Biochimica et Biophysica Acta, 637 (1981) 551–554
Elsevier/North-Holland Biomedical Press

BBA Report

BBA 41348

THE TRIPHASIC REDUCTION OF CYTOCHROME *b* IN THE SUCCINATE-CYTOCHROME *c* REDUCTASE

YUAN-ZHEN JIN [a], HAI-LUN TANG [b], SHU-LIAN LI [a] and CHEN-LU TSOU [a,*]

[a] *Institute of Biophysics, Beijing and* [b] *Shanghai Institute of Biochemistry, Academia Sinica, Shanghai (China)*

(Received March 12th, 1981)

Key words: Cytochrome b; Succinate-cytochrome c reductase; (Kinetics)

In the succinate-cytochrome *c* reductase, the reduction of cytochrome *b* has been found to be triphasic: an initial rapid partial reduction was followed first by a rapid oxidation and then finally by a slow reduction. The initial reduction of cytochrome *b* was faster than that of cytochrome c_1 and the final slow reduction of cytochrome *b* began when cytochrome c_1 reduction was approaching completion. In presence of the inhibitors antimycin A or HQNO the reduction of cytochrome *b* became monophasic. Hysteresis or a kinetic cooperative effect of a factor controlling cytochrome *b* oxidation has been suggested as a possible explanation for the triphasic reduction of cytochrome *b*.

Although the reduction and oxidation of cytochrome *b* have been extensively studied over a period of years, its specific role in the electron transfer through the ubiquinol-cytochrome *c* segment of the respiratory chain is still far from clear [1]. Chance [2] reported that the reduction of cytochrome *b* followed biphasic kinetics. In recent years, both King and co-workers [3] as well as Eisenbach and Gutman [4] studied this biphasic reduction and the latter authors suggested that the rate of reduction of cytochrome *b* is controlled by the oxidation-reduction level of an as yet unidentified factor. In the present paper, we report that the reduction of cytochrome *b* in the isolated succinate-cytochrome *c* reductase followed a triphasic course of reaction.

Succinate cytochrome *c* reductase was prepared from pig heart muscle preparations using the method described by Yu et al. [5]. It was kept in solid CO_2 and used within 1 week. The reduced minus oxidized spectrum and the contents of various components of this preparation were essentially the same as that obtained by Yu et al. [5] for the preparation from bovine heart. The rapid reduction and oxidation of cytochromes *b* and c_1 were followed in a Dionex stopped-flow apparatus at 562 and 553 nm, respectively. The enzyme preparation in phosphate buffer, pH 7.4, and the substrate, succinate, and inhibitor if present, also in the same buffer were placed in separate syringes before mixing. Spectral changes in time intervals longer than a few seconds were followed in a Hitachi 556 dual-wavelength spectrophotometer by repeated scanning between 575 and 545 nm. The reference and the sample cuvettes both contained 2 ml of the reductase at the same concentration as that for the stopped-flow experiments. After a baseline was recorded, the same volume of water and succinate was added, respectively, to the reference and sample cuvettes. Repeated scanning was started as soon as the substrate was added. The first scan of the reduced minus oxidized difference spectrum was usually obtained about 2.5 s after mixing and each scan took about 5 s. In some experiments, changes in absorbance at 562 nm with 575 nm as a reference

* To whom correspondence should be addressed.
Abbreviation: HQNO, 2-(*n*-heptyl)-4-hydroxyquinoline-*N*-oxide.

wavelength were also recorded and essentially the same results were obtained.

The initial stages of reaction of cytochromes *b* and c_1 after mixing of succinate with the enzyme preparation were followed by the increase in absorbance at 562 and 553 nm, respectively, in the stopped-flow apparatus. Their rates of reduction depended on the concentration of succinate added. At a succinate concentration (0.5 μM) of the same order of magnitude as those of cytochromes *b* and c_1, it can be seen from Fig. 1 that cytochrome *b* was reduced first, reaching a maximum value after about 5–10 s and then slowly oxidized, whereas cytochrome c_1 was gradually reduced while cytochrome *b* was being oxidized.

At higher succinate concentrations, a triphasic oxidation-reduction change of cytochrome *b* was unexpectedly observed. Fig. 2 shows the results obtained with a succinate concentration of 12.5 μM. Cytochrome *b* was again reduced faster than cytochrome c_1, reaching a maximum value at about 0.2 s. It was then oxidized while cytochrome c_1 was being reduced. After the reduction of cytochrome c_1 had nearly reached completion, cytochrome *b* began to be reduced again but at a slower rate than that in the initial phase. The results for still higher succinate concentrations were similarly triphasic but at a faster rate; the initial two phases lasted only 50 ms at 1 mM succinate.

Repeated spectral scanning from 545 to 575 nm under similar conditions showed spectral changes entirely consistent with the results presented in Figs. 1 and 2. The reduction of cytochromes *b* and c_1 by 12.5 μM succinate is shown in Fig. 3. Cytochrome c_1 was almost completely reduced as soon as observation commenced which was about 3 s after mixing. On the other hand, cytochrome *b* was reduced at a much slower rate, approaching a constant value in about 30 s. The final levels of reduction of the cytochromes by a high succinate concentration (25 mM) and by dithionite are also shown in Fig. 3. About 40% of the total cytochrome *b* present

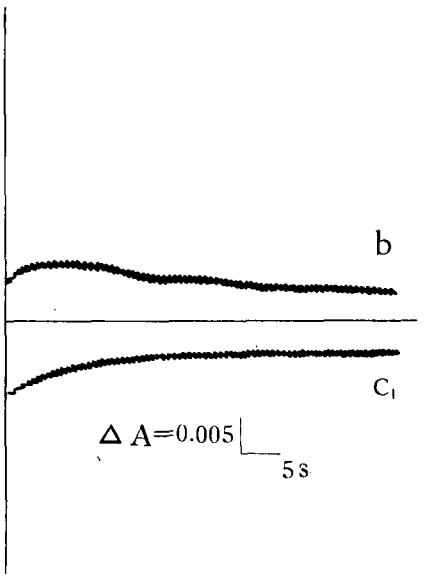

Fig. 1. The partial reduction of cytochromes *b* (b) and c_1 (c_1) at low succinate concentration. The reduction of cytochromes *b* and c_1 in the succinate-cytochrome *c* reductase preparation was followed at 562 and 553 nm, respectively, in the stopped-flow apparatus with a light path of 20 mm. Syringe 1 contained the enzyme and syringe 2 contained the substrate in the same buffer. The final concentrations of cytochromes *b* and c_1 were 2.3 and 1.1 μM, rspectively. Other concentrations were: succinate, 0.5 μM; phosphate buffer, pH 7.4, 70 mM; EDTA, 0.3 mM and sucrose, 0.23 M (23°C).

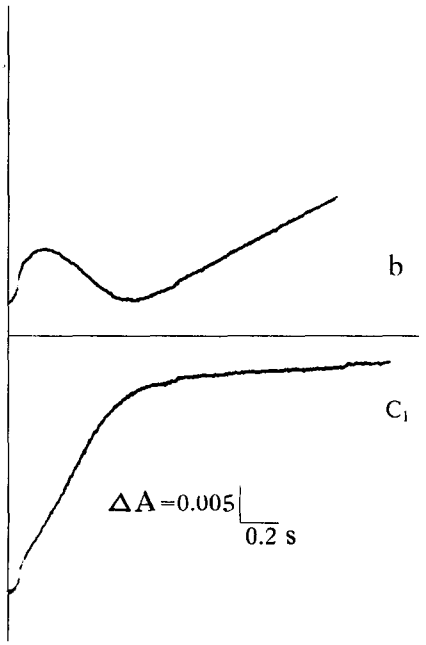

Fig. 2. The initial stages of the reduction of cytochromes *b* (b) and c_1 (c_1). Experimental conditions as in Fig. 1 except the final concentration of succinate was 12.5 μM.

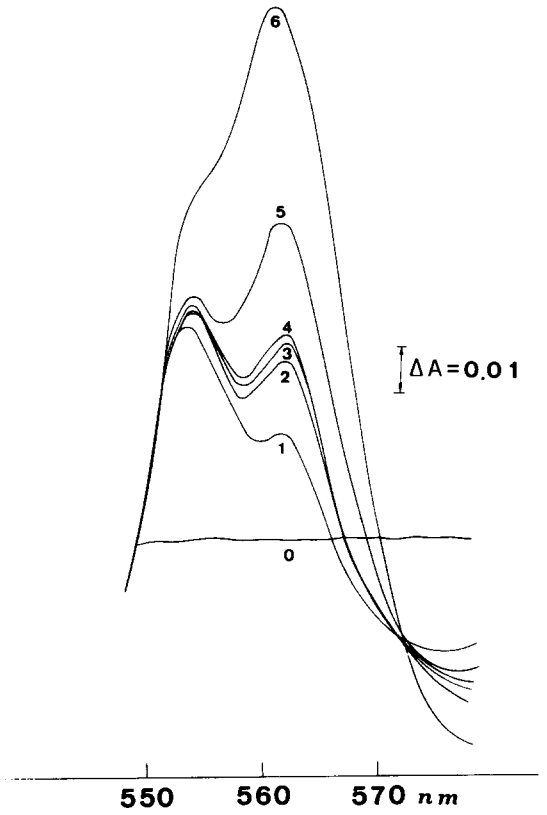

Fig. 3. Repeated spectral scan of the reduction of cytochromes *b* and c_1 by succinate. Experimental conditions as described in the text. Reaction mixture as in Fig. 2 except the final concentrations of cytochromes *b* and c_1 were 5.8 and 3.2 μM, respectively. Curves 0, 1, 2, 3 and 4 represent, respectively, the baseline and scanning 3, 7.5, 12 and 31 s after mixing with 12.5 μM succinate to start the reaction. Curve 5 represents the scan after reduction with 25 mM succinate and curve 6 after reduction with dithionite.

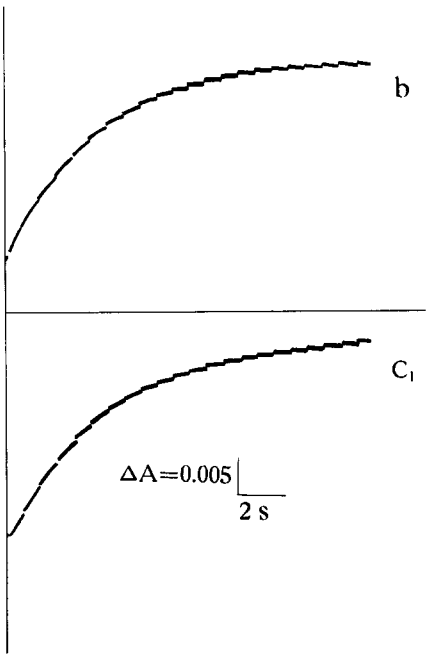

Fig. 4. Effect of antimycin A on the initial stages of the reduction of cytochromes *b* (b) and c_1 (c_1). The reaction mixture contained: cytochrome *b*, 2.8 μM; cytochrome c_1, 1.4 μM; succinate, 12.5 μM; antimycin A, 2.8 μM added together with succinate; KCN, 2 mM; phosphate buffer, pH 7.4, 70 mM; EDTA, 0.3 mM and sucrose, 0.23 M (23°C).

were reducible only by dithionite and not by succinate. The addition of KCN (2 mM) to the reaction mixture had little effect on the results in the experiments described above.

Antimycin A is a well known inhibitor of the respiratory chain with a site of action located between cytochromes *b* and c_1. The effect of antimycin A on the triphasic reaction of cytochrome *b* was examined. With increasing molar ratios of antimycin A/cytochrome *b*, the magnitude of the initial absorbance change in the initial reduction and oxidation phases became smaller and smaller until at a molar ratio of 1 (Fig. 4) the reduction of cytochrome *b* became monophasic. KCN (2 mM) was also present in the above reaction mixture. HQNO is another inhibitor believed to act in the same region [6] but at a different site [4]. In our hands, this inhibitor also caused the first and second phases of cytochrome *b* reduction and oxidation to disappear as shown in Fig. 5.

Since a dual-wavelength stopped-flow apparatus is not available to us, it has to be first ascertained that the triphasic nature of the cytochrome *b* reaction was not an artifact due to a faulty mixing device of the instrument. This possibility can be excluded by the fact that the reduction of cytochrome c_1 was always monophasic. Furthermore, traces of the changes in absorbance at both 562 and 575 nm as shown on the oscilloscope could be reproduced quite satisfactorily on repeated actuation and the calculated $A_{562-575}$ traces showed essentially the same shape as those at 562 nm alone. A triphasic reduction of cytochrome *b* was also reported with a preparation of cytochromes *b* and

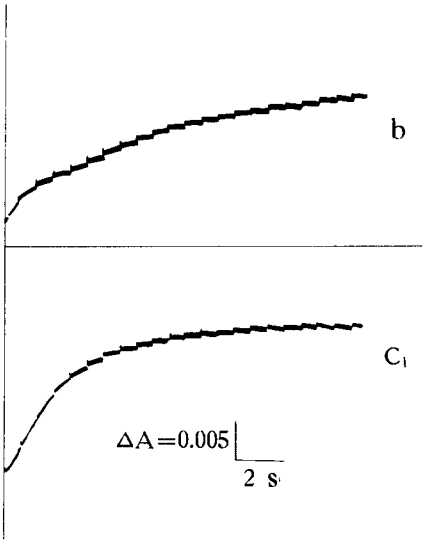

Fig. 5. Effect of HQNO on the initial stages of the reduction of cytochromes *b* (b) and c_1 (c_1). Experimental conditions as in Fig. 4 except HQNO, 2.8 μM was added instead of antimycin A.

c_1 and added succinic dehydrogenase by Yu et al. [7], however, their reaction was two orders of magnitude slower as compared to ours.

During the course of reduction of the cytochromes, their reduction state depend on the difference of their rates of reduction and oxidation. The occurrence of an oxidation phase would require that during this phase, the oxidation rate of cytochrome *b* actually exceeds its reduction rate. This could come about either by a marked increase in its oxidation rate, by a marked decrease in its reduction rate or both. A slower reduction rate was actually observed later but the observed oxidation rate of cytochrome *b* in the second phase was almost as fast as its overall reduction rate in the initial phase. A decrease of the reduction rate to the level of that in the third phase does not seem to be enough to account for the observed oxidation rate in the second phase. We would like, therefore, to suggest that the rate of oxidation of cytochrome *b* increases greatly after an initial lag phase or, in other words, the kinetics of cytochrome *b* oxidation show a positive cooperative or hysteretic effect [8]. The initial phase of rapid reduction of cytochrome *b* takes place during the lag phase of its oxidation, then its oxidation rate begins to increase and finally exceeds that for its reduction which has by this time slowed down somewhat. Both these changes contribute to the appearance of a transient oxidation phase of cytochrome *b*. Finally, since the passage of electrons to oxygen is blocked in the succinate-cytochrome *c* reductase, cytochrome *b* begins to be reduced again in the third phase when the reduction of cytochrome c_1 is nearing completion.

Previous authors working on the biphasic nature of cytochrome *b* reduction have postulated various factors controlling the electron flow through cytochrome *b*. Eisenbach and Gutman [9] postulated a factor which controlled the oxidation of cytochrome *b* and suggested that antimycin A and HQNO acted, respectively, before and after this factor. Although our results are in agreement with the suggestion that both antimycin A and HQNO inhibit the oxidation of cytochrome *b*, they seem to act at the same site.

If the change in oxidation rate is indeed involved in the triphasic reaction of cytochrome *b* described in the present paper, it should be recalled that both the kinetic cooperative model and the hysteretic model would require a conformational change of the factor controlling the oxidation of cytochrome *b*.

The authors wish to thank Professor E.C. Slater for many helpful suggestions and for sending preprints of his paper before publication. We also thank Professor T.E. King for his interest and Messrs. J.X. Xu, Q.S. Zhu and H.X. Wang for taking part in some of the experiments.

References

1 Von Jagow, G. (1980) Annu. Rev. Biochem. 49, 282–314
2 Chance, B. (1958) J. Biol. Chem. 233, 1233–1229
3 King, T.E., Yu, C.A., Yu, L. and Chiang, Y.L. (1975) in Electron Transfer Chain and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E.C. and Siliprandi, N., eds.), pp. 105–118, Elsevier, Amsterdam
4 Eisenbach, M. and Gutman, M. (1975) Eur. J. Biochem. 52, 107–116
5 Yu, C.A., Yu, L. and King, T.E. (1974) J. Biol. Chem. 249, 4905–4910
6 Van Ark, G. and Berden, J.A. (1977) Biochim. Biophys. Acta 458, 119–137
7 Yu, C.A., Nagoaka, S., Yu, L. and King, T.E. (1980) Arch. Biochem. Biophys. 204, 59–70
8 Ricard, J., Meunier, J.-C. and Buc, J. (1974) Eur. J. Biochem. 49, 195–208
9 Eisenbach, M. and Gutman, M. (1975) Eur. J. Biochem. 59, 223–230