molecules. Autoreduction of the aged enzyme may also contribute to the later red shift observed at 22°.

DISCUSSION

The spectral properties of the oxygenated compound are in agreement with those reported by other investigators[9,10,12,21]. The spectrum is similar to that of ferric cytochrome aa_3–cyanide which also possesses a γ-peak at 427 nm. The agreement between the difference spectrum oxidized cytochrome aa_3–CN *minus* oxidized cytochrome aa_3 (K. J. H. VAN BUUREN, unpublished observations) and that of oxygenated cytochrome aa_3 *minus* oxidized cytochrome aa_3 is especially striking. This may indicate that the oxygenated form like the cyanide complex has a more low-spin haem character than the oxidized enzyme. The similarity between the 830-nm band of oxidized and oxygenated cytochrome aa_3 as found by GILMOUR[24] was confirmed, although a closer look at the spectrum in this region revealed a slightly increased absorbance and a blue shift of the broad band after oxygenation.

The CD spectra of the oxidized and reduced cytochrome in the Soret region are similar to those reported by URRY AND VAN GELDER[25]. MYER AND KING[20] emphasize the difference between the spectrum taken in the presence of 0.25 % Emasol-1130 and with 2 % deoxycholate, and warn against the use of ionic detergents. However, our CD spectra of oxidized cytochrome aa_3, in the presence of 1 % cholate, are identical to the 'Emasol spectra' of KING and co-workers[19,20], both being characterized by the rather symmetrical γ-band and distinct negative peak at 570 nm (Table I). Our spectra of reduced cytochrome aa_3 show both the small negative extremum at 430 nm seen in KING's 'deoxycholate spectrum' and the high molar ellipticity of the α-band in the 'Emasol spectrum' (see Table I). The CD spectrum of the oxygenated compound in the γ-band region has no common isosbestic points with the curves of the reduced and oxidized forms, thus establishing its separate identity.

TABLE I

CD PARAMETERS OF CYTOCHROME *c* OXIDASE

State	*Ref.*	*Detergent present*	*CD extremum*		*CD extremum*	
			λ (*nm*)	$[\theta] \times 10^{-3}$ (*degrees·cm²·dmole⁻¹ haem*)	λ (*nm*)	$[\theta] \times 10^{-3}$ (*degrees·cm²·dmole⁻¹ haem*)
Oxidized	19, 20	Emasol	427	123	570	-4
		Deoxycholate	427	123	indistinct	
	25	Cholate	428–429	114	*	*
	This paper	Cholate	427–428	120	570	-4.6
					620	
Oxygenated	This paper	Cholate	430	132	570	-5.2
					626	
Reduced	19, 20	Emasol	445	197	608–611	22
		Deoxycholate	445	227	608	17
	25	Cholate	445–446	225	*	*
	This paper	Cholate	446	244	608–609	21

* Not determined.

No difference was found between the EPR spectra of oxidized and oxygenated cytochrome aa_3, either in the haem signals or in the copper region. This is not surprising since spectral changes in the ultraviolet (haem groups) and near infrared (copper) caused by the oxygenation are only slight.

Low concentrations of azide (400 μM) cause small spectral changes that are quite similar for oxidized and oxygenated preparations. This suggests different sites on the enzyme molecule for oxygenation and reaction with azide. However, since the primary point of attack by azide is unknown, this does not help in locating the oxygenation site.

A disagreement exists about the possible role of the oxygenated compound in the normal cytochrome *c* oxidase reaction. In their most recent papers LEMBERG and co-workers[13,26] emphasize the importance of the oxygenated form, whereas WHARTON AND GIBSON[21] place it on a slow sidepath. The matter is complicated by the uncertainty about the spectral properties of the oxidized enzyme. The intensity of the Soret peak of our oxygenated preparations exceeds that of the oxidized form, in agreement with WHARTON AND GIBSON[21]. In other papers the peak of the oxygenated form is equally[10] or even less intense[2,7,9] than that of the oxidized enzyme. In a few cases oxygenation is clearly incomplete. Where incomplete oxygenation can be excluded the difference may be caused by the values for the oxidized enzyme rather than for the oxygenated, as illustrated in Table II. The argument can also be reversed. The reasoning then becomes that the peak value for the oxidized enzyme does not vary, and the most reducible preparation, with the highest value for the reduced γ-band, shows the highest conversion to the oxygenated form. In any case, a clear correlation exists between the extinction coefficients for the γ-bands of the reduced and oxygenated forms.

TABLE II

RELATIVE INTENSITIES OF γ-PEAKS AS PERCENTAGE OF THE ABSORBANCE OF THE REDUCED ENZYME

Authors	*Reduced**	*Oxygenated*	*Oxidized*	*Oxidized γ-band (nm)*
LEMBERG AND GILMOUR[10]	100	79	79	418
WHARTON AND GIBSON[21]	100	79	72	419**
This paper	100	78	73.5	423

* Reducing agent $Na_2S_2O_4$.

** The Tris–HCl buffer used contains 60 mM chloride. Chloride like fluoride tends to shift the γ-peak to the blue.

Two different viewpoints also exist concerning the wavelength of the γ-band of the oxidized enzyme. LEMBERG and co-workers[12,27] and KING[28] consider 418–420 nm as the true position. Both groups report a shift to 424 nm on ageing or repeated freezing and thawing. On the other hand, the maximum absorption in our fresh preparations is at 423–424 nm, in agreement with the original observations of GRIFFITHS AND WHARTON[29], FOWLER *et al.*[15], OKUNUKI *et al.*[30] and YONETANI[17], and this always shifts on standing to lower wavelength, the shift being more pronounced with Tween 80 than with cholate as a detergent. The initial blue shift is slowly followed

by a change in the opposite direction. WHARTON AND GIBSON[21] take an intermediate position. They usually observed the peak at 418 nm but occasionally found it at 424 nm and stated that sometimes 424-nm preparations resulted from fresh mitochondria and also 418-nm preparations from aged mitochondria.

A possible resolution of the disagreement is provided by the results described in Fig. 7. After a few days incubation of our preparation in the presence of Tween 80 or the Emasol used by the groups of LEMBERG and KING the spectral properties become similar to those reported by the above mentioned authors. The Soret band lies at 420 nm or lower and gradually shifts to the red on further ageing. However, it is difficult to see why freshly prepared samples of others should be considered 'aged' as compared with our preparations. The word 'aged' must not be interpreted in this case as necessarily meaning 'inferior'. From Figs. 7 and 8 it can be seen that a preparation incubated for 6 days at 2° in the presence of Tween 80 showed a γ-band shift from 424 to 419 nm while the reducibility parameter changed only from 1.33 to 1.29. The remarkable preservation of reducibility under anaerobic conditions even at 22° may be explained by the impossibility of (phospho)lipid peroxidation (*cf.* ref. 31).

Although the rather big changes in peak position must reflect some structural difference it must not be forgotten that both types of preparation show about equal specific activities and identical spectra on reduction and oxygenation. Nevertheless the oxygenated form reverts to the original preparation on decomposition. In other words: the different oxygenated preparations with identical γ-band positions still contain the difference reflected in the wavelength variation of their oxidized precursors and products.

In the opinion of LEMBERG[26] the 424-nm preparations are partly oxygenated. However, the very fast and kinetically homogeneous reduction of our oxidized preparation by NADH *plus* phenazine methosulphate under anaerobic conditions and the absence of the normal decomposition reaction of the presumed oxygenated complex on standing are compelling evidence against this view. As described in another paper[11] the oxygenated cytochrome aa_3 is not reduced at all by NADH and phenazine methosulphate.

The rapid γ-band shift observed under anaerobic conditions provides evidence for the existence of different conformations of the oxidized enzyme, one of these being stabilized by the presence of O_2. This is not to be interpreted as O_2 occupying the sixth ligand position of the a_3 haem iron. In the first place the observed spectral changes are too small and, secondly, they are slow as compared with the rapid removal of O_2 upon evacuation. According to our view the absence of O_2 induces a small conformational change somewhere in the protein that only slightly affects the region of the haem groups. A binding of O_2 analogous to the xenon binding of hemoglobin or myoglobin[32] seems possible.

The relation between the 'anaerobic oxidized form' with its 419–420 nm peak and LEMBERG's '418-nm ferric form' is uncertain. They may not be identical since LEMBERG[26] observed a difference spectrum of the 424-nm compound *minus* the 418-nm enzyme essentially the same as his spectrum oxygenated *minus* oxidized. This is not true for the difference spectrum of our anaerobic *minus* aerobic sample, as shown in Fig. 6.

Summarizing it is possible to distinguish at least three different forms of the ferric cytochrome aa_3: (1) The oxidized form with a γ-peak at 424 nm and stabilized

by O_2. (2) The 420-nm form, stable in the absence of O_2. (3) The conformation with a peak at 428 nm, decomposing spontaneously and not reducible by NADH and phenazine methosulphate. This form is known as 'oxygenated compound'. Maybe the 'normal oxidized form or ferric oxidase' with the 418-nm peak as defined by LEMBERG[27] and KING[28] should be regarded as a separate fourth conformation.

A possible method for detecting conformational changes in proteins is analysis of α-helix and β-structure content by means of CD spectroscopy in the 200-nm region. However, as a preliminary experiment showed, denaturation of a small amount of the enzyme during the oxygenation might have a more profound effect than a conformational change induced by oxygenation.

ACKNOWLEDGEMENTS

We wish to thank Prof. E. C. Slater for his continuous interest and advice and Mr. K. J. H. Van Buuren for critical discussion. Our thanks are also due to Mr. R. Le Clercq for his skilful technical assistance. This work was supported by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

REFERENCES

1 K. OKUNUKI, B. HAGIHARA, I. SEKUZU AND T. HORIO, *Proc. Intern. Symp. Enzyme Chemistry, Tokyo and Kyoto*, Maruzen, Tokyo, 1958, p. 264.
2 I. SEKUZU, S. TAKEMORI, T. YONETANI AND K. OKUNUKI, *J. Biochem. Tokyo*, 46 (1959) 43.
3 Y. ORII AND K. OKUNUKI, *J. Biochem. Tokyo*, 53 (1963) 489.
4 K. OKUNUKI, in M. FLORKIN AND E. H. STOTZ, *Comprehensive Biochemistry*, Elsevier, Amsterdam, 1966, Vol. 14, p. 232.
5 K. MINNAERT, *Biochim. Biophys. Acta*, 35 (1959) 282.
6 A. J. DAVISON AND W. W. WAINIO, *Federation Proc.*, 23 (1964) 323.
7 W. W. WAINIO, in T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems*, Vol. 2, Wiley, New York, 1965, p. 622.
8 R. LEMBERG AND G. E. MANSLEY, *Biochim. Biophys. Acta*, 118 (1966) 19.
9 R. LEMBERG AND J. STANBURY, *Biochim. Biophys. Acta*, 143 (1967) 37.
10 R. LEMBERG AND M. V. GILMOUR, *Biochim. Biophys. Acta*, 143 (1967) 500.
11 R. H. TIESJEMA, A. O. MUIJSERS, M. F. J. BLOKZIJL, B. F. VAN GELDER AND E. C. SLATER, in B. CHANCE, T. YONETANI AND A. MILDVAN, *Probes for Membrane Structure and Function*, Vol. 2, Academic Press, New York, 1971, p. 601.
12 M. R. LEMBERG, M. V. GILMOUR AND M. E. CUTLER, in K. OKUNUKI, M. D. KAMEN AND I. SEKUZU, *Structure and Function of Cytochromes*, University of Tokyo Press, Tokyo, 1968, p. 54.
13 R. LEMBERG AND M. E. CUTLER, *Biochim. Biophys. Acta*, 197 (1970) 1.
14 Q. H. GIBSON AND D. C. WHARTON, in K. OKUNUKI, M. D. KAMEN AND I. SEKUZU, *Structure and Function of Cytochromes*, University of Tokyo Press, Tokyo, 1968, p. 5.
15 L. R. FOWLER, S. H. RICHARDSON AND Y. HATEFI, *Biochim. Biophys. Acta*, 64 (1962) 170.
16 D. H. MACLENNAN AND A. TZAGOLOFF, *Biochim. Biophys. Acta*, 96 (1965) 166.
17 T. YONETANI, *J. Biol. Chem.*, 236 (1961) 1680.
18 B. F. VAN GELDER, *Biochim. Biophys. Acta*, 118 (1966) 36.
19 F. C. YONG AND T. E. KING, *Biochem. Biophys. Res. Commun.*, 40 (1970) 1445.
20 Y. P. MYER AND T. E. KING, *Biochem. Biophys. Res. Commun.*, 33 (1968) 43.
21 D. C. WHARTON AND Q. H. GIBSON, *J. Biol. Chem.*, 243 (1968) 702.
22 G. R. WILLIAMS, R. LEMBERG AND M. E. CUTLER, *Can. J. Biochem.*, 46 (1968) 1371.
23 A. O. MUIJSERS, E. C. SLATER AND K. J. H. VAN BUUREN, in K. OKUNUKI, M. D. KAMEN AND I. SEKUZU, *Structure and Function of Cytochromes*, University of Tokyo Press, Tokyo, 1968, p. 129.
24 M. V. GILMOUR, *Federation Proc.*, 26 (1967) 455.
25 D. W. URRY AND B. F. VAN GELDER, in K. OKUNUKI, M. D. KAMEN AND I. SEKUZU, *Structure and Function of Cytochromes*, University of Tokyo Press, Tokyo, 1968, p. 210.

26 M. R. LEMBERG, *Physiol. Rev.*, 49 (1969) 48.
27 M. R. LEMBERG, in B. CHANCE, R. W. ESTABROOK AND T. YONETANI, *Hemes and Hemoproteins*, Academic Press, New York, 1966, p. 477.
28 T. E. KING, in B. CHANCE, R. W. ESTABROOK AND T. YONETANI, *Hemes and Hemoproteins*, Academic Press, New York, 1966, p. 495.
29 D. E. GRIFFITHS AND D. C. WHARTON, *J. Biol. Chem.*, 236 (1961) 1850.
30 K. OKUNUKI, I. SEKUZU, T. YONETANI AND S. TAKEMORI, *J. Biochem. Tokyo*, 45 (1958) 847.
31 Y. HATEFI AND W. G. HANSTEIN, *Arch. Biochem. Biophys.*, 138 (1970) 73.
32 B. P. SCHOENBORN, H. C. WATSON AND J. C. KENDREW, *Nature*, 207 (1965) 28.