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POLYPEPTIDE COMPOSITION OF PURIFIED QH_2:CYTOCHROME *c* OXIDOREDUCTASE FROM BEEF-HEART MITOCHONDRIA

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SUMMARY

1. The polypeptide composition of purified QH_2: cytochrome *c* oxidoreductase prepared by three different methods from beef-heart mitochondria has been determined. Polyacrylamide gel electrophoresis in the presence of dodecyl sulphate resolves eight intrinsic polypeptide bands; when, in addition, 8 M urea is present and a more highly cross-linked gel is used, the smallest polypeptide band is resolved into three different bands.

2. The identity of several polypeptide bands has been established by fractionation. The two heaviest polypeptides (bands 1 and 2) represent the so-called core proteins, band 3 the hemoprotein of cytochrome *b*, band 4 the hemoprotein of cytochrome c_1, band 5 the Rieske Fe-S protein, band 6 a polypeptide associated with cytochrome c_1 and identified with the so-called oxidation factor, and band 7 a polypeptide associated with cytochrome *b*.

3. The validity of molecular weight estimates for the polypeptides of the enzyme based on their mobility on dodecyl sulphate gels has been examined. The polypeptides of bands 1, 2 and 3 showed anomalous migration rates. The molecular weights of the other polypeptides have been estimated from their relative mobilities on either dodecyl sulphate gels or 8 M urea-dodecyl sulphate gels as 29 000, 24 000, 12 000, 8000, 6000, 5000 and 4000, respectively.

4. The stoicheiometry of the different polypeptides in the intact complex was determined using separate staining factors for the individual polypeptide bands.

INTRODUCTION

Although the behaviour of the redox components of the cytochrome b-c_1 region of the mitochondrial respiratory chain has been extensively studied, the mechanism of electron transfer and energy conservation in this region is still largely obscure. Moreover, isolated QH_2: cytochrome *c* oxidoreductase contains in addition to the redox components, cytochromes *b*, cytochrome c_1 and iron-sulphur, a number of

Abbreviation: SDS, sodium dodecyl sulphate.

polypeptides containing no prosthetic group. The presence of at least two of these is necessary for electron transfer through QH_2:cytochrome *c* oxidoreductase [1–3], and it has been proposed [3] that one of them (the oxidation factor) might have a function in the translocation of protons that is coupled to electron transfer. In order to gain a better insight into the mechanism of electron transfer and energy conservation in QH_2:cytochrome *c* oxidoreductase, a detailed knowledge of the polypeptide composition is essential.

The introduction of polyacrylamide gel electrophoresis of membranes dispersed in dodecyl sulphate [4] has opened up new possibilities for determining the polypeptide composition of the inner membrane of the mitochondrion and of enzymes or complexes isolated from it. Although, as described in recent reports [5–7], considerable progress has been made using this method concerning the polypeptide composition of QH_2:cytochrome *c* oxidoreductase, there still remain some uncertainties, especially concerning the assignment of the polypeptide(s) of the *b* cytochromes. The present study was undertaken in an attempt to resolve these uncertainties and also to determine the stoicheiometry of the different polypeptides in the intact complex.

METHODS

Beef-heart QH_2 : cytochrome *c* oxidoreductase was isolated by three different methods [8–10]. The amounts of cytochrome *b* (about 8 nmol/mg) and c_1 (about 4.2 nmol/mg) and the specific activity for QH_2-2 oxidation (about 20 μmol cytochrome *c* reduced per min per mg) were similar for all three preparations.

A preparation lacking the Fe-S protein was obtained as follows. Heart-muscle particles, prepared essentially as described by Keilin and Hartree [11], were diluted to 6 mg protein/ml with 5 mM Tris · HCl, 1 mM EDTA and 125 mM sucrose (pH 7.5) (TES buffer) and incubated with excess of antimycin for 5 min at room temperature. Triton X-100 (1.25 %, w/v) and 0.5 M KCl were then added, and after 10 min at room temperature, the suspension was centrifuged for 5 min at 200 000 × *g*. Subsequent operations were carried out at 0–4 °C. The pellet was discarded and the supernatant was diluted to 0.1 M KCl with TES buffer containing 1 % Triton X-100. A small amount of DEAE-cellulose (DEAE 23SH, Serva Heidelberg, art. No. 45051) was added and, after standing for 2 h, the DEAE-cellulose was collected by low-speed centrifugation and made into a column. After washing with 0.1 M KCl in TES buffer containing 1 % Triton X-100, elutions were carried out with increasing KCl concentrations; Fe-S protein-free QH_2 : cytochrome *c* oxidoreductase was eluted at about 0.25 M KCl. Most of the Triton X-100 could be removed from the preparation by gel filtration on a Sephadex G-100 column, equilibrated and eluted with TES buffer containing 1 % cholate.

ATPase F_1 was prepared by the method of Knowles and Penefsky [12].

Gel electrophoresis in the presence of dodecyl sulphate was performed essentially as described by Weber et al. [13], using stacking gels as described by Maurer [14]. Unless otherwise stated, the protein was dissolved to a concentration of 0.5 mg/ml in a mixture containing 1 % dodecyl sulphate, 10 % 2-mercaptoethanol, 50 mM Na_2CO_3, 1 mM iodoacetate and 10 % glycerol, and incubated for 5 min at room temperature before electrophoresis. Split gels were obtained by inserting a partition into the top of the stacking gel [15]. Gel electrophoresis in the presence of both

dodecyl sulphate and 8 M urea was performed as described by Swank and Munkres [16], using a 10 to 1 ratio of acrylamide to bisacrylamide, except that the protein was dispersed at room temperature. After electrophoresis the gels were stained with Coomassie Brilliant Blue as described by Swank and Munkres [16]. The gels were scanned either at 280 nm prior to staining or at 550 nm after staining, with an automatic scanning attachment connected to a Zeiss spectrophotometer equipped with a linear scale expander. Standard proteins used for molecular weight estimates were: bovine serum albumin, 67 000; catalase, 60 000; ovalbumin, 45 000; aldolase, 40 000; chymotrypsin, 25 000; myoglobin, 17 200; bovine cytochrome *c*, 12 300; bovine trypsin inhibitor, 6160; pig insulin B chain, 3400; pig insulin A chain, 2300.

Gel filtration in the presence of dodecyl sulphate was performed essentially as described by Yu et al. [17] using a column (65×2.2 cm or 90×4 cm) of Sephadex G-150 superfine, equilibrated and eluted with 0.1 % sodium dodecyl sulphate (SDS), 1 mM EDTA and 50 mM sodium phosphate buffer (pH 7.4).

Acid-acetone extraction of the haem of haemoglobin was performed as described by Rieske [18].

The cytochrome c_1 content was estimated from the difference spectrum of the ascorbate-reduced minus the ferricyanide-oxidized preparation, assuming that $A_{552.5\ nm} - A_{540\ nm} = 20.1\ mM^{-1} \cdot cm^{-1}$ [19]. The cytochrome *b* content was estimated from the difference spectrum of dithionite-reduced minus the ascorbate-reduced preparation, assuming that $A_{563\ nm} - A_{577\ nm} = 25.6\ mM^{-1} \cdot cm^{-1}$ [19]. Spectral measurements were carried out at room temperature in an Aminco spectrophotometer, model DW-2.

Protein was estimated by the biuret method in the presence of H_2O_2 [20].

RESULTS

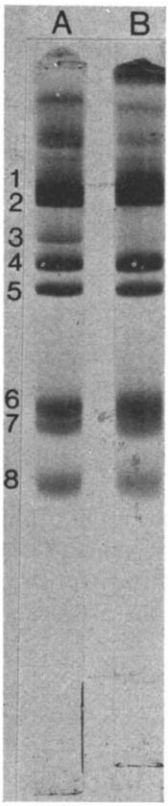
Number of polypeptides in QH_2 : cytochrome c oxidoreductase and their identity

Polyacrylamide gel electrophoresis of QH_2 : cytochrome *c* oxidoreductase dispersed in 1 % sodium dodecyl sulphate and 10 % mercaptoethanol at room temperature resolves 8 major polypeptide bands (Fig. 1A). If the protein is treated with dodecyl sulphate and mercaptoethanol at 100 °C for 4 min, as is recommended by Weber et al. [13], band 3 is missing and protein, presumably the aggregated polypeptide of band 3, appears on top of the gel (Fig. 1B). The position and intensity of the other bands was not affected by the heating step. Solubilization at 100 °C was improved when 3 % dodecyl sulphate was used. Solution at 37 °C leads to only partial resolution of band 3.

The numbering of the polypeptides following band 3 differs from that in previous publications [21, 22] since this band was not visible in the earlier gels which were run with protein treated with dodecyl sulphate and mercaptoethanol at 100 °C.

The eight polypeptides shown in Fig. 1A were present in the same relative amounts in preparations made in three different ways (Fig. 2), suggesting that they are intrinsic to QH_2 : cytochrome *c* oxidoreductase. The minor bands, the intensity of which differs from preparation to preparation, are probably derived from contaminants, since the cytochrome *b* and c_1 contents and the enzyme activity were similar in all three preparations (see Methods).

In order to identify the different polypeptides we subfractionated the protein



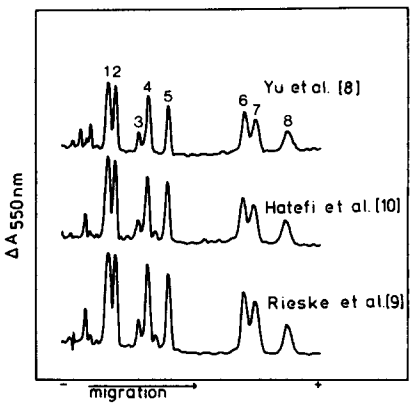


Fig. 1. Dodecyl sulphate-polyacrylamide gel patterns of purified QH_2: cytochrome *c* oxidoreductase using 15 % acrylamide gels. A, dispersion in 1 % dodecyl sulphate and 10 % mercaptoethanol at room temperature for 5 min; B, as in A, but at 100 °C for 4 min.

Fig. 2. Dodecyl sulphate-polyacrylamide gel patterns of purified QH_2: cytochrome *c* oxidoreductase prepared by three different methods using 15 % acrylamide gels.

into its individual components and coelectrophoresed the various subfractions together with the intact protein (Fig. 3).

The two largest polypeptides (Fig. 3B) correspond to the core proteins [23], which have no detectable prosthetic group. Gellerfors et al. [2] recently showed that alkylation with iodoacetamide of core protein-1 in the intact enzyme leads to a parallel inhibition of the enzyme activity, suggesting a functional role for this polypeptide in electron transfer through the enzyme. Fig. 4 shows, in agreement with Gellerfors and Nelson [5] but in disagreement with Hare and Crane [24], that neither polypeptide is associated with the α or β subunit of ATPase F_1. Yu et al. [17] and Capaldi [25] assign band 2 to cytochrome *b* rather than to core protein. However, in our hands, when the intact protein is split with cholate/ammonium sulphate in the absence of antimycin into cytochrome *b*- and cytochrome c_1-enriched fractions, most of bands 1 and 2 are found in the latter, with the same relative intensities as in the intact protein.

As shown by Rieske and co-workers [26] ammonium sulphate in the presence

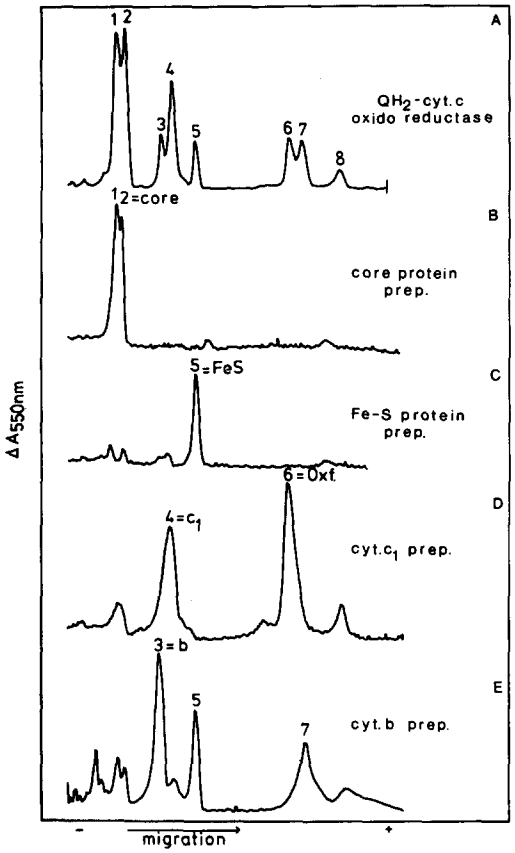
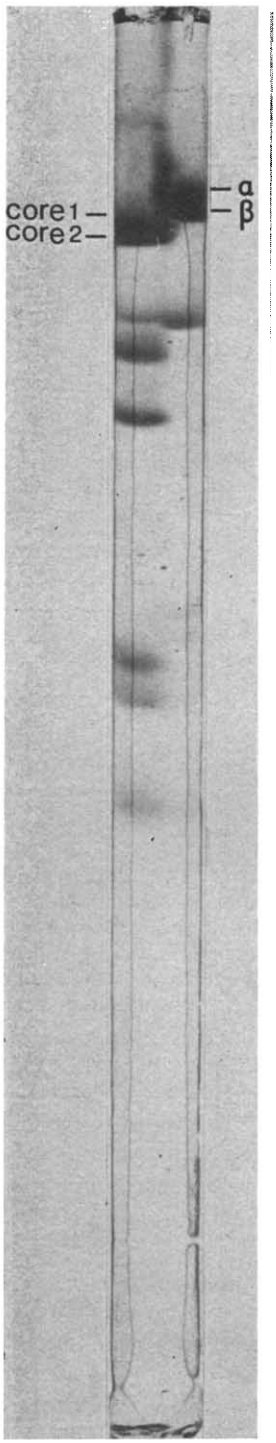
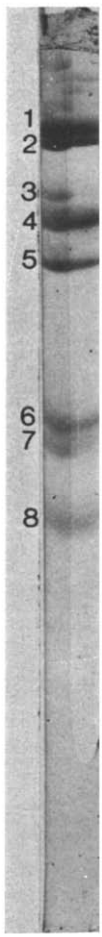


Fig. 3. Dodecyl sulphate-polyacrylamide gel patterns of the purified enzyme [8] and the isolated subfractions using 13.5 % acrylamide gels. (A) Purified enzyme. (B) Core proteins precipitated from the supernatant obtained after taurocholate and ammonium sulphate treatment by incubation in the presence of 1 mM mersalyl [23]. (C) Fe-S protein precipitated from the antimycin-treated enzyme by taurocholate and ammonium sulphate [23]. (D) Cytochrome c_1 preparation, the supernatant obtained after precipitation of core proteins in B. (E) Cytochrome *b* preparation, cleaved from the intact enzyme with 2 % cholate and 20 % ammonium sulphate [17].

of taurocholate precipitates the Fe-S protein from antimycin-treated QH_2 : cytochrome *c* oxidoreductase. Fig. 3C shows that the Fe-S protein corresponds to band 5 of the intact protein. Indeed, when QH_2 : cytochrome *c* oxidoreductase is treated with *o*-phenanthroline [24] prior to electrophoresis, a pink-coloured band is seen on the gel at a position corresponding to band 5, which indicates the presence of an *o*-phenanthroline · iron complex.

It has been reported that isolated cytochrome c_1 preparations from beef heart [27, 28] and yeast [29] show two bands in dodecyl sulphate-polyacrylamide gel electrophoresis. In our hands, cytochrome c_1 isolated by the method of Baum et al. [30] also shows two bands (see Fig. 3D). Since the haem of cytochrome c_1 is covalently linked to the polypeptide (unlike that of cytochrome *b*) and remains bound during electrophoresis, it could be established that band 4, which migrates as a red-coloured band and is detectable by scanning the unstained gel at 400 nm [31], contains the





Figs. 4 and 5. See opposite page for legend.

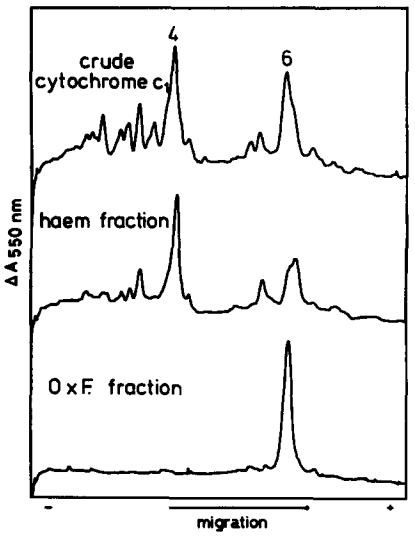


Fig. 6. Dodecyl sulphate gel patterns of the two fractions obtained when a crude cytochrome c_1 preparation, dissolved in 25 mM mercaptoethanol/0.2 M potassium phosphate (pH 7.4), is applied to a Sephadex G-100 column and eluted with the same buffer.

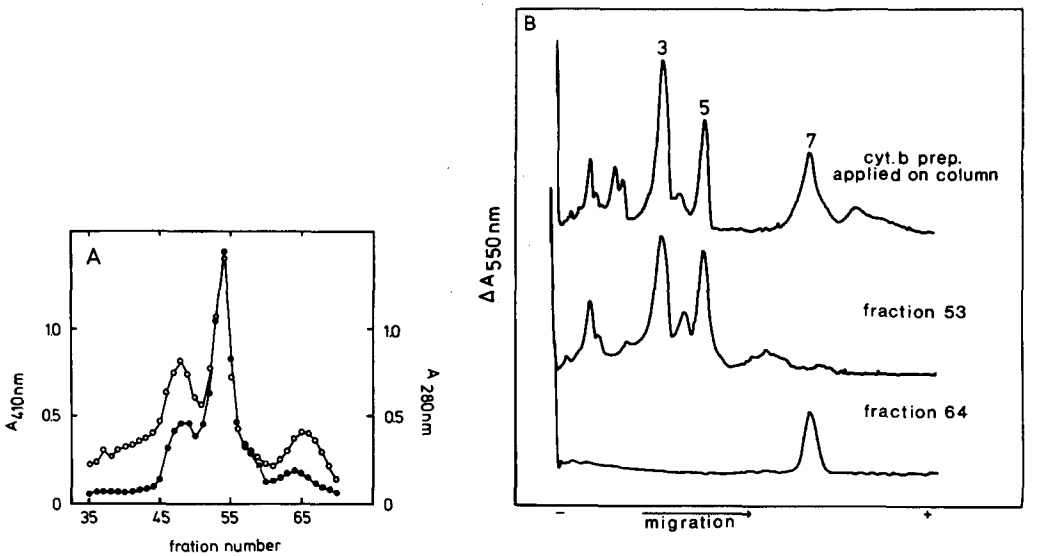


Fig. 7. (A) Elution profile of a cytochrome *b* preparation, obtained as described in Fig. 1E, applied to a Sephadex G-150 column in the presence of dodecyl sulphate (see Methods). Fraction volume, 1.5 ml. ●—●, $A_{410\ nm}$; ○—○, $A_{280\ nm}$. (B) Gel patterns of the cytochrome *b* preparation applied to the column and of the eluate fractions indicated.

Fig. 4. Dodecyl sulphate gel electrophoresis on 13 % acrylamide split gels of QH_2: cytochrome *c* oxidoreductase (shown on the left) and ATPase F_1 (shown on the right).

Fig. 5. Dodecyl sulphate gel electrophoresis on 13 % acrylamide split gels of the intact enzyme (shown on the left) and a crude cytochrome c_1 preparation (supernatant of Fig. 1E) (shown on the right).

mesohaem. Fig. 3D shows that the smaller polypeptide corresponds to either band 6 or 7 of the intact reductase. Fig. 5, which shows a split gel of the intact enzyme and a crude cytochrome c_1 preparation from which bands 3 and 7 have largely been removed, demonstrates that it comigrates with the sixth band. This is in agreement with the recent report of Bell and Capaldi [7], but in disagreement with Gellerfors and Nelson [5] and Das Gupta and Rieske [6], who associated this polypeptide with the seventh band of the intact protein. The reason for this discrepancy is not clear. Nishibayashi-Yamashita et al. [1] and recently Trumpower [3] have shown that a crude preparation of cytochrome c_1 loses its activity for reconstitution with cytochrome *b*-containing fractions on further purification, and regains it on adding a fraction that was called oxidation factor (OXF) [1]. By separating the colourless protein from the haem fraction in a similar crude preparation of cytochrome c_1, using the procedure described by Nishibayashi-Yamashita et al. [1] to isolate the oxidation factor, band 6 was clearly identified as OXF (Fig. 6).

The gel pattern of the cytochrome *b*-enriched preparation obtained by dissociating the protein with cholate and ammonium sulphate in the absence of antimycin (see above) is shown in Fig. 3E. It is clear that this fraction is enriched in the polypeptides of bands 3, 5 and 7. However, as discussed above, band 5 can be attributed to the Fe-S protein. Indeed, a preparation lacking only band 5 but with normal amounts of cytochrome *b* and c_1 has been isolated by Triton X-100 extraction in the presence of antimycin, followed by DEAE-cellulose chromatography (Riccio et al. [32] have also reported the absence of this band in a preparation lacking the Fe-S protein.) Thus the possibility that band 5 is composed of both the Fe-S protein and cytochrome *b* is excluded. This leaves bands 3 and 7 as possible candidates for cytochrome *b*.

The cytochrome *b* preparation was fractionated by gel filtration in the presence of dodecyl sulphate into fractions of different molecular weight (see Fig. 7A). The high molecular weight fraction contains the core proteins and some unfractionated material (not shown), the intermediate fraction shows bands 3 and 5, and the low molecular weight fraction band 7 (Fig. 7B).

Fig. 7A shows that the ratio $A_{410\ nm} : A_{280\ nm}$ is much higher in the fraction of intermediate molecular weight than in the low or high molecular weight fractions, indi-

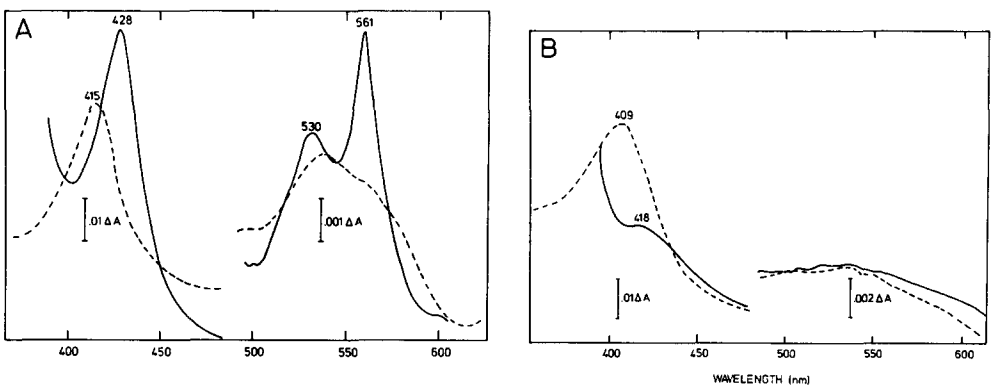


Fig. 8. Absolute spectra of the oxidized (- - - -) and dithionite-reduced (—) forms of the second (A) and third (B) fractions shown in Fig. 7A.

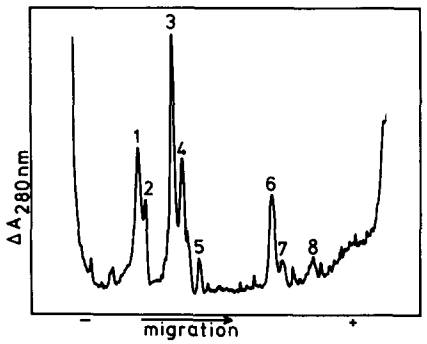


Fig. 9. Dodecyl sulphate gel pattern of QH_2 : cytochrome *c* oxidoreductase scanned at 280 nm prior to staining. 15 % acrylamide gels.

cating a higher concentration of haem in the intermediate fraction, since the contribution of the Fe-S centre to the 410 nm absorption is only very small compared with that of the cytochrome. Moreover, the absorption spectrum of the intermediate fraction after reduction with dithionite is a typical haemochrome (see Fig. 8A), whereas that of the low molecular weight fraction resembles that of free haem (Fig. 8B). The pyridine haemochrome spectra are the same in both fractions. Identical results were obtained by Yu et al. [17]. In one experiment (with Sephacryl S-200 instead of Sephadex G-150) the low molecular weight fraction did not contain any haem at all.

In model experiments with a mixture of haemoglobin and serum albumin, 85 % of the haem comigrated with the globin during gel filtration in the presence of dodecyl sulphate, and the remainder 15 % comigrated with the albumin. With a mixture of free haem and serum albumin all the haem comigrates with the albumin.

It is concluded, then, that band 3 belongs to the haem-containing peptide of cytochrome *b*. The low height of band 3 relative to the other bands of the enzyme in Fig. 3A is not because it is derived from a minor component, but is due to a relatively low staining of the polypeptide by Coomassie Brilliant Blue (cf. Table II). When scanned at 280 nm, prior to staining, band 3 appears as a major band (Fig. 9).

The identities of bands 7 and 8 have not been established.

Molecular weights

Estimations of the molecular weights of the polypeptides of QH_2 : cytochrome *c* oxidoreductase, based on the relative mobilities in a dodecyl sulphate-acrylamide gel, have been frequently reported [5–8]. Frank and Rodbard [33] have pointed out, however, that equality of Y_0 (the extrapolated relative mobility at zero gel concentration) for the polypeptide under investigation and the standards with which it is compared is a sine qua non for valid molecular weight estimates when using a single gel concentration.

In order to check this criterion for the polypeptides of QH_2 : cytochrome *c* oxidoreductase, plots have been constructed in which the logarithm of the relative mobility (R_F) for a single polypeptide is plotted against the gel concentration (T), expressed as the amount (g) of acrylamide plus the amount of bis-acrylamide (g) per 100 ml solution [34]. According to Ferguson [35], these are related by the equation:

$$\log R_F = \log Y_0 - K_R T,$$

where the coefficient K_R is defined as the retardation coefficient, and Y_0 is given by the value of R_F at zero T. Fig. 10 shows that bands 4–8 extrapolate to a common point on the ordinate, which was moreover the same as that obtained with the standard proteins (not shown). However, both core proteins and cytochrome *b* show an anomalous Y_0. Thus, molecular weights obtained for these polypeptides by comparison with standard proteins in gels at a single value of T are invalid. This is clearly shown for the cytochrome *b* polypeptide in Fig. 11, which describes an experiment in which the intact enzyme was run together with standard proteins at different values of T. At $T = 17$, the cytochrome *b* polypeptide has a lower mobility than cytochrome c_1; as the gel concentration decreases the relative mobility of cytochrome *b* increases until at $T = 10.5$ it has the same mobility as cytochrome c_1. It has been suggested that the relation between retardation coefficient K_R and molecular weight may be used to estimate molecular weights of anomalous polypeptides in dodecyl sulphate gel electrophoresis [33, 36], since this essentially corrects for any unusual free electrophoretic mobility. For the core proteins the K_R values found correspond to molecular weights of about 43 000 and 40 000, respectively, and for cytochrome *b* a value corresponding to about 55 000. However, for reasons to be considered in Discussion we shall not use this latter value for cytochrome *b* in the next section, but instead we shall accept the molecular weight of 28 000 per haem obtained by Goldberger et al. [37] for a pure cytochrome *b* preparation from beef-heart mitochondria.

Since the Ferguson plots of the other polypeptides of the enzyme have a common point of intersection with those of the standards when T is (or very nearly) zero, suggesting a normal migration behaviour on dodecyl sulphate gels, molecular weights of these polypeptides were estimated from the relative mobility at a single gel concentration. However, as Swank and Munkres [16] have pointed out, the linear correlation of molecular weight with migration distance on dodecyl sulphate gels with low cross-linkages displays a pronounced break at 12 000 leading to a lack of separation and large errors in estimating the molecular weight of the smaller peptides. Greatly improved separation is obtained with gels with higher cross-linkages and with 8 M

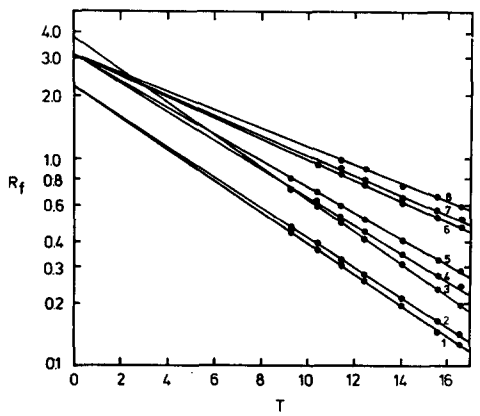


Fig. 10. Ferguson plots of the polypeptides of QH_2 : cytochrome *c* oxidoreductase. The numbers refer to bands in Fig. 1.

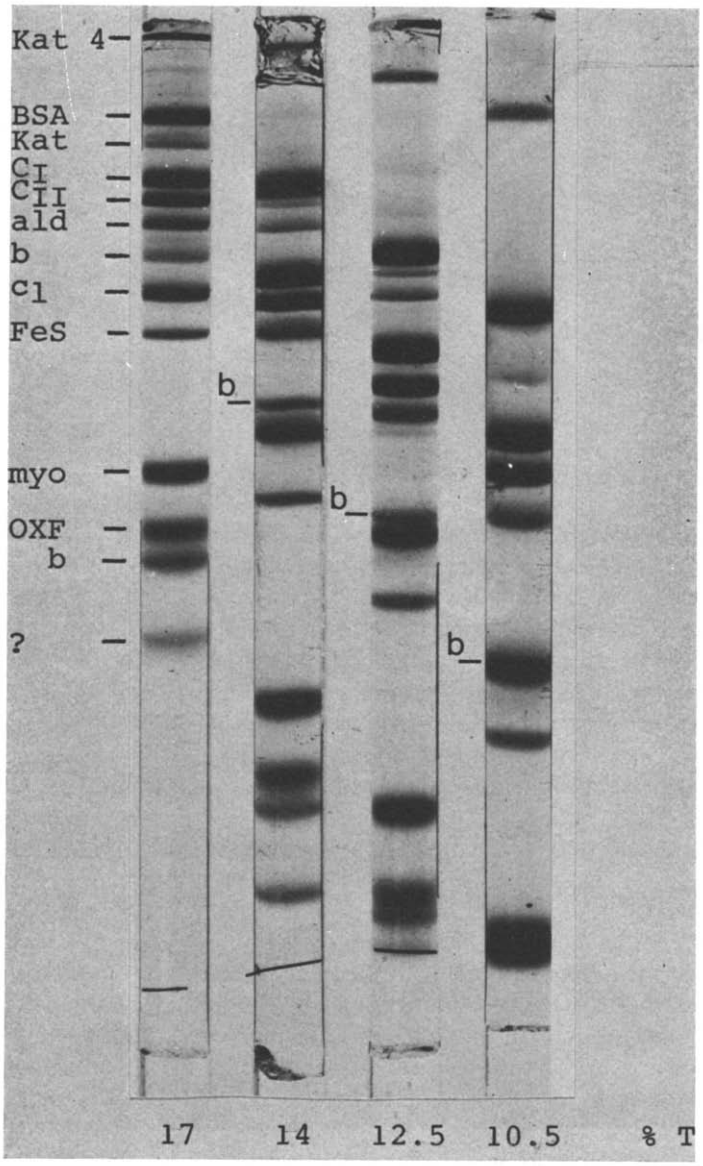


Fig. 11. Dodecyl sulphate gels of QH_2 : cytochrome *c* oxidoreductase together with some standards at four different gel concentrations. Kat 4, tetramer of catalase; BSA, bovine serum albumin; Kat, monomer catalase; C_I, core 1; C_{II}, core 2; ald, aldolase; b, cytochrome *b*; c_1, cytochrome c_1; FeS, Fe-S protein; myo, myoglobin; OXF, oxidation factor; $\sim b$, polypeptide tightly bound to cytochrome *b*; ?, smallest peptide of the intact protein.

urea present in addition to dodecyl sulphate. Fig. 12, a typical gel pattern of QH_2 : cytochrome *c* oxidoreductase using this system, shows two bands in addition to those seen in dodecyl sulphate gels (Fig. 3A). All 10 bands had the same relative intensities in all three preparations of the enzyme.

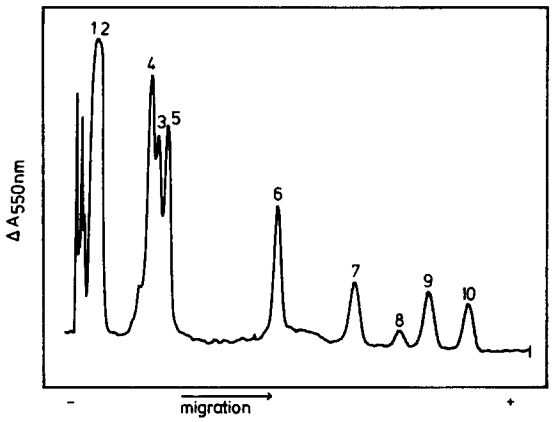


Fig. 12. Electrophoresis of intact enzyme in 8 M urea-dodecyl sulphate with high cross-linkage acrylamide gel (see Methods). 10 % acrylamide.

By extraction of the polypeptides from dodecyl sulphate gels of QH_2 : cytochrome *c* oxidoreductase and re-electrophoresis of the individual polypeptides on urea-dodecyl sulphate gels, band 8 of dodecyl sulphate gels was resolved into three different low molecular weight bands. In contrast to the report of Capaldi et al. [38] the relative positions of all bands were identical in both gel types, except for the cytochrome *b* polypeptide (see below). This is confirmed in Fig. 13 for the two polypeptides associated with cytochrome c_1 and *b*, respectively. Ferguson plots with urea-dodecyl sulphate gels gave similar results as with dodecyl sulphate gels. Fig. 14 shows the position of the cytochrome *b* band relative to the cytochrome c_1 band as a function of the gel concentration.

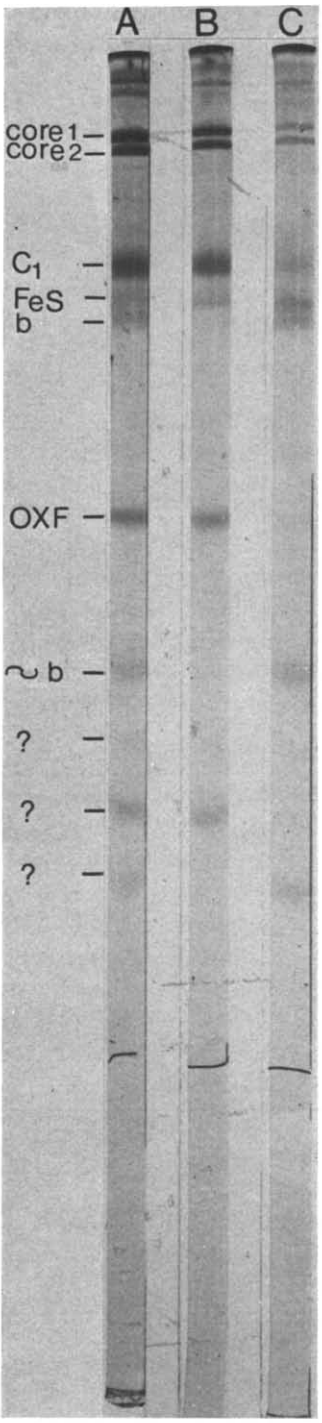
Table I gives a summary of the molecular weights estimated as described in this section.

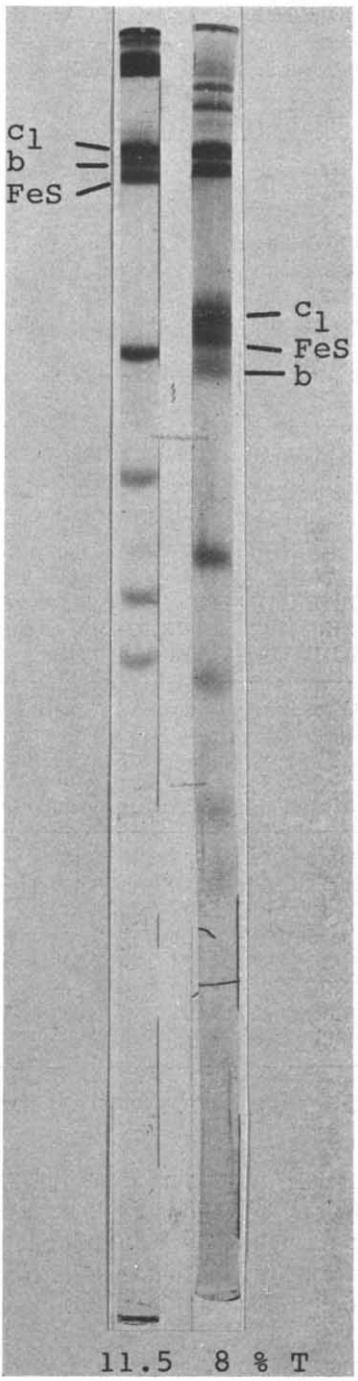
Stoicheiometry of the different polypeptides in the intact protein

The molar ratio of the different subunits in the intact protein was determined from the intensity of the Coomassie Blue staining. Since it is to be expected that the staining intensity is different for different proteins, this has been determined for each pure subunit loaded on the gel. The subunits were isolated either by the procedure described in Fig. 3 or by repeated gel filtration in the presence of dodecyl sulphate, as described in Methods. To avoid variations in the staining intensity caused by the staining procedure itself, the subunits were solubilized and electrophoresed in the same run, and stained and destained in the same vessel as used for the intact protein. Moreover, to check for possible deviations from Beer's law (cf. ref. 14) different amounts of each subunit were electrophoresed. With these precautions the relative intensities of the different polypeptides of QH_2 : cytochrome *c* oxidoreductase were constant, but without them considerable variations have been observed (especially for the cytochrome *b* polypeptide).

Fig. 13. Urea-dodecyl sulphate gels of: A, intact enzyme; B, cytochrome c_1 preparation (supernatant of Fig. 1E); C, cytochrome *b* preparation (Fig. 1E). 7.5 % acrylamide gels.

Fig. 14. Urea-dodecyl sulphate gels of the intact enzyme at two different gel concentrations.





Figs. 13 and 14. See opposite page for legend.

TABLE I

MOLECULAR WEIGHTS CALCULATED FROM ELECTROPHORESIS DATA

Polypeptide	Molecular weight ($\times 10^{-3}$) calculated from		
	R_F on SDS gel[a]	R_F on urea-SDS gel[b]	K_R on SDS gels[c]
Core-1	49	46	43
Core-2	45	43	40
Cytochrome *b*	34	28	55
Cytochrome c_1	29	29	
Fe-S	24	24	
OXF	12.7	12	
~ Cytochrome *b*	11	8	
?	9	6	
?		5	
?		4	

[a] Under conditions of Fig. 3A.
[b] Under conditions of Fig. 12.
[c] See Fig. 10.

TABLE II

DETERMINATION OF THE STOICHEIOMETRY OF THE POLYPEPTIDES IN QH_2: CYTOCHROME *c* OXIDOREDUCTASE USING SEPARATE STAINING FACTORS FOR THE INDIVIDUAL POLYPEPTIDES

Polypeptide	Mol. wt.	Isolated polypeptides	Intact QH_2: cytochrome *c* oxidoreductase			
		Staining* per μg protein (arbitrary units)	Staining* (arbitrary units)	Protein (μg)	Protein (pmol)	Stoicheiometry
Core 1	43	15	41	2.7	64	1
Core 2	40	15	36	2.4	60	1
Cytochrome *b*	28	4	13	3.3	116	2
Cytochrome c_1	29	16	28	1.8	60	1
Fe-S	24	11	16	1.5	60	1
OXF	12	20	29	1.5	121	2
Cytochrome *b*	8	23	22	1.0	120	2
?	5	17	12	0.7	141	2

* Area under the absorption peaks of the 550 nm gel scans.

The staining intensities per μg protein for each subunit and the calculation of their molar ratio in the intact enzyme are given in Table II, in which band 8 is treated as a single band, not taking into account its splitting into three different bands on urea-dodecyl sulphate gels. Moreover, it is assumed that the staining factors for both core proteins are identical.

DISCUSSION

Polypeptide analyses by polyacrylamide gel electrophoresis of QH_2 : cyto-

chrome *c* oxidoreductase dispersed in dodecyl sulphate have been reported by several investigators [5–7]. Despite the use of different preparations, the electrophoretic patterns of the major bands obtained by the several groups are quite similar. The principal disparity, apart from overestimation of the molecular weights of the smaller peptides when urea is not added to the solvent system for the electrophoresis and the degree of cross-linkage in the gel is insufficient, is in the identification of the *b* cytochrome.

Kinetic, potentiometric and spectral studies indicate the existence of at least two different *b*-type cytochromes [39]. It is, however, still not clear whether the multiplicity of cytochromes *b* is due to different haem-containing polypeptides or to the same polypeptide in different environments. Purification of the *b* cytochromes is accompanied by the loss of the native structure together with the loss of its distinct properties, which hampers the elucidation of the origin of the multiplicity of the *b* cytochromes. We have shown that a *b* haem-containing polypeptide migrates on gel electrophoresis in dodecyl sulphate as a single band in the 30 000 kilodalton region. It is striking that this is the band concerning the presence or absence of which there is the most disagreement in the literature. Our results indicate three possible explanations for this discrepancy, all related to the anomalous behaviour of cytochrome *b* in dodecyl sulphate gel electrophoresis. (i) The cytochrome *b* polypeptide tends to aggregate even in the presence of 1 % dodecyl sulphate when solubilization is attempted at temperatures higher than room temperature, but 3 % dodecyl sulphate improves the solubilization at 100 °C. This might explain the absence of a 34 000 band in a recent paper of Gellerfors et al. [2], in which they solubilized the enzyme in 2 % dodecyl sulphate at 100 °C, in contrast to an earlier report of these authors [5] in which they solubilized the enzyme at 37 °C and observed a 34 kilodalton band. (ii) The low staining intensity (see Table II) and the large variations in staining due to the staining procedure itself. (iii) The anomalous migration of the cytochrome *b* polypeptide on dodecyl sulphate gels (Fig. 11) explains probably the differences reported for the molecular weight of cytochrome *b* based on the migration on dodecyl sulphate gels [5, 8, 40] and even the absence of the band [6]. Since most reduced polypeptides bind large and equivalent amounts of dodecyl sulphate on a gram per gram basis, the charge of the sulphate moiety overshadowing the intrinsic charge of the polypeptides, and since the polypeptide-dodecyl sulphate complexes have a typically rod-like configuration whose length is a unique function of the molecular weight, the migration of these polypeptides in dodecyl sulphate gels is solely a function of their molecular weight [41]. However, there are several polypeptides described in literature that show mobilities not in accord with their molecular weight [33, 36]. Our results indicate that the cytochrome *b* polypeptide also shows such "anomalous" behaviour (Fig. 11), which is presumptive evidence that one or both of the conditions mentioned above that are necessary for valid molecular weight determinations have not been met. One possibility is that the polypeptide does not bind the same amount of dodecyl sulphate as the standards (and thus has not the same charge density and free electrophoretic mobility). If this were the only reason for its anomalous behaviour, the value of the retardation coefficient K_R (reflecting the hydrodynamic properties of the polypeptide-dodecyl sulphate complex) could be used to estimate its molecular weight since this value essentially corrects for any unusual free electrophoretic mobility. A molecular weight of about 55 000 may be calculated in this way. However, if this explanation for

the anomalous migration of cytochrome *b* on acrylamide gels is correct, the same value for the molecular weight should be obtained by gel filtration in the presence of dodecyl sulphate, but the value found by this method (by comparison of the elution pattern shown in Fig. 7A with standard proteins) was only 30 000. It must be concluded, then, that different binding of dodecyl sulphate is an insufficient explanation of the anomalous behaviour, and that the cytochrome *b*-dodecyl sulphate complex has abnormal hydrodynamic properties. Thus, neither the value of K_R nor gel filtration can be used to determine the molecular weight.

Groot and van Harten-Loosbroek [42] have found a similar anomalous behaviour with other mitochondrially synthesized polypeptides.

Goldberger et al. [37] reported as long ago as 1961 a minimum molecular weight of 28 000, based on haem content, for a purified preparation of beef-heart cytochrome *b*. Weiss and Ziganke [40] have reported a similar haem content for a cytochrome *b* polypeptide purified from *Neurospora crassa* mitochondria. Yu et al. [17] isolated a *b* haem-containing polypeptide with a minimum molecular weight of 37 000 based on haem content, but this value may well be overestimated since some loss of the protohaem from the polypeptide would be expected during gel filtration in the presence of dodecyl sulphate. Weiss and Ziganke [40] reported a molecular weight of about 30 000 based on gel filtration and gel electrophoresis in the presence of dodecyl sulphate, and values of 50 000–60 000 by ultracentrifugation and gel filtration in the presence of deoxycholate and/or cholate. They conclude from these results that cytochrome *b* exists as a dimer in the presence of deoxycholate or cholate but as a monomer in the presence of dodecyl sulphate. Our results on the anomalous migration of cytochrome *b* in dodecyl sulphate gels indicate that this conclusion might not be correct and that it is possible that also in the presence of dodecyl sulphate the migrating entity contains two molecules of haem, either as the result of association, even in the presence of dodecyl sulphate, of two identical or closely related polypeptide chains or because cytochrome *b* is a single polypeptide with two haems.

Katan et al. [43] identified a polypeptide, with an apparent molecular weight of 32 000 in dodecyl sulphate gel electrophoresis, as cytochrome *b* in yeast, on basis of its absorption spectrum and high hydrophobicity and because it is synthesized on the mitochondrial ribosomes [44]. Moreover, like the cytochrome *b* band in heart QH_2 : cytochrome *c* oxidoreductase, it penetrates the gel poorly. Bell and Capaldi [7] have shown that a polypeptide migrating in the apparent 30 000 molecular weight region is the most hydrophobic of those present in heart QH_2 : cytochrome *c* oxidoreductase and is enriched in a purified preparation of cytochrome *b* prepared by the method of Goldberger et al. [37].

Fig. 3E shows that, in addition to this *b* haem-containing polypeptide, a band corresponding to a smaller polypeptide (about 8 kdaltons, Table I) is intensified in a cytochrome *b*-enriched fraction. Other authors [30, 6, 17, 5] have also reported a concentration of a small polypeptide in such fractions and have, in fact, attributed it to cytochrome *b*. However, in our hands the bulk of the haem remained in the fraction containing polypeptides of intermediate molecular weight when a crude cytochrome *b* preparation was fractionated by gel filtration in the presence of dodecyl sulphate. Little haem (sometimes none) is found in the small polypeptide fraction. Moreover, the spectrum of such a fraction resembles that of free haem, in contrast to the typical haemochrome spectrum of the fraction containing most of the haem.

It seems likely, then, that a small amount of the haem becomes detached from the cytochrome *b* during gel filtration and comigrates with the small polypeptide. This conclusion is supported by a model experiment in which a mixture of haemoglobin and serum albumin was fractionated under the same conditions. Most of the haem remained with the haemoglobin, but some comigrated with the albumin. Thus, although the 8-kdalton polypeptide may very well be functionally related to cytochrome *b*, in the same way as the OXF factor seems to be functionally related to cytochrome c_1, it seems probable that, like the OXF factor, it does not contain haem, and therefore should not be designated as a cytochrome.

The relative stoicheiometry of the eight different polypeptides identified in this paper by dodecyl sulphate gel electrophoresis (Fig. 3A), determined from the staining intensities of the different bands using colour factors appropriate for each polypeptide, is one molecule of cytochrome c_1, one of each of the core proteins, two of cytochrome *b*, one of the Fe-S protein, two of OXF and two of each of the smaller polypeptides. The smallest has been resolved into three different polypeptides (Fig. 11). In preparations made by the method of Yu et al. [8] the amount of Fe-S protein varied between 0.4 and 1 per molecule of cytochrome c_1, probably due to loss of this polypeptide during preparation. The relative intensities of the other bands were the same in all three preparations.

The minimum molecular weight calculated from this stoicheiometry is 242 000. Since the molecular weight determined by ultracentrifugation corresponds to 250 000 [45], it appears that the intact protein is a monomer. The specific roles of the different polypeptides and their interactions are objects for future study.

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