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THE REACTIVITY OF THIOL GROUPS IN BOVINE HEART CYTOCHROME *c* OXIDASE TOWARDS 5,5′-DITHIOBIS(2-NITROBENZOIC ACID)

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Bovine heart cytochrome *c* oxidase consists of 12 stoicheiometric polypeptide chains of at least 11 different types. The enzyme contains 14–16 cysteine residues; the distribution of nearly all cysteine residues over the subunits has been established. In native cytochrome *c* oxidase two thiol groups reacted rapidly and stoicheiometrically with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). These thiol groups are located in subunits I and III, respectively. This implies that subunit I is not fully buried in the hydrophobic core of the enzyme. After dissociation of the enzyme by sodium dodecyl sulphate more thiol groups became available to DTNB, in addition to those in subunits I and III, at least one in subunit II, two in fraction V/VI and one to two in the smallest subunit fraction. It is shown that separation of the subunits of cytochrome *c* oxidase by gel permeation chromatography in the presence of sodium dodecyl sulphate depends on the pH of the elution medium. The elution volume of subunits I, III and VII is dependent on pH, that of the others independent.

Introduction

Cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) is a multi-subunit complex, asymmetrically located across the mitochondrial inner membrane [1]. Research on two-dimensional crystals by electron microscopy and image reconstruction has shown that cytochrome *c* oxidase resembles a lopsided 'Y'. Two domains which form the arms of the Y are located at the matrix side of the complex, whereas the stem of the Y protrudes far into the intermembrane space of the mitochondria [2,3].

Yeast cytochrome *c* oxidase is composed of seven subunits, the three largest of which are synthesized inside the mitochondrion, the others products of cytoplasmic protein synthesis [4,5]. There is still no concensus about the exact number of polypeptides of mammalian cytochrome *c* oxidase. Most authors, following the subunit nomenclature of Downer et al. [6], adhere to a seven-subunit model [6–10]. Yu and Yu [11] also accept a seven-subunit model, but their subunit nomenclature is different. Ludwig et al. [12] claimed that antibodies raised against a preparation of cytochrome *c* oxidase, which was almost free of their so-called impurities (a, b and c), immunoprecipitated an enzyme containing seven different subunits (I–VII) from detergent-solubilized mitochondria or submitochondrial particles. Other authors [13,14] purified a six-subunit enzyme. Saraste et al. [13] claimed that their purified six-subunit enzyme, lacking some impurities and subunit III, had similar properties to the crude enzyme, but it did not catalyze net proton translocation. Recent evidence, however, showed that the bovine enzyme is more complex than the yeast enzyme [15]. Buse [16] and Merle and Kadenbach

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulphate; PCMB, *p*-chloromercuribenzoate; IAEDANS, *N*-iodoacetylamidoethyl-1-aminonaphthalene-5-sulphonic acid.

[17] identified 12 polypeptide chains for bovine heart and rat liver cytochrome *c* oxidase, respectively. The latter group presented evidence that all 12 subunits are present in the complex at 1:1 stoicheiometric amounts. In a previous report [18], we showed that bovine heart cytochrome *c* oxidase contains 12 subunits, one copy each of subunits I–VI, two identical or non-identical chains in fraction VII and four chains in fraction VIII with three different amino termini (serine, isoleucine and phenylalanine).

Much work has been done to elucidate the structure of the multi-subunit complex cytochrome *c* oxidase. The membrane-impermeable reagent diazobenzene[^{35}S]sulphonate was used by Eytan et al. [19] and Ludwig et al. [12] to label polypeptides from cytochrome *c* oxidase exposed in intact mitochondria, in submitochondrial particles (inside-out particles) and the native enzyme. These studies showed amongst other things that subunit II was labelled from the cytoplasmic side of the mitochondrial inner membrane whereas subunit IV is located at the matrix side, confirming that the enzyme asymmetrically spans the membrane. Bisson et al. [20] incorporated cytochrome *c* oxidase into vesicles containing ^{14}C-labelled azidophospholipids. After irradiating the vesicles, the subunits surrounded by phospholipids and subunits projecting out of the membrane were identified. Although much insight was obtained from labelling experiments, knowledge of the spatial structure of the cytochrome *c* oxidase complex can be augmented by using protein-modifying reagents which are specific for a single type of amino acid.

We chose a modification of the cysteine residues because there are only a few such residues in cytochrome *c* oxidase [21] and these are scattered over the subunits. Moreover, cysteine residues in enzymes can be found either buried in a hydrophobic environment, or exposed at the surface of the protein. Few authors have studied the reactivity of the thiol groups in cytochrome *c* oxidase. The reagents used include PCMB [22], *N*-ethylmaleimide [23,24], IAEDANS [25] and $^{203}HgCl_2$ [26]. These studies do not give a uniform picture of the reactivity of thiol groups in cytochrome *c* oxidase.

As an absolute specific cysteine-modifying reagent, we used in our study DTNB (Ellman's reagent) [27]. From the results we conclude that bovine heart cytochrome *c* oxidase contains two highly reactive thiol groups, one in subunit I and one in subunit III, and several less reactive thiol groups which only become accessible to DTNB after dissociation of the enzyme by SDS.

Materials and Methods

Bovine heart cytochrome *c* oxidase was purified according to the method of Fowler et al. [28] as modified in our laboratory [29]. The final preparation was dissolved in 50 mM sodium phosphate (pH 7.8), 1% Tween 80 (polyoxyethylene sorbitan monooleate) (v/v). For determination of the concentration of cytochrome *c* oxidase a molar absorption coefficient was used of 24.0 $mM^{-1} \cdot cm^{-1}$ at 605 nm (reduced minus oxidized) [30].

Polyacrylamide gradient gel electrophoresis

Electrophoresis was carried out according to the method of Laemmli and Favre [31] adapted to the use of slab gels of $30 \times 15 \times 0.2$ cm containing a gradient running gel from 12 to 20% acrylamide, 0.39–0.65% *N,N'*-methylenebisacrylamide and stacking gel of 4% acrylamide, 0.13% *N,N'*-methylenebisacrylamide in a Bio-Rad GE-221 apparatus. The gels were stained with Coomassie brilliant blue.

Spectrophotometric determination of the amount of thionitrobenzoate released upon reaction of DTNB with cytochrome c oxidase

A stock solution of 50 mM DTNB in deoxygenated 50 mM sodium phosphate (pH 7.8), 1 mM EDTA and 1% Tween 80 was prepared. Cytochrome *c* oxidase was diluted with the same buffer. The amount of thionitrobenzoate released per mol cytochrome *c* oxidase was determined spectrophotometrically on a Cary 17 apparatus at 412 nm using tandem cells. One compartment of each cell (light path 2×0.437 cm) contained cytochrome *c* oxidase, the other DTNB. The contents of the measuring cell were mixed to initiate the reaction (the solutions in the reference cell were kept separate). The molar absorption coefficient used to determine the amount of released thionitrobenzoate was 13.6 $mM^{-1} \cdot cm^{-1}$ at 412 nm [27].

Labelling cytochrome c oxidase with [$^{14}C_2$]DTNB

100 μCi of 5,5′-dithiobis(2-nitro[*carboxyl*-^{14}C]benzoic acid) ([$^{14}C_2$]DTNB), purchased from Commisariat à l'Energie Atomique, France, with a specific activity of 19.2 mCi/mmol were dissolved in 1.0 ml 100 mM sodium phosphate (pH 7.8), and 2 mM EDTA under an N_2 atmosphere.

Fractions containing radioactivity were mixed with Packard Emulsifier Scintillator 299^{TM} and counted in a Packard TriCarb Liquid Scintillation Spectrometer. Radioactivity in cpm was converted to dpm with the aid of ^{14}C-labelled external standards quenched to various degrees by using the channel ratio method.

In order to determine the recovery of protein-bound thionitrobenzoate after SDS dissociation, cytochrome *c* oxidase was labelled at pH 7.8 with an 80-fold excess of [$^{14}C_2$]DTNB. After 30 min reaction at 25°C, unbound label was removed by the centrifuged-column procedure according to the method of Penefsky [32]. This procedure also exchanges cholate, if present in the enzyme preparation, for Tween 80. The protein was dissociated at 25°C by SDS (6 mg SDS/mg protein) at pH 7.8, 7.2, 6.9 and 6.5. After 4 h dissociation, the label released during dissociation of the protein by SDS was removed by the centrifuged-column procedure and the protein-bound label was counted.

Based on the results of these experiments, pH 6.5 was selected for dissociation of the enzyme by SDS. Further experimental details are given in the legends to Figs. 3 and 4.

Determination of amount of protein by amino acid analysis and from absorbance at 280nm

Protein was determined (after SDS removal) by amino acid analysis according to the method of Moore et al. [33,34] on a Beckman Multichrom M. The concentration of a subunit with a known number of tryptophan and tyrosine residues, and therefore the amount of that subunit in a particular fraction, was determined from the absorbance at 280 nm as described previously [18]. For subunits I and III absorption coefficients at 280 nm based on tryptophan and tyrosine contents (as derived from mtDNA sequence [35]) of 115 and 80 $mM^{-1} \cdot cm^{-1}$, respectively, were used. The results obtained with the two methods are in close agreement [18].

Determination of amino terminal residues

Edman degradation as modified by Tarr [36,37] was used, followed by identification of phenylthiohydantoin amino acids by thin-layer chromatography as described before [18].

Chemicals

Acrylamide, *N,N′*-methylenebisacrylamide, *N,N,N′,N′*-tetramethylethylenediamine, ammonium persulphate and Coomassie brilliant blue of electrophoretic purity were purchased from Bio-Rad. SDS, specially pure, was purchased from British Drug Houses, DTNB and Tween 80 from Sigma and Tris from Boehringer. Chemicals used for amino acid sequencing were from Pierce (sequanal grade). All other chemicals were of analytical grade.

Results

Cholate- and lipid-depleted cytochrome *c* oxidase can be dissociated by SDS into its subunits which are numbered according to their order of elution upon gel chromatography [18,38,39]. Some subunit fractions (e.g., VII), however, can be separated into more polypeptides, as shown on the SDS-polyacrylamide gradient gel (Fig. 1) where peak fractions of a subunit separation on an Ultrogel AcA 54 column eluted at pH 8.2 [18] are compared with the subunits of the cytochrome *c* oxidase as obtained by gel electrophoresis.

Previously, we calculated the M_r of cytochrome *c* oxidase, which is close to 200000 [18]. After performic acid oxidation [40,41], we found 0.94 mol% cysteic acid in the purified cytochrome *c* oxidase preparation. This is equivalent to the presence of 14–16 cysteine residues in the enzyme.

Sequence data (protein and mtDNA) [35,42–48] and our cysteic acid determinations in isolated subunits enable us to determine the distribution of cysteine residues in the subunits as shown in Table I. The table demonstrates that nearly all cysteine residues can be assigned to the various subunits.

The SDS-polyacrylamide gradient gel depicted in Fig. 1 shows that our fraction VII, containing two stoicheiometric polypeptide chains [18], separates into two bands and thus represents two different polypeptides, referred to as VIIa and

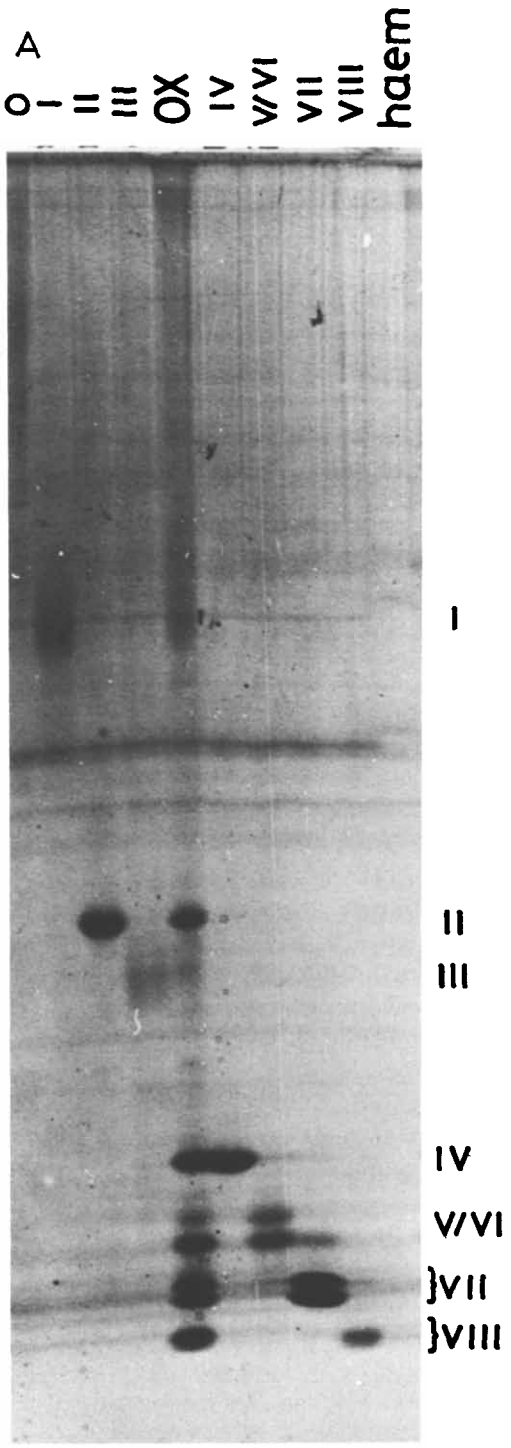
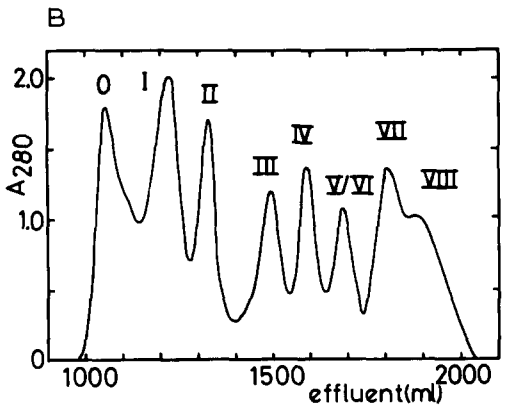


Fig. 1. (A) Polyacrylamide gradient gel electrophoretogram of the subunits of cytochrome *c* oxidase obtained by gel chromatography at pH 8.2. (B) Subunit separation on Ultrogel AcA 54 (for details see Ref. 18). At OX the dissociated holoenzyme was applied. Conditions of the gradient gel are given in Materials and Methods.



VIIb. This extends the conclusion of our report on the stoicheiometry to 12 stoicheiometric polypeptide chains being present of at least 11 different types.

Polypeptide VIIa, corresponding to subunit VII of the group of Buse [46], contains four cysteine residues. We find only 2.32 mol% cysteic acid in polypeptide VIIa plus VIIb (Table I), which would imply that polypeptide VIIb does not contain cysteine.

The question arises as to which of these cysteine residues in cytochrome *c* oxidase are exposed as thiol groups at the surface of the molecule, which are hidden inside the complex and whether cytochrome *c* oxidase contains any disulphide bonds. To solve this problem we did experiments in which cytochrome *c* oxidase reacted with the water-soluble thiol reagent DTNB [27].

In order to establish the number of exposed thiol groups we measured the release of thionitrobenzoate during reaction of detergent-solubilized cytochrome *c* oxidase with DTNB at pH 7.8. The increase in absorbance at 412 nm as a function of time was followed spectrophotometrically (Fig. 2A) and the number of thiol groups that reacted per cytochrome *c* oxidase molecule was plotted as a function of the DTNB concentration (Fig. 2B). As can be concluded from Fig. 2A, two thiol groups rapidly reacted with DTNB; additional thiol groups only tended to react when higher concentrations of DTNB were added.

In a specific experiment, we compared the release of thionitrobenzoate upon reaction of DTNB with cytochrome *c* oxidase with the amount of radioactivity incorporated into the protein when

TABLE I

DISTRIBUTION OF CYSTEINE RESIDUES IN THE SUBUNITS OF BOVINE HEART CYTOCHROME *c* OXIDASE

n.d., not determined.

Subunit	M_r ($\times 10^{-3}$)	Number of cysteine residues	Data obtained from
I (fMet-)	57 [35,52]	1	cysteic acid
II (fMet-)	26	2	sequence [43]
III (Met-)	30 [35,42]	2	cysteic acid
IV (Ala-)	17	0	sequence [44]
V [a] (Ala-)	12	1	sequence [45]
VI [a] (Ala-)	12	2–3	cysteic acid
VIIa [b] (NacAla-)	10	4	sequence [46]
VIIb [b] (Ala-)	10	0	cysteic acid
VIIIa (Ser-)	5	0	sequence [47]
VIIIb (Ile-)	5	0	cysteic acid
VIIIc (Phe-)	6	n.d.	–

[a] 1.64 mol% cysteine in V/VI fraction.

[b] 2.32 mol% cysteine in VII fraction.

[$^{14}C_2$]DTNB was used. The total content of the spectroscopic cell was passed through a Sephadex G-25 superfine column and the protein-bound.label was counted. With the spectrophotometric method an average of 2.75 thiol groups per cytochrome *c* oxidase molecule was found and with the label incorporation method 2.65 thiol groups per cytochrome *c* oxidase molecule. The amount of released thionitrobenzoate equals that of protein-bound reagent which shows that there is no aspecific binding of DTNB to cytochrome *c* oxidase.

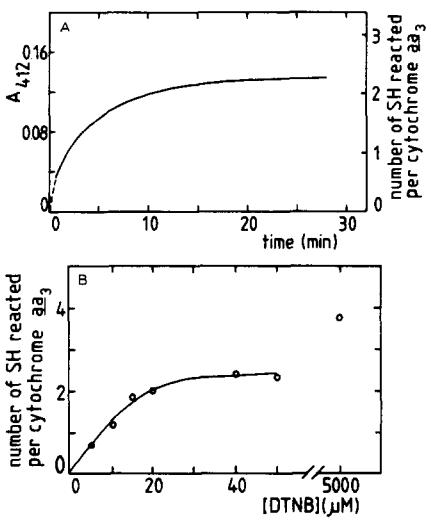


Fig. 2. (A) Rate of formation of thionitrobenzoate anion determined spectrophotometrically using tandem cells. 5 μM cytochrome *c* oxidase was incubated with 50 μM DTNB in 50 mM sodium phosphate (pH 7.8), 1 mM EDTA and 1% Tween 80 (v/v). (B) Number of detectable thiol groups per cytochrome *c* oxidase determined after 45 min as a function of DTNB concentration. Conditions as in A.

In order to locate the subunits containing the two highly reactive thiol groups, the stability of the formed mixed disulphides during dissociation by SDS had to be investigated. For this purpose, samples of cytochrome *c* oxidase were labelled with a large excess of [$^{14}C_2$]DTNB at pH 7.8 and dissociated by SDS at several pH values, after which the amount of label retained in the protein fraction was counted. Table II shows that the

TABLE II

STABILITY OF PROTEIN-THIONITROBENZOATE BOND AS A FUNCTION OF THE pH AFTER 4 h DISSOCIATION OF CYTOCHROME *c* OXIDASE BY SDS

pH of dissociation by SDS	% protein-bound label retained after SDS dissociation
7.8	49
7.2	78
6.9	82
6.5	90

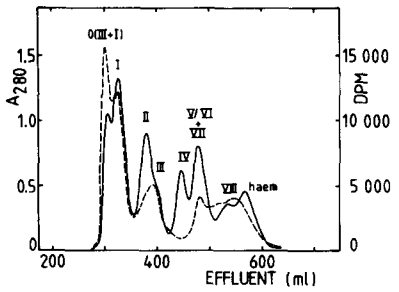


Fig. 3. Labelling pattern (- - - - - -) after reaction of native cytochrome *c* oxidase with an 80-fold excess of [$^{14}C_2$]DTNB at pH 7.8 and 25°C as shown by the chromatogram of the subunits of cytochrome *c* oxidase (———) on Ultrogel AcA 54 (2.5×150 cm column), pretreated as described previously [18]. Unbound label was removed prior to dissociation of the protein by SDS (6 mg SDS/mg protein, dissociation during 4 h at pH 6.5 and 25°C). Sample: 66 mg protein in 6.5 ml. Elution with 3% SDS, 50 mM sodium phosphate (pH 6.5), 1 mM EDTA and 0.01% NaN_3 at 2.3 cm/h.

bound label was most stable when the dissociation of the protein by SDS was performed at pH 6.5, the lowest pH at which cytochrome *c* oxidase can be kept in solution for several hours. A possible explanation for the loss of label during dissociation by SDS at the higher pH values is that thiol groups which become available upon dissociation of the enzyme by SDS cause protein cross-links with release of thionitro[^{14}C]benzoate. This reaction requires a negatively charged thiol group and is, therefore, enhanced at high pH values [49].

It was decided to perform the label experiments for identification of the reactive thiol groups in the subunits with the aid of column chromatography and not with SDS-polyacrylamide gel electrophoresis for the following reasons:

The mixed disulphides formed are not likely to be stable at high pH; SDS-polyacrylamide gel electrophoresis is normally performed at pH 8.8, whereas columns can be eluted at pH 6.5;

working with larger amounts of protein facilitates accurate determination of the ratio of binding of thionitrobenzoate per subunit;

it would be possible to isolate the labelled subunits and to locate the site of the label in the sequence.

In order to locate the two reactive thiol groups as well as the less reactive thiol groups, experiments were performed in which cytochrome *c* oxidase reacted with excess [$^{14}C_2$]DTNB. After the reaction the enzyme was dissociated with SDS at pH 6.5 and the subunits were separated on Ultrogel AcA 54 at pH 6.5. Fig. 3 shows the absorbance elution profile at 280 nm and the ^{14}C-label profile of the cytochrome *c* oxidase subunits. Elution at pH 6.5 causes overlap of some subunit peaks which are clearly separated when elution is performed at pH 8.2, as will be shown later.

A large amount of label was found in the aggregate fraction, subunit I contained label and

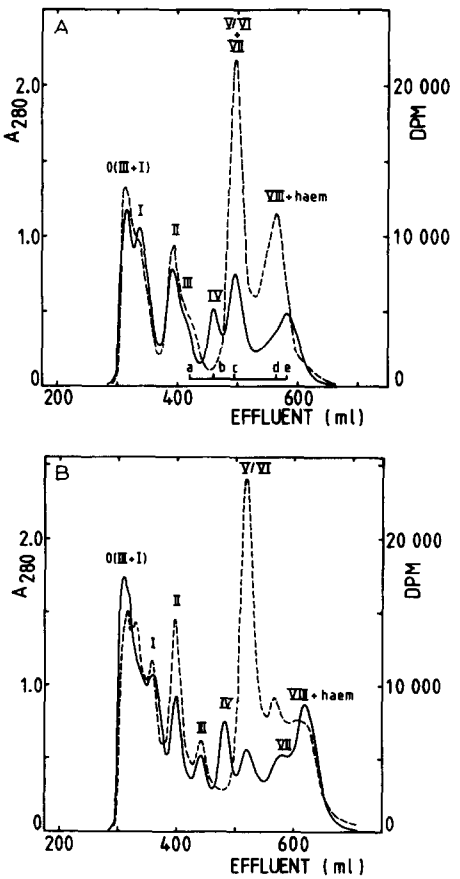


Fig. 4. Labelling pattern (- - - - -) after reaction of SDS-dissociated cytochrome *c* oxidase (6 mg SDS/mg protein, dissociation during 4 h at pH 7.8 and 25°C) with an 80-fold excess of [$^{14}C_2$]DTNB at pH 7.8 and 25°C as shown by the chromatogram of the subunits of cytochrome *c* oxidase (———) on Ultrogel AcA 54 (2.5×150 cm column), pretreated as described previously [18]. (A) Sample: 63 mg protein in 5.2 ml. Elution with 3% SDS, 50 mM sodium phosphate (pH 6.5), 1 mM EDTA and 0.01% NaN_3 at 2.5 cm/h. Fractions a–e were used for the polyacrylamide gel depicted in Fig. 5. (B) Sample: 108 mg protein in 6.9 ml. Elution with 3% SDS, 50 mM Tris-H_2SO_4 (pH 8.2), 1 mM EDTA and 0.01% NaN_3 at 1.5 cm/h.

TABLE III

mol THIONITROBENZOATE BOUND PER mol SUBUNIT UNDER DIFFERENT CONDITIONS OF REACTION OF [$^{14}C_2$]DTNB WITH CYTOCHROME *c* OXIDASE

Calculations were based on the experiments depicted in Figs. 3 and 4. The given pH values refer to the pH of the elution buffer used in the subunit separation. Two methods for calculation were used: (A) via planimetry, label area per subunit as fraction of the total label area; and (P) in peak fractions, mol thionitrobenzoate bound per mol subunit in that fraction. n.d., not determined

Incubation sequence	pH	Calculation method	Subunit								Oxidase sample before separation
			O+I+III	I	III	II	IV	V+VI	VII	VIII+haem	
Cytochrome *c* oxidase		A	2.0			0.3	0.1	0.3		0.7	3.5
DTNB	6.5										
SDS		P		0.9	0.8	0.2	0.1	n.d		n.d	
Cytochrome *c* oxidase	6.5	A	2.1			0.9	0.1	2.2		2.0	7.3
		P		1.0	1.0	0.6	0.1	1.9		1.6	
SDS	8.2	A	2.4			1.0	0.2	1.8		1.8	7.2
DTNB		P		1.2	1.0	0.9	0.2	n.d.		n.d.	

in subunit III there is a label maximum. The latter subunit elutes at pH 6.5 as a shoulder after subunit II. Some label was found scattered over the smaller subunits. In a previous paper [18], we showed that the aggregate fraction (peak 'O' in Fig. 3) mainly contains subunit III, whereas under more rigorous conditions during dissociation of the enzyme by SDS the amount of subunit I in the aggregate fraction increases.

Under the conditions used, 3.5 mol thionitrobenzoate remained bound per mol cytochrome *c* oxidase, which is reflected in the total area of the label profile. Using planimetry, the label area per subunit as a fraction of the total label area can be calculated and thus the ratio of bound thionitrobenzoate per subunit, taking into account the 1 : 1 ratio for the subunits [18]. An alternative calculation was made for some peak fractions where the ratio of bound thionitrobenzoate per subunit in that particular fraction was determined (thionitrobenzoate by the total amount of radioactivity in the peak fraction and protein by amino acid analysis and from absorbance at 280 nm).

The results are given in the upper part of Table III. We conclude that 2 mol thionitrobenzoate are bound per mol cytochrome *c* oxidase, 1 mol in subunit I and 1 mol in subunit III, whereas much smaller amounts of thionitrobenzoate are bound to subunit II and the smaller subunits.

It is evident that after dissociation of the enzyme by SDS more thiol groups become exposed to DTNB. Cholate-depleted cytochrome *c* oxidase was dissociated by SDS and subsequently incubated with labelled DTNB at pH 7.8. Two samples treated in this way were applied to Ultrogel AcA 54 columns; one was eluted at pH 6.5 (Fig. 4A), the other at pH 8.2 (Fig. 4B). In both cases a large amount of label was found in the aggregate fraction and in subunits I and III. There is more label in subunit II as compared to the reaction of DTNB with the holoenzyme and the amount of label in the smaller subunits has also increased (label was found in fraction V/VI and also in subunit VIII).

Determination of the ratio of bound thionitrobenzoate per subunit was performed as described before and the results are summarized in the lower part of Table III. We conclude that 7.2–7.3 mol thionitrobenzoate are bound per mol cytochrome *c* oxidase, again 1 mol in subunit I and 1 mol in subunit III, a significant amount of label being bound to subunit II. 2 mol thionitrobenzoate are bound to fraction V/VI and 1–2 mol in fraction VIII. The amount of label preserved, in contrast to

what might be expected, was not dependent on the pH at which the elution was performed, which would indicate that the label is only lost during dissociation of the enzyme by 12% SDS at pH 7.8 (Table II) and not during elution in the presence of 3% SDS at pH 8.2. It is clear that after dissociation of the enzyme by SDS the number of thiol groups reactive to DTNB has increased, but still half of the total cysteine content of the enzyme cannot be accounted for, either because the cysteine residues are inaccessible or because they are present as disulphide bridges, or because of both reasons. Elution at pH 8.2 as shown in Fig. 4B gives a better resolution of subunit III and therefore strengthens the evidence for binding of DTNB to subunit III.

Comparison of the elution profiles at 280 nm at pH 6.5 and 8.2 (Fig. 4A and B) reveals that some subunits show pH-dependent elution behaviour upon gel permeation chromatography. Analysis of a large number of gel filtration experiments between pH 6.5 and 9.6 showed that the elution volumes of subunits II and IV were pH independent (not shown). The migration velocity and therefore the apparent molecular mass of subunits I and III was found to be larger upon elution at pH 6.5 than at pH 8.2. At pH 6.5 subunit I overlaps with the aggregate fraction (peak O) and monomeric subunit III with subunit II, whereas at pH 8.2 subunits I and III are both resolved. Anomalous behaviour of the very hydrophobic subunits I and III has also been demonstrated on SDS-polyacrylamide gel electrophoresis [4,7,50–52]. The absorbance at 280 nm associated with fraction V/VI was much higher at pH 6.5 than at pH 8.2, which suggests that one of the smaller subunits also has a pH-dependent elution behaviour.

Fig. 5 shows an SDS-polyacrylamide gradient gel of some fractions of the column depicted in Fig. 4A. The shoulder after subunit II (lane a) clearly is subunit III. It is evident that the fraction normally called V/VI (lane c) in this case also contains subunits VIIa and VIIb (and even a trace of VIII). Lane d only contains subunit VIII, whereas the fraction called e only contains haem which in this experiment elutes after the smallest subunits. Capaldi et al. [53] showed that in SDS-polyacrylamide gel electrophoresis in the presence

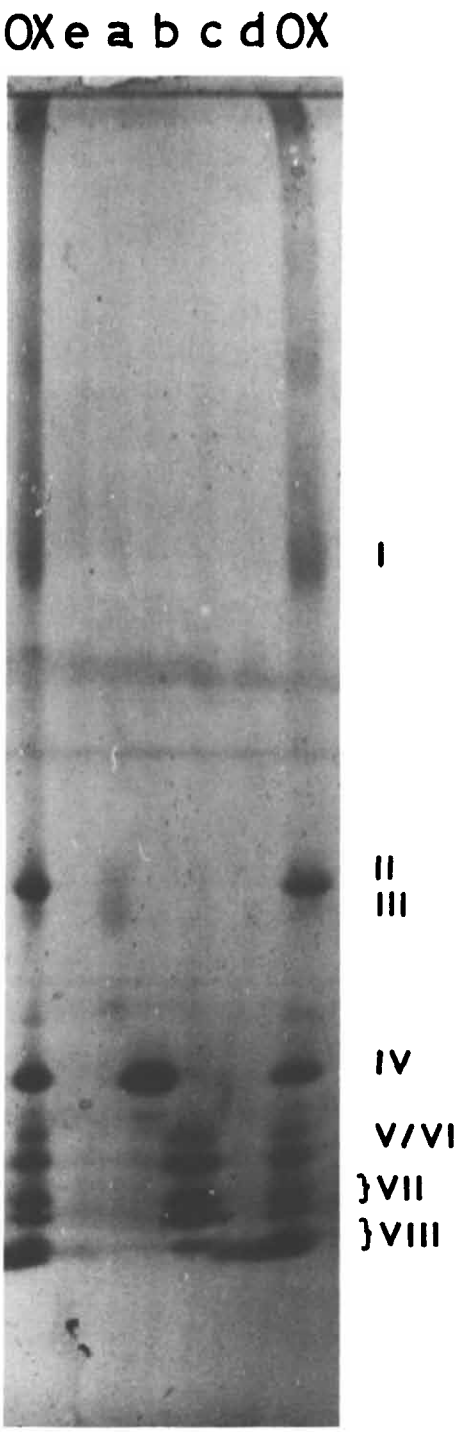


Fig. 5. Polyacrylamide gradient gel electrophoresis of relevant peak fractions of the separation shown in Fig. 4A. The sample slots are marked by letters a–e corresponding to those in Fig. 4A. At OX the dissociated holoenzyme was applied. Conditions of the gradient gel are given in Materials and Methods.

or absence of urea, his subunits V and VI exchange positions. Recently, Jones et al. [54] depicted an SDS-polyacrylamide electrophoretogram showing that the mobility of their component c (our VIIa) was pH dependent. We now show that this phenomenon is not restricted to SDS-polyacrylamide gels but can also be observed on gel permeation chromatography.

As an additional proof for the assignment of the smaller subunits, we analyzed amino terminal residues of the fractions called c, d and e from the column depicted in Fig. 4A. For fraction c, the first Edman degradation cycle resulted in alanine and serine, the second in histidine and serine. For fraction d, the first Edman cycle resulted in serine, isoleucine and phenylalanine, whereas glutamic acid and histidine were found for the second cycle. In fraction e, no amino terminal residue was found, consistent with the absence of Coomassie blue stain in Fig. 5. According to the SDS-polyacrylamide gradient gel from Fig. 5, lane c contains subunits V, VI and VIIa and VIIb; this is in agreement with the results of the Edman degradation [16,45,46]. Lane d, which shows an unresolved band on the SDS-polyacrylamide gradient gel, contains the three polypeptide chains which are known to constitute the heterogeneous subunit fraction VIII [18,38,39,47]. These data indicate that the elution behaviour of subunit fraction VII, like that of subunits I and III, is dependent on pH. At pH 6.5, subunit fraction VII comigrates with fraction V/VI whereas at pH 8.2 it is more retarded. Since the amount of label found in fraction V/VI is approximately the same at both pH values, it is concluded that subunit VIIa, which contains four cysteine residues, is not involved in DTNB binding.

Discussion

Reactive thiol groups in native and SDS-dissociated cytochrome c oxidase

The total number of 14–16 cysteine residues which we determined for cytochrome aa_3 is in agreement with an early report of Matsubara et al. [21] who found seven cysteine residues per haem *a*. A subsequent study of the group of Okunuki [55] showed that some thiol groups were more reactive towards $AgNO_3$, $HgCl_2$ and PCMB than others. Furthermore, the number of titratable groups increased upon addition of SDS; this was also observed in our experiments with DTNB. Now that the distribution of cysteine residues over the subunits of cytochrome *c* oxidase is largely known (Table I), the question is raised in which subunits the reactive thiol groups are located.

Recently, several authors [23,24,26] studied the reactivity of thiol groups in cytochrome *c* oxidase by trace labelling the subunits, whereas we labelled the thiol groups under conditions where quantitative modification of cysteine-containing subunits could be obtained, thus facilitating their identification. Upon reaction of native bovine heart cytochrome *c* oxidase with DTNB, the hydrophobic subunits I and III were both rapidly and stoicheiometrically labelled, whereas only a low amount of label was found in subunit II and the smaller subunits. After dissociation of the enzyme by SDS, subunit II bound up to 1 mol thionitrobenzoate.

McGeer et al. [24] labelled isolated and membrane-bound bovine heart cytochrome *c* oxidase with *N*-ethylmaleimide. When the reaction was performed with the solubilized enzyme they found label in subunit III and in their fraction VIa (VI in our nomenclature). With the membrane-bound enzyme, label was also found in subunit I and relatively more label was observed in subunit III, but none in fraction VIa. The amount of label, however, was not stoicheiometric. According to an earlier report of Kornblatt et al. [56], label was also found in subunit I when the isolated enzyme was labelled with *N*-ethylmaleimide. The results of these authors are in fair agreement with ours obtained with DTNB.

Fraction V/VI, reacting with a sub-stoicheiometric amount of DTNB in the native enzyme, bound 2 mol thionitrobenzoate after SDS dissociation. This shows that these thiol groups are hardly accessible in the intact enzyme. At pH 6.5, subunits VIIa and VIIb comigrated V/VI without leading to an increase in detectable thiol groups, indicating that the four cysteine residues in subunit VIIa are either present as disulphide bridges or remain buried in the protein. The first possibility is more likely because Darley-Usmar and Wilson [26] found that this fraction bound labelled mercury after reduction with 2-mercaptoethanol. As mentioned before, subunit VIIb does not con-

tain cysteine (Table I) and therefore cannot bind DTNB. Subunit V has one cysteine residue [45] and, according to our cysteic acid determination, fraction VI contains at least two. The latter is in agreement with reports of other investigators [24,26,57]. It cannot yet be decided which of the polypeptides of fraction V/VI is involved in DTNB binding.

After SDS dissociation a ratio of up to 2.0 mol thionitrobenzoate per mol oxidase was found in fraction VIII. Two of the different polypeptide chains in fraction VIII do not contain cysteine (Table I). The third polypeptide with amino terminal phenylalanine must contain at least one cysteine residue, in agreement with the observation of Darley-Usmar and Wilson [26] that the smallest subunit fraction binds label upon the reaction of $^{203}HgCl_2$ with native bovine heart cytochrome *c* oxidase. These authors found a high amount of label in subunit II and in their component a (our VI). The label in subunit II is probably due to displacement of copper by mercury. Moderate labelling was found in their fraction VII (our VIII) and a low amount of label in subunits III and V, whereas no label was found in subunit I. The discrepancy between their results and ours might be attributed to different properties of $HgCl_2$ and DTNB as thiol reagents.

The position of cysteine residues in the mitochondrially synthesized subunits I, II and III

The position of cysteine residues in the large subunits is known from sequence data (protein and mtDNA). The group of Tzagoloff [58–60] sequenced yeast mtDNA. For the bovine enzyme the protein sequence of subunit II was determined [43] and used for alignment with the mtDNA sequence [48]. The mtDNA sequences of human cytochrome *c* oxidase became available through the work of the group of Sanger [42] who also determined the base sequence of bovine subunits I and III [35]. With these sequence data Table IV was composed, which shows the distribution of cysteine residues in the sequences of yeast, bovine and human subunits I, II and III, respectively.

We found a reactive thiol group in bovine sub-

TABLE IV

DISTRIBUTION OF CYSTEINE RESIDUES IN THE SEQUENCES OF SUBUNITS I, II AND III OF YEAST, BOVINE AND HUMAN CYTOCHROME *c* OXIDASE, RESPECTIVELY

Data for yeast subunits I, II and III are taken from Refs. 58–60, for bovine subunit II from Ref. 43, for human subunits I, II and III from Ref. 42 and data for bovine subunits I and III from Ref. 35.

		NH_2							COOH
		1	110						510
Subunit I	yeast	fM—	C—						—S
		1							498 514
	bovine	fM—							—C—K
		1							498 513
	human	fM—							—C—S
		1	23	107			221 225		251
	yeast	fM—	C—	C—			C—C		—Q
		1					196 200		227
Subunit II	bovine	fM—					C—C		—L
		1	35				196 200		227
	human	fM—	C—				C—C		—L
		1		123	184				269
	yeast	fM—		C—	C—				—V
		1		115			218		261
Subunit III	bovine	M—		C—			C—		—S
		1					218		261
	human	M—					C—		—S

unit I, which is not present in yeast [23,25], the cysteine residue in bovine subunit I being located in the carboxy terminal region of the protein, whereas the corresponding yeast subunit has its cysteine residue near the amino terminal part of the sequence. In contrast to the wide-spread opinion that the hydrophobic subunit I is buried inside the protein complex [12,19,20], we conclude that at least the carboxy terminal part is exposed. This is in agreement with the results of Kawato et al. [61] who reported that subunit I of bovine heart cytochrome *c* oxidase is reactive towards a maleimide derivative. From labelling experiments with diazobenzenesulphonate and *N*-(4-azido-2-nitrophenyl)-2-aminoethane sulphonate with the bovine enzyme, Prochaska et al. [62] concluded that part of subunit I is exposed, but that this part does not contain cysteine. McGeer et al. [24] found label in subunit I after reaction of *N*-[^{14}C]ethylmaleimide with the membrane-bound bovine cytochrome *c* oxidase but not when the solubilized enzyme was used.

Yeast, bovine and human subunits II have an invariable region around the two cysteine residues in the carboxy terminal region; one of these residues is a putative copper ligand [43]. Subunit II is far exposed at the cytoplasmic side of the membrane [12,19,62], but for the bovine enzyme only reacts with DTNB after dissociation of the enzyme by SDS, which displaces copper. This is in line with an earlier study of Tsudzuki et al. [22] who showed that SDS was required to displace copper from a thiol group in the enzyme. In yeast cytochrome *c* oxidase the most reactive thiol group is located in subunit II [23,25,63], in contrast to the results found for the bovine heart enzyme. Subunit II of yeast cytochrome *c* oxidase is able to bind *N*-ethylmaleimide, iodoacetamide [23] and the fluorophore IAEDANS [25]. This difference between the yeast and the bovine enzyme is explained by the presence of two additional cysteine residues in the sequence of yeast subunit II.

Subunit III of bovine cytochrome *c* oxidase binds 1 mol DTNB; the second cysteine residue in subunit III is not labelled even after SDS dissociation of the enzyme. Apparently, the latter cysteine residue is not accessible to the reagent. Yeast subunit III also contains two cysteine residues, but these are not reactive towards thiol reagents, although one of them could be coupled to a disulphide derivative of yeast iso-1-cytochrome *c* [23,63]. One of the two cysteine residues in subunit III of bovine heart cytochrome *c* oxidase is in a position that is the same as that of yeast subunit III, which suggests that the other (position 218) is the reactive one. This can be investigated by studying the reaction of DTNB with human cytochrome *c* oxidase, because human subunit III lacks one of the two cysteine residues present in bovine subunit III. To answer the question as to whether the reactive cysteine residues are relevant to the catalytic mechanism of the enzyme, the interaction of cytochrome *c* with thiol-modified cytochrome *c* oxidase will be studied.

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