Volume 233, number 1, 25–30 FEB 05898 June 1988

# The location of $Cu_A$ in mammalian cytochrome *c* oxidase

Peter R. Rich, Ian C. West and Peter Mitchell

*Glynn Research Institute, Bodmin PL30 4AU, England*

Received 5 April 1988

Imposition of a protonmotive force across the inner membrane of coupled cyanide-inhibited, beef heart mitochondria by addition of ATP causes reduction of cytochrome *c* and $Cu_A$ with concomitant oxidation of haem $a_A$. The data are consistent with previous demonstrations of an intramembrane location of haem $a_A$ but further indicate that $Cu_A$ is very close to the cytosolic surface of the membrane. The implications of this finding for electron transfer route and the site of the proton pumping chemistry are discussed.

Cytochrome *c* oxidase; Energisation; ATP effect; Midpoint potential; Proton pumping

## 1. INTRODUCTION

Although most groups now accept the proton pumping action of cytochrome *c* oxidase as originally demonstrated by Krab and Wikström [1], speculations as to its site and mechanism are extremely varied. $Cu_A$ [2,3], haem $a_A$ [4] (nomenclature of [5]), and the binuclear centre [3,6,7] have all been invoked as possible participants, in a range of direct [8] and indirect [9,10] chemiosmotic mechanisms [11].

Identification of the component(s) which contribute(s) towards the proton pumping action is complicated by the additional electrogenic reactions associated with oxygen reduction, a process which is thought to use electrons from cytochrome *c* and protons from the matrix space. Hinkle and Mitchell [12] demonstrated the effects of electric field on the apparent midpoint potential of haem $a_A$ in the presence of carbon monoxide and concluded that it was located approx. 50% through the dielectric barrier. It has been argued on the basis of kinetic measurements [13] that haem $a_A$ is the initial acceptor of electrons from cytochrome *c* and that $Cu_A$ might accept electrons from haem $a_A$. This would suggest that electron transfer between cytochrome *c* and $Cu_A$ should also be electrogenic. However, a recent model from known amino acid sequences and structural prediction methods suggests that $Cu_A$ is close to the docking site for cytochrome *c* [14]. This implies that $Cu_A$ ought to be the first acceptor of electrons from cytochrome *c*, an order consistent with the report that $Cu_A$ has a midpoint potential which is not affected by ATP in coupled pigeon heart mitochondria [15]. Location of $Cu_A$ relative to cytochromes *c* and *a* is relevant to those models which involve redox cycling of $Cu_A$ as the agent of proton pumping [2,3]. An electron transfer sequence of cyt *c* $\longrightarrow$ $Cu_A$ $\longrightarrow$ haem $a_A$ would argue a proton pumping role for $Cu_A$, since the pumping would cause a midpoint potential shift in haem $a_A$ on energisation which is far greater than the observed 40–50 mV [16] and might also be expected to cause an ATP-induced shift in the measured midpoint potential of $Cu_A$.

In this report we attempt to estimate the position of $Cu_A$ in relation to haems of cytochromes *c* and *a*, and conclude that dielectrically $Cu_A$ is within 10% of the cytosolic surface of the inner mitochondrial membrane. We further conclude that $Cu_A$ cannot be acting as a proton pump under the conditions of these experiments.

*Correspondence address:* P.R. Rich, Glynn Research Institute, Bodmin PL30 4AU, England

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*

## 2. MATERIALS AND METHODS

Coupled beef heart mitochondria were isolated from Nagarse-treated fresh beef heart muscle following a method for isolation from rat heart [17]. Their respiratory control index with succinate as substrate in a magnesium-free medium was typically between 2.5 and 3.8. Coupled rat liver mitochondria were prepared as described elsewhere [18].

Optical measurements were made with a single beam scanning spectrophotometer capable of serial sampling at multiple wavelengths and programmed so that any combination of single, double or triple wavelength records could be plotted. The time between measurement of sample and reference wavelengths was small compared to the rate of redox changes, as indicated by the fact that reversing the order of wavelength measurements made insignificant difference to results. Cytochromes *c* and *a* were monitored in the same sample by serial measurements at 550, 575, 605 and 630 nm and the changes 550–575 nm and 605–630 nm were attributed to cytochromes *c* and *a*, respectively. Where necessary, corrections were made for mutual overlap. Detection was with a photomultiplier screened with a Schott glass GG495 filter and the grating had 300 nm blaze and 1200 lines/mm.

In intact mitochondria, quantitative monitoring of $Cu_A$ proved to be impossible when using only two wavelengths; different results were obtained if the reference wavelength was chosen to be above or below the sample wavelength of 825 nm. This arose from the large light scattering changes which often occurred on changes of energisation and from the need to have a widely separated reference wavelength to measure the very broad $Cu_A$ signal. Instead, a triple wavelength technique was used. This involved the use of two reference wavelengths at 725 nm and 925 nm which were equidistant from, but on opposite sides of, the sample wavelength of 825 nm. Signals attributable to $Cu_A$ were then taken to be the difference between changes at the sample wavelength minus the average change of the two reference wavelengths. Detection was with a 1 $cm^2$ photodiode which was screened with GG475 and RG695 glass filters and the monochromator grating had a blaze wavelength of 1000 nm and 600 lines/mm.

## 3. RESULTS

### 3.1. *Detection of $Cu_A$ in intact beef heart and rat liver mitochondria*

Fig.1A illustrates a spectrum of beef heart mitochondria in the 540–1000 nm region. The spectrum is the difference of a sample treated with ascorbate, TMPD and KCN minus an aerobic, untreated sample. Full reduction of cytochromes *c*, *a* and $Cu_A$ is expected from this treatment. Assuming a 1:1 ratio of haem $a_A/Cu_A$ and a haem $a_A$ extinction coefficient at 605–630 nm of 21.85 $mM^{-1} \cdot cm^{-1}$ (average value measured from the data in [19–21]), the millimolar extinction coefficient of $Cu_A$ in a triple wavelength assay (825 nm − 0.5 × (725 nm + 925 nm)) was measured to be close to 1 $mM^{-1} \cdot cm^{-1}$, in reasonable agreement with published spectra of the purified enzyme e.g. [21].

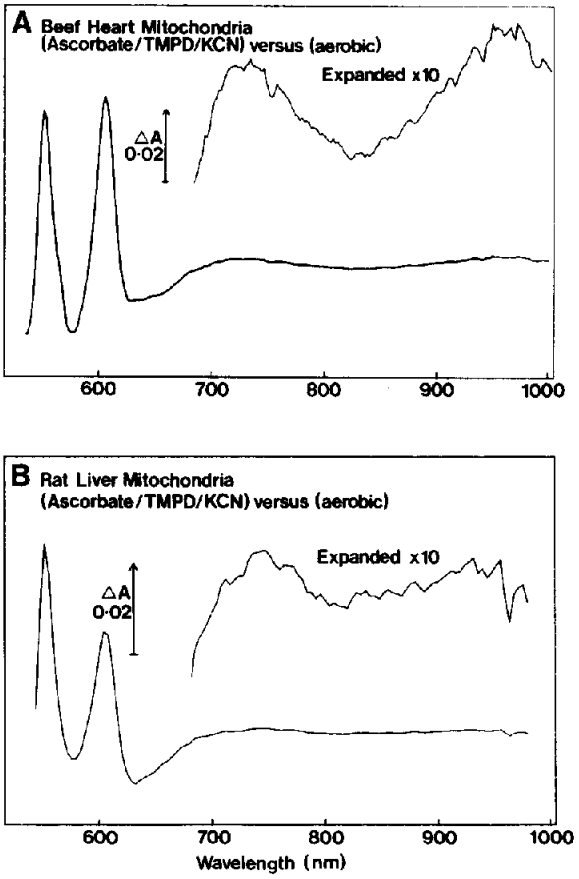


Fig.1. The relative contributions of cytochrome *c*, haem $a_A$ and $Cu_A$ in beef heart and rat liver mitochondria. Mitochondria were resuspended in 100 mM KCl, 50 mm Hepes and 1 mM EGTA at pH 7.0 and 23°C. An aerobic baseline was recorded. 1 mM KCN, 10 mM sodium ascorbate and 10 μM TMPD were then added. After 5 min a further spectrum was recorded. The spectra illustrated are the difference between these and the corresponding aerobic baseline. (A) Beef heart mitochondria at 10 mg/ml (average of 2 two-way scans at 2 nm intervals); (B) rat liver mitochondria at 11.5 mg/ml (average of 3 two-way scans at 4 nm intervals).

A similar extinction coefficient for $Cu_A$ was obtained with coupled rat liver mitochondria with the triple wavelength technique. However, the full spectrum (fig.1B) was distorted by a contaminating component, possibly haemoglobin, which was always present in our preparations. This factor, which also caused distortions in the 540–630 nm region, together with the smaller

amounts of components present in the rat liver mitochondria, made them unsuitable for accurate assay of $Cu_A$ redox changes.

### 3.2. *Energy-linked responses of cytochrome c, haem $a_A$ and $Cu_A$ in beef heart mitochondria*

In the experiments of fig.2, 75 $\mu$g/ml of nigericin was present throughout to minimise $\Delta$pH effects, although essentially the same result was obtained in the absence of nigericin. Addition of 0.4 mM KCN caused an approximately synchronous reduction of cytochrome *c* and haem $a_A$ and $Cu_A$ over several minutes. Comparison of the signal sizes with those obtained by reduction with sodium dithionite showed that the components became fully reduced with KCN (see fig.2A). Subsequent addition of myxothiazol to the cyanide-inhibited mitochondria then caused partial reoxidation of all three components. On addition of ATP, cytochrome *c* and $Cu_A$ became further reduced and haem $a_A$ became more oxidised; these effects were reversed by FCCP. That these infrared changes were caused by redox state changes of $Cu_A$ was confirmed by taking complete spectra, as in fig.3. This spectrum, the difference between a sample with ATP minus the same sample after addition of FCCP, showed a broad trough at around 825 nm, characteristic of a 10–15% extra reduction of $Cu_A$ when ATP alone was present.

Complementary experiments were performed in the presence of valinomycin, in order to minimise electric field and increase $\Delta$pH. In this case, rather less shift in relative midpoint potentials of components was observed, indicating that $pH_I$ or $\Delta$pH were less effective than $\Delta\psi$ in modulating relative midpoint potentials. Accurate quantitation and interpretation of the shifts in the presence of valinomycin was, however, hampered by large light scattering changes which always occurred on addition of ATP, and by a residual remaining electric field component.

### 3.3. *Quantitation of the electronic redistribution on addition of ATP*

Apparent midpoint potentials of $Cu_A$ relative to cytochrome *c* could be calculated from these data, assuming that both components behaved as $n = 1$ species. In the de-energised state ($\pm$ nigericin, $\pm$ FCCP) it was calculated that the apparent midpoint potential of $Cu_A$ was 16 mV (SD $\pm$ 2.4 mV; $n = 6$) more positive than the midpoint potential of cytochrome *c*. In the energised state (i.e. + ATP and $\pm$ nigericin), the apparent midpoint potential of $Cu_A$ remained almost unchanged at +20 mV

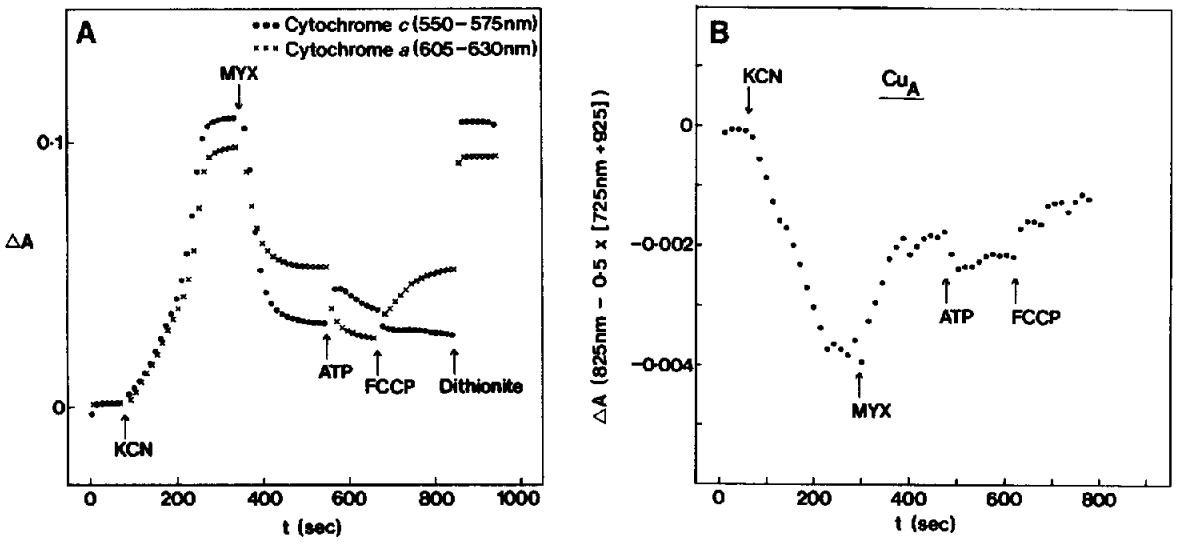


Fig.2. Energy-linked responses of cytochrome *c*, haem $a_A$ and $Cu_A$ in coupled beef heart mitochondria. Mitochondria were resuspended to 14 mg/ml in 100 mM KCl, 50 mM Hepes and 1 mM EGTA at pH 7.0 and 23°C. 75 $\mu$g/ml nigericin were then added. At the points indicated the following additions were made: 0.4 mM KCN, 18 $\mu$M myxothiazol, 4 mM ATP and 1 $\mu$M FCCP. Spectral changes were plotted as 550–575 nm, 605–630 nm and 825 – (0.5 × (725 + 925)) nm for cytochrome *c*, haem $a_A$ and $Cu_A$, respectively.

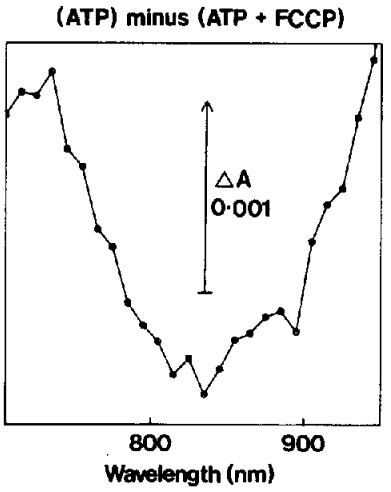


Fig.3. ATP-induced infra-red difference spectrum in coupled beef heart mitochondria. Coupled beef heart mitochondria were resuspended to 26 mg/ml protein in 100 mM KCl, 50 mM Hepes and 1 mM EGTA at pH 7.0 and 75 ng/ml nigericin were added and a baseline was recorded. The redox poise of $Cu_A$ was brought to around 50% with 0.4 mM KCN, followed 3 min later by 18 $\mu$M myxothiazol (cf. fig.2). After a further 3 min, 4 mM ATP was added and a spectrum was recorded. This was followed by addition of 1 $\mu$M FCCP whereupon a further spectrum was taken. The figure shows the difference between these two spectra i.e. the difference spectrum (ATP) minus (ATP plus FCCP).

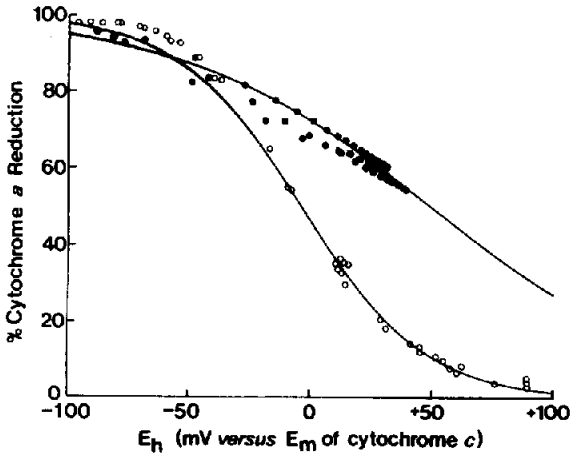


Fig.4. Redox titrations of haem $a_A$ in the presence of cyanide in energised and de-energised beef heart mitochondria. Mitochondria were resuspended to around 10 mg/ml in 130 mM choline chloride, 10 mM Hepes and 5 mM KCl at pH 7.4. Cytochromes *c* and haem $a_A$ were monitored concurrently and allowance was made for their mutual spectral overlap. 0.4 mM KCN was added which caused full reduction of both components. Either 4 mM ATP (open circles) or 1 $\mu$M FCCP plus 1 $\mu$M valinomycin (closed circles) were added to obtain the energised and de-energised data, respectively. Titrations were then performed by stepwise additions of varying amounts of myxothiazol and antimycin A. Potential difference from the midpoint potential of cytochrome *c* ($E_h$) was calculated from the redox state of the cytochrome *c*, using $E_h = 59\log_{10}(c_{ox}/c_{red})$. Two ideal curves are plotted for comparison of $E_m = -3$ mV, $n = 1$ and $E_m = 50$ mV, $n = 0.5$.

(SD ± 2.7 mV; $n = 5$) more positive than cytochrome *c*.

Calculation of a relative midpoint potential shift of haem $a_A$ on energisation was less useful since in the presence of cyanide it titrates in a manner that approximates to an $n = 0.5$ component [22], presumably because of its redox interaction with other centres. It was found that the titration became very close to an $n = 1$ curve in the presence of ATP (fig.4). Hence the apparent midpoint potential shift was dependent on the initial redox poise of components.

Because electron donation into and out of the $c/Cu_A/a_A$ region was severely inhibited by a combination of inhibitors, it was possible to equate the number of electrons lost from haem $a_A$ with those appearing on $c/Cu_A$. The changes were measured at the point of maximum cytochrome *c* reduction after ATP addition (see fig.2), at which time any leakage through the inhibitory blocks should have been negligible. For these experiments, KCN was used at 1.6 mM and a combination of antimycin A (5 mol/mol $bc_1$ complex) and stigmatellin (2.5 mol/mol $bc_1$ complex) were used instead of myxothiazol. These minimised the possibility of reversed electron transfer through centre o of the $bc_1$ complex and also raised the midpoint potential of the iron sulphur centre above +500 mV [23] so that it could not change redox state during the experiment. Extinction coefficients of 21.85 [19–21], 21.9 [24] and 1 $mM^{-1} \cdot cm^{-1}$ (see above) were used to quantitate the optical changes of $a_A$, *c*, and $Cu_A$, respectively. In addition, the changes of cytochromes *c* and *a* were corrected for their small mutual overlap ($\epsilon$ of oxidase at 550–575 nm = +3.1 $mM^{-1} \cdot cm^{-1}$; $\epsilon$ of cytochrome *c* at 605–630 nm = −0.3 $mM^{-1} \cdot cm^{-1}$). On this basis, the number of electrons which appeared on cytochrome *c* and $Cu_A$ combined was always somewhat in excess of those lost from haem $a_A$. In an average of three experiments with mitochondria resuspended to 5 $\mu$M oxidase, energisation with

ATP caused a 27% oxidation of haem $a_A$ (equivalent to 1350 nM electrons) whereas cytochrome *c* and $Cu_A$ became 18% and 30% more reduced, respectively (a total reduction equivalent to 2400 nM electrons, although the electrons appearing on $Cu_A$ were possibly underestimated by up to 500 nM because of opposing infra-red changes from cytochrome *c*). Presumably, the extra electrons (1050–1550 nM) originate from oxidation of $Cu_B$.

## 4. DISCUSSION

The relative midpoint potential shift of haem $a_A$ relative to cytochrome *c* has been rationalised in terms of haem $a_A$ residing in a position approx. 50% across the membrane dielectric barrier with cytochrome *c* being in full contact with the external medium [12]. Wikström [25] pointed out that the behaviour is also consistent with a proton well connected to haem $a_A$ which opens into the matrix space, particularly in view of the reported $pH_I$ dependency of the haem $a_A$ midpoint potential [22]. However, if the $pH_I$ dependency of haem $a_A$ is absent in the present of carbon monoxide [25], it is likely that the electric field is the dominant force. This is supported qualitatively by our finding that $\Delta\psi$ is more effective than a $pH_I$ change in causing the haem $a_A$ shift.

The present data indicate that the midpoint potential of $Cu_A$ shifts by less than 10 mV on energisation, in agreement with [15] and with the recent discussion in [11]. Since the electric potential generated by ATP addition to the mitochondria is of the order of 100 mV [12], and since $Cu_A$ has a pH-independent midpoint potential, the results indicate that $Cu_A$ is less than 10% into the membrane. Hence, electron transfer from $Cu_A$ to haem $a_A$ should be electrogenic. This location favours $Cu_A$ being closer to cytochrome *c* than to haem $a_A$ and favours an ordering of components of $c \longrightarrow Cu_A \longrightarrow a_A$. Although this is consistent with recent structural predictions [14] and with the lack of ATP effect on the measured midpoint potential of $Cu_A$ [15], it is in contrast to a large body of kinetic data which have been interpreted as favouring haem $a_A$ as the immediate acceptor from cytochrome *c*, reviewed in [13]. However, at least some of these kinetic data might be explained by the slightly lower midpoint potential of $Cu_A$ compared to haem $a_A$ and from the complex redox titration curve of haem $a_A$ in the absence of carbon monoxide ([22] and fig.4). Nevertheless, the present experiments cannot rule out the other somewhat unlikely possibility that haem $a_A$ is the initial acceptor from cytochrome *c* with $Cu_A$ accepting electrons from haem $a_A$ in a reaction which would be 'reverse electrogenic', i.e. would produce an electric field which is negative on the cytosolic side.

The magnitude of the ATP-induced shift in measured midpoint potential of haem $a_A$ in the presence of cyanide (fig.4) or carbon monoxide [12] is around 50 mV, and is more responsive to a $\Delta\psi$ than to a $\Delta$pH. These observations, when coupled with the likely order of reactants of $c \longrightarrow Cu_A \longrightarrow a_A$, would mean that $Cu_A$ cannot be acting as a proton pump under the conditions of our experiments; a proton pumping role of $Cu_A$ between cytochromes *c* and *a* would cause a far larger shift in the redox titration curve of haem $a_A$ which would be equally responsive to $\Delta\psi$ and $\Delta$pH. However, it is not clear why we fail to see at least a $-60$ mV shift in the midpoint potential of haem $a_A$ caused simply by a change of $pH_I$ (i.e. when ATP is added in the presence of valinomycin), as has been reported by Artzatbanov et al. [22].

Although it is expected that haem $a_3$ remains fully oxidised in the experiments with KCN, the observation that ATP does not, apparently, cause a larger flow of electrons from $Cu_B$ to haem $a_A/Cu_A$ is notable in view of the location of $Cu_B$ on the oxygen side of haem $a_A$, its midpoint potential of around $+340$ mV [26–28] and the popular notion of the site of the proton pump at haem $a_A$ or between haem $a_A$ and $Cu_B$/haem $a_B$. The extent of oxidation of $Cu_B$ is of the same order as that of haem $a_A$, whereas $Cu_B$ might have been predicted to change from almost full reduction to almost full oxidation on ATP addition. The result is also inconsistent with data of Lindsay et al. [27,28] which indicated an ATP-independent midpoint potential of $Cu_B$ in the presence of carbon monoxide. Goodman [26] has suggested that the midpoint potential of $Cu_B$ is dependent on the redox state of haem $a_A$, and this could form a partial basis for our results as it is possible that $Cu_B$ is mostly oxidised before ATP addition in our experiments. The behaviour of $Cu_B$ requires further investigation. This may clarify the reason for distortion of the haem $a_A$

redox titration curve in the absence of carbon monoxide or ATP and may define the site of action of the protonmotive chemistry.

*Acknowledgements:* This work was funded by a CEC Research Contract (no.ST2P-0056-1-UK) and by the benefactors named in the brochure of the UK Registered Charity called The Glynn Research Foundation Ltd. It was aided by useful discussions with Mr R. Mitchell and Dr A.J. Moody and by the able technical assistance of Ms S. Madgwick, Mr A. Jeal and Mr R. Harper. I.C.W. is on leave of absence from the University of Newcastle upon Tyne.

## REFERENCES

[1] Krab, K. and Wikström, M. (1979) Biochim. Biophys. Acta 548, 1–15.
[2] Gelles, J., Blair, D.F. and Chan, S.I. (1986) Biochim. Biophys. Acta 853, 205–236.
[3] Mitchell, P. (1987) FEBS Lett. 222, 235–245.
[4] Wikström, M. and Casey, R.P. (1985) J. Inorg. Biochem. 23, 327–334.
[5] Miki, T. and Orii, Y. (1986) J. Biochem. 100, 735–745.
[6] Mitchell, P. (1987) Glynn Biol. Res. Rep. 3, 1–7.
[7] Chance, B. and Powers, L. (1985) Curr. Top. Bioenerg. 14, 1–19.
[8] Mitchell, P., Mitchell, R., Moody, A.J., West, I.C., Baum, H. and Wrigglesworth, J.M. (1985) FEBS Lett. 188, 1–7.
[9] Malmström, B.G. (1985) Biochim. Biophys. Acta 811, 1–12.
[10] Penttila, T. (1983) Eur. J. Biochem. 133, 355–361.
[11] Krab, K. and Wikström, M. (1987) Biochim. Biophys. Acta 895, 25–39.
[12] Hinkle, P. and Mitchell, P. (1970) Bioenergetics 1, 45–60.
[13] Brunori, M., Antonini, G., Malatesta, F., Sarti, P. and Wilson, M.T. (1988) Adv. Inorg. Biochem. 7, 93–153.
[14] Holm, L., Saraste, M. and Wikström, M. (1987) EMBO J. 6, 2819–2823.
[15] Erecinska, M., Chance, B. and Wilson, D.F. (1971) FEBS Lett. 16, 284–286.
[16] Mitchell, P. and Moyle, J. (1979) in: Cytochrome Oxidase (King, T.E. et al. eds) pp. 361–372, Elsevier/North Holland, Amsterdam.
[17] Chappell, J.B. and Hansford, R.G. (1969) in: Subcellular Components – Preparation and Fractionation (Birnie, G.D. and Fox, S.M. eds) pp.43–56, Butterworths, London.
[18] Mitchell, P. and Moyle, J. (1967) Biochem. J. 104, 588–600.
[19] Yonetani, T. (1961) J. Biol. Chem. 236, 1680–1688.
[20] Vanneste, W.H. (1966) Biochemistry 5, 838–848.
[21] Kuboyama, M., Yong, F.K. and King, T.E. (1972) J. Biol. Chem. 247, 6375–6383.
[22] Artzatbanov, V.Yu., Konstantinov, A.A. and Skulachev, V.P. (1978) FEBS Lett. 87, 180–185.
[23] Von Jagow, G. and Ohnishi, T. (1985) FEBS Lett. 185, 311–315.
[24] Margoliash, E. and Frohwirt, N. (1959) Biochem. J. 71, 570–572.
[25] Wikström, M. and Krab, K. (1979) Cation Flux Across Biomembranes (Mukohata, Y. and Packer, L. eds) pp.321–329, Academic Press, London.
[26] Goodman, G. (1984) J. Biol. Chem. 259, 15094–15099.
[27] Lindsay, J.G., Owen, C.S. and Wilson, D.F. (1975) Arch. Biochem. Biophys. 169, 492–505.
[28] Lindsay, J.G. (1974) Arch. Biochem. Biophys. 163, 705–715.