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THE ABSORBANCE COEFFICIENT OF BEEF HEART CYTOCHROME c_1

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Isolated cytochrome c_1 contains endogenous reducing equivalents. They can be removed by treating the protein with sodium dithionite followed by chromatography. This treatment has no effect on the reaction with cytochrome c, nor does it alter the optical spectrum, or the polypeptide or amino acid composition of the protein. Both the titration of dithionite-treated ferrocytochrome c_1 with potassium ferricyanide and the anaerobic titration of dithionite-treated ferricytochrome c_1 with NADH in the presence of phenazine methosulphate lead to the same value for the absorbance coefficient of cytochrome c_1: 19.2 $mM^{-1} \cdot cm^{-1}$ at 552.4 nm for the reduced-minus-oxidised form. This value was also obtained when the haem content was determined by comparing the spectra of the reduced pyridine haemochromes of cytochrome c and cytochrome c_1. Comparison of the optical spectra of cytochrome c and cytochrome c_1 by integration shows equal transition moments for the transitions in the porphyrin systems of both proteins. A set of equations is presented with which the concentration of the cytochromes aa_3, b, c and c_1 can be calculated from one reduced-minus-oxidised difference spectrum of a mixture of these proteins.

Introduction

As part of the ubiquinol-cytochrome c oxidoreductase complex (complex III), cytochrome c_1 is involved in the transfer of electrons through the mitochondrial respiratory chain: the cytochrome catalyses the electron transfer from the iron-sulphur protein to cytochrome c. The kinetic parameters of the reaction between cytochrome c and bovine cytochrome c_1, as part of complex III [1–4] or in the isolated form [5], have been studied. In order to calculate the concentration of cytochrome c_1 it is important to have an accurate absorbance coefficient for this protein. In contrast to the absorbance coefficients of cytochrome c [6] and cytochrome c oxidase [7], which are well defined, the values for cytochrome c_1 reported in the literature show a considerable variation [8–11], ranging from 15.4 [10] to 17.8 $mM^{-1} \cdot cm^{-1}$ [8] at 552.4 nm for the reduced-minus-oxidised form. The methods which have been used are reductive and oxidative titrations [8–10], determination of iron content [8] and antimycin binding [11]. In all cases these values are significantly lower than the coefficient of cytochrome c [6], a protein which has a spectrum similar to that of cytochrome c_1.

Recently, we described a procedure for the isolation of cytochrome c_1 from beef heart [12]. In the resulting preparation the protein is in the monomeric state and it contains only one polypeptide, in contrast to the preparation of Yu et al. [8] which is isolated as a pentamer and which contains two polypeptide chains. The kinetic behaviour of the isolated monomeric cytochrome c_1 in the reaction with cytochrome c [13] and with modified cytochrome c preparations [14] has been described in detail. The calculations in the kinetic experiments were performed using an absorbance coefficient of 19.2 $mM^{-1} \cdot cm^{-1}$ at 552.4 nm which is considerably higher than those reported in the literature. In this paper, a detailed account is given of the three different

methods by which this value of 19.2 $mM^{-1} \cdot cm^{-1}$ was obtained. Furthermore, during the study it was found that most isolated cytochrome c_1 preparations are likely to contain considerable amounts of mercaptans which interfere with some reactions of the protein.

Materials and methods

Ferrocytochrome c_1 was isolated from beef heart according to the method of König et al. [12]. Ferricytochrome c_1 was prepared by gel filtration on Sephadex G-25 after incubation with potassium ferricyanide. Dithionite-treated cytochrome c_1 was prepared by incubating ferrocytochrome c_1 with an excess of sodium dithionite, after which the preparation was passed through a Sephadex G-25 column.

Horse heart cytochrome *c* was prepared by the method of Margoliash and Walasek [15]. Ferrocytochrome *c* was obtained by gel filtration after incubation with ascorbate.

Anaerobic titrations were carried out in Thunberg cells, having two side bulbs, one for NADH and one for phenazine methosulphate. The latter bulb was covered with black plastic in order to protect phenazine methosulphate from light. The cuvette was deaerated by repeated evacuation and flushed with helium. To be able to correct for the decrease in volume caused by these manipulations, spectra were recorded before and after evacuation. The concentration of NADH was determined at 340 nm using an absorbance coefficient of 6.22 $mM^{-1} \cdot cm^{-1}$ [16].

Titrations of reduced cytochrome c_1 with potassium ferricyanide were carried out in a 1 cm cell by adding subsequent amounts of a 500 μM potassium ferricyanide solution. The concentration of this solution was determined in a similar titration of horse heart ferrocytochrome *c*. Concentrations of cytochromes were determined by using the following absorbance coefficients for the reduced-minus-oxidised proteins: 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm for cytochrome *c* oxidase [7], 21.1 $mM^{-1} \cdot cm^{-1}$ at 549.5 nm for cytochrome *c* [6] and 28.0 $mM^{-1} \cdot cm^{-1}$ for cytochrome *b* at 562 minus 577 nm [11].

Pyridine haemochromes were prepared according to the method of Lemberg et al. [17]. Iron was determined after extensive dialysis against 1 mM EDTA on a Jarrell–Ash inductively coupled argon-plasma atom-emission spectrophotometer as described in Refs. 18 and 19. All other spectrophotometric measurements were carried out on a Cary 17 spectrophotometer at room temperature. Measured absorbance coefficients are given as arithmetical means with standard deviations.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS) was performed essentially according to the method of Weber and Osborn [20]. Amino acid compositions were determined on a Beckman Multichrom-M amino acid analyser, using the method of Spackman et al. [21].

Second-order rate constants were determined in stopped-flow experiments, following the absorbance change at 547.5 nm on a Durrum-Gibson instrument. Signal handling and calculations were carried out as described before [22].

NADH (Grade I) was a product of Boehringer. Pyridine (sequenal grade) was obtained from Pierce. Phenazine methosulphate and Tween 20 were from Sigma Chemimal Co. All other chemicals were purchased from British Drug Houses and were of the highest available puritty.

Results

Titration of ferricytochrome c_1 with NADH *in the presence of phenazine methosulphate*

Ferricytochrome c_1 could only be reduced by NADH when phenazine methosulphate was present as a redox mediator. It appeared that at phenazine methosulphate concentrations of 1% of the reducing equivalents added – which are normally used in these type of titrations – the reaction rate was too low. Therefore, the titration was performed anaerobically at a phenazine methosulphate concentration of about 20% of the cytochrome c_1 concentration used. It was found that cytochrome c_1 preparations contain reducing equivalents, which can be transferred to the haem when a redox mediator is present. Within 24 h ferricytochrome c_1 is fully reduced in the absence of oxygen, solely upon addition of phenazine methosulphate at a concentration of 1% of the cytochrome c_1 concentration.

A possible source of reducing equivalents may be reduced sulphydryl groups, since the protein is isolated with the use of a high concentration of β-mercaptoethanol [12]. Therefore, the thiol was removed as described in Materials and Methods.

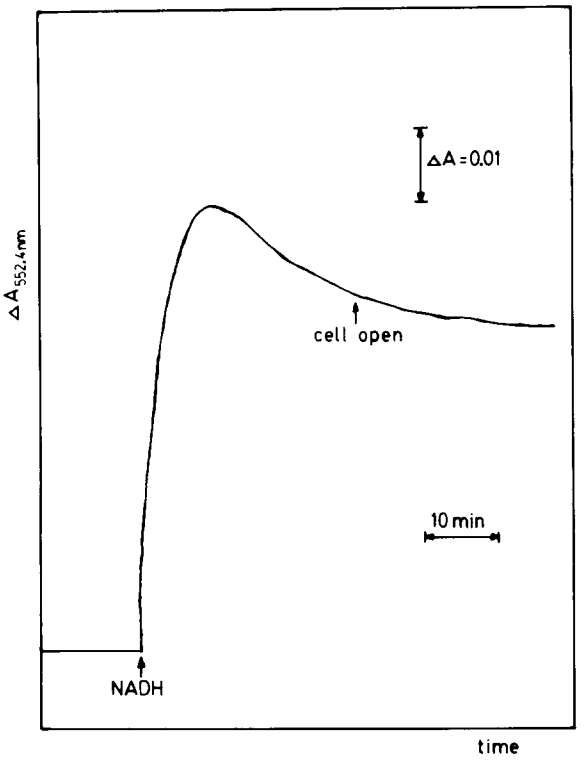

Fig. 1. Reduction of dithionite-treated ferricytochrome c_1 with NADH in the presence of phenazine methosulphate. Absorbance change at 552.4 nm was recorded after the addition of 2 μM NADH as indicated by the vertical arrow. The cell was opened at the time indicated by the second arrow. Ferricytochrome c_1, treated with sodium dithionite as described in Materials and Methods, was present at a concentration of 10 μM in 50 mM potassium phosphate (pH 6.5), 1% Tween 20 and 2 μM phenazine methosulphate.

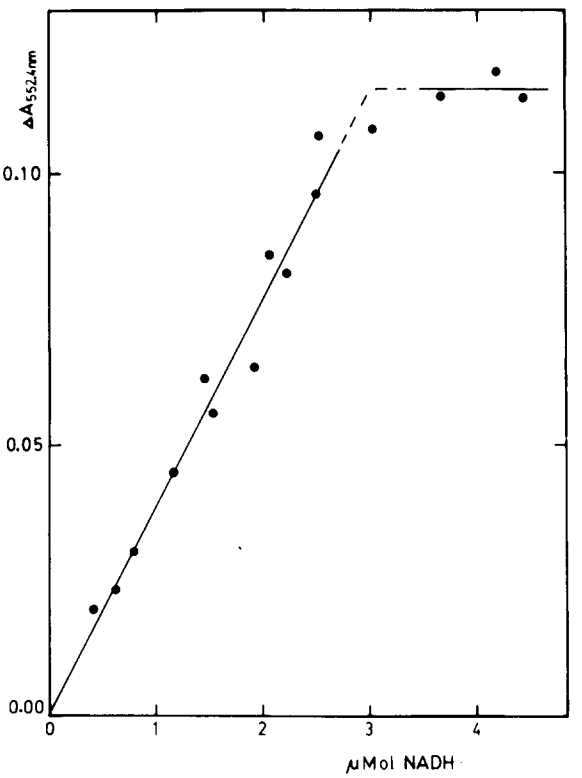

Fig. 2. Anaerobic titration of dithionite-treated ferricytochrome c_1 with NADH in the presence of phenazine methosulphate. Reaction conditions: 6 μM dithionite-treated ferricytochrome c_1, 1.2 μM phenazine methosulphate in 50 mM potassium phosphate (pH 6.5) and 1% Tween 20.

Fig. 1 shows a trace of an anaerobic titration of dithionite-treated ferricytochrome c_1 with NADH in the presence of phenazine methosulphate. The trace consists of a rapid reduction, after which a rearrangement of electrons takes place, resulting in a curve which is the sum of two exponential functions. The reoxidation is not caused by leakage of oxygen into the Thunberg cell, because opening of the cell does not accelerate this reaction. The second part of the curve was extrapolated to the time of mixing. In this way the NADH-induced absorbance change could be calculated for each amount of NADH added, resulting in the titration curve given in Fig. 2. From the curve an absorbance coefficient of 19.2 ± 0.6 $mM^{-1} \cdot cm^{-1}$ at 552.4 nm for reduced-minus-oxidised cytochrome c_1 can be calculated. The protein can be fully reduced by NADH, compared to the degree of reduction obtained by adding an excess of sodium dithionite.

The removal of β-mercaptoethanol, as described in Materials and Methods, had no effect on the spectrum of the protein in the visible region. Polyacrylamide gel electrophoresis and amino acid determinations revealed that neither the polypeptide nor the amino acid composition had changed compared to the untreated protein. The second-order rate constant of the reaction between dithionite-treated cytochrome c_1 and ferricytochrome c was found to be $2.6 \cdot 10^5\ M^{-1} \cdot s^{-1}$ (450 mM potassium phosphate, pH 7.0, 1% Tween 20), similar to the value for untreated cytochrome c_1 [13]. Furthermore, the protein no longer showed any autoreduction in the presence of redox mediators.

Titration of ferrocytochrome c_1 with potassium ferricyanide

A second approach to determine the absorbance coefficient was by titrating dithionite-treated cytochrome c_1 with potassium ferricyanide. The second-order rate constants for the reaction between ferricyanide and cytochromes c and c_1 are reported to be $3.6 \cdot 10^4$ [5] and $6.7 \cdot 10^6\ M^{-1} \cdot s^{-1}$ [23], respectively, and indeed in both cases the reaction was completed within the time of mixing, after which the absorbance stayed constant. The decrease in absorbance upon addition of potassium ferricyanide was linearly dependent on the amount of ferricyanide added, except in the case where the protein was more than 80% oxidised, so that these data were not taken into account.

The results of the titration are presented in Fig. 3. From the slope of the line an absorbance coefficient of $19.2 \pm 0.2\ mM^{-1} \cdot cm^{-1}$ for reduced-minus-oxidised cytochrome c_1 at 552.4 nm can be calculated.

Pyridine haemochrome spectra

A third method to establish the absorbance coefficient of cytochrome c_1 is comparison of the pyridine haemochrome spectra of cytochrome c and cytochrome c_1 (not shown). The spectra of the oxidised forms of the haemochromes show a small difference in peak position. Since these spectra are affected by the amount of potassium ferricyanide added to oxidise the haemochrome, it is conceivable that ferricyanide or cyanide binds as a ligand to haem. The spectra of the reduced haemochromes are identically shaped, showing an absorption maximum at 550 nm and a minimum at 535 nm.

Since the concentration of cytochrome c is known, the absorbance coefficient of the reduced haemochrome at 550 minus 535 nm can be calculated. The value was found to be 22.1 $mM^{-1} \cdot cm^{-1}$. This absorbance coefficient was used to calculate the concentration of the haemochrome of cytochrome c_1. With this concentration the absorbance coefficient of reduced-minus-oxidised cytochrome c_1 could be determined, yielding a value of $19.2 \pm 0.3\ mM^{-1} \cdot cm^{-1}$ at 552.4 nm.

Iron content of cytochrome c_1

The absorbance coefficient of 19.2 $mM^{-1} \cdot cm^{-1}$ was used to determine the content of haem iron in our cytochrome c_1 preparation. Chemical iron determinations revealed that the preparation contained 65.7 mg iron/mmol haem. This corresponds to an amount of 15% of non-haem iron.

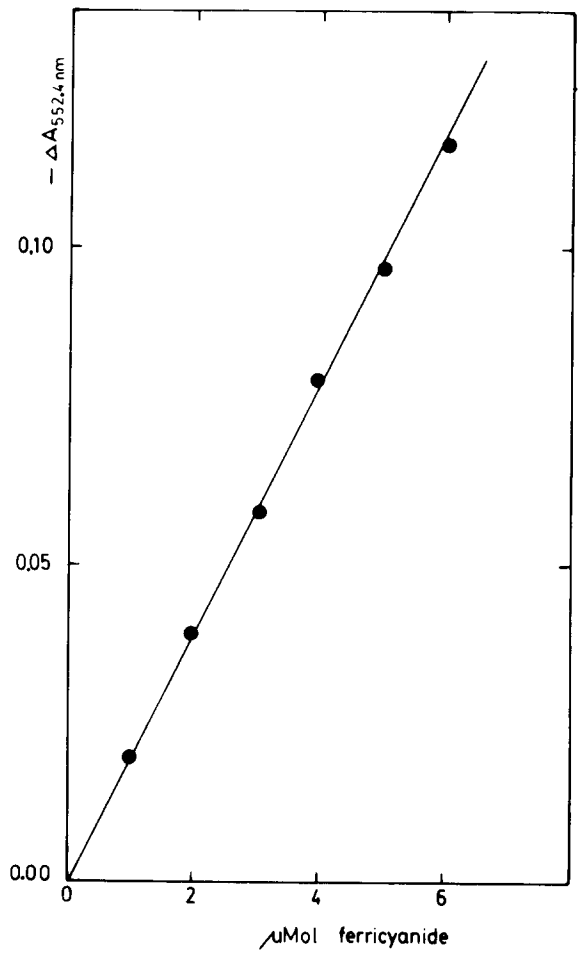

Fig. 3. Titration of dithionite-treated cytochrome c_1 with potassium ferricyanide. Reaction conditions: 8 µM dithionite-treated cytochrome c_1 in 50 mM potassium phosphate (pH 7.2) and 1% Tween 20.

Comparison of the spectra of cytochrome c_1 and cytochrome c

The optimal spectra of reduced cytochrome c_1 and reduced cytochrome c were compared by integration, both for the α, β-band and for the Soret region. For this purpose the wavelength scale in the spectra was converted to a scale of wavenumbers (σ) and the spectra were manually integrated.

TABLE I

INTEGRATION OF THE SPECTRA OF REDUCED CYTOCHROME *c* AND REDUCED CYTOCHROME c_1

Integration interval, 1 nm. $\Sigma\epsilon(\sigma) \cdot \Delta\sigma = \Sigma A(\sigma) \cdot \Delta\sigma$/concentration. Oscillator strength: $f = 4.33 \cdot 10^{-9} \cdot \Sigma\epsilon(\sigma) \cdot \Delta\sigma$ [24].

	Cytochrome *c* band		Cytochrome c_1 band	
	$\alpha + \beta$	Soret	$\alpha + \beta$	Soret
Wavelength limits for integration (nm)	490–575	375–460	490–575	375–460
$\Sigma\epsilon(\sigma) \cdot \Delta\sigma$ ($M^{-1} \cdot cm^{-1}$) ($\times 10^{-7}$)	2.80	23.3	2.84	24.1
Oscillator strength	0.121	0.99	0.123	1.02

Because the absorbance between two peaks never returns completely to zero, the wavelength limits had to be selected arbitrarily. This may introduce a certain inaccuracy in the calculation of the total area under a certain peak. From the integral, the oscillator strength for the transitions corresponding to the α, β-band of cytochrome c_1 could be calculated and this value turned out to be only 2% larger than that of cytochrome *c*; in the Soret region the difference was 3% (Table I). In both cases the difference was found to be within experimental error.

Calculation of the concentration of cytochromes in a mixture

Table II summarises the millimolar absorbance coefficients of cytochromes *c*, c_1, aa_3 and *b* at relevant wavelength pairs in the reduced-minus-oxidised difference spectrum. These values have been calculated from the spectra of purified cytochromes *c*, c_1 and aa_3. The values for cytochrome *b* were deduced from the difference spectrum (dithionite-reduced minus ascorbate-reduced) of purified complex III. It is hard to determine an accurate absorbance coefficient for cytochrome *b* for the wavelength pair 552–540 nm since several forms of cytochrome *b* with slightly different absorption maxima exist in the various preparations of complex III [11]. As 552 nm is situated on the slope of the α-absorption peak of cytochrome *b*, a small shift in peak position results in a large effect on the value $A^{red-ox}_{552nm} - A^{red-ox}_{540nm}$. This value, stated in Table II, is an average value calculated from values ranging between 1.8 and 3.2 $mM^{-1} \cdot cm^{-1}$. When high concentrations of cytochrome *b* are present it is more accurate to calculate the concentrations of cytochromes *c* and c_1 from the difference spectrum ascorbate-reduced minus oxidised.

The spectrum of each of the cytochromes mentioned did not alter when they were mixed (provided no electron transfer took place). This allows us to compose a set of equations, derived from the data listed in Table II, with which the concentration of each component in a mixture of cytochromes can be calculated. These equations are presented in Table III. They form an independent set with four unknowns, which can be solved after introducing the proper

TABLE II

ABSORBANCE COEFFICIENT FOR CYTOCHROMES *b*, *c*, c_1 AND aa_3 IN THE REDUCED-MINUS-OXIDISED DIFFERENCE SPECTRUM AT RELEVANT WAVELENGTH PAIRS

The values were calculated from difference spectra using the following absorbance coefficients (reduced minus oxidised): cytochrome *c*, 21.1 $mM^{-1} \cdot cm^{-1}$ at 549.5 nm [6]; cytochrome c_1, 19.2 $mM^{-1} \cdot cm^{-1}$ at 552.4 nm (this paper); cytochrome aa_3, 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm [7]; cytochrome *b*, 28.0 $mM^{-1} \cdot cm^{-1}$ at 562-577 nm [11], n.d., not determined.

Wavelength pair (nm)	Absorbance coefficients for cytochrome (red–ox) ($mM^{-1} \cdot cm^{-1}$).			
	c	c_1	*b*	aa_3
549–534	25	10.0	−3.3	1.22
552–540	15.8	21.0	3.0	1.98
553–540	11.2	20.8	n.d	n.d
562–577	−1.28	−0.21	28.0	−1.4
605–625	−0.29	−0.33	−1.15	24.95

TABLE III

EQUATIONS FOR THE CALCULATION OF THE CONCENTRATION OF CYTOCHROMES *b*, *c*, c_1 AND aa_3 IN A MIXTURE OF CYTOCHROMES

c, c_1, *b* and aa_3 represent the concentrations (in mM) of the corresponding cytochromes. The equations are derived from the data in Table II.

Mixture of cytochromes *c*, c_1, *b*, and/or aa_3
ΔA (549–534 nm) = $25c + 10.0c_1 - 3.3b + 1.22aa_3$
ΔA (552–540 nm) = $15.8c + 21.0c_1 + 3.0b + 1.98aa_3$
ΔA (562–577 nm) = $-1.28c - 0.21c_1 + 28b - 1.4aa_3$
ΔA (605–625 nm) = $-0.29c - 0.33c_1 - 1.15b + 24.95aa_3$
Mixture of only cytochromes *c* and c_1
ΔA (549–534 nm) = $25c + 10.0c_1$
ΔA (553–540 nm) = $11.2c + 20.8c_1$

data derived from one difference spectrum. In experiments in which only cytochromes *c* and c_1 change in redox state, it is better to use the wavelength pair 553–540 nm instead of 552–540 nm because the former gives a better resolution. The equations for this particular combination are also listed in Table III.

Discussion

It is clear that isolated cytochrome c_1 contains endogenous reducing equivalents which can be transferred to the haem, a process catalysed by phenazine methosulphate. Autoreduction of cytochrome c_1 was also shown to be induced by light, after which process the number of titratable sulphydryl groups in the protein decreased by one [25].

It is likely that preparations of cytochrome c_1 contain bound thiols, since in most isolation procedures high concentrations of mercaptans are used [8,10,12,26]. The reaction by which the bound mercaptan is removed on treating the protein by sodium dithionite remains as yet unclear. Apparently, the thiol is not bound in the vicinity of the binding site for cytochrome *c*, as the removal by sodium dithionite does not affect the kinetic parameters of the reaction between these two proteins.

As revealed by polyacrylamide gel electrophoresis in the presence of SDS [12], the slight reoxidation observed in the anaerobic titration of dithionite-treated cytochrome c_1 cannot be caused by contaminating proteins. It is conceivable that the non-haem iron present in the preparation, which in that case must have a high redox potential, acts as an acceptor of electrons from the haem of cytochrome c_1.

Comparison between the pyridine haemochrome spectra of cytochromes *c* and c_1 can be used as a method to determine the absorbance coefficient of cyrochrome c_1, since the haem groups in both proteins are likely to be equal [8,27]. This method leads to the same value for the absorbance coefficient of cytochrome c_1 as that found by both redox titrations. This justifies the conclusion that dithionite-treated cytochrome c_1 does not contain notable amounts of redox components other than haem which react rapidly with phenazine methosulphate or ferricyanide. The rather low values for the absorbance coefficients of cytochrome c_1 given in the literature must be attributed either to the presence of bound mercaptans, affecting the results of redox-titrations, or, in the case of calculations from the iron content, to contamination of the preparations with non-haem iron as was shown to be present in preparations of cytochrome c_1.

The integration of the optical spectra of reduced cytochromes *c* and c_1 indicates that the transition moments in the porphyrin systems of both proteins are equal. This is even the case for the forbidden transitions leading to the α,β-bands. However, some evident differences exist between the spectra of the two proteins. In the spectrum of ferrocytochrome c_1 all maxima show a red shift of 2–3 nm compared to those in cytochrome *c*. If the haem groups in both proteins are indeed equal as proposed [8,27], this difference may be caused by a difference in one of the axial ligands of the haem. The broad absorption band, observed at 695 nm in the spectrum of ferricytochrome c_1 has, like that for cytochrome *c*, been attributed to a methionine residue as the sixth ligand of the haem [28]. From the effect of *p*-chloromercuribenzenesulphonic acid on the spectrum of cytochrome c_1, it has been proposed by Berden [10] that cysteine acts as the fifth ligand in cytochrome c_1. However, in all known types of cytochromes *c*, histidine and methionine have been shown to be the ligands of the haem [29]. The spectra of both ascorbate- and dithionite-reduced cytochrome c_1 show a distinct peak at 530 nm at room temperature

[12]. Splitting of the β-band in the spectrum of ferrocytochrome *c* has only been observed at low temperatures [30]. Recently, we found that the presence of the band at 530 nm in the spectrum of ferrocytochrome c_1 is indicative of the native conformation of the protein, and that it is absent in the presence of an SH reagent. (Tervoort, M.J., unpublished data). Whether this effect of SH reagents can be ascribed to binding to a cysteine residue in the vicinity of the haem or to induced conformational changes is as yet unclear.

The method, developed for calculating the concentrations of cytochromes in a mixture, provides a useful tool in performing several experiments without the need for digitalized equipment. The concentrations of individual cytochromes in a mixture or, e.g., particles, can be accurately determined from one reduced-minus-oxidised difference spectrum. The procedure can also be used for spectrophotometric studies on redox reactions between cytochromes, the spectra of which are very closely related like those of cytochromes *c* and c_1.

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