Volume 231, number 1, 247–252 FEB 05781 April 1988

Protonic sidedness of the binuclear iron-copper centre in cytochrome oxidase

Mårten Wikström

Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10A, SF-00170 Helsinki 17, Finland

Received 22 February 1988

The oxidised (ferric-cupric) binuclear centre of cytochrome oxidase is converted into two other states, presumably ferryl-cupric (F) and ferric-peroxy-cupric (P), by energy-dependent reversed electron transfer from the centre (and water) to cytochrome *c* [(1981) Proc. Natl. Acad. Sci. USA 78, 4051–4054; (1987) Chem. Scr. 27B, 53–58]. This sequence of events represents a partial reversal of the O_2 reduction catalysed by the centre. Here it is shown that the strong pH-dependence of these reactions is exerted specifically from the matrix (M) side of the inner mitochondrial membrane. This proves unequivocally that the binuclear centre generates protonmotive force by means of its vectorial accessibility for electrons and protons.

O_2 reduction; Respiratory chain; Proton pumping; Protonmotive force; Oxidative phosphorylation; Proton stoichiometry

1. INTRODUCTION

Oxygen reduction to water, the most essential step in cell respiration, is catalysed by the binuclear iron-copper centre of cytochrome oxidase. The centre accepts electrons from cytochrome *c*, which is located on the outside of the inner mitochondrial membrane. Cytochrome *a* and Cu_A are involved in this electron transfer, which is coupled to translocation of 1 H^+/e^- across the membrane by a so far unknown mechanism [1–3].

Mitchell and Moyle [4] first proposed that the four protons required in reduction of O_2 to water would be taken up from the matrix (M) side of the inner mitochondrial membrane. Uptake of electrons and protons in the reduction of O_2 to water would thus occur from opposite sides of the membrane, which would render this reaction protonmotive.

The finding that cytochrome oxidase catalyses proton translocation [5] reopened this question. Suppose that the proton pump functions with a $2H^+/e^-$ stoichiometry. Since there is experimentally net release of one proton per electron on the cytoplasmic side of the membrane, this alternative would mean that the 'water protons' would have to be taken up from the cytoplasmic side [5].

Certain subsequent findings have been interpreted to support the notion of uptake of the water protons from the matrix.

Treatment of cytochrome oxidase with DCCD was demonstrated to abolish net proton release on the cytoplasmic side of the membrane [6]. Similarly, the removal of subunit III was reported in some conditions to abolish net proton-pumping [7], although later results have shown that some proton translocation can remain after removal of this subunit [8–10]. In all these cases the vesicular cytochrome oxidase preparations retained respiratory control, indicating that cytochrome oxidase retained ability to generate protonmotive force without net proton pumping. Penttilä [7]

Correspondence address: M. Wikström, Department of Medical Chemistry, University of Helsinki, Siltavuorenpenger 10A, SF-00170 Helsinki 17, Finland

Abbreviations: DCCD, dicyclohexyl carbodiimide; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; FerriCN, potassium ferricyanide; KP_i, potassium phosphate; NEM, *N*-ethylmaleimide; oligo, oligomycin

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

showed that in preparations depleted of subunit III, and devoid of net proton translocation, the charge translocation ratio fell from its normal value of 2 charges per electron to about 1.

These and other related data have generally been interpreted to mean decoupling or 'slipping' of the proton pump from its linkage to electron transfer, and hence to demonstrate that the binuclear centre must catalyse the remaining charge separation and consequently take up the water protons from the matrix space of the mitochondrion.

However, though very widely accepted, this interpretation is not conclusive. If, for example, the pump consisted of two sequential $1H^+/e^-$ translocation steps of different mechanism (and the water protons were taken up from the cytoplasmic side), decoupling of one, but not the other, would yield results indistinguishable from those just described.

Konstantinov et al. [11] recently showed that the pH-sensitive binding of cyanide to the binuclear centre depends specifically on pH in the matrix space. This was interpreted to show that the binuclear centre is in protonic contact with the matrix, and that the water protons are taken up from the matrix during respiration. But again it cannot be excluded that this is an indirect effect where protonation of an acidic group in equilibration with the matrix, but far away from the binuclear centre, controls the accessibility or reactivity of the binuclear centre with cyanide. Clearly more direct data on the protonic sidedness of the binuclear centre is required to fully understand whether it exhibits protonmotive function.

A direct approach to this problem is possible from a study of the pH-dependence of the partial catalytic steps of O_2 reduction at the binuclear centre. As we have shown [12–14], it is possible to reverse this reaction. Electron transfer from the binuclear centre (plus water) to ferricytochrome *c* can be observed under the influence of a high protonmotive force. In such conditions the originally ferric/cupric state (O) of the centre is converted into a 'ferryl' (F), and the latter into a 'peroxy' (P) intermediate, both reactions being strongly favoured by high pH (fig.1). Over most of the pH range studied both reactions appear to be linked to the release of two protons [13,14]. As these steps are very likely the ones in which protons are taken up for formation of water in the forward reaction,

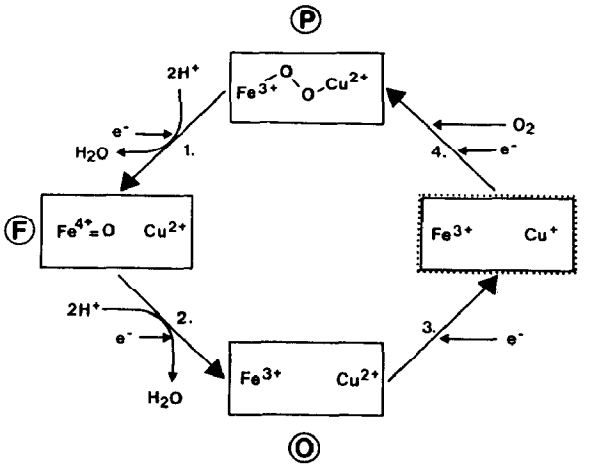

Fig.1. Suggested main intermediates in the reduction of O_2 catalysed by the binuclear centre of cytochrome oxidase (from [14]; cf. [1,12,13]). Fe and Cu represent haem iron of cytochrome a_3 and Cu_B of the binuclear centre, respectively. O is the oxidised ferric/cupric state. F and P are spectrophotometrically distinct states which are formed from O by transfer of one and two electrons to ferricytochrome *c*, respectively, in conditions that favour reversed electron transfer [1,12–14].

the possible sidedness of the pH-dependence can be studied directly.

2. MATERIALS AND METHODS

Rat liver mitochondria were prepared by a conventional procedure [5] in 0.25 M sucrose–0.1 mM EGTA, and suspended in 0.25 M sucrose at a concentration corresponding to 10 μM cytochrome aa_3. The latter was determined from the absorbance difference upon reduction with dithionite at 605–630 nm, using a millimolar absorptivity of 27 cm^{-1}.

Dual wavelength spectrophotometry was performed at room temperature with a DBS-1 spectrophotometer (Johnson Research Foundation Workshops, University of Pennsylvania), using conventional glass cuvettes with 1 cm light path.

Spectrophotometric measurements of intermediates F and P were performed at 583–630 and 607–630 nm. Intermediate F has a broad absorption maximum at 583 nm in the difference spectrum against oxidised enzyme (intermediate O), with a millimolar absorptivity of approx. 6 cm^{-1}. Intermediate P absorbs maximally at 607 nm (relative to O), with a millimolar absorptivity of approx. 12 cm^{-1}. The spectra of F and P overlap [12,13]. The contribution of each is obtained by measurement at both wavelength couples and spectral deconvolution.

The precise experimental conditions are described in the legends to the figures.

The FCCP was a gift by Dr P.G. Heytler. Myxothiazol was obtained through the courtesy of Drs G. von Jagow and H. Reichenbach. Other reagents were commercial products of the highest purity available.

3. RESULTS

In previously published experiments the F and P forms of the binuclear centre were generated in isolated mitochondria using ATP hydrolysis as the source of energy. In figs 2 and 3 the protonmotive force is generated by coupled oxidation of β-hydroxybutyrate by ferricyanide. Excess ferricyanide keeps cytochrome *c* highly oxidised in the steady state, which, in addition to protonmotive force, is required to reverse the dioxygen reaction [12]. In the presence of excess ferricyanide there is no electron flux from substrate to dioxygen [15].

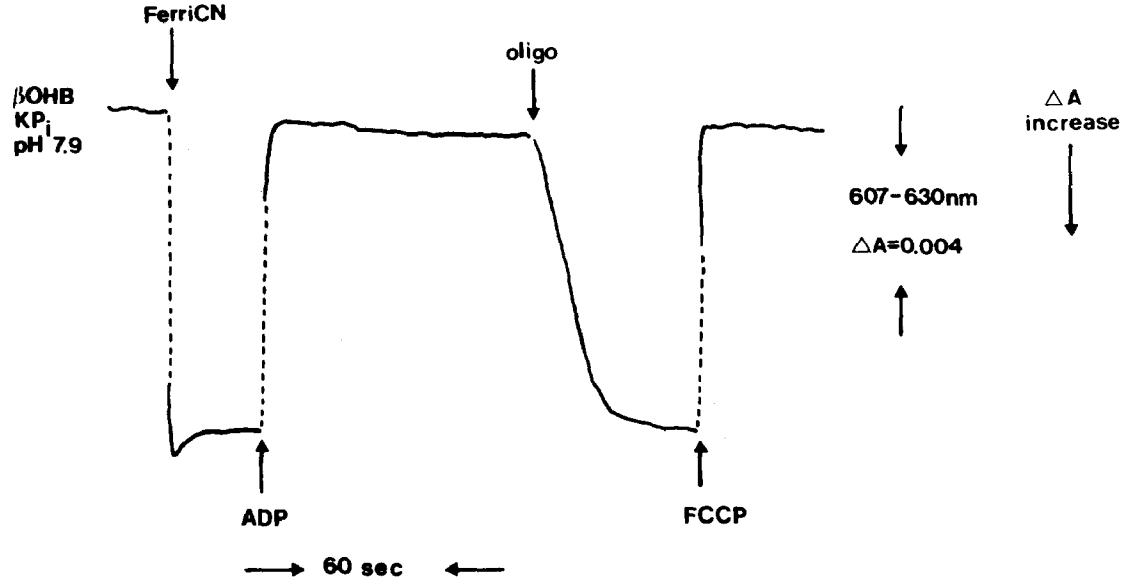

Fig.2. Formation of P intermediate driven by protonmotive force generated by coupled oxidation of β-OH-butyrate by ferricyanide. Rat liver mitochondria (0.85 μM cytochrome aa_3) were suspended in 200 mM sucrose–20 mM KCl–20 mM Tris-Hepes medium (pH 7.9), in the presence of 0.1 mM EGTA, 5 mM potassium phosphate, and 7.7 mM β-OH-butyrate. Additions shown in the figure are: 3.1 mM FerriCN, 1.5 mM ADP, 3.9 μg/ml oligomycin, and 0.5 μM FCCP.

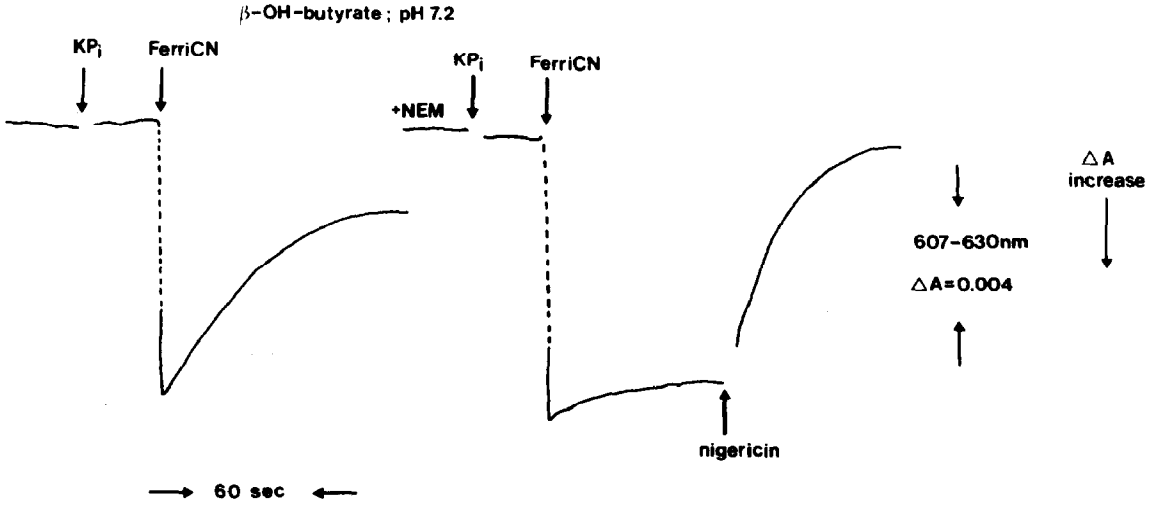

Fig.3. Formation of P driven by protonmotive force generated by coupled oxidation of β-OH-butyrate by ferricyanide. Conditions as in fig.2, except that pH = 7.2, and that the potassium phosphate addition is shown in the figure (KP_i). FerriCN is added as in fig.2. In the experiment to the right 0.25 mM NEM is added before the phosphate, and nigericin (38 ng/ml) is added as shown.

When electron transfer is initiated from the NAD-linked substrate to ferricyanide by addition of the latter, the originally ferric-cupric binuclear centre (state O) is converted into intermediate P (fig.2; see section 2). As shown [12–14], maximum formation of P is optimal at a high pH of the medium. Interestingly, the addition of ADP, which in the presence of inorganic phosphate initiates oxidative phosphorylation and a slight decline in the steady state protonmotive force, reverses the formation of P. P is again formed when oxidative phosphorylation is inhibited by oligomycin (fig.2). Evidently, the formation of P from O is very sensitive to relatively small changes in the protonmotive force.

Fig.3 shows a similar experiment, but now at a much lower extramitochondrial pH. Addition of ferricyanide initially yields a similar amount of P as in fig.2, but now its formation is transient and it returns to a much lower steady state level. As also shown in fig.3, this spontaneous decay of P can be prevented by NEM. Nigericin, which catalyses electroneutral K^+/H^+ exchange across the membrane, induces relaxation of the binuclear centre back to the O state. The latter is not the case at high extramitochondrial pH (not shown, but see below).

These experiments confirm that formation of P from O is extensive and stable at high extramitochondrial pH. At low extramitochondrial pH P is formed extensively in the steady state only provided that pH equilibration across the membrane is prevented by blocking the phosphate carrier with NEM. The transient formation of P at low extramitochondrial pH (fig.3) is presumably due to a transient rise of intramitochondrial pH when redox-coupled proton translocation is initiated. Subsequently, electroneutral pH equilibration takes place across the membrane, catalysed mainly by the phosphate/proton symporter [16].

These results suggest that the reversed electron transfer reaction from the binuclear centre to cytochrome *c*, which is associated with formation of intermediate P from intermediate O (fig.1), is sensitive specifically to pH of the matrix space.

Intramitochondrial pH may be modulated in the presence of nigericin without changing the pH of the well-buffered outside medium, and without appreciably affecting the protonmotive force generated by ATP hydrolysis. The potassium ion concentration inside rat liver mitochondria is about 120 mM. At low extramitochondrial potassium concentrations nigericin will thus induce intramitochondrial acidification. However, this should not take place at an extramitochondrial potassium concentration comparable to that inside.

Fig.4 shows the ATP-linked formation of P (607 nm) and F (585 nm) at the appropriate wavelength couples (see section 2), with 120 mM (traces A and B) and 20 mM (traces C and D) potassium in the medium, and at a high extramitochondrial pH. Lowering the extramitochondrial potassium concentration displaces the P/F equilibrium strongly towards F (cf. fig.1). It should be noticed that there is spectral overlap between F and P (cf. section 2). When this is deconvoluted it is found that there is an approx. 30-fold decrease in the P/F ratio when the

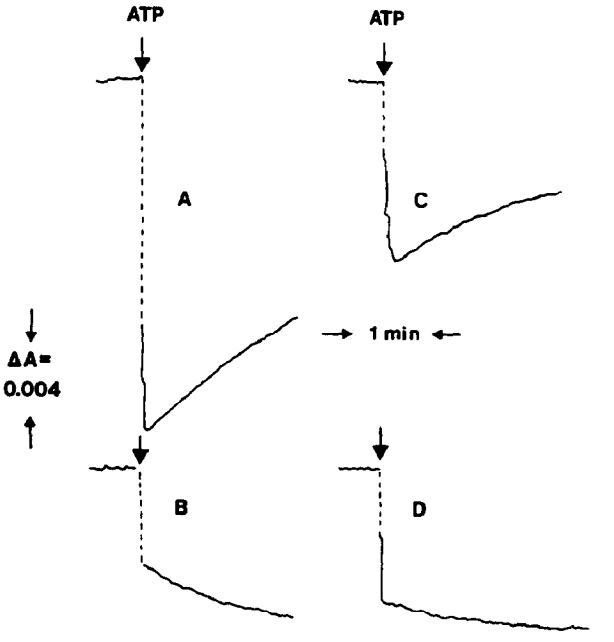

Fig.4. Effect of intracellular pH on the equilibrium between P and F. Rat liver mitochondria (1.6 μM cytochrome aa_3) were suspended in 120 mM KCl–20 mM Tris-Hepes (traces A and B) or in 200 mM sucrose–20 mM KCl–20 mM Tris-Hepes (traces C and D), at pH 8.1, with further additions of 3 μM rotenone, 0.1 mM EGTA, 0.19 μg/ml antimycin, 7 μM myxothiazole, 5 mM sodium ferricyanide, and 38 ng/ml nigericin. 3 mM ATP was added, as shown. Traces A and C were recorded at 607–630 nm; traces B and D at 585–630 nm. Downward deflection reflects an increase in absorption at the 607 and 585 nm relative to the respective reference.

potassium concentration is dropped from 120 to 20 mM. This phenomenon is not seen in the absence of nigericin (not shown, but cf. below).

Fig.5 shows an analogous experiment in conditions that favour observation of the F/O equilibrium (see fig.1), i.e. lower pH and lower redox potential of cytochrome *c*. Here all traces are recorded at 583–630 nm where absorption of intermediate F predominates. Measurements at 607 nm (not shown) revealed that in these conditions there is no significant formation of intermediate P.

In the absence of nigericin (fig.5; traces A,B) a decrease in the potassium concentration has only a small influence on the F/O equilibrium. But with nigericin present (traces C,D) the decrease in the potassium concentration causes a dramatic displacement of the F/O equilibrium towards O. Note here that the baseline before addition of ATP

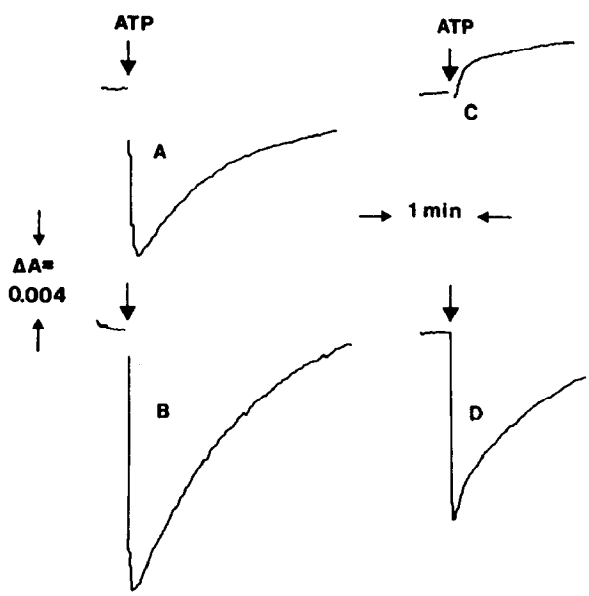

Fig.5. Effect of intracellular pH on the equilibrium between F and O. Rat liver mitochondria (2.3 μM cytochrome aa_3) were suspended in either 240 mM sucrose–20 mM Hepes (traces A and C) or in 120 mM KCl–20 mM Hepes (traces B and D), at pH 7.2, and in the further presence of 3 μM rotenone, 0.1 mM EGTA, 0.19 μg/ml antimycin, 7 μM myxothiazole, 4.2 mM sodium ferrocyanide and 2.1 mM sodium ferricyanide. Nigericin (42 ng/ml) was further present in the experiments shown in traces C and D, but not in those of traces A and B. All measurements were done at 583–630 nm; downward deflection of the trace reflects an increase in absorption at 583 nm relative to the reference.

represents maximum occupancy of the ferric/cupric intermediate O.

Clearly both the P/F and F/O equilibria are specifically dependent on pH of the matrix space. The quantitative aspects of these pH-dependences have been reported elsewhere [13,14].

4. DISCUSSION

The results show that the catalytic events at the binuclear centre are in protonic equilibrium specifically with the intramitochondrial (matrix) space. This proves that the binuclear centre possesses unique functional sidedness or accessibility for protons. Together with the electronic sidedness this makes the function of the binuclear centre protonmotive. Consequently, it can now also be concluded that the proton pump functions with a H^+/e^- stoichiometry of unity (cf. section 1).

Experiments on removal of subunit III or DCCD-treatment of the enzyme that have shown abolishment of net proton translocation, have consistently been interpreted as effects on the proton pump. But in the light of the information presented in this paper we should stress that this interpretation is equivocal.

Loss of net proton release on the cytoplasmic side of the membrane, decrease of proton uptake on the matrix side, and a decrease in the number of translocated charge equivalents, need not necessarily be due to effects on the proton pump. Such results would follow also if the chemical/structural perturbation of the native enzyme resulted in a significant increase in the protonic conductance from the cytoplasmic (C) side into the binuclear centre. In fact, there is no finding reported to date that would discount this possibility.

A relative increase in protonic conductance between the C phase and the binuclear centre could, in fact, easily account for the observations of non-integer H^+/e^- ratios. It seems unlikely that the artificially induced conductance from the C side could become higher than that from the M side as a result of a chemical or structural perturbation of the enzyme. However, in the presence of protonmotive force the driving force on the proton is expected to be higher from the C side into the binuclear centre than from the M side. In such conditions the velocity of proton uptake from the

former side could be comparable to that from the latter.

Accordingly, protonic conduction into the binuclear centre from the C side, if significant, would be expected to be highly dependent on the magnitude of the protonmotive force.

Speculations on the molecular background of a 'slip' in the proton pump of cytochrome oxidase are clearly premature before it has been shown that the observed decrease in H^+/e^- ratio is not simply the result of an increased proton current from the C side of the protein into the binuclear centre of cytochrome oxidase.

Acknowledgements: This work was supported by grants from the Sigrid Juselius Foundation and the Finnish Academy (Medical Research Council). The technical assistance of Hilkka Vuorenmaa, and helpful comments by Moshe Finel, Tuomas Haltia, Anne Puustinen and Matti Saraste are gratefully acknowledged.

REFERENCES

[1] Wikström, M., Krab, K. and Saraste, M. (1981) Cytochrome Oxidase – A Synthesis, Academic Press, London.
[2] Malmström, B.G. (1982) Annu. Rev. Biochem. 51, 21–59.
[3] Naqui, A., Chance, B. and Cadenas, E. (1986) Annu. Rev. Biochem. 55, 137–166.
[4] Mitchell, P. and Moyle, J. (1967) in: Biochemistry of Mitochondria (Slater, E.C., Kaniuga, Z. and Wojtczak, L. eds) pp.53–74, Academic Press, London and PWN, Warsaw.
[5] Wikström, M. (1977) Nature 266, 271–273.
[6] Casey, R.P., Thelen, M. and Azzi, A. (1980) J. Biol. Chem. 255, 3994–4000.
[7] Penttilä, T. (1983) Eur. J. Biochem. 133, 355–361.
[8] Puettner, I., Carafoli, E. and Malatesta, F. (1985) J. Biol. Chem. 260, 3719–3723.
[9] Sarti, P., Jones, M.G., Antonini, G., Malatesta, F., Colossimo, A., Wilson, M.T. and Brunori, M. (1985) Proc. Natl. Acad. Sci. USA 82, 4876–4880.
[10] Finel, M. and Wikström, M. (1986) Biochim. Biophys. Acta 851, 99–108.
[11] Konstantinov, A., Vygodina, T. and Andreev, I.M. (1986) FEBS Lett. 202, 229–234.
[12] Wikström, M. (1981) Proc. Natl. Acad. Sci. USA 78, 4051–4054.
[13] Wikström, M. (1987) Chem. Scr. 27B, 53–58.
[14] Wikström, M. (1988) Chem. Scr., in press.
[15] Sottocasa, G.L. and Sandri, G. (1970) Biochim. Biophys. Acta 223, 36–45.
[16] Lanoue, K.F. and Schoolwerth, A.C. (1984) in: Bioenergetics (Ernster, L. ed.) pp.221–268, Elsevier Science Publishers, Amsterdam.