CYTOCHROME *c* OXIDASE IS A PROTON PUMP

A rejoinder to recent criticism

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1. Introduction

Based on experimental evidence from this laboratory, we have suggested that mitochondrial cytochrome *c* oxidase (EC 1.9.3.1.) functions as a redox-linked proton pump [1–5], and not merely as an electron translocator as postulated by Mitchell [6]. The evidence for our proposal has been found in experiments with intact mitochondria, sonicated submitochondrial particles, and with phospholipid vesicles inlaid with the isolated and purified enzyme. In all these systems the results have been consistent, suggesting that the cytochrome *c* oxidase reaction may be characterised by the equation:

$$\text{cyt. } c^{2+} + 1/4\ O_2 + 2\ H^+_M \rightarrow$$

$$\text{cyt. } c^{3+} + 1/2\ H_2O + H^+_C \qquad (1)$$

The subscripts M and C refer to the matrix and cytoplasmic sides of the membrane, respectively. In artificial phospholipid vesicles the C-side refers to the side from which added cytochrome *c* is allowed to react with the enzyme.

According to eq. (1) the cytochrome *c* oxidase reaction is linked to net transport of 1 H^+/e^- all across the membrane. In addition 1 H^+/e^- is consumed from the M-side in the formation of water. In contrast, according to Mitchell's postulate, only the latter consumption of protons takes place without any true transport of H^+ across the membrane.

Moyle and Mitchell [7] criticised our proposal of a proton-pumping function of cytochrome *c* oxidase. The criticism mainly concerned our interpretation of proton ejection from isolated rat-liver mitochondria during respiration with ferrocyanide [1,2]. Since ferrocyanide specifically reduces cytochrome *c* in mitochondria [8] initiating respiration, we interpreted the proton ejection as being linked to cytochrome *c* oxidase activity. This interpretation was, however, rejected in [7], where H^+ ejection was suggested to be an artefact due to oxidation of an unidentified hydrogenated mitochondrial reductant by the ferricyanide formed during respiration with ferrocyanide. Moyle and Mitchell [7] proposed further that the reduction of formed ferricyanide by the endogenous reductant is not only linked to mere release of H^+, but is in addition linked to the activity of a proton-motive system catalysing proton translocation across the mitochondrial membrane. We may interject here that such a process would have to be insensitive to both rotenone (which inhibits NADH dehydrogenase [9]) and antimycin (which inhibits electron transfer in the cytochrome b–c_1 complex [10]), since the proton ejection observed during ferrocyanide oxidation is not sensitive to these inhibitors [1,2].

In support of the proposal in [7], the consumption of H^+ during uncoupled respiration with ferrocyanide, in many conditions, was reported not to match the rate of oxygen consumption. In cases uncomplicated by side reactions, eq. (2) described overall ferrocyanide oxidation by uncoupled mitochondria:

Abbreviations: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; FIC, ferricyanide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonate

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$$(Fe^{II}(CN)_6)^{4-} + 1/4\ O_2 + H^+ \rightarrow$$

$$(Fe^{III}(CN)_6)^{3-} + 1/2\ H_2O \quad (2)$$

and predicts as H^+/O quotient of proton consumption of 2.0. As described [7], this H^+/O quotient often fell 20–30% short of the expected value when measured by comparison of pH and O_2 electrode traces. In the following we will refer to this finding as the 'shortfall' phenomenon.

Moyle and Mitchell [7] interpreted this finding to indicate that 'extra oxygen', not accounted for by eq. (2), was consumed by a side reaction without simultaneous H^+ consumption, i.e., extra oxygen reduction by a hydrogen donor. As indicated above, this was explained as being the result of reduction of ferricyanide, formed during ferrocyanide respiration, by a hydrogenated reductant with release of protons to the medium, the electrons being shuttled to oxygen via cytochrome *c* oxidase. Hence, in the proposed overall process, the 'shortfall' in the H^+/O quotient would result from an 'extra' consumption of oxygen that is not associated with H^+ consumption.

The 'shortfall' phenomenon did not occur under all experimental conditions. It was stressed [7] that the phenomenon correlated completely with H^+ ejection linked to ferrocyanide oxidation by coupled mitochondria. Thus, in conditions where no 'shortfall' was observed, it was claimed [7] that there was also no H^+ ejection. It was therefore concluded that the H^+ ejection observed under coupled conditions is an artefact, which is not linked to the cytochrome *c* oxidase reaction as proposed by us.

Finally, Moyle and Mitchell [7] reported that oxidation of added ferrocytochrome *c* by mitochondria is not linked to proton ejection (transport), although proton ejection would certainly be expected on the basis of our proposal of a proton-pumping function of cytochrome *c* oxidase (eq. (1)).

In this paper we will demonstrate the following:

(i) The interpretation [7] of the 'shortfall' in the H^+/O quotient is incorrect.

(ii) There is no correlation between the occurrence of this phenomenon and H^+ ejection from mitochondria linked to respiration with ferrocyanide. Thus H^+ ejection can readily be observed under conditions where the 'shortfall' phenomenon is absent.

(iii) The oxidation of added ferrocytochrome *c* by rat-liver mitochondria is linked to proton translocation, which is readily observable under appropriate experimental conditions.

We retain our proposal of a proton-pumping function of mitochondrial cytochrome *c* oxidase. The criticism raised by Moyle and Mitchell [7] is found to be invalid on all points.

2. Materials and methods

Rat-liver mitochondria were isolated as in [11] and finally gently homogenised into a small volume of 0.25 M sucrose–0.2 mM EDTA, pH 6.8, to final conc. ~40 mg protein/ml. Protein was measured as in [12] with human serum albumin as standard.

pH changes in mitochondrial suspensions were recorded using a combination electrode (Ingold) connected to an Instrulab IM 555 pH-meter and a Goerz Servogor strip chart recorder. The reaction chamber was open and stirred vigorously with a magnetic flea. The temperature was thermostated to 24 ± 0.1°C. pH changes were calibrated to yield changes in proton equivalents produced or consumed using standard HCl and/or oxalate solutions.

O_2 consumption was measured polarographically with a Clark electrode equipped with an extra thin membrane (50% response in less than 1 s), and inserted into a closed glass vessel. The vessel contents were stirred with a magnetic flea and thermostated by the same system as the pH-electrode setup (24 ± 0.1°C). The O_2 meter electronics was designed and constructed at the Johnson Research Foundation Workshops, Philadelphia. Changes in O_2 concentration were carefully calibrated using two independent techniques. The null point (anaerobiosis) was adjusted after dissolving a few grains of sodium dithionite into aqueous medium in the oxygraph. Equilibration of the electrode system with pure water equilibrated with air at 24°C resulted in a signal which was taken to be equal to 263 μM O_2 [13]. Secondly, known concentrations of NADH were added to aerobic suspensions of submitochondrial particles, and the O_2

scale was calibrated according to the O_2 consumed by NADH oxidation (see [14]). The two methods of calibration were found to agree closely with one another.

The rate of production of ferricyanide was measured spectrophotometrically at the wavelength couple 420–500 nm with an Aminco DW-2 dual wavelength spectrophotometer using 1 cm cuvettes thermostated at 24°C. The changes in ferricyanide concentration were carefully calibrated by additions of small aliquots of fresh standard ferricyanide solutions to the experimental solution containing the mitochondria.

The three kinds of measurement (pH, O_2 and ferricyanide) were performed simultaneously in three different reaction chambers, but, to avoid pipetting errors, the same solution of medium with necessary constituents plus mitochondria was divided into the three reaction vessels.

Reagents were of highest grade available commercially. Horse-heart cytochrome *c* was purchased from Boehringer and reduced as in [2]. The concentration was assayed by oxidation with ferricyanide at 550 nm, using an extinction coefficient (reduced minus oxidised) of 21 $mM^{-1}cm^{-1}$ [15]. Potassium ferro- and ferricyanide (hexacyanoferrate, II and III) were obtained from Merck (Darmstadt). Ferrocyanide was added as small aliquots of a fresh 0.2 M solution in water (adjusted to pH 7 with HCl), with a calibrated microsyringe. Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift by Dr P. G. Heytler, and the extra thin O_2 electrode membrane was kindly donated by Dr A. Kemp, jr.

3. Results and discussion

As an essential control of the suggestion by Moyle and Mitchell [7] that the 'shortfall' in H^+ consumption with respect to O_2 consumption would be due to reduction of formed ferricyanide by an endogenous mitochondrial hydrogen donor, it was considered necessary to compare the rates of oxygen consumption and ferricyanide production during oxidation of ferrocyanide by mitochondria. Table 1 summarises data on this point for various conditions. It may be seen that the initial rates of production of ferricyanide and consumption of O_2 match each other closely (on a one-electron basis) under all conditions. We

Table 1
Stoichiometry of oxygen consumption, proton consumption and production of ferricyanide during uncoupled respiration with ferrocyanide as substrate

Medium	Initial rates in μM/min			
	$-4(\frac{dO_2}{dt})$	$\frac{dFIC}{dt}$	$-\frac{dH^+}{dt}$	'Shortfall' (%)
Sucrose 150 mM KCl 50 mM Hepes 1 mM	13.5 (± 1.25 SD) $n = 3$	13.3 (± 0.57 SD) $n = 3$	11.8 (± 0.44 SD) $n = 3$	12
KCl 150 mM Hepes 1 mM	19.2 (± 3.07 SD) $n = 3$	19.2 (± 2.40 SD) $n = 3$	16.9 (± 3.19 SD) $n = 3$	12
Sucrose 150 mM KCl 50 mM EDTA 5 mM Hepes 1 mM	19.1 (± 1.44 SD) $n = 3$	19.7 (± 1.80 SD) $n = 4$	15.9 (± 0.57 SD) $n = 4$	18

The media were supplemented with 0.4 μM rotenone, 0.08 μg/ml antimycin, 0.025 μg/ml valinomycin, 0.3 μM FCCP and 1 mg/ml of rat-liver mitochondria. pH 7.1, temp. 24 ± 0.1°C. The reaction was started by addition of 0.8 mM potassium ferrocyanide. % 'Shortfall' (see text) is calculated from the mean values of $-\frac{dH^+}{dt}$ and $-4(\frac{dO_2}{dt})$. SD, standard deviation; *n*, no. expts

have in fact not observed any significant deviation from this result in several dozens of experiments under different conditions, whether the 'shortfall' effect was present or not. This control, which was lacking in [7], unequivocally rules out their interpretation of the basis of the 'shortfall' effect. It is clear that during oxidation of ferrocyanide by rat-liver mitochondria, there is no significant further reduction of formed ferricyanide by endogenous reductants; all oxygen reduced can be quantitatively accounted for by the amount of ferrocyanide oxidised. Hence the tabulation and extensive discussion in [7] of an 'extra amount of oxygen' reduced by a hypothetical hydrogen donor via the ferri-/ferrocyanide couple, is misleading.

As also shown in table 1, we have been able to reproduce the 'shortfall' phenomenon described in [7]. In this particular series of experiments the H^+ consumption fell 12–18% short of the expected value. In our hands this phenomenon is, however, highly variable with the preparation of mitochondria, and also to some extent with the composition of the medium. It is essential that under some conditions (see below and [7]) this phenomenon is entirely absent. It may be added that we have not been able to confirm the finding by Moyle and Mitchell [7] that *N*-ethylmaleimide (NEM) would increase or induce the 'shortfall' effect.

Since we can demonstrate a complete stoichiometric fit between rates of oxygen consumption and production of ferricyanide (table 1) under all tested conditions, it must be concluded that the 'shortfall' in the H^+/O quotient of proton consumption must be the result of some highly variable artefactual H^+-producing (or OH^--consuming) reaction, which is synchronous to oxidation of ferrocyanide by mitochondria.

Based on the dependence of pH on concentration of aqueous solutions of potassium ferro- and ferricyanide, it is likely that $(Fe^{II}(CN)_6)^{4-}$ attract H^+ and/or H_3O^+ more readily into the primary ionic shell than is the case for the ferric anions. On dilution of a stock potassium ferrocyanide solution (adjusted to pH 7.0 with HCl) into an aqueous medium of the same pH, an abrupt and persistent alkalinisation is observed (see e.g. fig.1). This is presumably the result of dissociation of the salt and attraction of H^+ and/or H_3O^+ by the complex anions, an effect that is not

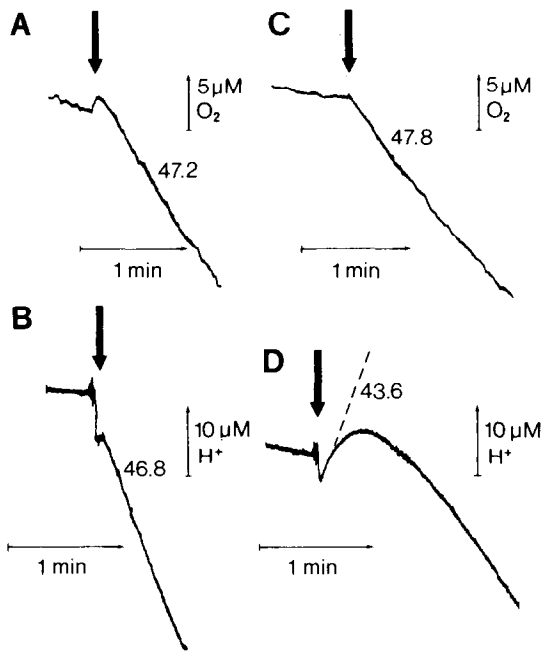

Fig.1. Proton ejection from rat-liver mitochondria during respiration with ferrocyanide. 110 mM KCl–1 mM Hepes (pH 7.0) was supplemented with 5.5 μM rotenone, 0.118 μg/ml antimycin, 0.055 μg/ml valinomycin and ~2.5 mg/ml rat-liver mitochondrial protein. Temp. 24°C. The reaction was started by addition of 0.8 mM potassium ferrocyanide (at the arrow). In A and B 0.5 μM FCCP was also present. Numbers adjacent to the traces are initial rates expressed as μM electrons or protons per minute. Traces (A,C) oxygen consumption. Traces (B,D) pH changes (see section 2).

observed with the ferric salt. Reduction of cytochrome *c* by ferrocyanide in aqueous solution is a fast process [16], which occurs without uptake or release of protons. However, the reduction of added cytochrome *c* which is bound to mitochondrial membranes is slow and associated with release of H^+ (M.K.F. W. and K. K., unpublished observations). This release of protons is accelerated by mitochondria, but the extent is unaffected by the mitochondrial concentration. It is thus possible that ferrocyanide anions loose their primary ionic shell containing H^+ and/or H_3O^+ on approaching membrane-bound cytochrome *c* and, since ferricyanide anions attract protons less readily, the oxidation of ferrocyanide will be associated with some acidification. Such an effect is expected to be highly variable depending on

the exact experimental conditions, and is suggested to be the basis of the 'shortfall' in the H^+/O quotient.

It is, however, much more important in the present context that under some experimental conditions the 'shortfall' effect is absent. In such cases, Moyle and Mitchell [7] claimed that there was also no observable proton ejection coupled to oxidation of ferrocyanide. We tested this essential statement in experiments such as those shown in fig.1.

In fig.1A,B, uncoupled mitochondria are pulsed with ferrocyanide, and it is clear that the H^+/O quotient of proton consumption shows no significant sign of the 'shortfall' phenomenon under these conditions (see also [7]). On addition of the ferrocyanide, there is an abrupt alkalinisation of the reaction mixture whether an uncoupler is present or not (fig.1B,D). This effect, which also occurs in the absence of mitochondria (not shown), was discussed in some detail above. In the presence of an uncoupler (fig.1B), this alkalinisation artefact is followed by steady akalinisation at a rate which agrees quantitatively with O_2 consumption according to eq. (2). However, in the absence of an uncoupler (fig.1D) respiration is associated initially with net production of protons at a maximum rate of nearly 1 H^+/e^- (0.91 in fig.1). This is in good agreement with our previous reports ([1,2] but see [7]), and we cannot but conclude that the proton ejection is observable under conditions where the 'shorfall' effect is absent. We also cannot reproduce the results by Papa et al. [17,18], who reported that the H^+/e^- quotient of proton ejection would amount only to 0.5 when electron transport is measured with an oxygen electrode as opposed to measuring ferricyanide production spectrophotometrically. In our hands the two methods agree within experimental error (cf. [1,2] and fig.1C,D).

Since it is clear from our data in fig.1 that the proton ejection is unrelated to the 'shortfall' phenomenon, and that the former can be observed in the absence of the latter, our position with respect to this question is diametrically opposite to that of Moyle and Mitchell [7]. Since so much emphasis is placed in [7] on a complete correlation between proton ejection and the 'shortfall' phenomenon, and largely based their criticism of our proposal on this point, we are obliged to point out that in very similar experiments reported by Mitchell and Moyle more than 10 years ago (fig.8C,D in [19]), the results were quite comparable to those shown here in fig.1B,D. However, presumably as a result of a low expansion of the pH scale and, in particular, due to the use of a much lower concentration of ferrocyanide (resulting in a low respiratory rate), Mitchell and Moyle [19] then dismissed their result as a 'small, reproducible pH wobble'. Nevertheless, a close examination of their experiment demonstrates both the fast alkalinisation artefact on addition of ferrocyanide and, subsequently, the net production of H^+, which they also show is abolished by uncoupling agents (cf. fig.1B,D here with fig.8C,D in [19]). In connection with this experiment, Mitchell and Moyle [19] stated that 'in the presence of CFCCP, the rate of uptake of protons can be shown to correspond to the rate of ferrocyanide oxidation', implying the absence of any 'shortfall' phenomena. These findings agree very well with ours (fig.1) but not with their own more recent report [7].

It should be stressed that the conditions of fig.1 may be much improved to increase the size of H^+ ejection. Thus, for instance, the inclusion of 3–5 mM of $MgCl_2$ speeds up respiration with ferrocyanide [20], and increases the rate and extent of H^+ ejection. Figure 1 is reproduced here mainly for comparative purposes.

Figure 2 shows that H^+ ejection coupled to cytochrome *c* oxidase activity may be observed also when reduced cytochrome *c* is used as a substrate. In contrast to the artefact mentioned in [7], the proton ejection shown in fig.2 is abolished in the presence of an uncoupler. It is also dependent on the presence of valinomycin plus K^+ (or Ca^{2+}) as would be expected from transmembrane proton movement (see e.g. [2]), and is inhibited by cyanide (not shown). Note that the overall consumption of protons is the same whether an uncoupler is present or not (fig.2). This fact, which was also shown for the cytochrome *c* oxidase vesicle system [2,5], is consistent with the idea of the proton ejection being the result of proton transport, and rules out possible artefacts due to net production of protons in the system such as the one suggested in [7].

Proton ejection from rat-liver mitochondria may also be observed with cytochrome *c* plus ascorbate, ferrocyanide plus ascorbate and TMPD plus ascorbate as respiratory substrates (to be reported separately) with a stoichiometry close to 1 H^+ transported/e^-

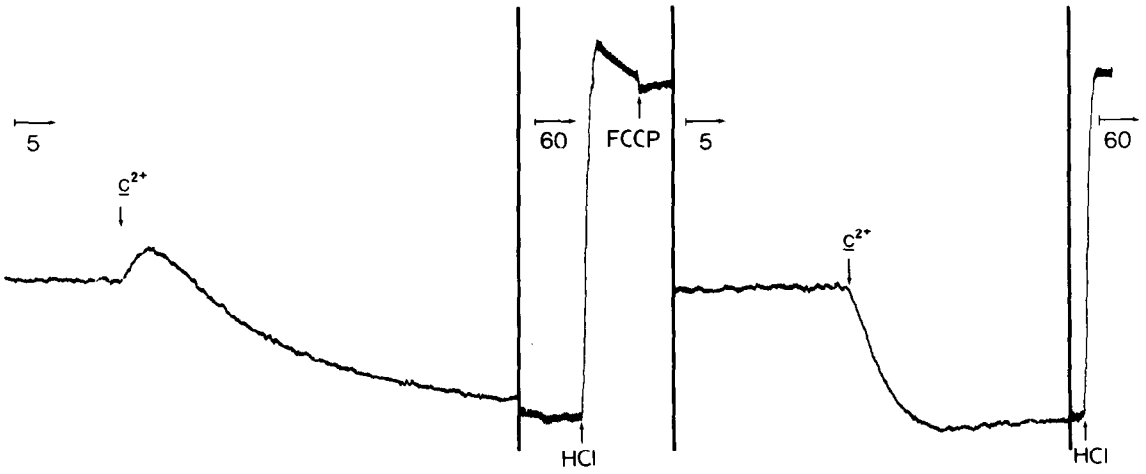

Fig.2. Proton ejection from rat-liver mitochondria coupled to oxidation of added ferrocytochrome *c*. 120 mM KCl–0.5 mM Hepes (pH 7.05) was supplemented with 6 μM rotenone, 0.13 μg/ml antimycin, 0.08 μg/ml valinomycin and ~2 mg/ml of rat-liver mitochondrial protein. Temp. 24°C. c^{2+}, HCl and FCCP indicate additions of 4.3 μM ferrocytochrome *c*, 10 μM HCl and 1 μM FCCP, respectively. The numbers next to the horizontal arrows give the time scale in seconds. The vertical lines indicate changes in recorder speed.

transferred. This is in good agreement with our results using ferrocyanide ([1,2] and fig.1).

4. Conclusion

We conclude that the redox activity of mitochondrial cytochrome *c* oxidase is likely to be linked to a true proton-pumping function of this respiratory enzyme. Based on experiments with intact mitochondria using several artificial and natural substrates, experiments with sonicated submitochondrial particles, and with cytochrome oxidase vesicles ([1–5] and this paper), the stoichiometry of the proton pump may be described by eq. (1). The criticism raised in [7] can be dismissed by experiment on all points.

Our finding of a proton-pumping function of cytochrome *c* oxidase is in agreement with the re-evaluated H^+/O quotients of respiratory chain-linked proton translocation of Brand et al. [21] in contrast to the quotients proposed [22–25]. It seems necessary to study the stoichiometric aspects of mitochondrial proton translocation further by using different independent techniques, since knowledge of the true stoichiometries is essential for any mechanistic description of the process in the future. It is hoped that the causes of the present controversies will also be unravelled by such studies.

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