

# The cytochrome chain of mitochondria exhibits variable $H^+/e^-$ stoichiometry

S. Papa, N. Capitanio, G. Capitanio, E. De Nitto and M. Minuto

*Institute of Medical Biochemistry and Chemistry, University of Bari, Bari, Italy*

Received 26 March 1991

A study is presented of the  $\leftarrow H^+/e^-$  stoichiometry for  $H^+$  pumping by the cytochrome chain in isolated rat liver mitochondria under level-flow and steady-state conditions. It is shown that the  $\leftarrow H^+/e^-$  stoichiometry for the cytochrome chain varies under the influence of the flow rate and transmembrane  $\Delta\mu H^+$ . The rate-dependence is shown to be associated with cytochrome *c* oxidase, whose  $\leftarrow H^+/e^-$  ratio varies from 0 to 1, whilst the  $\leftarrow H^+/e^-$  ratio for the span covered by cytochrome *c* reductase is invariably 2.

Mitochondria; Cytochrome chain;  $H^+$  pumping; Cytochrome *c* oxidase; Cytochrome *c* reductase

## 1. INTRODUCTION

Electron flow down the cytochrome chain of mitochondria is compulsorily linked to vectorial  $H^+$  translocation from the inner to the outer aqueous space [1,2]. The mechanism by which  $\Delta\mu H^+$  is generated by mitochondrial respiration and utilized to make ATP [3] is, however, not yet completely understood. Critical in this respect is knowledge of the  $H^+/e^-$  and  $H^+/ATP$  stoichiometries (for review see [4,5]).

The  $\leftarrow H^+/e^-$  stoichiometry for electron flow in the cytochrome *c* reductase (span from ubiquinol to cytochrome *c*) is reported to be 2 [4,5]. The  $\leftarrow H^+/e^-$  stoichiometry for  $H^+$  ejection by cytochrome oxidase (span from cytochrome *c* to oxygen) is, on the contrary, still debated [6]. Some authors maintain that the  $\leftarrow H^+/e^-$  ratio is invariably 1 [5,7,8]. Others have reported  $\leftarrow H^+/e^-$  ratios for  $H^+$  ejection by the oxidase lower [4,6] or higher [9,10] than 1. In this paper an analysis is presented of the phenomenological  $\leftarrow H^+/e^-$  stoichiometry for respiratory  $H^+$  pumping in rat-liver mitochondria under level flow and steady-state conditions [11]. For level flow, that is, conditions of negligible transmembrane  $\Delta\mu H^+$  (see [11]), spectrophotometric measurements of the initial rates of  $e^-$

flow [12] and  $H^+$  translocation elicited by substrate addition were used. The  $\leftarrow H^+/e^-$  ratios at the steady-state were measured with a method our group had previously introduced [13].

The results show that the  $\leftarrow H^+/e^-$  stoichiometry for succinate respiration varies from around 2 to 3 under the influence of the rate of electron flow and  $\Delta\mu H^+$ . The rate-dependence is shown to be specifically associated with  $H^+$  pumping the cytochrome oxidase. These observations seem to solve the controversy so far registered for the  $\leftarrow H^+/e^-$  stoichiometry in the oxidase.

## 2. MATERIALS AND METHODS

Rat-liver mitochondria were isolated as in [14].  $H^+$  translocation was measured either potentiometrically [15] or by dual-wavelength spectrophotometry at 558–593 nm with the pH indicator phenol red. Oxygen uptake was measured either electrometrically by fast responding  $O_2$  electrode or spectrophotometrically by human hemoglobin as in [12] (see also [16]). Deoxygenation of  $HbO_2$  was monitored by dual wavelength spectrophotometry at 577–568 nm ( $\Delta\epsilon$  (mM) for hemoglobin deoxygenation was 6.3). The rate of electron transfer was obtained by multiplying the rate of  $HbO_2$  deoxygenation (in nmol heme) by 4 and a correction factor (*f*). This was calculated polarographically and spectrophotometrically as in [16] (see also legend to Fig. 1). Measurements were carried out in thermostatically controlled glass cells equipped with  $O_2$  electrodes (4004 YSI, Yellow Spring, OH), coated with a high sensitivity membrane (YSI 57776), and a combination glass electrode (Beckman 39532) or in spectrophotometric cuvettes, both provided with rapid stirring and sealed with a perspex plug with a 2 cm long, thin channel filled with the sample, for insertion of microsyringe needles. Mitochondria were suspended in: 130 mM LiCl, 1 mM KCl, 1 mM HEPES, 30 nmol NEM/mg protein, 0.1  $\mu$ g valinomycin/mg protein, 0.5  $\mu$ g rotenone/mg protein, pH 7.4.

## 3. RESULTS

Fig. 1 shows spectrophotometric measurements of

**Abbreviations:** TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; CCP, carbonyl cyanide-*m*-chlorophenylhydrazine; NEM, *N*-ethylmaleimide; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; Hb, deoxyhemoglobin;  $HbO_2$ , oxyhemoglobin;  $\leftarrow H^+/e^-$ , number of  $H^+$  equivalents released from mitochondria per equivalent  $e^-$  transfer.

**Correspondence address:** S. Papa, Institute of Medical Biochemistry and Chemistry, University of Bari, Piazza Giulio Cesare, 70124 Bari, Italy. Fax: (39)(80)278429.

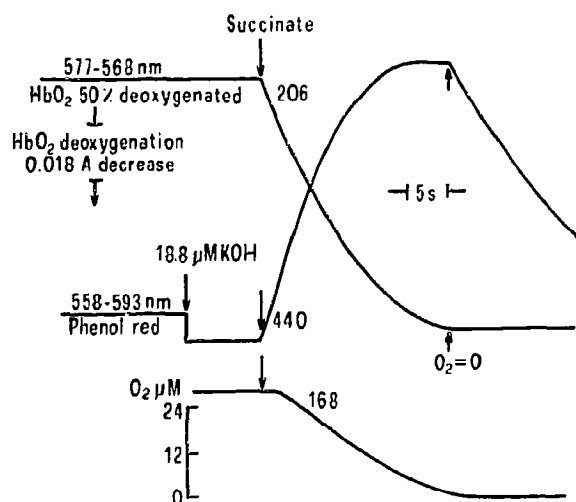


Fig. 1. Measurement of level flow  $\text{H}^+/\text{e}^-$  ratios for succinate respiration in rat liver mitochondria. Mitochondria (2.5 mg protein · ml) were suspended in the reaction medium described in section 2.  $\text{O}_2$  was removed from the mitochondrial suspension in the glass cell and spectrophotometric cuvette by gentle argon flow, after which both cells were sealed with the  $\text{O}_2$ -proof plug. When the  $\text{O}_2$  concentration was lowered to 27  $\mu\text{M}$ , and 25  $\mu\text{M}$   $\text{HbO}_2$ , added to the suspension in the spectrophotometric cuvette, was 50% deoxygenated, respiration was started by addition of 1 mM succinate. Controls showed that hemoglobin had no effect per se on the initial rate of  $\text{O}_2$  consumption measured polarographically.  $\text{H}^+$  translocation was measured spectrophotometrically on a sample of the mitochondrial suspension treated used for spectrophotometric measurement of respiration, with the only difference that hemoglobin was replaced with 50  $\mu\text{M}$  phenol red, pH, 7.4, at 25°C. The figures on the  $\text{O}_2$  and Hb traces represent initial respiratory rates in equivalents  $\text{e}^- \cdot \text{min}^{-1} \cdot \text{mg}$  protein. Those on the pH traces represent initial rates of  $\text{H}^+$  translocation, equivalents  $\text{H}^+ \cdot \text{min}^{-1} \cdot \text{mg}$  protein. The measured  $K_m$  for  $\text{O}_2$  of the Hb preparation sampled used in the experiment was 28  $\mu\text{M}$  and the correlated factor 'f' at 25  $\mu\text{M}$  Hb was 2.1 (see [12,16]).

respiration and  $\text{H}^+$  ejection in rat liver mitochondria supplemented with rotenone and valinomycin plus  $\text{K}^+$ . Succinate addition to the mitochondrial suspension, when  $[\text{O}_2]$  had been pre-lowered so as to cause 50% deoxygenation of  $\text{HbO}_2$ , resulted in an immediate deoxygenation of hemoglobin, from which the initial respiratory rate was calculated as in [12]. It can be noted that the  $\text{O}_2$  electrode underestimated the initial respiratory rate. The respiratory burst was accompanied by immediate  $\text{H}^+$  translocation which was monitored on separate samples where hemoglobin was replaced by phenol red. The  $\text{H}^+/\text{e}^-$  ratio obtained from initial rates of  $\text{e}^-$  flow and  $\text{H}^+$  release at level-flow was, in this experiment, 2.1. The same ratio was obtained when  $\text{H}^+$  release was measured potentiometrically (not shown) (see also [12]).

In the experiment of Fig. 2, mitochondria supplemented with succinate were left to become anaerobic. After 5 min equilibration, respiration was activated by addition of  $\text{H}_2\text{O}_2$  in the presence of added catalase. An immediate acidification took place which reached a steady-state level in about 1 min, when

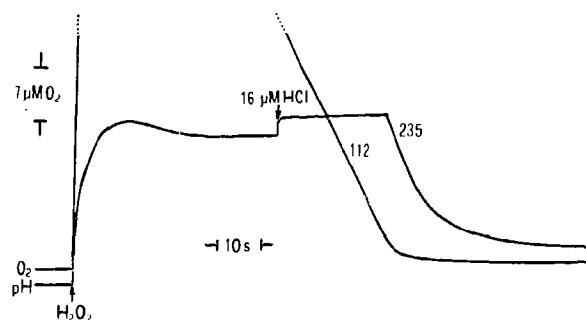


Fig. 2. Measurement of steady-state  $\text{H}^+/\text{e}^-$  ratios for succinate respiration in rat liver mitochondria. Mitochondria (2.5 mg protein · ml) were suspended in the medium described in Section 2 and supplemented with 10 mM succinate. The suspension was left to become anaerobic in a closed glass cell thermostated at 25°C. Once anaerobiosis was reached, 0.1 mg/ml of purified catalase (Boehringer, Mannheim) was added and after 5 min equilibration, respiration was started by addition of 4  $\mu\text{l}/\text{ml}$  of 0.2%  $\text{H}_2\text{O}_2$ . The  $\text{H}^+/\text{e}^-$  ratio was calculated from the steady-state  $\text{e}^-$  flow rate and the initial rate of anaerobic  $\text{H}^+$  influx.

respiratory rate adjusted itself at a constant steady rate. Upon anaerobiosis immediate alkalization occurred. At the steady-state the rate of  $\text{H}^+$  efflux is equal to that of  $\text{H}^+$  influx; the latter is obtained by measurement of the initial rate of anaerobic  $\text{H}^+$  influx [13]. The steady-state  $\text{H}^+/\text{e}^-$  ratio amounted, under these conditions, to 2.1.

Fig. 3 presents a comparative analysis of  $\text{H}^+/\text{e}^-$  ratios for  $\text{H}^+$  pumping associated to aerobic oxidation of succinate and ascorbate plus TMPD as a function of the respiratory rate. Under level-flow conditions the  $\text{H}^+/\text{e}^-$  ratio for succinate respiration was practically 2 at the higher rates of  $\text{e}^-$  flow (Fig. 3A, curve a). As the rate of  $\text{e}^-$  flow was depressed by adding, in different samples, increasing concentrations of malonate, the  $\text{H}^+/\text{e}^-$  ratio first increased up to around 2.8, then further inhibition of  $\text{e}^-$  flow resulted in progressive decrease of the ratio until at extremely low rates it returned to the value of 2.

Under steady-state conditions (Fig. 3A, curve b) the  $\text{H}^+/\text{e}^-$  ratio exhibited practically the same rate-dependence observed at level-flow. The steady-state  $\text{H}^+/\text{e}^-$  ratios, however did not reach the values found at level-flow for intermediate flow rates. The highest  $\text{H}^+/\text{e}^-$  values observed at the steady-state were around 2.4, and thus significantly lower than the level-flow  $\text{H}^+/\text{e}^-$  ratio of around 2.8 observed at optimal flow rates.

Differently from what was observed for overall electron flow from succinate to  $\text{O}_2$ , the  $\text{H}^+/\text{e}^-$  ratio measured for electron flow from succinate to ferricyanide (cytochrome c reductase span) was constantly 2 over the same range of  $\text{e}^-$  transfer rates in which succinate respiration exhibited a variable  $\text{H}^+/\text{e}^-$  ratio.

In Fig. 3B the observed  $\text{H}^+/\text{e}^-$  ratios for ascorbate respiration, as a function of the respiratory rate,

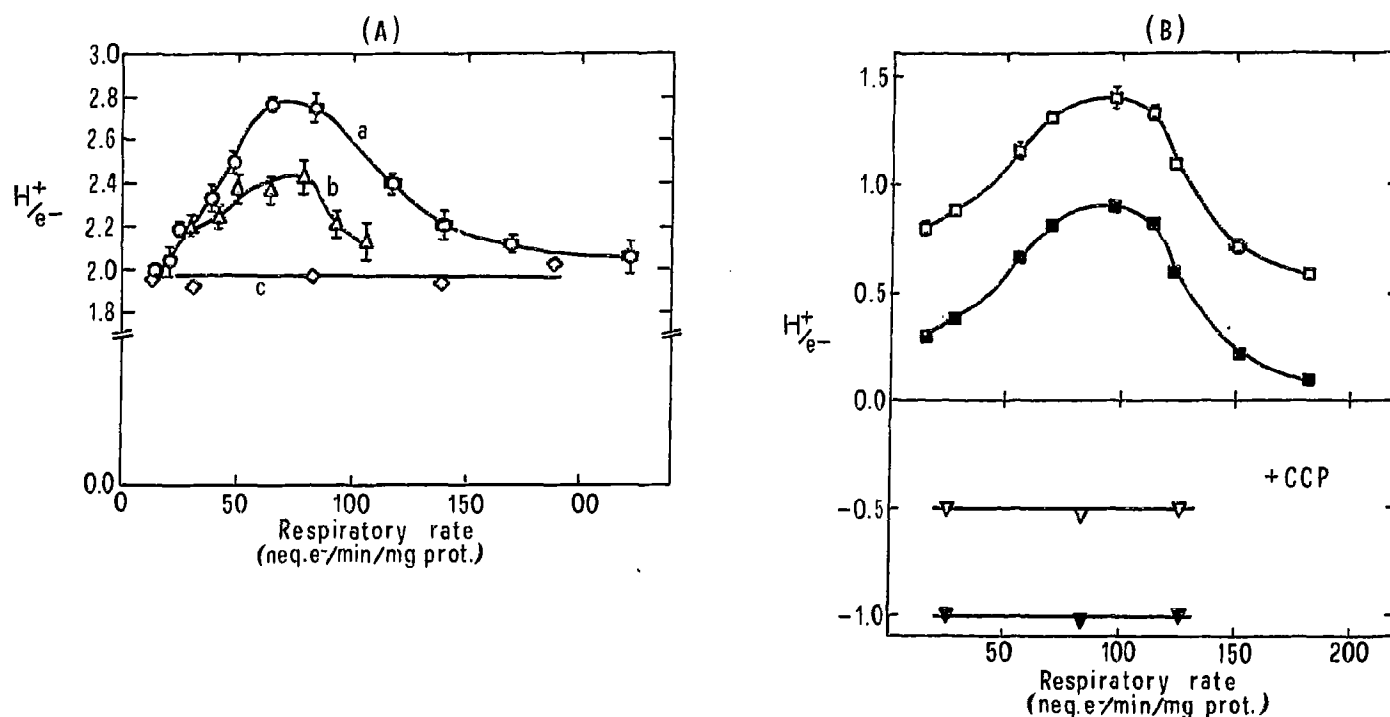


Fig. 3. Analysis of the  $H^+/e^-$  ratios for redox linked  $H^+$  translocation as a function of the rate of electron transfer in rat liver mitochondria. **Panel A.** (a) Level flow  $H^+/e^-$  ratios for succinate respiration, mean of 11 experiments  $\pm$  SEM; the respiratory rate was varied by the addition in different samples of increasing amounts of malonate; for other experimental conditions see legend to Fig. 1. (b) Steady-state  $H^+/e^-$  ratios for succinate respiration (8 experiments), the respiratory rate was varied by malonate, for other experimental conditions see legend to Fig. 2. (c) Level flow  $H^+/e^-$  ratios associated with electron flow from succinate to ferricyanide. (2 experiments). Mitochondria (2.5 mg protein/ml) were suspended in the medium described in Section 2 supplemented with 1 mM KCN. Ferricyanide reduction was measured spectrophotometrically at 420–500 nm;  $H^+$  release was measured on another sample of the same suspension supplemented with 50  $\mu$ M phenol red. Reduction of ferricyanide and  $H^+$  release were initiated by the addition of 1 mM succinate. The rate of electron flow was varied by malonate. **Panel B.** (□—□) level flow  $H^+/e^-$  ratios associated with ascorbate oxidation (4 experiments). Mitochondria (2.5 mg protein/ml) were suspended in the medium described in Section 2 supplemented with 0.05  $\mu$ g antimycin A/mg protein. The initial rate of  $H^+$  translocation and  $e^-$  flow were measured as described in Section 2 and in the legend to Fig. 1. Respiration was activated by addition of 1 mM ascorbate plus various concentrations of TMPD ranging from 5 to 160  $\mu$ M. For other details see Fig. 1, pH 7.4; 25°C. (▽—▽)  $H^+/e^-$  ratios for proton consumption associated with ascorbate oxidation in the presence of 3  $\mu$ M CCP. The experimental conditions were the same as in the coupled state. (▣—▣) and (▽—▽) refer to  $H^+/e^-$  ratios corrected for the scalar  $H^+$  release contributed by the oxidation of ascorbate to dehydroascorbate. Where not shown the SEM bars fall inside the symbols.

adjusted by varying TMPD concentration, are presented. The  $H^+/e^-$  ratio calculated from initial rates at level-flow exhibited a rate dependence similar to that observed for succinate respiration. With ascorbate plus TMPD the  $H^+/e^-$  ratios varied from around 0.6 at the highest rates explored, increased up to a maximum value of 1.4 at intermediate  $e^-$  flow rate, and then decreased to around 0.8 as the rate of  $e^-$  flow was further depressed. The  $H^+/e^-$  ratio corrected for the scalar production of 0.5  $H^+/e^-$  associated to oxidation of ascorbate to dehydroascorbate, varied, correspondingly, from 0.1 to 0.90 and back to 0.3. The same rate-dependent changes of the  $H^+/e^-$  ratio have been observed in purified cytochrome c oxidase reconstituted in liposomes (see accompanying paper [17]). The  $H^+/e^-$  ratio for  $H^+$  consumption associated to aerobic oxidation of ascorbate plus TMPD in the presence of CCP was 0.5 (1.0 after correction for the scalar  $H^+$  production associated to oxidation of ascor-

bate to dehydroascorbate) independent of the respiratory rate.

#### 4. DISCUSSION

The data presented show that in isolated mitochondria the phenomenological  $H^+/e^-$  stoichiometry for the cytochrome chain varies under the influence of the respiratory rate and transmembrane  $\Delta\mu H^+$ .

At level-flow, in the absence of significant  $\Delta\mu H^+$  and changes in  $H^+$  conductance, the  $H^+/e^-$  ratio for succinate respiration varied from minima of around 2, at extreme high (cf. [12]) and low respiratory rates, to about 3 at intermediate rates. Under the same conditions the  $H^+/e^-$  ratio for ascorbate respiration varied from minima of around 0 to 1 at intermediate respiratory rates. The  $H^+/e^-$  ratio for electron flow from succinate to ferricyanide was, on the contrary, invariably 2 over all the rate range explored.

The same rate-dependence of the  $\leftarrow H^+/e^-$  ratio in the oxidase was observed in the purified bovine heart enzyme reconstituted in liposomes, whilst the  $\leftarrow H^+/e^-$  ratio for reconstituted cytochrome *c* reductase remained constant (see [17,18]).

It is, thus, clear that the observed rate-dependence of the  $\leftarrow H^+/e^-$  ratio for  $H^+$  pumping by the mitochondrial respiratory chain is associated with electron flow in the oxidase.

Comparison of the  $\leftarrow H^+/e^-$  ratios for succinate respiration at level-flow and steady-state shows that the highest ratios attainable at the steady-state, at intermediate flow rates, were significantly lower than those observed for the same rates at level-flow. This provides direct evidence that, besides the flow rate, also the magnitude of  $\Delta\mu H^+$  affects the  $\leftarrow H^+/e^-$  stoichiometry. Murphy and Brand ([19], however see [20]) have reported that  $\Delta\mu H^+$  depressed the charge/ $e^-$  ratio in cytochrome oxidase. In their experiments  $\Delta\mu H^+$  was lowered by inhibiting steady-state electron flow, thus distinction between effects of  $\Delta\mu H^+$  and flow rate could not be made.

The systematic analysis of factors affecting the  $\leftarrow H^+/e^-$  stoichiometry for  $H^+$  pumping by cytochrome oxidase which our group has carried out, (see also [17,21]) seems to solve the controversy so far registered for the protonmotive activity of the oxidase [4-8]. The variability of the  $\leftarrow H^+/e^-$  stoichiometry documented, provides an explanation for the different values previously observed.

Dependence of the phenomenological  $\leftarrow H^+/e^-$  stoichiometry of cytochrome *c* oxidase on flow rate and  $\Delta\mu H^+$  seems, in principle, to favour indirect [2,22-24] over direct [1,8] pumping models. Examination of this aspect is, however, beyond the scope of the present paper.

**Acknowledgements:** This work was in part supported by Grant 89.00317.75 of Consiglio Nazionale delle Ricerche, Italy.

## REFERENCES

- [1] Mitchell, P., (1966) *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research, Bodmin.
- [2] Papa, S. (1976) *Biochim. Biophys. Acta* 456, 39-84.
- [3] Boyer, P.D., Chance, B., Ernster, L., Mitchell, P., Racker, E. and Slater, E.C. (1977) *Annu. Rev. Biochem.* 46, 955-1026.
- [4] Papa, S., Capitanio, N., Izzo, G. and De Nitto, E., (1988) in: *Advances in Membrane Biochemistry and Bioenergetics* (Kim, C.H., Tedeschi, H., Diwan, J.J. and Salerno, J.C. eds.) pp. 333-345, Plenum, New York.
- [5] Wikström, M. and Saraste, M. (1984) in: *Bioenergetics* (Ernster, L. ed.) pp. 49-94, Elsevier, Amsterdam.
- [6] Papa, S. (1988) in: *Oxidases and Related Redox Systems*, (Mason, H.S. ed.) pp. 707-730, Alan R. Liss, New York.
- [7] Wikström, M. (1987) in: *Cytochrome Systems* (Papa, S. et al., eds.) pp. 377-383, Plenum Press, New York.
- [8] Mitchell, P. (1987) *FEBS Lett.* 222, 235-245.
- [9] Azzone, G.F., Pozzan, T. and Di Virgilio, F. (1979) *J. Biol. Chem.* 254, 10206-10212.
- [10] Reynafarje, B., Alexandre, A., Davies, P. and Lehninger, A.L. (1982) *Proc. Natl. Acad. Sci. USA* 79, 7218-7222.
- [11] Westerhoff, H.V. and Dam, K., (1987) *Thermodynamics and Control of Biological Free-Energy Transduction*, Elsevier, Amsterdam.
- [12] Papa, S., Capuano, F., Markert, M. and Altamura, N. (1980) *FEBS Lett.* 111, 243-248.
- [13] Papa, S., Guerrieri, F., Lorusso, M. and Quagliariello, E., (1970) *FEBS Lett.* 10, 295-298.
- [14] Lorusso, M., Capuano, F., Boffoli, D., Stefanelli, R. and Papa, S. (1979) *Biochem. J.* 182, 133-147.
- [15] Papa, S., Guerrieri, F. and Rossi Bernardi, L. (1979) *Methods Enzymol.* 55, 614-627.
- [16] Barzu, O. (1978) *Methods Enzymol.* 54, 485-498.
- [17] Capitanio, N., Capitanio, G., De Nitto, E., Villani, G. and Papa, S. (1991) *FEBS Lett.* 288, 179-182.
- [18] Lorusso, M., Gatti, D., Boffoli, D., Bellomo, E. and Papa, S. (1983) *Eur. J. Biochem.* 137, 413-320.
- [19] Murphy, M.P. and Brand, M.D. (1988) *Eur. J. Biochem.* 173, 645-651.
- [20] Brown, G.C. (1989) *J. Biol. Chem.* 264, 14704-14709.
- [21] Papa, S., Capitanio, N. and De Nitto, E., (1987) *Eur. J. Biochem.* 164, 507-516.
- [22] Wikström, M., Krab, K. and Saraste, M. (1981) *Cytochrome Oxidase, A synthesis*, Academic Press, New York.
- [23] Blair, D.F., Gelles, J. and Chan, S.I. (1986) *Biophys. J.* 50, 713-733.
- [24] Malmström, B.G., (1989) *FEBS Lett.* 250, 9-21.