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The effect of deletion of the genes encoding the 40 kDa subunit II or the 17 kDa subunit VI on the steady-state kinetics of yeast ubiquinol–cytochrome-*c* oxidoreductase

Peter J. Schoppink, Wieger Hemrika and Jan A. Berden

Laboratory of Biochemistry, Faculty of Chemistry, University of Amsterdam, Amsterdam (The Netherlands)

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Yeast ubiquinol–cytochrome *c* oxidoreductase is still active after inactivation of the genes encoding the 40 kDa Core II protein or the 17 kDa subunit VI (Oudshoorn et al. (1987) Eur. J. Biochem. 163, 97–103 and Schoppink et al. (1988) Eur. J. Biochem. 173, 115–122). The steady-state levels of several other subunits of Complex III are severely reduced in the 40 kDa^0 mutant. The level of spectrally detectable Complex III cytochrome *b* in the mutant submitochondrial particles is about 5% of that of the wild type. However, when the steady-state activity of Complex III with respect to the cytochrome *c* reduction was examined, similar maximal turnover numbers and K_m values were found for the mutated and the wild-type complexes, both when yeast cytochrome *c* and when horse-heart cytochrome *c* was used as electron acceptor. We therefore conclude that the Core II subunit of yeast Complex III plays no role in the binding of cytochrome *c* and that it has no major influence on the overall electron transport and on the binding of ubiquinol by the enzyme. Absence of the 17 kDa subunit VI of yeast Complex III, the homologous counterpart of the hinge protein of the bovine heart enzyme, resulted in a decrease in the rate of reduction of both horse-heart cytochrome *c* and yeast cytochrome *c* by Complex III under conditions of relatively high ionic strength. However, under conditions of optimal ionic strength, no difference could be seen in the maximal turnover numbers and K_m values, neither with horse-heart cytochrome *c* nor with yeast cytochrome *c* between Complex III deficient in the 17 kDa protein and the wild-type complex. Binding of ATP to ferricytochrome *c* inhibits its reduction by Complex III under conditions of relatively high ionic strength. But when the 17 kDa protein is absent, this inhibition is also observed under optimal ionic-strength conditions. These results can be explained by assuming a stimulating role for the acidic 17 kDa protein in the association of basic cytochrome *c* with Complex III. This association is (part of) the rate-limiting step in the reduction of cytochrome *c* by Complex III under conditions of relatively high ionic strength or when this association is hindered, for instance, by binding of ATP. This means that the rate of reduction of cytochrome *c* by Complex III deficient in the 17 kDa subunit VI is similar to that of the wild-type complex under conditions of optimal ionic strength, but lower under conditions of high ionic strength or when ATP is bound to ferricytochrome *c*. The results obtained with mitochondria (Schoppink, P.J., Hemrika, W., Reynen, J.M., Grivell, L.A. and Berden, J.A. (1988) Eur. J. Biochem. 173, 115–122) suggest, then, that in mitochondria from aerobically grown yeast the concentration of cytochrome *c* is well below the K_m value for Complex III, probably due to a high ionic strength.

Introduction

The yeast ubiquinol–cytochrome-*c* oxidoreductase, or Complex III, consists of at least nine different subunits [1–3]. Except for the mitochondrially encoded

Abbreviations: Q_2, 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol; SMP, submitochondrial particles.

Correspondence: J.A. Berden, Laboratory of Biochemistry, P.O. Box 20151, 1000 HD Amsterdam, The Netherlands.

cytochrome *b*, all are encoded by the nuclear genome. Three subunits, cytochrome *b*, cytochrome c_1 and the Rieske Fe-S protein with molecular masses of 44.0, 27.4 and 20.1 kDa, respectively, contain prosthetic groups and play an essential role in the transfer of electrons from ubiquinol to cytochrome *c*, catalysed by the complex.

The remaining subunits, as far as known, are two Core proteins (I and II) of 44 and 40 kDa, respectively, and four smaller polypeptides of 17, 14, 11 and 7.2 kDa, as measured by SDS-polyacrylamide gel electrophoresis [1–3]. Until recently, only little information was available about a role for these proteins in the biogenesis and activity of Complex III, and comparative analysis with the Complex III from other organisms was mostly used to attribute possible functions to some of these. Since then a more direct approach has been started to examine their function, by specifically inactivating the genes coding for these subunits. Analysis of the mutants has shown that disruption of the genes encoding the 11, 14 and 44 kDa subunits results in inactivation of Complex III [4–6]. Severe reduction at the protein level, but not at the mRNA level, is found for apocytochrome *b* the Rieske Fe-S protein and the 11 and 14 kDa proteins, suggesting that the different mutations cause improper assembly of the remaining complex. The most profound effect is observed for holocytochrome *b*, which cannot be detected by spectral analysis in the mutant mitochondria. This means that the absence of ubiquinol–cytochrome-*c* oxidoreductase activity in these mutants does not have to be a direct consequence of the absence of the respective subunit, but is more likely caused by a defective assembly of Complex III.

The null mutants deficient in the genes encoding the Core II protein [7] or the 17 kDa subunit VI [8] are not respiratory deficient, which implies that Complex III is still active. However, the growth of the 40 kDa^0 mutant on non-fermentable carbon sources is considerably slower than that of the wild type, reflecting the low Complex III activity present in this mutant [7]. Just as in the mutants mentioned above, the steady-state levels of apocytochrome *b*, the Rieske Fe-S protein and the 11 and 14 kDa subunits are severely reduced, but holocytochrome *b* can be detected by spectral analysis of mitochondria. We concluded [7] that the assembly of Complex III in the absence of the Core II protein is hampered but suggested that possibly the remaining complex has a turnover rate comparable to that of the wild-type complex.

In contrast, the 17 kDa^0 mutant grows on non-fermentable carbon sources just as well as the wild type and also the other subunits of Complex III are present at a wild-type level in the mutant mitochondria [8], implying that the 17 kDa subunit VI is neither required for a proper assembly of the remaining complex, nor for electron transport.

Under conditions, however, that the complex is unable to assemble because of a secondary mutation, it was shown that the presence of cytochrome c_1 in yeast mitochondria is stabilised by the 17 kDa protein. In agreement with this is the observation that the homologous counterpart of this polypeptide in the bovine heart enzyme, the so-called 'hinge protein' [9], protects cytochrome c_1 against auto-oxidation [10].

No effect of inactivation of the gene encoding the 17 kDa protein was detected in well-coupled mitochondria, neither on the energy-transducing activity of Complex III as measured by the *P/O* ratio, nor on the electron-transport activity of the whole respiratory chain under conditions of either State 4 or State 3. In uncoupled mitochondria, the NADH : O_2 or succinate : O_2 activity appeared to be approx. 50% lower in the mutant than in the wild type. In correspondence with this, it was shown that the Complex III activity in the mutant mitochondria was about half that of the wild type when the ubiquinol analogue 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol, (Q_2H_2), and horse-heart cytochrome *c* were used as substrates and a 100 mM potassium phosphate buffer was employed in the assay. We therefore concluded previously that the 17 kDa protein stimulates but is not essential for the electron transfer from cytochrome c_1 to cytochrome *c*. Similar conclusions have been drawn for its analogue, the hinge protein of the bovine heart enzyme. It was shown that electron transfer from cytochrome c_1 to cytochrome *c* is possible in the absence of the hinge protein [11,12], but that the protein stimulates the transfer by promoting the forward reaction, i.e., the reduction of cytochrome *c* by cytochrome c_1, under conditions of low ionic strength ($I = 20$ mM) [13].

In this paper we present a more detailed study, with attention to the reduction of cytochrome *c*, of the steady-state kinetics of yeast Complex III lacking the Core II protein or the 17 kDa subunit VI in comparison with the wild-type and the bovine heart complex.

Materials and Methods

Strains and growth conditions

Saccharomyces cerevisiae strain HR2 (α, his4, leu2, trp1) was used previously to obtain the mutants lacking the 40 kDa Core II protein (α, his4, Core II::LEU2, trp1) [7], or the 17 kDa subunit VI (α, his4, 17 kDa::LEU2, trp1) [8], by the one-step gene disruption procedure [14].

HR2 wild-type and 17 kDa^0 mutant cells were grown in lactate medium, containing 0.5% (w/v) yeast extract, 0.2% (w/v) magnesium sulphate, 0.6% (w/v) ammonium phosphate, 2% sodium lactate (70%, w/v), and 1.3% lactic acid (75%, w/v). The pH was adjusted to 5 with 10 M potassium hydroxide. 40 kDa^0 mutant cells were grown in maltose medium, containing 1% (w/v)

yeast extract, 2% (w/v) bactopeptone, and 2% (w/v) maltose.

Liquid cultures were incubated at 28°C and were always inoculated with cells grown on selective plates.

Isolation of mitochondria and submitochondrial particles

Yeast mitochondria were isolated as described previously [8]. Bovine heart mitochondria, kindly supplied by Dr. A. van Hoek, were isolated essentially as described [15]. Submitochondrial particles (SMP) were prepared by sonication as described [16], except that before sonication KCl was added to the buffer at a concentration of 250 mM to minimise the amount of endogenous cytochrome *c* in the SMP. Mitochondria were diluted before sonication to a protein concentration of 10–15 mg/ml. Protein concentrations were determined by the Lowry method [17].

Spectral analysis and assay of NADH : Q_2 oxidoreductase

Spectral measurements were carried out at room temperature in an Aminco dual-wavelength spectrophotometer, model DW-2. Concentrations of cytochromes were determined using the following absorbance coefficients and wavelength pairs for the reduced-minus-oxidised proteins: 21.3 $mM^{-1} \cdot cm^{-1}$ at 605 minus 630 nm for cytochromes aa_3 [18,19], 20.1 $mM^{-1} \cdot cm^{-1}$ at 550 minus 540 nm for cytochromes $c + c_1$ [20], and 28.5 $mM^{-1} \cdot cm^{-1}$ at 562 minus 575 nm for cytochrome *b* [21].

The NADH : Q_2 oxidoreductase activity was measured as described [22].

The Q_2H_2 : cytochrome-c oxidoreductase assay

The ubiquinol-cytochrome-*c* oxidoreductase activity of mitochondria and submitochondrial particles was assayed spectrophotometrically at 25°C by measuring the reduction of ferricytochrome *c* at 550 nm by 25 μM 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol (Q_2H_2). The types and amounts of cytochrome *c* used are indicated in the Results section. The buffer employed contained 2 mM EDTA, 0.25 mM KCN and the concentrations of potassium phosphate indicated in the Results section. The ionic strength given by a certain potassium phosphate concentration was calculated with the aid of the formula given in Ref. 23. The pH was 7.5, except when the dependency of the activity of Complex III on the pH was examined.

Miscellaneous

All chemicals used were of the highest purity available. Yeast cytochrome *c*, type VIIIb, was obtained from Sigma (St. Louis, MO, U.S.A.) and had to be oxidised before use in the Complex III assay. For this, potassium ferricyanide was added in a 1 : 1 ratio with cytochrome *c*, after which the oxidised cytochrome *c* was obtained by separation on a Sephadex G25 column eluted with 25 mM potassium phosphate (pH 7.4). The concentrations of oxidised cytochrome *c* were determined spectrophotometrically by reduction with sodium dithionite. An absorption coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$ at 550 nm was used for reduced cytochrome *c* [24]. Q_2 was synthesised by A.F. Hartog in this laboratory, according to Ref. 25.

Results

Analysis of mitochondria and submitochondrial particles

Yeast mitochondria were isolated from cells grown on lactate medium for the HR2 wild type and the 17 kDa^0 mutant, or on maltose medium (to avoid glucose repression) in the case of the 40 kDa^0 mutant, since this latter mutant grows poorly on media containing non-fermentable carbon sources.

Reduced-minus-oxidised spectra were recorded to measure the cytochrome content of the mitochondria and SMP. The NADH : Q_2 oxidoreductase activity was measured in the yeast mitochondria and SMP as a control for enzyme inactivation during their preparation. The total cytochrome *b* content was deduced from dithionite-reduced minus ascorbate-reduced or ferricyanide-oxidised spectra. The Complex III cytochrome *b* level was measured by succinate-reduced (in the presence of antimycin) minus ferricyanide-oxidised spectra. Antimycin was added to obtain complete reduction of the Complex III cytochrome *b* hemes. The levels of Complex III cytochrome *b* in the various SMP are shown in Fig. 1. In the figure the spectrum of a yeast mutant lacking the 14 kDa subunit [5] is also given. This mutant contains no spectrally detectable cytochrome *b*. The total result of the spectral analysis and enzyme activity is given in Table I.

Similar cytochrome aa_3 levels and NADH : Q_2 oxidoreductase activities have been measured in the different yeast mitochondria and SMP, indicating that no enzyme inactivation has taken place during sonication.

It can be seen that in mitochondria and SMP from the 17 kDa^0 mutant the level of Complex III cytochrome *b* is somewhat lower than in the wild type. We assume that Complex III lacking the 17 kDa protein is less stable than the wild-type complex, which results in some loss of cytochrome *b* during the preparation of mitochondria and SMP. The mitochondria and SMP from the 40 kDa^0 mutant contain approx. 5% Complex III cytochrome *b* relative to the wild type.

In the kinetic analysis of Complex III (see below), the enzyme activity is expressed in turnover rates rather than in specific activities, to be able to make a better comparison of the activity in the mutant SMP with that in the wild-type and the bovine heart SMP. For this the amount of Complex III present in the various SMP was deduced from their Complex III cytochrome *b* content,

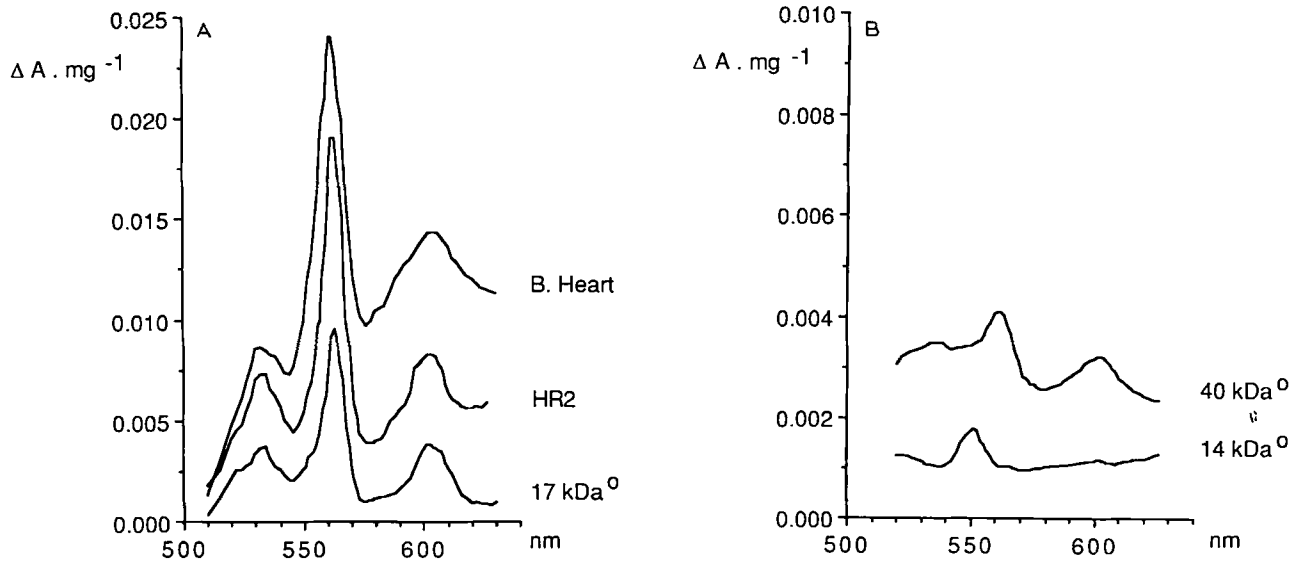

Fig. 1. Spectral analysis of Complex III cytochrome *b* content in submitochondrial particles. Submitochondrial particles of bovine heart, yeast HR2 wild type, the 17 kDa^0 mutant (A), the 40 kDa^0 mutant and a yeast mutant lacking the 14 kDa subunit VII (B) described elsewhere [5], were suspended in a buffer containing 100 mM potassium phosphate (pH 7.4), 0.25 M sucrose and 0.5% (w/v) sodium cholate and used for recording difference spectra at room temperature. Shown are the succinate-reduced (in the presence of antimycin) minus ferricyanide-oxidized spectra, obtained after correlation of the absorption difference to the respective protein concentrations.

taking into account that the Complex contains two cytochrome *b* hemes per monomer.

pH and ionic strength dependency of ubiquinol–cytochrome-c oxidoreductase

It is well known that the rate of reduction of cytochrome *c* by ubiquinol–cytochrome-*c* oxidoreductase is dependent on both the pH and the ionic strength of the medium in which the assay is performed (see e.g. Refs. 13 and 28). This dependency has been thoroughly examined for the bovine heart enzyme with horse-heart cytochrome *c* as electron acceptor. To see whether Complex III of wild-type yeast HR2 and especially that of the two mutant yeast strains derived from this wild type behave similarly as the bovine heart enzyme; as far as the reaction with horse-heart and yeast cytochrome *c* is concerned, we have measured the ir activity in SMP under different conditions.

With respect to the pH dependency, both horse-heart and yeast cytochrome *c* were more rapidly reduced by both the bovine heart and yeast enzyme as the pH increased, under conditions of low ionic strength. The buffer employed contained a variable amount of potassium phosphate in order to keep the ionic strength at 33

TABLE I

Spectral analysis and NADH:Q_2 oxidoreductase activity of mitochondria and submitochondrial particles of HR2 wild type, the 17 and 40 kDa^0 yeast mutants and bovine heart

	Cytochrome $c + c_1$ (mmol·mg^{-1})	Total cytochrome *b* (nmol·mg^{-1})	Complex III cytochrome *b* (nmol·mg^{-1})	Cytochrome aa_3 (nmol·mg^{-1})	NADH:Q_2 oxidoreductase (μmol·min^{-1}·mg^{-1})
Mitochondria					
HR2	0.65	0.34	0.29	0.21	8.4
17 kDa^0	0.88	0.25	0.21	0.19	8.5
40 kDa^0	0.81	– [a]	0.02	0.18	8.4
Bovine heart	0.41	0.45	0.42	0.50	– [b]
SMP					
HR2	0.67	0.64	0.56	0.37	13.8
17 kDa^0	0.49	0.40	0.34	0.32	14.5
40 kDa^0	0.58	– [a]	0.035	0.32	17.4
Bovine heart	0.32	0.56	0.53	0.53	– [b]

[a] When dithionite-reduced minus ascorbate-reduced or ferricyanide-oxidised spectra were recorded, a broad absorption band around 558 nm was observed, possibly from cytochrome b_2 [26] or cytochrome b_5 [27]. A similar result has been obtained in a mutant deficient in the gene encoding the 14 kDa subunit of Complex III [5].

[b] Bovine heart mitochondria contain Complex I instead of rotenone-insensitive NADH:Q oxidoreductase. The Complex I activity has not been determined.

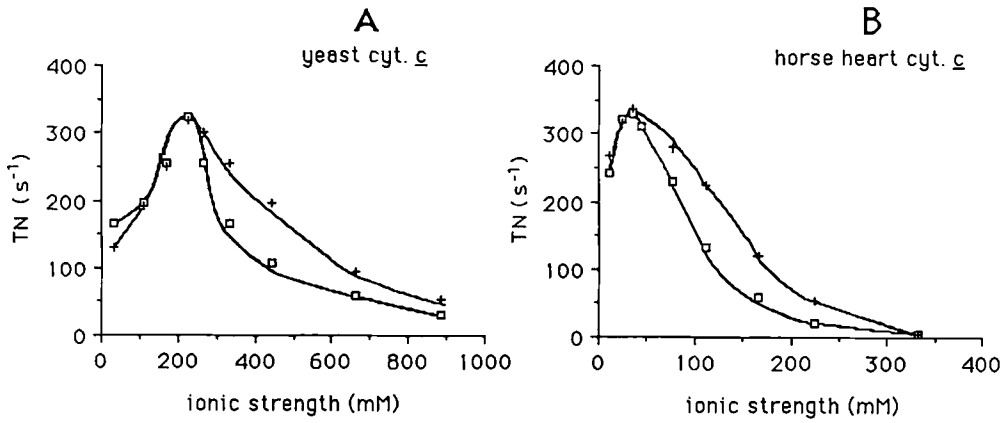

Fig. 2. Ionic strength dependency of the reduction of cytochrome *c* by Complex III using yeast (panel A) or horse-heart cytochrome *c* (panel B). The ubiquinol–cytochrome-*c* oxidoreductase activity present in submitochondrial particles of yeast HR2 wild type and the 17 kDa^0 mutant was assayed as described in Materials and Methods at various concentrations of potassium phosphate (pH 7.5). Both types of cytochrome *c* were used at a concentration of 15 μM. TN, turnover rate; +, HR2 wild type; □, 17 kDa^0 mutant.

mM. Cytochrome *c* was added to a concentration of 15 μM and the reaction was started with 25 μM Q_2H_2. The range from pH 6 to pH 8.5 was examined. The optimal activity with both types of cytochrome *c* and for either the yeast or the bovine heart enzyme was observed close to pH 8, as described before for the bovine heart enzyme with horse-heart cytochrome *c* as acceptor [28]. It is worth mentioning that in this respect the yeast enzyme lacking the 17 kDa subunit did not behave differently from the wild-type enzyme. Below pH 7, the activity of Complex III in SMP from the 40 kDa^0 mutant was too low to be measured accurately.

Because of the fact that the non-enzymic reduction of both horse-heart and yeast cytochrome *c* by Q_2H_2 increased rapidly with increasing pH (see also Ref. 28), we decided to characterise the Complex III activity in the various SMP at pH 7.5.

We have measured the Complex III activity in SMP from bovine heart, yeast HR2 wild type and the 17 kDa^0 mutant under conditions of variable ionic strength. The results for the last two are shown in Fig. 2. Because of the low Complex III activity present in the SMP from the 40 kDa^0 mutant, it turned out to be very difficult to make accurate measurements of this activity outside the relatively sharp ionic strength optimum found for yeast Complex III.

It is clear that the optimal ionic strength for the Q_2H_2–cytochrome-*c* oxidoreductase assay is dependent on the kind of cytochrome *c* used. The activity of both bovine-heart and yeast Complex III shows an optimum at $I = 33$ mM with horse-heart cytochrome *c*, while this optimum is found at $I = 225$ mM with yeast cytochrome *c*. Interestingly, no difference in the turnover rates of yeast Complex III with or without the 17 kDa protein is observed for either yeast or horse-heart cytochrome *c* as acceptor under conditions of optimal ionic strength. However, at ionic strength values higher than the relative optima, the activity of the mutated enzyme is substantially lower, both when horse-heart and yeast cytochrome *c* are used. This suggests that the yeast analogue of the hinge protein stimulates the electron transfer from cytochrome c_1 to cytochrome *c* under conditions of high ionic strength.

Equivalent results as shown here were obtained when KCl was used instead of potassium phosphate to vary the ionic strength, indicating that the results are not specifically influenced by the presence of varying amounts of inorganic phosphate.

Determination of K_m and TN_{max} of Complex III in SMP from bovine heart, yeast HR2 wild type and the 17 and 40 kDa^0 mutants

We have reported previously that the Complex III activity in mitochondria of the 17 kDa^0 mutant is about half that of the wild type [8]. As can be seen above, however, this activity was measured under conditions far from optimal, using horse-heart cytochrome *c* at $I = 225$ mM in the assay.

To further characterise the Complex III activity in both the 17 and 40 kDa^0 mutants we determined the K_m and the TN_{max} for both horse-heart and yeast cytochrome *c* under conditions of optimal ionic strength and at $I = 225$ mM for horse cytochrome *c*, and compared these values with those of the bovine-heart and wild-type yeast Complex III.

The result when horse-heart cytochrome *c* was used is shown in Fig. 3 for $I = 33$ mM and in Fig. 4 for $I = 225$ mM. For yeast cytochrome *c* at $I = 225$ mM, the result is depicted in Fig. 5. The left-hand panels of the figures show the turnover rates measured at various cytochrome *c* concentrations, the right-hand panels show the Hanes plots [29] used for the calculation of the respective K_m values and the maximal turnover numbers given in Table II.

No significant difference was seen between the K_m and TN_{max} values of HR2, the 17 and 40 kDa^0 mutants

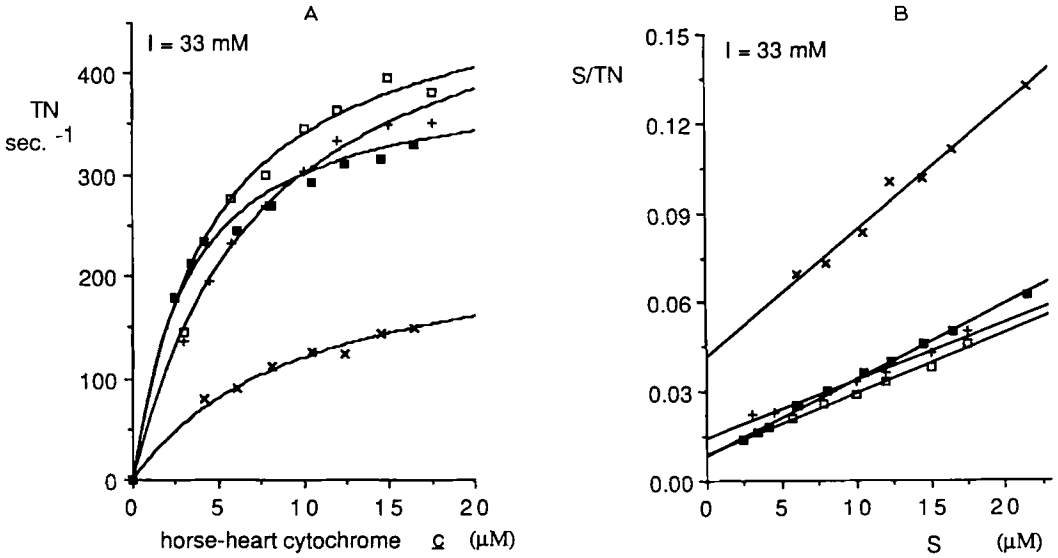

Fig. 3. Determination of the K_m value for cytochrome *c* and the maximal turnover number of Complex III at an ionic strength of 33 mM with horse-heart cytochrome *c* as acceptor. The ubiquinol–cytochrome-*c* oxidoreductase activity present in submitochondrial particles of bovine heart, yeast HR2 wild type, the 17 and 40 kDa^0 mutants was assayed as described in Materials and Methods. The activity is expressed in turnover rates (A), with the aid of the results given in Table I. In (B) the Hanes plots [29] are shown from which the K_m values and maximal turnover rates given in Table II were deduced. TN, turnover rate; S, cytochrome *c* concentration; +, HR2 wild type; □, 17 kDa^0 mutant; ■, 40 kDa^0 mutant; ×, bovine heart.

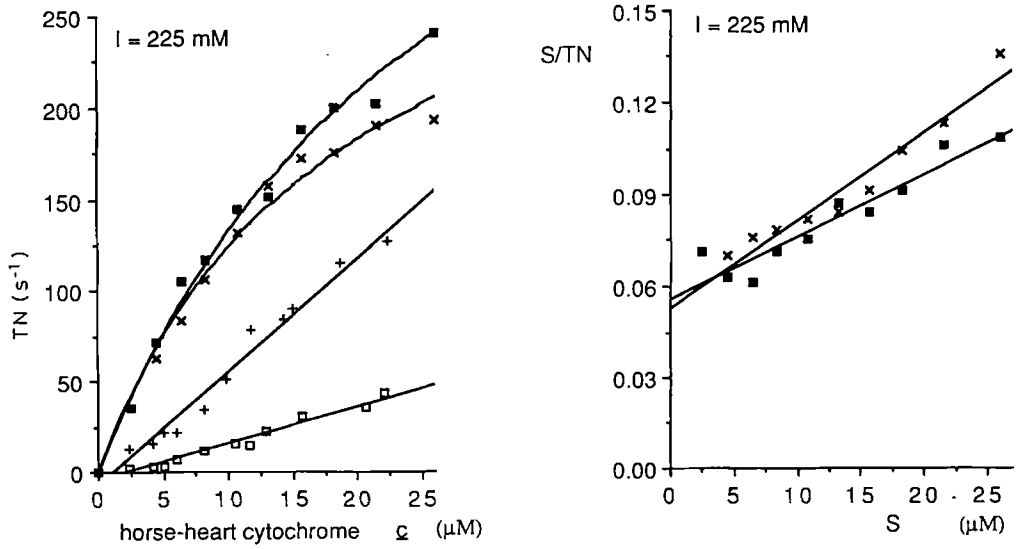

Fig. 4. Determination of the K_m value for cytochrome *c* and the maximal turnover number of Complex III at an ionic strength of 225 mM with horse-heart cytochrome *c* as acceptor. For further details, see legend to Fig. 3.

TABLE II

Kinetic parameters of the interaction of horse-heart and yeast cytochrome c with Complex III of bovine heart, yeast HR2 wild type, the 17 kDa^0 mutant and the 40 kDa^0 mutant SMP

The assay conditions are given in the Materials and Methods section and the Legends to Figs. 3–5.

Ionic strength:	33 mM		225 mM		225 mM	
Electron acceptor:	horse-heart cytochrome *c*		horse-heart cytochrome *c*		yeast cytochrome *c*	
	K_m (μM)	TN_{max} (s^{-1})	K_m (μM)	TN_{max} (s^{-1})	K_m (μM)	TN_{max} (s^{-1})
HR2	7.6	530			30.4	1000
17 kDa^0	4.7	500			35.8	1120
40 kDa^0	3.3	400	27.9	500	20.0	830
Bovine heart	9.9	240	18.3	350		

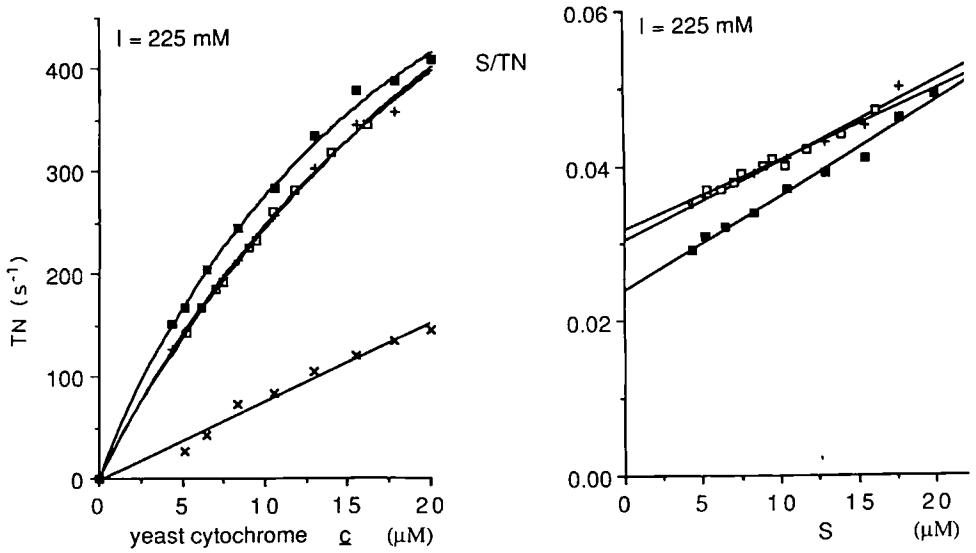

Fig. 5. Determination of the K_m value for cytochrome *c* and the maximal turnover number of Complex III at an ionic strength of 225 mM with yeast cytochrome *c* as acceptor. For further details, see legend to Fig. 3.

when horse-heart cytochrome *c* was used at an ionic strength of 33 mM. The affinity of the bovine heart enzyme for horse-heart cytochrome *c* was comparable to that of the yeast complex and its mutated versions but its maximal turnover number appeared to be lower.

The finding that the 17 kDa protein, which is thought to have a similar function as the hinge protein in the mammalian enzyme [18], did not stimulate the electron transfer from cytochrome c_1 to cytochrome *c* under these conditions was unexpected. However, when the ionic strength was raised, the Complex III activity in SMP from the 17 kDa^0 mutant was substantially lower than that in SMP of the wild type. As can be seen in Fig. 4, it was not possible to calculate the K_m and TN_{max} of either the wild-type or the 17 kDa-lacking yeast enzymes at an ionic strength of 225 mM with horse-heart cytochrome *c* as acceptor. The increase in Complex III activity was linearly related to the increase in cytochrome *c* concentration, at concentrations up to 25 μM. The lower activity observed with the 17 kDa^0 mutant under these conditions indicates that under conditions of (relatively) high ionic strength the 17 Da protein stimulates the electron transfer from cytochrome c_1 to cytochrome *c*, as has already been suggested by the results presented in Fig. 2.

We have also deduced, at an ionic strength of 225 mM, the TN_{max} and the K_m for horse-heart cytochrome *c* of yeast Complex III lacking the Core II protein (Fig. 3, Table II), but since the absolute Complex III activity in SMP from the 40 kDa^0 mutant was very low when measured with horse-heart cytochrome *c* as acceptor at an ionic strength of 225 mM, we do not want to exclude the possibility that the results for the 40 kDa^0 mutant under these conditions are too inaccurate to give a reliable value for the K_m and TN_{max}.

The K_m and TN_{max} of the bovine-heart enzyme increase with ionic strength, as reported before [28]. Our value for TN_{max} is comparable with the reported value, but the K_m given here is about an order of magnitude higher than measured before at an ionic strength of 100 mM [28].

When the Complex III activity was measured in the various SMP using yeast cytochrome *c* as electron acceptor at an ionic strength of 225 mM (Fig. 5), similar results were obtained as for horse-heart cytochrome *c* at $I = 33$ mM. Neither the affinity of the yeast complex for cytochrome *c* nor its maximal turnover number is affected by the absence of the 40 kDa subunit or the 17 kDa subunit.

When the measurements under conditions of optimal ionic strength for horse-heart cytochrome *c* and for yeast cytochrome *c* are compared, it appears that for the yeast enzymes the K_m for yeast cytochrome *c* is about 5-fold higher than that for horse-heart cytochrome *c*, while the maximal turnover numbers are approximately 2-fold higher when the yeast cytochrome is used.

ATP inhibition

Recently it has been reported that ATP is able to bind specifically to ferricytochrome *c* [31]. ADP and AMP also show affinity for ferricytochrome *c* but much less than the triphosphate. It is to be expected that the binding of ATP, being negatively charged at neutral pH, weakens the binding of the basic cytochrome *c* to the acidic moiety of Complex III, cytochrome c_1 together with the 17 kDa protein (yeast) or the hinge protein (bovine heart).

We have examined whether the binding of ATP results in an effect on the Q_2H_2 : cytochrome-*c*

TABLE III

Inhibition of cytochrome c reduction by Complex III by ATP as measured in yeast mitochondria of HR2 wild type, the 17 kDa^0 mutant and the 40 kDa^0 mutant

The assay conditions of ubiquinol–cytochrome-*c* oxidoreductase are given in the Materials and Methods section. The buffer employed contained 100 mM potassium phosphate (pH 7.5) ($I = 225$ mM). Both types of cytochrome *c* were used at a concentration of 15 μM. ATP and ADP were added to the incubation mixture at a concentration of 5 mM.

	Horse-heart cytochrome *c* (activity in μmol·min^{-1}·mg^{-1})			Yeast cytochrome *c* (activity in μmol·min^{-1}·mg^{-1})		
	no addition	+ATP	+ADP	no addition	+ATP	+ADP
HR2	1.20	0.81	1.13	2.26	2.17	2.17
17 kDa^0	0.26	0.15	0.23	1.94	1.29	1.66
40 kDa^0	n.d. [a]			0.20	0.18	0.18

[a] The Complex III activity measured under these conditions is too low in mitochondria of the 40 kDa^0 mutant to quantify the effect of ATP.

oxidoreductase activity in yeast mitochondria from HR2 wild type, the 17 kDa^0 mutant and the 40 kDa^0 mutant. Measurements were made with both horse-heart and yeast cytochrome *c* at concentrations of approx. 15 μM, at $I = 225$ mM. This means that when yeast cytochrome *c* was used, Complex III was assayed just below its K_m, while in the case of horse-heart cytochrome *c* the concentration was well below the K_m (see previous section). Some of the results are shown in Table III.

It can be seen that Complex III lacking the 17 kDa protein behaves differently from both the wild type and the Core II protein-deficient complex. When yeast cytochrome *c* is used, addition of ATP in a concentration of 5 mM only results in inhibition of the 17 kDa-deficient complex. In contrast, when horse-heart cytochrome *c* is used, the activities of both the wild-type and the 17 kDa-deficient Complex III are lowered in the presence of ATP. The inhibition observed for the wild-type HR2 enzyme (32%) is lower than that found for the 17 kDa-deficient enzyme (43%). When ADP was added to the assay, the activity of Complex III was only slightly inhibited.

Apparently the effect of binding of ATP to ferricytochrome *c* is more strongly reflected in the rate of electron transfer when Complex III is devoid of the 17 kDa protein. Inhibition of Complex III activity by ATP can be observed for the wild-type complex only when the cytochrome *c* concentration is well below the K_m (at $I = 225$ mM and with horse-heart cytochrome *c* as electron acceptor in our case), but when the 17 kDa protein is missing, this inhibition is already noticeable at cytochrome *c* concentrations close to the K_m value.

Discussion

In the present study we have analysed the Complex III activity present in the mitochondria and SMP from the 40 and 17 kDa^0 mutants and compared these with the activity of wild-type yeast and bovine heart Complex III.

The amount of Complex III in mutant and wild-type mitochondria and SMP was determined by the level of succinate-reducible cytochrome *b* in the presence of antimycin. The Complex III activity in the various SMP, expressed in turnover numbers, was assayed under varying conditions of ionic strength and with different types of cytochrome *c* used as electron acceptors.

Comparison of bovine heart Complex III with the wild-type yeast enzyme revealed that they do not differ in their sensitivity to pH and ionic strength of the assay mixture, except that the dependency on ionic strength is less strong for the bovine heart enzyme. Analysis, as far as possible, of the K_m values for horse-heart and yeast cytochrome *c* showed that the affinity of the bovine heart complex for horse-heart cytochrome *c* is higher, while the affinity of the yeast enzyme for yeast cytochrome *c* is higher. The maximal turnover numbers of yeast Complex III are about 2-fold higher when either yeast or horse-heart cytochrome *c* is used at optimal ionic strength. Still, the differences between bovine and yeast Complex III, as far as analysed, are small. This suggests that comparison of the possible roles of analogous subunits of both complexes may be fruitful.

Yeast Complex III lacking the Core II protein did not differ significantly from the wild-type complex under conditions of optimal ionic strength in its affinity for either horse-heart or yeast cytochrome *c*.

Considering the localization of the Core II protein at the matrix side of the mitochondrial inner membrane [32] it is not unexpected that this subunit has no direct role in the binding of cytochrome *c*. In line with this is the fact that the addition of ATP in the assay has no effect on the reduction of yeast cytochrome *c* under conditions of optimal ionic strength, just as seen for the wild-type complex and in contrast to the complex lacking the 17 kDa protein.

When the real maximal turnover numbers are compared, the yeast complex without the Core II protein appears to be only slightly slower that the wild-type enzyme. This is true with both types of cytochrome *c* as

electron acceptor. Since the absolute Complex III activity and the cytochrome *b* content in the mutant SMP are relatively low, approx. 5% of the wild-type level, we consider this difference within experimental error. The data show that the activity of yeast Complex III lacking the Core protein II, under saturating conditions for both substrates and at low cytochrome *c* concentrations, is similar to that of wild-type yeast, indicating that also no other reaction in the complete electron pathway from Q_2H_2 to cytochrome *c* has become rate-limiting in the mutant.

It has been reported [33] that removal of both Core proteins leads to inactivation of *Neurospora crassa* Complex III, and it was proposed that the Core complex stabilises the bound ubisemiquinone. The results presented here suggest that the Core II subunit of yeast Complex III has no major function in the overall electron transport, nor in the binding of cytochrome *c*, and has no important role in the binding of quinol or quinone.

Compared to the Core II protein, much more is known about a possible role of the 17 kDa subunit VI in the electron-transport activity of Complex III. Especially the role of its homologous counterpart in the bovine heart enzyme, the 'hinge protein' [9], has been studied extensively [10–13]. The results obtained in those studies are in agreement with the data on the 17 kDa yeast protein as far as analysed [8]. Both proteins interact with cytochrome c_1 and stimulate the electron transfer from cytochrome c_1 to cytochrome *c*.

The results shown in the present study indicate that this stimulating activity of the 17 kDa protein is dependent on the ionic strength of the medium in which the Complex III activity is studied, in correlation with the type of cytochrome *c* used. Under optimal conditions ($I = 33$ mM for horse heart and $I = 225$ mM for yeast cytochrome *c*), the absence of this polypeptide does not affect the steady-state kinetic parameters of yeast Complex III. However, when the ionic strength is raised, the rate of reduction of both types of cytochrome *c* is lower in SMP of the 17 kDa^0 mutant than in SMP of the wild type. No K_m values or maximal turnover numbers could be determined for the wild-type and mutant enzyme when horse-heart cytochrome *c* was used as electron acceptor at high ionic strength ($I = 225$ mM), implying that the K_m is much higher than the cytochrome *c* concentrations used. A similar result was obtained for the bovine-heart enzyme when yeast cytochrome *c* was used under ionic strength conditions of $I = 225$ mM.

The slower reduction of horse-heart and yeast cytochrome *c* by yeast Complex III lacking the 17 kDa polypeptide under conditions of relatively high ionic strength, illustrates that binding of the basic ferricytochrome *c* at neutral pH is stimulated by the presence of the acidic 17 kDa protein. Obviously, this binding is note rate-limiting under conditions of low ionic strength, but when the ionic strength is raised, the rate of association of ferricytochrome *c* becomes rate-limiting, resulting in slower reduction of cytochrome *c* in the absence of the 17 kDa protein.

This supposed role for the 17 kDa protein is supported by the experiments in which ATP was added to the incubation mixture. Under conditions of optimal ionic strength, $I = 225$ mM when yeast cytochrome *c* is used, the reduction of cytochrome *c* was inhibited by the presence of ATP in the case of the mutant, but not when the wild-type yeast Complex III was studied. Under conditions of high ionic strength, $I = 225$ mM when horse-heart cytochrome *c* is used, the reaction is inhibited by ATP with both yeast complexes, although to a somewhat lesser extent in the case of the wild type.

The presence of a binding site for ATP on ferri- but not on ferrocytochrome *c* has been described recently [31]; ADP was also able to bind but much less firmly. It is to be expected that this binding of anionic ATP, at neutral pH, will reduce the association of ferricytochrome *c* to Complex III. Under conditions of optimal ionic strength, however, this step in the electron transfer from cytochrome c_1 to cytochrome *c* is not rate-limiting, as can be seen for the wild-type complex. But the absence of the 17 kDa protein has already decreased this association in the mutant and as a consequence the binding of ATP makes this event (part of) the rate-limiting step in the electron transfer. When the ionic strength is relatively high, the association rate of ferricytochrome *c* is reduced while the rate of dissociation of reduced cytochrome *c* is increased. As a consequence the association becomes rate-limiting for both the wild-type complex and the complex lacking the 17 kDa protein and this is reflected by the inhibition of both enzymes by ATP when horse-heart cytochrome *c* is used at $I = 225$ mM.

A stimulation by the 17 kDa protein of the association rate between cytochrome *c* and cytochrome c_1 has also been reported for the hinge protein [13]. The authors, however, concluded that this effect manifests itself only under conditions of low ionic strength. The discrepancy with our conclusions may be the result of the way the electron transfer from cytochrome c_1 to cytochrome *c* was studied. We have studied electron transfer through the intact Complex III as present in the mutant SMP, while the role of the hinge protein was studied with isolated one-band and two-band cytochrome c_1 (the second band being that of the hinge protein). On the other hand, if at low ionic strength an effect on the rate of association is reflected in the rate of electron transfer, it should do even more so at high ionic strength.

We have shown earlier that the electron-transport activity of the whole respiratory chain in uncoupled mitochondria lacking the 17 kDa protein is reduced [8].

The results shown here suggest that Complex III in yeast mitochondria functions under conditions where its K_m is well above the cytochrome *c* concentration, most likely because of conditions of high ionic strength present in yeast mitochondria ($I > 225$ mM), and consequently the rate of association of ferricytochrome *c* to Complex III (partly) controls the overall rate of the respiratory chain in uncoupled mitochondria.

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