DEMONSTRATION OF THE ACTIVE AND THE SLUGGISH FORMS OF CYTOCHROME *b* IN ISOLATED *b*-c_1 COMPLEXES OF THE RESPIRATORY CHAIN

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1. Introduction

Previous studies on cytochrome *b* carried in our laboratory [1–4], showed the existence of a dynamic control mechanism, operating in mitochondria as well as in sonicated submitochondrial particles (ETP_H). A regulatory group, called 'Y' (to distinguish it from 'X' [3,5,6] participating in the thermodynamic control – the variation of the reducibility of cytochrome *b*), controls the rate of reduction of the *b*-type cytochromes. It was also shown that cytochrome *b* assumes alternatively one of its two kinetic forms, active (b_A) or sluggish (b_S). Cytochrome b_A is favoured by oxidized Y, while cytochrome b_S predominates when Y is reduced [1]. Redox titration and inhibition studies deliniates Y from any recognized components of the respiratory system. Y is a comparatively slowly reacting component [1] located between the antimycin and 2-heptyl-4-hydroxyquinoline *N*-oxide (HOQNO) inhibition sites [3] with a midpotential higher than that of cytochrome *c* [2].

In the present study we looked for this control mechanism in complex (II + III) or complex III, so to determine whether the regulatory function is an integral part of the respiratory chain.

2. Materials and methods

Succinate-cytochrome *c* reductase (complex II + III) was made of fresh beef-heart mitochondria [7] by the method of Yamashita and Racker [8]. The cholate was removed by dialyzing for 24 h (4°C) against 200 vols of 0.1 M phosphate buffer pH 7.4. Complex III was prepared from these particles as described by King [9].

Durohydroquinone (DQH_2) was prepared by reducing duroquinone in slightly acidic ethanol (2% v/v glacial acetic acid) by aqueous solution of $NaBH_4$; the reduction was carried only to 85–90% so as to ensure absence of $NaBH_4$. Kinetic analyses was carried as described before [1]. Protein was determined by the Biuret method [10].

3. Results and discussion

To test the operation of the dynamic control mechanism, it is best to compare the kinetics of the reduction of cyt. *b* under the two extreme conditions, when cyt. *b* is completely in either the active or the sluggish form. For the first case, Y should be kept oxidized and for the second case Y is pre-reduced Thus, we studied the rate of reduction of cyt. *b* of complex (II + III) or complex III, in presence of antimycin (plus O_2) or after pretreatment with ascorbate plus *NNN'N'*-tetramethy *p*-phenylendiamine (TMPD).

The succinate cytochrome *c* reductase preparation contained cytochrome *b* and c_1 1.5 and 0.9 nmol/mg respectively, with specific activity (measured according to Tisdal [11]) of 0.34 μmol $min^{-1} \times mg^{-1}$ at room temperature. Similar values were obtained by other groups [12]. The cyt. *c* reduction was complete-

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ly blocked by antimycin (0.36 nmol/mg) or by HOQNO (13.4 μM). No spectral evidence for cyt. *c* or $a + a_3$ was observed. Yet, by addition of KCN (2 mM) the rate of reduction of external cyt. *c* was slightly enhanced (10%) and the amount cyt. c_1 aerobically reduced by succinate, was increased 3-fold. Thus we have to conclude that the preparation was not free of traces of cytochrome oxidase. It should be emphasized that 3 mM ascorbate plus 50 μM TMPD kept cyt. c_1 in a fully reduced state. Thus this treatment is justified for reduction of Y.

Typical results are given in table 1. The first line demonstrates that in the presence of antimycin and O_2 the reduction of cyt. *b* is rapid and that the rate constant (measured at 2°C) is comparable to that obtained for submitochondrial particles under similar conditions [1]. Pretreatment with ascorbate plus TMPD, changed the reduction rate to a value typical for cyt. b_S [1,3]. The same was observed for reduction by DQH_2. The reduction of cyt. b_A is extremely rapid, instantanous with respect to our instrumentation. The reaction of cyt. b_S is slow and easily followed at 24°C. Consequently we used the 100-fold difference in the rate of reduction of b_A vs. b_S by DQH_2 as a diagnostic test for the existence of the active and sluggish forms of cyt. *b* in complex III.

Complex III: the amount of native cyt. *b* in complex III was determined by reduction with $Na_2S_2O_4$ and bleaching the denatured cyt. *b* by CO [13]. In complex III, only 59% of cyt. *b* was native, but all of the fraction reduced by DQH_2 was native. This fraction, as determined by absorbance spectrum, was cyt. b_{561}. The rest of cyt. *b* could be reduced by DQH_2 in a transient way upon addition of $K_3Fe(CN)_6$ [5,14,15]. As shown in the third line in table 1 and also demonstrated in fig.1, curve B, the addition of DQH_2 caused an immediate reduction of cyt. *b*. Treatment with ascorbate plus TMPD, brought a slow reduction of cyt. *b* (distinguishing it from complex (II + III)) but after this fraction was reduced, addition of DQH_2 caused a further reduction of cyt. *b* – in a very slow reaction (fig.1, curve A). Thus the ascorbate-TMPD treatment did transform b_A to b_S in isolated complex III.

In order to ascertain the above conclusion we looked also for the reversibility of this transition. In the submitochondrial particles, the $b_S \rightarrow b_A$ transition was achieved by adding $K_3Fe(CN)_6$ [3]. This

Table 1
Kinetic parameters of the reduction of cyt. *b* in complex (II + III) and complex III. The reduction was followed at 563–675 nm and the rate constants were calculated as described before [1]

Batch	Reagents in the solution			Antimycin only		Antimycin+ascorbate + TMPD	
	Experimental conditions						
	substrate	complex type	temp. °C	$k(sec^{-1})$	ΔA	$k(sec^{-1})$	ΔA
1[ac]	Succinate (6.7 mM)	II + III	2	0.16	0.0135	0.027[ef]	0.0133
2[bc]	DQH_2 (0.5 mM)	II + III	24	>>0.6	0.0050	0.018[fh]	0.0045
3[ad]	DQH_2 (0.5 mM)	III	0.5	>>0.6	0.0084	0.004[eg]	0.0048

[a] 0.18 M Sucrose, 50 mM Tris Acetate, 5 mM $MgSO_4$ pH 7.4.
[b] 0.1 M K phosphate buffer pH 7.4.
[c] 1 mg protein/ml.
[d] 0.75 mg protein/ml.
[e] 3 mM ascorbate.
[f] 1.5 mM ascorbate.
[g] 50 μM TMPD.
[h] 30 μM TMPD.

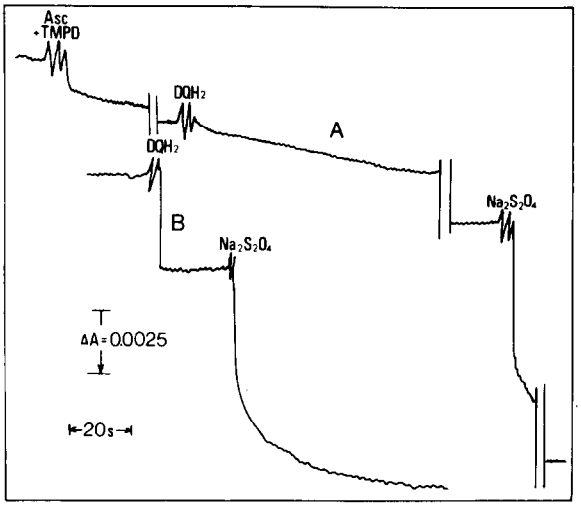

Fig.1. The reversibility of $b_S \rightarrow b_A$ transition in complex III. (A) Complex III vesicles in 0.18 M sucrose, 5 mM $MgSO_4$ 50 mM Tris acetate pH 7.4, 1 mg protein/ml were treated by KCN (2 mM) and antimycin (4 nmol/mg). Ascorbate (300 μM) and TMPD (50 μM) were added as indicated, followed by DQH_2 (400 μM) and $Na_2S_2O_4$. The reaction was carried at 23°C and followed at 561.5–575 nm. (B) Complex III vesicles were treated batchwise by KCN, antimycin ascorbate and TMPD as described in (A). After 1 min (23°C) the ascorbate was oxidized by titration with $K_3Fe(CN)_6$ till blue colour of TMPD reappeared. The vesicles were spun down, resuspended in the same buffer, supplemented by KCN (2 mM) and antimycin (4 nmol/mg) and the reduction by DQH_2 followed as before. The total reduction by $Na_2S_2O_4$ of sample (B) is somewhat smaller compared to (A). This is because of some loss in complex III during centrifugation.

method is not applicable in the present case; unlike mitochondria or ETP_H where, due to presence of O_2, cyt. b is fully reducible in presence of antimycin [6, 14,16–18]. With complex III, O_2 cannot act as an oxidant and $K_3Fe(CN)_6$ must be used to induce full reduction of cyt. b_{565} [5,6,14,19]. Consequently, addition of $K_3Fe(CN)_6$ in our case will yield a complex response reflecting both kinetic and thermodynamic control. To overcome this difficulty, we divided the experiment in two steps. Complex III, treated with ascorbate plus TMPD to convert it to its b_S form, (as in fig.1A), was titrated by $K_3Fe(CN)_6$ untill the blue colour of the oxidized TMPD appeared. The complex III vesicles were sedimented by centrifugation, and the reduction of cyt. b was measured in presence of KCN and antimycin. In this case DQH_2 caused an immediate reduction of cyt. b_{561} (fig.1, line B) indicating that the sluggish form was reversed to the active one.

It is evident that the control group Y is tightly bound to the respiratory carriers and follows the b–c_1 segment of it. Furthermore, the operation of the dynamic control in complex III with DQH_2 as reductant eliminates the possibility that the mechanism of this control functions via CoQ. Thus the dynamic control is not a consequence of some partial reactions of the quinone cycle [20].

The results described above are in accord with Trumpower and Katki [21], who reported an inhibitory effect of ascorbate on the reduction of cyt. b in succinate cytochrome c reductase particles, a phenomenon identical to the dynamic control described before [1–4]. We wish to point out that though qualitatively their results are similar to ours, there are some quantitative discrepencies, especially the rate of reduction of the sluggish form, 0.0005 sec^{-1} in their case and 0.027 sec^{-1} in this study. Presently, there is no explanation of this 60-fold difference but, it might be due to the presence of contamination by cytochrome oxidase in their preparation. As mentioned in our case the steady state reduction of cyt. c by succinate was only 30% of that obtained in presence of KCN, a control missing in Trumpower and Katki experiment. A continuous leak of electrons to oxygen might cause a major change in the observed rate constant of reduction of b_S which is inherently slow. Being aware of this, 2 mM KCN was present in all of our studies. On the other hand its absence in the studies of Trumpower and Katki studies might lead to underestimation of the rate of reduction of b_S.

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