REDOX BOHR-EFFECTS IN ISOLATED CYTOCHROME bc_1 COMPLEX AND CYTOCHROME c OXIDASE FROM BEEF-HEART MITOCHONDRIA

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1. Introduction

The midpoint redox potential (E_m) of b cytochromes [1–4] and the Rieske Fe–S protein g = 1.90 [5] of the cytochrome bc_1 complex, as well as that of hemes a and a_3 [3,6–8], vary with pH, i.e., decrease as the pH of the medium is raised within certain ranges.

This property, which is unexpected for electron carriers, indicates the occurrence of cooperative linkage between electron transfer by the metal and proton transfer by organic protolytic groups (in the apoproteins or also in the prosthetic groups) owing to reversible increase of their pK upon reduction [3,9–11].

As a result of these phenomena, which by analogy to those described for hemoglobin [12,13] are denominated redox Bohr-effects [11,14], at pH-values between the pK_a of the oxidized and that of the reduced form, the respiratory carriers function partly as electron carriers and partly as effective hydrogen-carriers [10,11].

Mechanisms have been put forward, on the basis of which Bohr effects could participate in vectorial proton translocation in the cytochrome system of mitochondria and other coupling membranes [10,11,15–18].

We have used a direct approach, based on measurement of scalar proton transfer associated with net redox transitions of respiratory carriers, to verify the occurrence of redox Bohr-effects [19]. It was possible, in this way, to demonstrate the occurrence of redox Bohr effects in the cytochrome system of mitochondria.

Here, data are presented on the occurrence and characteristics of redox Bohr effects in isolated cytochrome bc_1 complex and cytochrome c oxidase.

2. Methods

The cytochrome bc_1 complex (complex III) was isolated from beef-heart mitochondria as in [20]. The content of b cytochromes was measured from $\Delta A_{562-575}$ caused by reduction with dithionite using a $\Delta\epsilon_{mM}$ of 20 [21], that of cytochrome c_1 at 552–540 nm with a $\Delta\epsilon_{mM}$ of 17.5 [22]. The cytochrome bc_1 preparations contained ~7 nmoles cytochromes b and 3.5 nmol cytochrome c_1/mg protein [20,23]. The V_{max} for ubiquinol-2 cytochrome c reductase activity was 150–220 μmol cytochrome c reduced . min^{-1} . mg protein^{-1} (cf. [23]). The complex contained traces of succinate dehydrogenase, succinate cytochrome c reductase activity of 0.1 μmol cytochrome c reduced . min^{-1} . mg protein^{-1} (cf. [23]).

Cytochrome c oxidase (complex IV) was isolated from beef-heart mitochondria as in [24]. The heme a content was determined from ΔA caused by reduction with dithionite using a $\Delta\epsilon_{mM}$ of 14 at 605–630 nm [25]. The preparations contained ~10 nmol heme $a + a_3$/mg protein. Cytochrome c oxidase contained traces of cytochrome bc_1 complex (0.05–0.1 mol cytochrome c_1/mol heme a,a_3). The specific activity of complex IV was 30 35 μgatom oxygen consumed . min^{-1} . mg protein^{-1}, assayed with 22 μM cytochrome c, 100 μM, N,N,N',N'-tetramethyl-p-phenylenediamine and 30 mM ascorbate (pH 7.2) [26].

Oxido–reductions of cytochromes and pH changes were monitored on the same sample in a thermostated, 25 ± 0.1°C, spectrophotometric cuvette under a stream of nitrogen. Oxido–reductions were monitored with a dual wavelength spectrophotometer, pH with a combination electrode and a differential

electrometer. The circuit used allowed the pH to be measured with a precision of 0.001 pH unit [27]. Bovine heart ferricytochrome *c* (M_r 12 327, type IV) from Sigma Chemical Co.

3. Results

Fig.1 shows oxygen consumption by isolated cytochrome bc_1 complex with succinate as reductant. This caused detectable oxygen consumption, provided that traces of exogenous cytochrome *c* and isolated cytochrome *c* oxidase were added.

Oxidation cycles of the cytochrome bc_1 complex supplemented with traces of added oxidase and cytochrome *c* were thus produced by pulsing with oxygen the enzyme brought to anaerobic reduction by succinate respiration (see fig.2).

Fig.2A shows that net aerobic oxidation of *c* and *b* cytochromes was accompanied, as expected, by H^+ consumption for protonation of reduced oxygen to H_2O. Net re-reduction of the cytochromes by succinate, which took place upon exhaustion of oxygen, resulted in release of the same amount of H^+ taken up upon oxygenation. It can, however, be seen that the H^+ taken up and subsequently released were much less than the sum of electron carriers undergoing oxido–reduction (table 1). The difference amounted to 5.2 ngion H^+/mg protein. Aerobic oxidation of electron carriers has to result in the consumption of an equivalent amount of H^+ and their reduction by

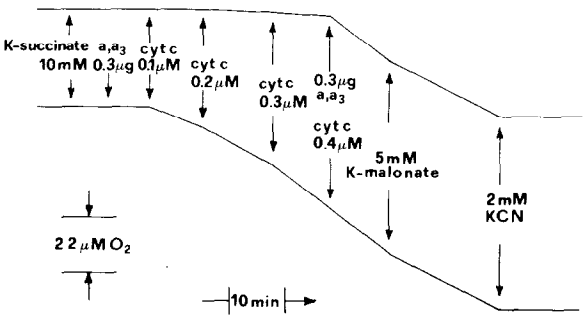

Fig.1. Succinate-supported oxygen consumption by isolated cytochrome bc_1 complex supplemented with exogenous cytochrome *c* and purified cytochrome *c* oxidase. Oxygen consumption was measured polarographically with a Clark electrode. Complex III (0.43 mg/ml) was incubated for 5 min in: 200 mM sucrose; 30 mM KCl (pH 7.2). Final vol. 1.8 ml; temp. 25°C.

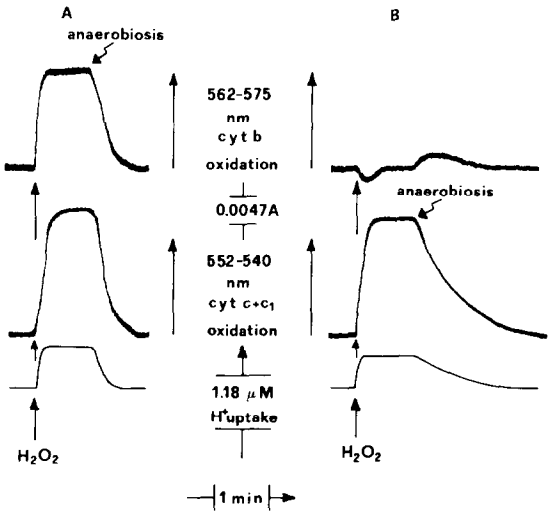

Fig.2. Redox transitions of electron carriers and associated scalar H^+-transfer reactions induced by oxygen pulses of anaerobic isolated cytochrome bc_1 complex supplemented with exogenous cytochrome *c* and cytochrome *c* oxidase. Complex III (0.43 mg/ml) was incubated in: 200 mM sucrose, 30 mM KCl, 0.3 μg purified complex IV, 0.24 μM cytochrome *c*, 9 μg purified catalase (pH 7.2); vol. 2.25 ml. The incubation was done in a stoppered spectrophotometric cuvette for 20 min under a continuous stream of argon to lower concentration of dissolved oxygen, then 1 mM K-succinate was added. Anaerobiosis was reached in ~10 min. Under these conditions cytochrome c_1 was reduced by ~80%, *b* cytochromes by ~50%. Oxygenation was brought about by repetitive addition of 1 μl 0.1% H_2O_2, in the presence of 0.5 mM K-malonate. (A) Control; (B) + 2.8 μg antimycin A/mg protein.

hydrogenated reductants in the production of the same amount of H^+. Thus the observed deficit in the consumption and production of H^+ is, respectively, a measure of H^+ release and H^+ binding at protolytic groups in the cytochrome bc_1 complex, whose pK_a decreases upon oxidation of the redox centers.

The E_m of cytochrome c_1 is pH-independent [4]. Thus the deficit of H^+ transfer has to be referred to the redox transition of *b* cytochromes and Rieske Fe–S protein and exhibit an overall H^+/e^- coupling number of 1.3 (table 1).

In table 1B a concentration of antimycin A, which prevented any net redox change of *b* cytochromes, was added. Also under these conditions H^+ transfer was <1/2 the sum of electron carriers undergoing oxido–reduction. The deficit of H^+ transfer gave, when referred to redox transitions of the Rieske Fe-S protein, an H^+/e^- coupling number of 1.5.

Table 1
Analysis of scalar H^+-transfer reactions associated to redox transitions of respiratory carriers in isolated cytochrome bc_1 complex

	A		B
(i) H^+ uptake	2.06		1.60
cyt. *b*	1.92		–
cyt. c_1	2.38		2.19
FeS $g = 1.90$	2.38		2.19
cyt. *c*	0.56		0.56
(ii) Σe^-	7.24		4.94
(iii) Deficit H^+ = (ii – i)	5.18		3.34
iii/cyt. *b*, Fes	1.20	iii/FeS	1.52
(iv) H^+ Release for oxidation of QH_2 to $Q^{\cdot-}$	1.64		1.51
(v) Bohr H^+ (iii – iv)	3.54		
Bohr H^+/cyt. *b*, FeS	0.82		
(vi) Fraction *b* cytochromes acting as e^- carriers, 27% of value in line 2 (cf. [34])	0.52		
(vii) Σe^- corrected for (vi)	5.84		
(viii) Bohr H^+ (FeS) [vii – (i + iv)]	2.14	Bohr H^+ (FeS) (iii – iv)	1.83
Bohr H^+ (FeS)/FeS	0.90		0.84

For experimental conditions see legend to fig.2. Experiments with the addition of 1 μM FCCP or 0.2% Emasol produced the same results. The nmol cytochrome c_1 were corrected for the contribution of cytochrome *c* oxido–reduction at 552–540 nm (Δc_{mM} of 14); the Fe S protein $g = 1.90$ was taken as equivalent to cytochrome c_1 [23,28]. The values are expressed in ngion or nmol/mg protein. Expt. (A) control, Expt. (B) + 2.8 μg antimycin A/mg protein. For the data in (iv) and below section 4

Statistical evaluation of H^+/e^- stoicheiometry numbers

Ratio	H^+/e^-	SEM
Deficit H^+/cyt. *b*, FeS	1.32	±0.02
Bohr H^+/cyt. *b*, FeS	0.88	±0.02
Bohr H^+/FeS	0.97	±0.03

Means of 14 expt described in fig.2A and table 1A

In fig.3 (see also table 2) an experiment on oxidation cycles of isolated cytochrome *c* oxidase is presented. The oxidase was brought to anaerobic reduction by oxidation of duroquinol in the presence of a trace of phenazine–methosulfate, or by oxidation of ethanol, in the presence of purified alcohol-dehydrogenase, and traces of NAD^+ and phenazine–methosulfate. The aerobic oxidation of hemes *a* and a_3 was accompanied by oxidation of a small amount of

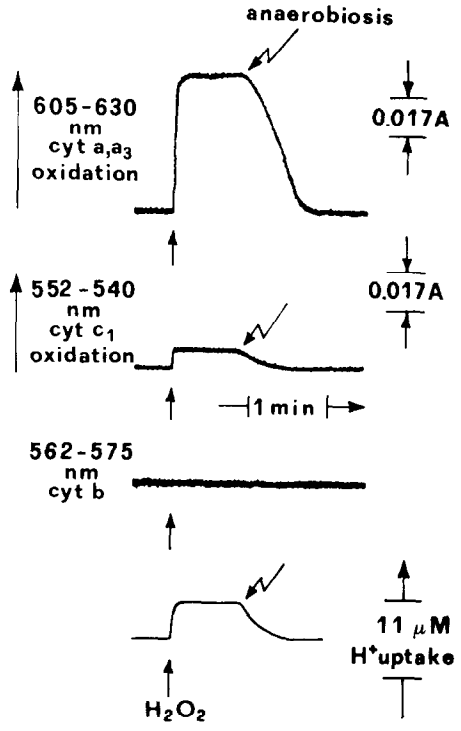

Fig.3. Redox transitions of electron carriers and associated scalar H^+-transfer reactions induced by oxygen pulses of anaerobic isolated cytochrome *c* oxidase. Purified cytochrome *c* oxidase (0.41 mg/ml) was incubated in: 200 mM sucrose, 30 mM KCl, 10 μg purified catalase and 0.2 μM phenazine–methosulphate (PMS) (pH 7.2); vol. 2.25 ml. The incubation was carried out in a stoppered spectrophotometric cuvette for 20 min under a continuous stream of argon. Then 1 mM duroquinol was added. Anaerobiosis was reached in ~5 min. Under these conditions hemes *a* and a_3 were practically completely reduced. Oxygenation was brought about by addition of 1 μl of 0.1% H_2O_2.

cytochrome c_1 from contaminating cytochrome bc_1 complex. No redox change attributable to *b* cytochromes was detected. Aerobic oxidation of cytochrome oxidase was accompanied by uptake of H^+ which were all released when added oxygen was exhausted. Proton transfer was significantly lower than the sum of electron carriers undergoing oxido–reduction. The two reductant-systems gave quantitatively the same results. The deficit of H^+ transfer amounted to 11 ngion H^+/mg protein, which referred to hemes *a* and a_3 undergoing oxido–reduction (it should be recalled that the E_m of the oxidase copper is pH-independent [3]) gave an H^+/e^- ratio of 1 (see table 2).

Table 2
Analysis of scalar H^+-transfer reactions associated to redox transitions of electron carriers in isolated cytochrome *c* oxidase

	A	B
(i) H^+ uptake	12.07	11.59
Hemes $a + a_3$	10.50	10.55
Copper	10.50	10.55
Cyt. c_1	1.18	1.23
PMS	0.49	0.49
(ii) Σe^-	22.67	22.82
(iii) Deficit H^+ = (ii − i)	10.60	11.23
Deficit $H^+/a + a_3$	1.01	1.06
(iv) Bohr H^+ of $b - c_1$ complex (1.52 . c_1)	1.79	1.87
(v) Bohr H^+ of complex IV (iii − iv)	8.81	9.36
Bohr $H^+/a + a_3$	0.84	0.89

Experimental conditions: (A) see legend to fig.3; (B) experimental conditions as reported for (A) except that instead of duroquinol, 10 mM ethanol was used as reductant in the presence of 30 units alcohol-dehydrogenase (Boehringer), 0.5 μM NAD^+ and 0.2 μM phenazine-methosulfate. The amount of copper oxidized was taken as equivalent to hemes $a + a_3$ [29]. The values reported in the table are expressed as ngion or nmol/mg protein

Statistical analysis of H^+/e^- stoicheiometry numbers

Ratio	A		B	
	H^+/e^-	SEM	H^+/e^-	SEM
Bohr $H^+/a + a_3$	0.86	±0.03	0.92	±0.08

The values are the means of 18 expt (A) and 5 expt (B)

Correction of the deficit of H^+ transfer for that contributed by contaminating cytochrome bc_1 complex (see table 1B) gave an H^+/e^- coupling number for redox Bohr effects in cytochrome oxidase, with reference to hemes *a* and a_3, of 0.9.

4. Discussion

This work provides a direct demonstration of the occurrence of redox Bohr effects in isolated cytochrome *c* oxidase and cytochrome bc_1 complex. Hemes *a* and a_3, the *b* cytochromes and the Rieske Fe–S protein of the cytochrome bc_1 complex appear to be involved in the Bohr effects. What is reported here is generally consistent with the observations on the pH-dependence of the E_m of these respiratory carriers in the membrane [1–3,5–8]. However, the H^+/e^- coupling numbers obtained in this work are higher than those one would expect on the basis of the pK_a calculated with the potentiometric method for the oxidized carriers in the native membrane. It is not clear in the light of the inherent ambiguities of redox titrations of membrane bound systems [30], whether these measurements can be applied to the isolated enzymes.

An H^+/e^- coupling number of 1.5 at pH 7.2 for the Rieske Fe–S protein (table 1B) is, in particular, much higher than that expected from the reported pH-dependence of its E_m, which appears to decrease by 60 mV/pH unit increase above 7.9 [5].

In the transition of the cytochrome bc_1 complex from the reduced to the oxidized state net H^+ release could, however, also derive from oxidation of endogenous ubiquinone, present in all the preparations of the cytochrome bc_1 complex [23]. It amounts in the preparation used here to 0.7 mol/mol cytochrome c_1 [20,23]. Endogenous ubiquinone of cytochrome bc_1 complex is likely to be associated to quinone binding protein(s), isolated from the complex [31]. Aerobic oxidation of ubiquinol to protein-stabilized bound ubisemiquinone [32,33], for which $pK \sim 6$ is reported [33], could result in the net production of protons according to:

$$2\,QH_2 + 1/2\,O_2 \rightarrow 2(Q^{\cdot -})n + 2\,(H^+)n + 2\,(Q^{\cdot}H)\,(1-n) + H_2O \qquad (1)$$

where *n* represents the percentage of the semiquinone anion at the given pH.

Assuming that endogenous ubiquinol (in the proportion of 0.7 mol/mol cytochrome c_1 oxidized) is oxidized to ubisemiquinone according to eq. (1), one can calculate from the data in table 1 the H^+ release by this reaction. The remaining H^+ production can be ascribed to deprotonation of ionizable groups in *b* cytochromes and Rieske Fe–S protein. The overall H^+/e^- coupling number for redox Bohr effects in these respiratory carriers results to be 0.88 (table 1A).

When the deficit of H^+ transfer in antimycin-treated cytochrome bc_1 complex is corrected for H^+ released by eq. (1) and H^+/e^- ratio of 0.84 for redox Bohr effect in the Rieske Fe–S protein is obtained (table 1B). An H^+/e^- ratio of 0.97 can be computed for Bohr effects in the Rieske Fe–S protein from the

experiment in the absence of antimycin, when the observed oxidation of *b* cytochromes is corrected for the extent of effective hydrogen conduction by this carrier which is estimated to amount to 73% at pH 7.2 [34].

Acknowledgements

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