ELSEVIER

Bioelectrochemistry 55 (2002) 139–143

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

Short communication

Electron transfer dynamics of cytochrome *c* bound to self-assembled monolayers on silver electrodes

P. Hildebrandt, D.H. Murgida*

Instituto de Tecnologia Química e Biológica, ITQB, Apartado 127, Av. Da República, 2780 Oeiras, Portugal
Max-Planck-Institut für Strahlenchemie, Stiftstr. 34–36, D-45470 Mülheim, Germany

Received 1 June 2001; accepted 5 September 2001

Abstract

Cytochrome *c* (Cyt-*c*) was electrostatically immobilised on Ag electrodes coated with self-assembled monolayers (SAM) that are formed by ω-carboxyl alkanethiols with different alkyl chain lengths (C_x). Surface enhanced resonance Raman (SERR) spectroscopy demonstrated that electrostatic binding does not lead to conformational changes of the heme protein under the conditions of the present experiments. Employing time-resolved SERR spectroscopy, the rate constants of the heterogeneous electron transfer (ET) between the adsorbed Cyt-*c* and the Ag electrode were determined for a driving force of zero electronvolts. For SAMs with long alkyl chains (C_{16}, C_{11}), the rate constants display a normal exponential distance dependence, whereas for shorter chain lengths (C_6, C_3, C_3), the ET rate constant approaches a constant value (ca. 130 s^{-1}). The onset of the non-exponential distance-dependence is paralleled by an increasing kinetic H/D effect, indicating a coupling of the redox reaction with proton transfer (PT) steps. This unusual kinetic behaviour is attributed to the effect of the electric field at the Ag/SAM interface that increasingly raises the energy barrier for the PT processes with decreasing distance of the adsorbed Cyt-*c* from the electrode. The distance-dependence of the electric field strength is estimated on the basis of a simple electrostatic model that can consistently describe the redox potential shifts of Cyt-*c* as determined by stationary SERR spectroscopy for the various SAMs. At low electric fields, PT is sufficiently fast so that rate constants, determined as a function of the driving force, yield the reorganisation energy (0.217 electronvolts) of the heterogeneous ET. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Cytochrome *c*; Electron transfer; Electrode; Self-assembled monolayer; Electric field; Surface enhanced resonance Raman spectroscopy

1. Introduction

Cytochrome *c* (Cyt-*c*), a soluble heme protein, acts as an electron shuttle in the respiratory chain of aerobic organisms [1]. Prior to the electron transfer (ET), an electrostatically stabilised complex is formed between Cyt-*c* and its membrane-bound partner proteins. Thus, the interprotein ET takes place under the influence of electrostatic field originating from local Coulombic interactions between oppositely charged amino acids in the protein binding domains as well as from the transmembrane potential gradient. In order to elucidate possible effects of such electrostatic fields on the ET dynamics and mechanism, we have studied the redox process of Cyt-*c* bound to self-assembled monolayers (SAM) on an Ag electrode that are formed by ω-carboxyl alkanethiols. As a particular advantage of this model system, it is possible to employ surface enhanced resonance Raman (SERR) spectroscopy that selectively probes the vibrational spectrum of the heme group exclusively of the adsorbed proteins [2–4]. This technique can be extended to the time-resolved domain by monitoring the SERR spectra subsequent to a potential jump [3–5]. In this way, it is possible to obtain detailed information about the interfacial processes and the active sites structures of the adsorbed species that are involved. Furthermore, changing the thickness of the SAM, i.e., the length of the alkyl chain, the electric field strength at the protein binding site can be varied in a systematic manner, which is turn is a prerequisite for analysing possible electric field effects on the equilibria and kinetics of the interfacial processes.

---

* Corresponding author. Instituto de Tecnologia Química e Biológica, ITQB, Apartado 127, Av. Da República, 2780 Oeiras, Portugal. Tel.: +351-21-446-9741; fax: +351-21-441-1277.
E-mail address: murgida@itqb.unl.pt (D.H. Murgida).

PII: S1567-5394(01)00154-2

2. Experimental section

2.1. Chemicals

Mercaptoacetic acid (C_2), 3-mercaptopropionic acid (C_3), 11-mercaptoundecanoic acid (C_{11}) and 16-mercaptohexadecanoic acid (C_{16}) were purchased from Sigma and used without further purification. 6-Mercaptohexanoic acid (C_6) was synthesised according to published procedures [6], and purified by HPLC. Horse heart cytochrome *c* (Cyt-*c*; Sigma) was purified as previously published [7].

2.2. SERR spectroscopy

SERR spectra were measured with 413-nm cw-excitation using a Kr-ion laser with a power of ca. 50 mW at the sample, using the experimental set described in detail elsewhere [5]. The spectral bandwidth and the increment per data point were 4 cm^{-1} and 0.53 cm^{-1}, respectively. The total accumulation time of the stationary and time-resolved SERR spectra was ca. 10 and 5 s, respectively. SERR experiments were carried out with a rotating electrode to avoid degradation processes caused by laser-induced heating. All potentials cited in this work refer to the saturated calomel electrode (SCE). A detailed description of the principles of the time-resolved SERR method as well as of the set-up developed in our laboratory is given elsewhere [4,5].

The preparation of the SER-active SAM-coated Ag electrodes was described elsewhere [2]. Modified electrodes were placed into the electrochemical cell containing 0.2 μM Cyt-*c* and 12.5 mM potassium phosphate buffer and 12.5 mM K_2SO_4 as supporting electrolyte, either in H_2O or D_2O at pH 7.0. The electrode was kept at open circuit for 45 min to establish adsorption equilibrium. After background subtraction, the SERR spectra were subjected to a component analysis in which complete spectra of the individual species are fitted to the measured spectra [8].

3. Results and discussion

Resonance Raman (RR) spectra of heme proteins display a vibrational band pattern that is characteristic of the oxidation, spin, and ligation state of the porphyrin [9]. For ferric six-coordinated low spin (6cLS) Cyt-*c* in neutral aqueous solution, typical marker bands are at 1371 (ν_4) and 1501 cm^{-1} (ν_3), whereas in the ferrous 6cLS species they are shifted to 1360 and 1491 cm^{-1}, respectively [2–5,7]. Immobilisation of the protein on a SAM-coated Ag electrode that is in contact with solution containing 0.2 μM Cyt-*c* leads to SERR spectra that are essentially indistinguishable from the RR spectra of Cyt-*c* in solution [2]. These findings imply that under these conditions electrostatic adsorption of Cyt-*c* occurs without any structural changes in the heme pocket.

The redox potential of the adsorbed Cyt-*c* is determined on the basis of potential-dependent stationary SERR measurements [2]. Simulating these SERR spectra by a superposition of the component spectra of ferric and ferrous Cyt-*c*, allows the quantitative analysis of the redox equilibrium. The redox potentials E^{o} obtained in this way reveal a systematic negative shift with increasing chain length of the SAM (Table 1). This shift can be rationalised in terms of an electrostatic model that takes into account the potential drops across the Ag/SAM/protein interfaces [2]. It consistently describes the magnitude of these shifts and also yields values for the $\mathrm{p}K_\mathrm{A}$ of the carboxylate head groups and the protein coverage, which compare very well with experimental data derived from electrochemical studies. Furthermore, the model allows estimating the changes of the electric field strength at the protein-binding site as a function of the SAM length (Table 1).

The dynamics of the heterogeneous ET of the adsorbed Cyt-*c* was analysed by employing time-resolved SERR spectroscopy [3,4]. Following potential jumps from the initial potential E_i to a final potential E_f, SERR spectra were measured at variable delay times δ. None of the spectra includes contributions from Cyt-*c* species other than the native ferrous and ferric states (denoted as B1) as shown in Fig. 1 for Cyt-*c* adsorbed on a C_{16}-coated Ag electrode with $E_\mathrm{i} = +0.105$ V and $E_\mathrm{f} = -0.240$ V, corresponding to an overpotential of -0.2 V. Therefore, it is concluded that the relaxation process exclusively involves the redox reaction of the adsorbed Cyt-*c* with the electrode. Setting $E_\mathrm{f} = E^\mathrm{o}$ and $E_\mathrm{i} = (E^\mathrm{o} - 0.1)$ V, the heterogeneous ET reaction occurs with a driving force of zero electronvolts ($\Delta G = 0$ eV). Then the quantitative analysis of these time-resolved SERR spectra in terms of a one-step relaxation process yields the relaxation constant τ that is related to the formal heterogeneous rate constant k^0_ET according to

$$\tau = \frac{1}{2k^0_\mathrm{ET}}. \tag{1}$$

The rate constants determined in this way for the various SAM-coatings are listed in Table 1. At long chain lengths,

Table 1
Redox potentials and kinetic parameters of the heterogeneous ET of Cyt-*c* on SAM-coated Ag electrodes[a]

d, Å	E^o, V (ΔE^o, V)	E_f, $\mathrm{V \cdot m^{-1}}$	k^0_ET, $\mathrm{s^{-1}}$	k^0_ET (H_2O)/ k^0_ET (D_2O)
24.0	0.0025 (− 0.0075)	$1.2 \cdot 10^9$	0.073	1.0
19.0	− 0.0096 (− 0.0196)	$1.5 \cdot 10^9$	43	1.0
11.5	− 0.0142 (− 0.0242)	$2.0 \cdot 10^9$	134	1.2
7.6	− 0.0310 (− 0.0410)	$2.1 \cdot 10^9$	128	2.0
6.3	− 0.0390 (− 0.0490)	$2.4 \cdot 10^9$	132	4.0

[a] SAM lengths (*d*), redox potentials (E^o), shifts of the redox potential with respect to the redox potential in solution (ΔE^o), and electric field strengths (E_F) are taken from Ref. [2]. ET rate constants are adopted from Ref. [4] and were measured at $\Delta G = 0$ eV.

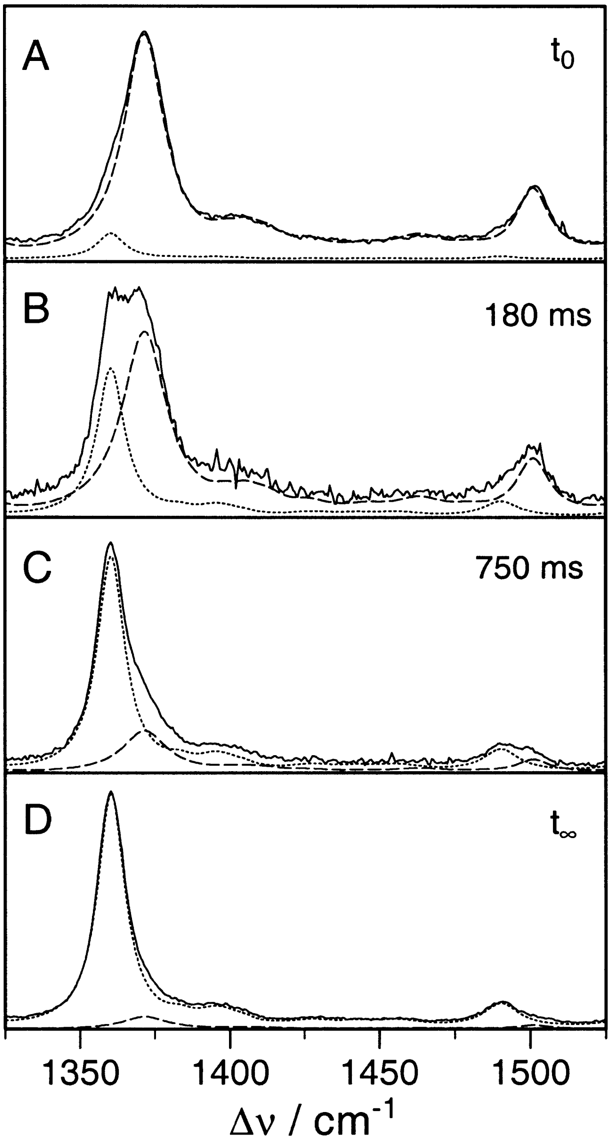

Fig. 1. Stationary and time-resolved SERR spectra of Cyt-*c* adsorbed on a C_{16}-coated Ag electrode measured with 413-nm excitation. (A) Stationary SERR spectrum measured at the initial potential of $E_i = +0.105$ V; (B, C) time-resolved SERR spectra measured at different delay times δ subsequent to the potential jump from $E_i = +0.105$ V to $E_f = -0.240$ V; (D) stationary SERR spectrum measured at the final potential of $E_f = -0.240$ V. The component spectra of the reduced and oxidized forms of Cyt-*c* are represented by the dotted and dashed lines, respectively.

the values display the expected exponential distance dependence of the ET. The increase of k_{ET}^0 from C_{16} to C_{11} corresponds to an electronic tunneling parameter (β) of 1.28 Å^{-1} and a pre-exponential factor (A) of 1.45×10^{12} s^{-1} that are close to those determined for long range ET reactions of and in proteins [4,10]. However, at C_6-SAM (11.5 Å) there is an increase of k_{ET}^0 only by a factor of ca. 3, and moreover, k_{ET}^0 remains essentially constant upon further reducing the chain lengths to 6.3 Å (C_2). Carrying out the measurements with the electrode in contact with a D_2O solution under otherwise identical conditions, the rate constants $k_{ET}^0(D_2O)$ for C_{16} and C_{11} were found to be identical to those obtained in H_2O [k_{ET}^0 (H_2O)]. In contrast, k_{ET}^0 (D_2O) was determined to be lower than k_{ET}^0 (H_2O) for C_6, C_3 and C_2 corresponding to a kinetic isotope effect that increases with decreasing chain length (Table 1). These findings imply that a process involving a proton transfer (PT) becomes rate limiting a short chain lengths, paralleling the onset of the non-exponential distance dependence of k_{ET}^0. This process has been attributed to the reorganisation of the hydrogen bonding network in the heme pocket of Cyt-*c* that is required to adapt the protein structure to the change in the charge distribution associated with the ET [4]. Since a non-exponential distance dependence in conjunction with a kinetic H/D effect has not been observed for electron-transferring proteins in solution, this kinetic behaviour must be related to the specific reaction conditions at the Ag/SAM interface. Whereas, regardless of the alkyl chain length of the monolayer, immobilisation of Cyt-*c* occurs via electrostatic binding between the lysine-rich domain around the heme crevice of the protein and the carboxylate head groups of the SAM in the same way, the electric field strength increases with decreasing separation of the protein binding site from the Ag surface (Table 1) [2]. Thus, it is concluded that the energy barrier for the PT processes of the adsorbed Cyt-*c* is raised by the electric field, which increases upon reducing the distance to the electrode, until nuclear tunneling becomes the rate-limiting step of the redox process [4]. The increase of this energy barrier slows down deuteron tunneling more strongly than proton tunneling so that this interpretation also accounts for the distance-dependent kinetic H/D effect.

Conversely, only for long chain lengths electron tunneling is the rate-limiting step. Thus, we have chosen a C_{16}-SAM coated electrode to determine the reorganisation energy λ. In these experiments, E_i was set $\geq E^\circ$ whereas E_f was lowered stepwise to increase the driving force ΔG of the heterogeneous ET that is given by

$$\Delta G = F(E_f - E^\circ). \tag{2}$$

ET reactions of molecular species at electrodes can be adequately described by Marcus theory that takes into account the energy distribution of electrons at the Fermi level [11]. Then, the ratio of the rate constants $k_{ET}^{\Delta G}$ (for $\Delta G \neq 0$) and k_{ET}^0 (for $\Delta G = 0$) is given by

$$\frac{k_{ET}^{\Delta G}}{k_{ET}^0} = \frac{1 - \operatorname{erf}\dfrac{\Delta G + \lambda}{2\sqrt{\lambda RT}}}{1 - \operatorname{erf}\dfrac{\lambda}{2\sqrt{\lambda RT}}} \tag{3}$$

where R and T denote the gas constant and the temperature, respectively. The best fit of Eq. (3) to the experimental data that were derived from the time-resolved SERR spectra leads to $\lambda = 0.217$ eV (Fig. 2). On the basis of cyclic voltammetric experiments on Cyt-*c* electrostatically immobilised on SAM-coated Au electrodes, a somewhat higher reorganisation energy (0.35 eV) has been estimated previ-

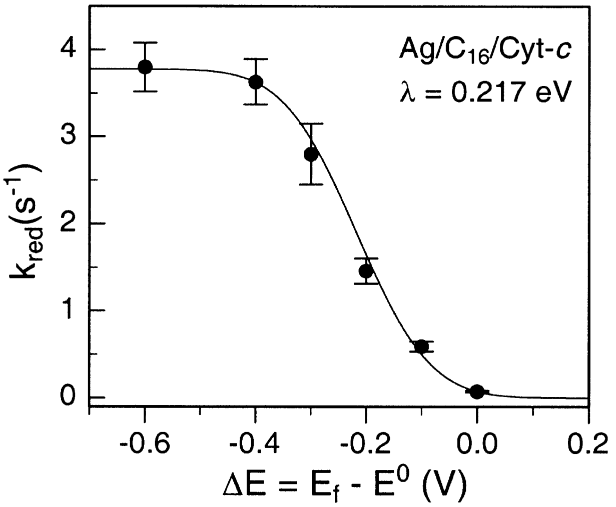

Fig. 2. Driving-force dependence of the ET rate constant for Cyt-*c* adsorbed on the C_{16}-coated Ag electrode. The solid line represents the best fit ($\lambda = 0.217$ eV) of Eq. (3) to the experimental data.

ously [12]. However, this value has been considered with caution in view of the limited accuracy of the method and the striking discrepancy compared to a reorganisation energy of ca. 0.6 eV that has been obtained from photoinduced ET reactions of Cyt-*c* in solution or by cyclic voltammetry for the non-immobilised protein [1,13]. Instead, the present time-resolved SERR spectroscopic approach allows a very precise determination of the reorganisation energy demonstrating that λ is substantially lowered when Cyt-*c* is immobilised via electrostatic interactions.

In general, the reorganization energy of redox proteins includes contributions of the structural reorganization of the redox site (λ_{RC}) and the protein (λ_P) as well as of the orientational reorganization of the surrounding solvent molecules (λ_S) which may constitute a large contribution to the total reorganization energy for Cyt-*c* in solution [14]. Solvent reorientation should predominantly occur in the vicinity of the exposed heme edge at the front surface of the protein. This part of the protein, however, constitutes the binding domain to anionic SAMs so that it is shielded against the solvent for electrostatically adsorbed Cyt-*c* [2]. Consequently, λ_S is expected to be much smaller for such adsorbed proteins, which in turn may account the overall lowering of the experimentally determined reorganisation energy compared to Cyt-*c* in solution. Therefore, the reorganisation energy of adsorbed Cyt-*c* should largely result from λ_{RC} and λ_P.

4. Conclusions

During the natural redox process, Cyt-*c* binds to carboxylate residues in the interaction domain of cytochrome *c* oxidase (CcO) [1] so that electrostatic interactions are similar to those upon immobilisation on anionic SAMs. Thus, most likely also the reorganisation energy of Cyt-*c* in the intra-complex ET to CcO is close to the value of 0.217 eV determined in this work. Consequently, a proper alignment of the partner proteins in the reactive complex may (also) be essential for an efficient exclusion of solvent molecules from the protein–protein interface and, hence, for the lowering of the reorganisation energy. In view of the low driving force (ca. 0 eV) for the ET from Cyt-*c* to the primary acceptor of CcO, such a low reorganisation energy may be required to enable a fast intracomplex ET, for which the rate constant is estimated to be ca. $4 \cdot 10^4$ s^{-1} or even higher [15,16]. In fact, within semiclassical Marcus theory [17], the intracomplex ET rate constant that can readily be reproduced on the basis of $\lambda = 0.217$ eV and the values for A ($1.45 \cdot 10^{12}$ s^{-1}) and β (1.28 $Å^{-1}$) determined for the "normal" exponential distance-dependence of the heterogeneous ET of Cyt-*c* adsorbed on SAM-coated electrodes at weak electric fields ($d > 12$ Å). However, also the dynamics of the redox process in the non-exponential regime of the Ag/SAM/Cyt-*c* assembly, that is at strong electric fields ($d < 12$ Å), may have an impact for understanding the biological ET of Cyt-*c*. In vivo, CcO is embedded in the mitochondrial membrane, so that the electric field strength at the Cyt-*c* binding site sensitively depends on the trans-membrane potential difference. In fact, evidence has been provided that high potential differences may efficiently slow down intra- and intermolecular ET reactions of the Cyt-*c*/CcO redox couple [18]. Thus, in analogy to the electrochemical system, it may well be that under physiological conditions the ET dynamics of Cyt-*c* is modulated by electric fields, which might represent a feedback control mechanism for the enzymatic process of CcO.

Acknowledgements

This work was supported by the Volkswagen-Stiftung (I/75567).

References

[1] R.A. Scott, A.G. Mauk (Eds.), Cytochrome *c*. A Multidisciplinary Approach, University Science Books, Sausalito, CA, 1995.

[2] D.H. Murgida, P. Hildebrandt, The heterogeneous electron transfer of cytochrome *c* adsorbed on coated silver electrodes, Electric field effects on structure and redox potential, J. Phys. Chem. B 105 (2001) 1578–1586.

[3] D.H. Murgida, P. Hildebrandt, Molecular structure and dynamics of immobilized cytochrome *c* on self-assembled monolayers on silver electrodes probed by time-resolved surface enhanced resonance spectroscopy, Angew. Chem., Int. Ed. 40 (2001) 728–731.

[4] D.H. Murgida, P. Hildebrandt, Proton coupled electron transfer in cytochrome *c*, J. Am. Chem. Soc. 123 (2001) 4062–4068.

[5] H. Wackerbarth, U. Klar, W. Günther, P. Hildebrandt, A novel time-resolved surface enhanced (resonance) Raman spectroscopic technique for studying the dynamics of interfacial processes, Application to

the electron transfer reaction of cytochrome *c* at a silver electrode, Appl. Spectrosc. 53 (1999) 283–291.

[6] C.D. Bain, E.B. Troughton, Y.T. Tao, E. Evall, G.M. Whiteside, R.G. Nuzzo, Formation of monolayer films by the spontaneous assembly of organic thiols from solution onto gold, J. Am. Chem. Soc. 111 (1989) 321–335.

[7] P. Hildebrandt, M. Stockburger, Cytochrome *c* at charged interfaces: 1. Conformational and redox equilibria at the electrode/electrolyte interface probed by surface enhanced resonance Raman spectroscopy, Biochemistry 28 (1989) 6710–6721.

[8] S. Döpner, P. Hildebrandt, A.G. Mauk, H. Lenk, W. Stempfle, Analysis of vibrational spectra of multicomponent systems, Application to a resonance Raman spectroscopic study of cytochrome *c*, Spectrochim, Acta Part A, Biomol. Spectrosc. 51 (1996) 573–584.

[9] N. Parthasarathi, C. Hansen, S. Yamaguchi, T.G. Spiro, Metalloporphyrin core size resonance Raman marker bands revisited, Implications for the interpretation of hemoglobin photoproduct Raman frequencies, J. Am. Chem. Soc. 109 (1987) 3865–3871.

[10] R.J.P. Williams, Electron transfer and proton coupling in proteins, J. Solid State Chem. 145 (1999) 488–495.

[11] C.E.D. Chidsey, Free-energy and temperature-dependence of electron-transfer at the metal-electrolyte interface, Science 251 (1991) 919–922.

[12] S. Song, R.A. Clark, E.F. Bowden, Characterization of cytochrome *c*/alkanethiolate structures prepared by self assembly on gold, J. Phys. Chem. 97 (1993) 6564–6572.

[13] J. Cheng, S. Terretaz, J.I. Blankman, C.J. Miller, B. Dangi, R.D. Guiles, Electrochemical comparison of heme proteins by insulated electrode voltammetry, Isr. J. Chem. 37 (1997) 259–266.

[14] M. Fedurco, Redox reactions of heme-containing metalloproteins: dynamics effects of self-assembled monolayers on thermodynamics and kinetics of cytochrome *c* electron-transfer reactions, Coord. Chem. Rev. 209 (2000) 263–331.

[15] L.M. Geren, J.R. Beasley, B.R. Fine, A.J. Saunders, S. Hibdon, G.J. Pielak, B. Durham, F. Millet, Design of a ruthenium-cytochrome c derivative to measure electron transfer to the initial acceptor in cytochrome *c* oxidase, J. Biol. Chem. 270 (1995) 2466–2472.

[16] K. Wang, Y. Zhen, R. Sadoski, S. Grinnell, L. Geren, S. Ferguson-Miller, B. Durham, F. Millet, Definition of the interaction domain for cytochrome *c* on cytochrome *c* oxidase, J. Biol. Chem. 274 (1999) 38042–38050.

[17] R.A. Marcus, N. Sutin, Electron transfer in chemistry and biology, Biochim. Biophys. Acta 811 (1985) 265–322.

[18] P. Sarti, G. Antonini, F. Malatesta, M. Brunori, Respiratory control in cytochrome *c* oxidase vesicles is correlated with the rate of internal electron transfer, Biochem. J. 284 (1992) 123–127.