Biochimica et Biophysica Acta, 682 (1982) 315–321
Elsevier Biomedical Press

BBA 41187

MULTIPHASIC OXIDATION-REDUCTION OF CYTOCHROME *b* IN THE SUCCINATE-CYTOCHROME *c* REDUCTASE

CHEN-LU TSOU [a], HAI-LUN TANG [b], DA-CHENG WANG [a] and YUAN-ZHEN JIN [a]

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

(Received June 4th, 1982)

Key words: Cytochrome b reduction; Succinate-cytochrome c reductase; Respiratory chain

The triphasic course previously reported for the reduction of cytochrome *b* in the succinate-cytochrome *c* reductase by either succinate or duroquinol has been shown to be dependent on the redox state of the enzyme preparation. Prior reduction with increasing concentrations of ascorbate leads to partial reduction of cytochrome c_1, and a gradual decrease in the magnitude of the oxidation phase of cytochrome *b*. At an ascorbate concentration sufficient to reduce cytochrome c_1 almost completely, the reduction of cytochrome *b* by either succinate or duroquinol becomes monophasic. Owing to the presence of a trace amount of cytochrome oxidase in the reductase preparation employed, the addition of cytochrome *c* makes electron flow from substrate to oxygen possible. Under such circumstances, the addition of a limited amount of either succinate or duroquinol leads to a multiphasic reduction and oxidation of cytochrome *b*. After the initial three phases as described previously, cytochrome *b* becomes oxidized before cytochrome c_1 when the limited amount of added substrate is being used up. However, at the end of the reaction when cytochrome c_1 is being rapidly oxidized, cytochrome *b* becomes again reduced. The above observations support a cyclic scheme of electron flow in which the reduction of cytochrome *b* proceeds by two different routes and its oxidation controlled by the redox state of a component of the respiratory chain.

Introduction

It has been reported in previous papers [1,2] that the reduction of cytochrome *b* in succinate-cytochrome *c* reductase by succinate or duroquinol follows a triphasic course, i.e., an initial rapid reduction followed by a rapid oxidation and finally a slow reduction phase which begins when the reduction of cytochrome c_1 is approaching completion. It has also been proposed that the second, oxidation phase is primarily due to a kinetic hysteretic effect of a factor which controls the oxidation of cytochrome *b*.

During their studies on the biphasic course of cytochrome *b* reductions, Eisenbach and Gutman [3–5] and Van Ark et al. [6] have produced evidence to show that the redox level of an intermediate controls the biphasic nature of cytochrome *b* reduction. That the redox status of cytochrome c_1 or an unknown factor controls the reduction of cytochrome *b* has also been proposed by Trumpower and Katki [7]. During our study on the triphasic reduction of cytochrome *b*, it has been our experience that although the triphasic course of cytochrome *b* reduction is quite reproducible, the height of the initial jump varies from preparation to preparation. This appears to be related to the presence of endogenous reducing substances in the enzyme preparations as indicated by the appearance of reduced cytochrome c_1 to various ecxtents in different preparations before the addition of substrates.

It is now shown in the present paper that the triphasic nature of the course of cytochrome *b*

0005-2728/82/0000–0000/$02.75

reduction indeed depends on the redox state of the enzyme preparation. Furthermore, in the presence of a small amount of added cytochrome *c*, cytochrome *b* follows an even more complex, multiphasic course of oxidation and reduction.

Materials and Methods

The succinate-cytochrome *c* reductase [8], cytochrome *c* and duroquinol used were all described as before [1,2]. Sodium ascorbate and potassium ferricyanide were local products of analytical grade used without further purification.

The reaction system, unless otherwise specified, always contained 70 mM phosphate buffer, pH 7.4; 0.23 M sucrose, 0.3 mM EDTA, and other additions as indicated. Spectral measurements were carried out in a Cary 219 Spectrophotometer. For reductive titrations of the enzyme preparations, the same enzyme solution were placed in the reference and sample beams, respectively, and a baseline was scanned. A small volume of ferricyanide solution was then added to the cuvette in the reference beam to a final concentration of 4 μM, the same volume of water added to the sample cuvette and the spectrum scanned. Occasionally, a small peak at 553 nm was observed to indicate the partial reduction of cytochrome c_1. Different amounts of sodium ascorbate were then added to the sample cuvette to obtained enzyme preparations reduced to different extents. After the difference spectrum was recorded, the enzyme was directly used in the stopped-flow experiments carried out essentially as described previously [1,2]. When cytochrome *c* was also present, it was added in the enzyme syringe.

Results

Effect of sodium ascorbate on the triphasic reduction of cytochrome b

The effect of the addition of different amounts of sodium ascorbate to the succinate-cytochrome *c* reductase preparation on the reduced-oxidized difference spectra was compared with its effect on the triphasic nature of cytochrome *b* reduction as followed at 562 nm (Fig. 1). The enzyme preparation used in the experiments shown in Fig. 1 appeared to be fully oxidized originally as the addition of

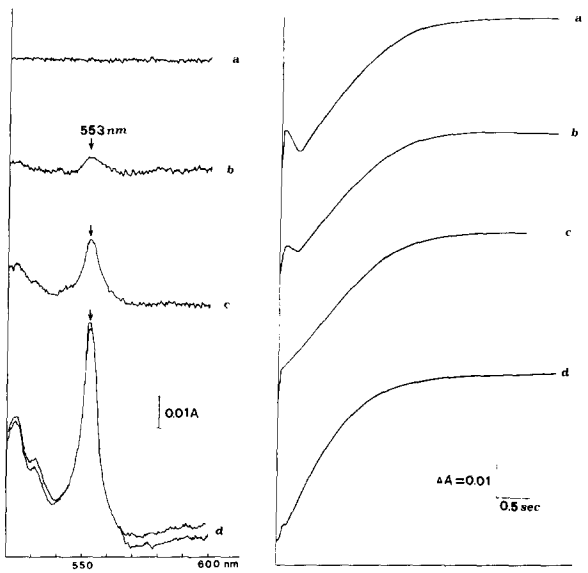

Fig. 1. Effect of sodium ascorbate on the initial course of cytochrome *b* reduction. The reduced-minus-oxidized spectra are shown on the left and the corresponding kinetic traces on the right. Concentrations of cytochrome *b* and c_1 were 7.1 and 3.4 μM, respectively, during spectral scan. The reductase after each addition of ascorbate was then mixed with an equal volume of the substrates, 50 μM duroquinol in the same buffer solution, to give the corresponding kinetic trace on the right. For the difference spectra shown in d, the preparation giving the lower spectrum was used in the kinetics trace. The upper spectrum was obtained after addition of an excess of solid sodium ascorbate. For details, see text.

ferricyanide produced no appreciable change. When ascorbate was added to produce approx. 10% reduction of cytochrome c_1, the triphasic nature of cytochrome *b* reduction was already much weakened and when a little more sodium ascorbate was added so that the reduction of cytochrome c_1 was about 30%, cytochrome *b* reduction became apparently biphasic. Finally, when sodium ascorbate was further added to reduce cytochrome c_1 nearly completely cytochrome *b* reduction was essentially monophasic with a rate approaching that for the slow reduction phase in its triphasic course of reduction.

Effect of substrate concentration on the reaction rates

With the enzyme preparation in the fully oxidized state, the effects of substrate concentration on the reaction rates of cytochrome *b* during the three different phases as well as the reduction

rate of cytochrome c_1 were examined. The results with duroquinol as the substrate are shown in Fig. 2. The rates corresponding to all three phases increased with duroquinol concentration approaching maximal values at 400 μM. It should also be noted that the initial reduction rate of cytochrome *b* was by far the fastest of the reactions and the third phase reduction rate the slowest.

Similar results were obtained with succinate as the substrate.

Effect of added cytochrome c on the reduction of cytochromes b and c_1

Our preparation of the succinate-cytochrome *c* reductase contained a little cytochrome oxidase but was completely devoid of cytochrome *c* and the oxidation of the reduced components was therefore blocked without added cytochrome *c*. However, with the addition of cytochrome *c*, some, although very weak, succinate oxidase activity could be observed. It therefore appears that with the fairly concentrated enzyme preparation employed, the addition of cytochrome *c* would lead to the complete oxidation of any intermediate originally in a partially reduced state. It can be seen from Fig. 3 that the addition of cytochrome *c* led

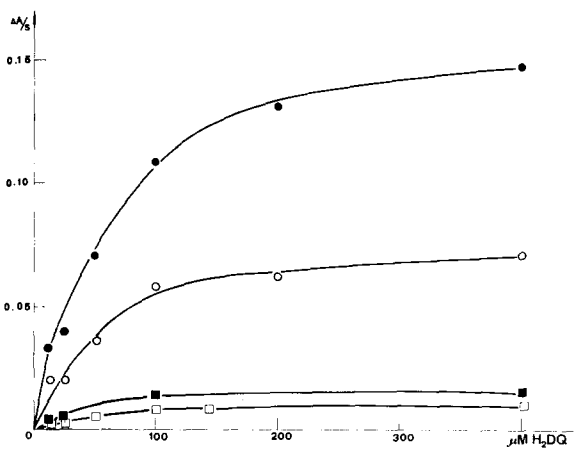

Fig. 2. Effect of duroquinol concentration on the reduction rates of cytochromes *b* and c_1. Reaction rates were followed in the Dionex stopped-flow apparatus at 24°C. The reaction mixture contained 2.1 mg protein/ml and final concentrations of cytochromes *b* and c_1 were 3.57 and 1.73 μM, respectively. (●) Initial rapid reduction of cytochrome *b*; (■) second oxidation phase of cytochrome *b*; (□) final reduction phase of cytochrome *b*; (○) reduction of cytochrome c_1.

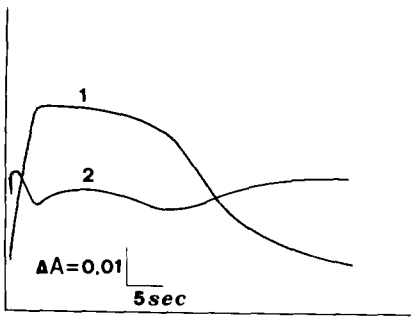

Fig. 3. Multiphasic oxidation-reduction of cytochrome *b* in the presence of added cytochrome *c* during the oxidation of succinate. The reaction mixture contained 1.84 mg succinate-cytochrome *c* reductase/ml and final concentrations of cytochromes *b* and c_1 were 3.67 and 1.71 μM, respectively. The reaction mixture also contained 12.5 μM succinate and added cytochrome *c* to a final concentration of 0.31 μM. The rate of succinate oxidation by oxygen in the presence of added cytochrome *c* was about 1/30 the rate of oxidation by cytochrome *c*. In the absence of added cytochrome *c* no succinate oxidase activity could be detected. Lines 1 and 2 represent tracings at 553 and 562 nm, respectively.

to a multiphasic reduction-oxidation of cytochrome *b* with succinate as the substrate. The initial phases of reactions of the cytochromes were as in the case of the reaction without added cytochrome *c*. However, after both cytochromes *b* and c_1 were reduced in the third reduction phase of cytochrome *b*, the latter was again oxidized followed by the oxidation of c_1. Finally, concurrent with the rapid oxidation of cytochrome c_1, the A_{562} trace increased again, suggesting the reduction of cytochrome *b*. Similar results were obtained with duroquinol as the substrate as are shown in Fig. 4a and b. After the initial reduction-oxidation and reduction phases, cytochrome *b* began to be gradually oxidized while cytochrome c_1 still remained in the reduced state. The final increase in A_{562} again coincided with the oxidation of cytochrome c_1. It has also been shown that both the height and the length of the middle reduction phase increased with increasing substrate concentration. As our enzyme preparation contained some cytochrome oxidase activity, electron flow to oxygen was made possible with added cytochrome *c*. The oxidation of both cytochromes was most probably due to the exhaustion of the limited amount of substrates originally added. Repeated spectral scan experi-

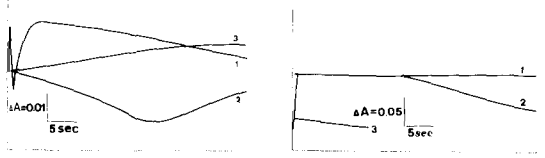

Fig. 4. Multiphasic oxidation-reduction of cytochrome *b* with added cytochrome *c* during the oxidation of duroquinol. Final concentrations of cytochrome *b* and cytochrome c_1 originally present in the enzyme preparation were 3.1 and 1.7 μM, respectively. Duroquinol, 12.5 μM; added cytochrome *c* 1.55 μM. (a) A_{562} tracings, (b) A_{553} tracings. Lines 1, 2 and 3 are sequential tracings of the same experiment on the oscilloscope.

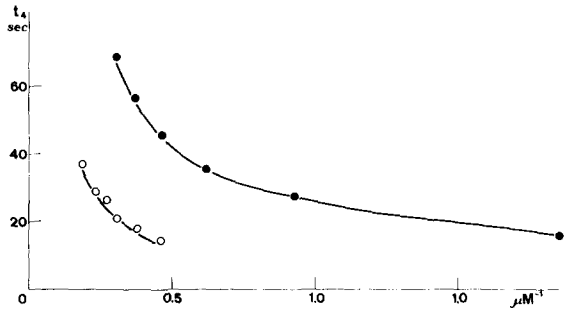

Fig. 5. Effect of added cytochrome *c* on the length of the middle reduction phase of cytochrome *b*. Final concentrations of cytochromes *b* and c_1 were 3.67 and 1.71 μM, respectively. Succinate concentrations were (●——●) 25 μM and (○——○) 12.5 μM.

ments were also carried out at a lower temperature (11°C). The final reduction of cytochrome *b* taking place concurrently with the rapid oxidation of cytochrome c_1, as seen in the increase in A_{562} with the stopped-flow apparatus, was confirmed by the appearance of an absorption peak at 562 nm in the repeated spectral scan experiments taken at 11°C.

Effect of cytochrome c concentration on the multiphasic reaction course of cytochrome b

Addition of cytochrome *c* at increasing concentrations to succinate-cytochrome *c* reductase preparations increased the rate of electron flow from either succinate or duroquinol to oxygen. The increased rate of substrate consumption appears to be responsible for the shortening of the middle reduction phase of cytochrome *b* as is also the case at a lower succinate concentration (Fig. 5). An increased rate of substrate oxidation at higher cytochrome *c* concentrations also leads to a lowering of the extent of cytochrome *b* reduction (Fig. 6). However, it should be pointed out that both the initial 'jump' and the final extent of reduction of cytochrome *b* were very much more marked at higher cytochrome *c* concentrations as is also shown in Fig. 6. Similar results were obtained with duroquinol as substrate. Not shown in Fig. 6, in all cases, is the final reduction phase of cytochrome *b* which always takes place at the same time as cytochrome c_1 is being rapidly oxidized.

This phenomenon is undoubtedly related to the well known oxidant-induced reduction of cytochrome *b* first reported by Erecinska et al. [9] in the presence of antimycin with a pulse of oxygen to oxidized the *c* cytochromes. Later, it has been shown that this linked reduction of cytochrome *b* also takes place in the succinate-cytochrome *c* reductase with an artificial oxidant such as ferricyanide in the absence of antimycin [10] or with cytochrome *c* plus cytochrome oxidase in the presence of antimycin [11]. In our experiments, a substrate pulse was used and the oxidation of cyto-

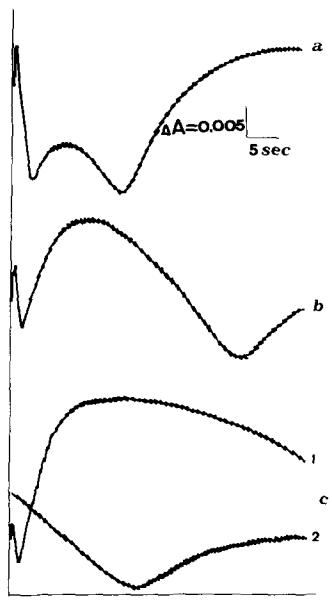

Fig. 6. Effect of added cytochrome *c* on the extent of reduction of cytochrome *b*. Concentrations of cytochrome *c* added were a, 6.85 μM; b, 0.62 μM; c, 0.31 μM. In c, a second lining on the oscilloscope was necessary to show the complete course of the reaction. The amount of succinate-cytochrome *c* reductase used was as for Fig. 5 and succinate concentration was 25 μM.

chrome c_1 through cytochrome *c* and cytochrome oxidase is again linked to cytochrome *b* reduction upon the exhaustion of substrate in the absence of an inhibitor such as antimycin.

It should also be noted from Fig. 6 that both the heights of the initial jump and the final level of reduction of cytochrome *b* increased with increasing concentrations of added cytochrome *c* and hence the overall rate of electron flow to oxygen.

Effect of antimycin A

We have reported in previous papers [1,2] that antimycin A abolishes the triphasic nature of the initial course of cytochrome *b* reduction by either succinate or duroquinol in the absence of added cytochrome *c*. The reduction of c_1 is hardly affected as has also been reported by Bowyer and Trumpower [12]. A monophasic course of reduction was observed in the presence of added cytochrome *c* as well as antimycin A for both cytochromes *b* and c_1. In the presence of added cytochrome *c*, the reduction rates of both cytochromes *b* and c_1 were noticeably inhibited by antimycin A without greatly affecting the final level of reduction reached. Upon prolonged reaction slow oxidation of both cytochromes was observed.

Discussion

Before a discussion on the multiphasic course of cytochrome *b* reduction, it would be appropriate to discuss first whether tracing of absorbance changes at a single wavelength, i.e., 562 nm, as is used in the present series of studies [1,2] could reflect accurately the oxidation-reduction state of cytochrome *b*. The usual practice has been the use of a dual-wavelength instrument with the wavelength pairs 562 vs. 575 nm [3–7]. In succinate-cytochrome *c* reductase preparations, the only species which absorb significantly in the 550–580 nm region are cytochromes *b* and c_1. Ideally, to follow the reduction of cytochrome *b*, an isosbestic point of the oxidized and reduced cytochrome c_1 absorption spectra should be chosen as the reference weavelength in the dual-wavelength measurements. However, the wavelength chosen, 575 nm, is quite some distance from such an isosbestic point [13] and the absorbance change at 562 nm vs. that at 575 nm as usually employed for the measurement of cytochrome *b* oxido-reduction would therefore include the oxido-reduction changes of cytochrome c_1. In a recent publication, Tervoort et al. [14] gave the absorbance coefficients of the cytochromes at relevant wavelength pairs in the reduced-minus-oxidized difference spectrum commonly employed for the calculation of oxidation-reduction of these cytochromes. The value they gave for the wavelength pair 562–577 nm for cytochrome c_1 was negligible compared either to the wavelength pair 553–540 nm for cytochrome c_1 or 562–577 nm for cytochrome *b*. However, although the reduction of cytochrome c_1 does lead to a slight decrease in absorbance at 562 nm [13,15], it is most unlikely that the observed oxidation phase of cytochrome *b* described in the present series of papers [1,2] could be due to the decrease in absorbance at 562 nm caused by the reduction of cytochrome c_1. The magnitude of the decrease in absorbance at this wavelength is usually much greater than would be expected by the reduction of cytochrome c_1 within the same time period. Moreover, the absorption spectra of oxidized and reduced cytochrome c_1, as present in the succinate-cytochrome *c* reductase preparation, might be slightly different from those of the purified pigment. Preliminary experiments have shown that addition of ascorbate causes very little absorbance change at 562 nm in the succinate-cytochrome *c* reductase. On the other hand, the reduction of cytochrome *c* would lead to a more marked decrease in absorbance at 562 nm and experiments with added cytochrome *c* at high concentrations may well contain significant contributions of the reduction of cytochrome *c* at this wavelength.

In their biphasic reduction of cytochrome *b*, Eisenbach and Gutman proposed that this cytochrome might exist in two states differing in their rates of reduction by succinate and the equilibrium between these two different forms of cytochrome *b* depending on the redox state of an unknown factor, Y. However, as a triphasic reduction course has now been observed, one has to assume that the rapidly reduced form of cytochrome *b* is also rapidly oxidized before its transformation to the slowly reducible form. The rate of the third reduction phase of cytochrome *b* is not greatly affected by the redox state of the enzyme complex.

As for the unknown factor whose redox state controls the multiphasic course of cytochrome *b* reduction, the following possibilities might be considered: the Rieske iron-sulfur protein [16,17], a bound form of ubiquinone [18,19] or cytochrome c_1. These constitute all the known redox components of the ubiquinol-cytochrome reductase complex [20]. In addition, Gutman [21] has also proposed that this control might be exerted not through a molecular entity but through a particular mitochondrial state. From the results obtained in the present study in the purified succinate-cytochrome *c* reductase complex devoid of a membrane system, it seems clear that even if a particular conformational state is responsible for the control of cytochrome *b* oxidation-reduction rates, it has to be brought about by the oxidation-reduction of a particular molecular entity.

Although the redox state of cytochrome c_1 has been shown to be related to the initial rapid reduction and oxidation phases of cytochrome *b* during succinate or ubiquinol oxidation, it does not necessarily mean that the triphasic behavior of cytochrome *b* is directly controlled by the redox state of cytochrome c_1 which may serve merely as an 'indicator' for the redox state of the factor responsible. Trumpower [18,22] has suggested that the iron-sulfur protein is involved in the reduction of cytochrome *b*, directly required for the oxidation of this cytochrome [17,23] and also takes part in the oxidant-induced reduction of cytochrome *b* [11]. Since a slight reduction of cytochrome c_1 produces a marked effect on the triphasic nature of cytochrome *b* reduction, the redox potential of iron-sulfur protein [17] would also place it in a more favorable position to play such a role than ubiquinone [18]. The involvement of the iron-sulfur protein does not necessarily exclude the participation of ubiquinone; both components may play complementary roles.

The triphasic reduction of cytochrome *b* still remains to be explained. It is interesting to note that both antimycin A and the prior reduction of an unknown factor abolish the triphasic nature of the reaction and the resulting monophasic reduction rate of cytochrome *b* corresponds to that for the third slow phase of cytochrome *b* reduction. It could be that the reduction of cytochrome *b* proceeds via two different routes as suggested by Trumpower [17,23], one antimycin sensitive the other insensitive as is depicted in a number of variations [6,12] of Mitchell's [20,21] cyclic scheme. It is possible that through this faster, antimycin-sensitive route of cytochrome *b* reduction an oxidant is being gradually accumulated and an intermediate gradually reduced resulting finally to oxidation and then a shift to the slower rate of reduction of cytochrome *b*.

Ubiquinone has been implicated in both of the routes for cytochrome *b* reduction [17,23]. The rapid phase 2 oxidation can be visualized as a reversal of the fast, antimycin-sensitive reduction. Both ubiquinone [17,18] and the semiquinone [6, 25] have been postulated as the oxidant of reduced cytochrome *b*. The initial rapid reduction of cytochrome *b* followed by rapid oxidation would seem to suggest the buildup of an oxidant. As evidence is accumulating to show the presence of different bound forms of ubiquinone, presumably with different proteins [18,19,26–28], it is conceivable that either the gradual accumulation of the semiquinone or its translocation from one protein to another or both might be responsible for the hysteretic behavior of cytochrome *b* oxidation [1,2].

Acknowledgements

We wish to thank Drs. B.F. Van Gelder, T.E. King and Y. Li for sending absorption spectra of oxidized and reduced cytochrome c_1.

References

1 Jin, Y.-Z., Tang, H.-L. Li, S.-L. and Tsou, C.-L. (1981) Biochim. Biophys. Acta 637, 551–554
2 Tang, H.-L. Jin, Y.-Z. and Tsou, C.-L. (1981) Biochem. Int. 3, 327–332
3 Eisenbach, M. and Gutman, M. (1974) FEBS Lett 46, 368–371
4 Eisenbach, M. and Gutman, M. (1975) Eur. J. Biochem. 52, 107–116
5 Eisenbach, M. and Gutman, M. (1975) Eur. J. Biochem. 59, 223–230
6 Van Ark, G., Raap, A.K., Berden, J.A. and Slater, E.C. (1981) Biochim. Biophys. Acta 637, 34–42
7 Trumpower, B.L. and Katki, A.G. (1975) Biochem. Biophys. Res. Commun. 65, 16–23
8 Yu, C.-A., Yu, L. and King, T.E. (1974) J. Biol. Chem. 249, 4905–4910
9 Erecinska, M., Chance, B., Wilson, D.F. and Dutton, P., L. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 50–54

10 Erecinska, M. and Wilson, D.F. (1972) FEBS Lett 24, 269–272
11 Bowyer, J.R., Edwards, C.A. and Trumpower, B.L. (1981) FEBS Lett. 126, 93–97
12 Bowyer, J.R. and Trumpower, B.L. (1981) J. Biol. Chem. 256, 2245–2251
13 König, B.W., Schilder, L.T.M., Tervoort, M.J. and Van Gelder, B.F. (1980) Biochim. Biophys. Acta 621, 283–295
14 Tervoort, M.J., Schilder, L.T.M. and Van Gelder, B.F. (1981) Biochim. Biophys. Acta 637, 245–251
15 Li, Y., Leonard, K. and Weiss, H. (1981) Eur. J. Biochem. 116, 199–205
16 Rieske, J.S. (1967) Methods Enzymol. 10, 357–362
17 Trumpower, B.L. (1981) Biochim. Biophys. Acta 639, 129–155
18 Trumpower, B.L. (1981) J. Bioenerg. Biomembranes 13, 1–24
19 Yu, C.-A. and Yu, L. (1981) Biochim. Biophys. Acta 639, 99–128
20 Mitchell, P. (1981) in Biomedical and Clinical Aspects of Coenzyme Q (Folkers, K. and Yamamura, Y., eds.), Vol. 3, pp. 3–15, Elsevier, Amsterdam
21 Gutman, M. (1980) Biochim. Biophys. Acta 594, 53–84
22 Trumpower, B.L and Katki, A.G. (1979) in Membrane Proteins in Energy Transduction (Capaldi, R.A., ed.), pp. 89–200, Marcel Dekker, New York
23 Edwards, C.A., Bowyer, J.R. and Trumpower, B.L. (1982) J. Biol. Chem. 257, 3705–3713
24 Mitchell, P. (1976) J. Theor, Biol. 62, 327–367
25 Wikstrom, M.K.F. and Berden, J.A. (1972) Biochim. Biophys. Acta 283, 403–420
26 King, T.E. (1978) FEBS Symp. 45, 17–31
27 De Vries, S., Berden, J.A. and Slater, E.C. (1980) FEBS Lett. 122, 143–148
28 De Vries, S., Albracht, S.P.J., Berden, J.A. Slater, E.C. (1981) J. Biol. Chem. 256, 11996–11998