Biochimica et Biophysica Acta 809 (1985) 39–43
Elsevier

BBA 41807

A new method for the determination of the buffer power of artificial phospholipid vesicles by stopped-flow spectroscopy

Paolo Sarti [a], Francesco Malatesta [b], Giovanni Antonini [b], Alfredo Colosimo [b] and Maurizio Brunori [a,*]

[a] *Institutes of Chemistry and Biochemistry and the Centre of Molecular Biology (C.N.R.), University of Rome 'La Sapienza' and* [b] *Department of Experimental Medicine and Biochemical Sciences, University of Rome 'Tor Vergata', Rome (Italy)*

(Received April 10th, 1985)

Key words: Liposome; Ionophore; Rapid kinetics; Membrane permeability

Energy-transducing membranes generate protonchemical gradients, and extrused protons have been measured either potentiometrically or spectroscopically by means of a pH indicator. The determination of the stoichiometry of proton pumps (H^+/e^-), such as that of cytochrome *c* oxidase, is affected by the membrane proton permeability and the intrinsic buffer capacity of the system. In order to assess the buffer power of the system (and therefore allow a better estimate of the H^+/e^- ratio for cytochrome *c* oxidase), a new calibration method which can be employed directly in the stopped-flow apparatus has been introduced. The method makes use of the trypsin-catalysed hydrolysis of *N*-α-tosyl-L-arginyl-*O*-methyl ester producing 1 H^+/mol at a velocity which can be calibrated (from milliseconds to seconds) changing trypsin concentration. The buffer capacity of phospholipid vesicles in the presence and absence of ionophores has been determined allowing to distinguish between the outer and inner layers of the artificial phospholipid membrane. The determinations acquire significance with reference to the time course of proton permeability of small unilamellar vesicles in the presence of suitably chosen ionophores.

Introduction

Electron flow through the mitochondrial respiratory chain generates a transmembrane electrochemical gradient which is coupled to the synthesis of ATP and transport of metabolites and ions across the phospholipid bilayer [1]. The number of protons translocated per electron flowing through the redox center (the so called H^+/e^- stoichiometry) is of crucial importance for a correct estimate of $\Delta\tilde{\mu}_{H^+}$ and of energy transduction efficiency [2].

Cytochrome *c* oxidase (EC 1.9.3.1) is an intrinsic membrane protein whose functions include: (A) transfer of electrons from reduced cytochrome *c*, on the cytoplasmic side of the mitochondrial inner membrane, to molecular oxygen with the uptake inside the matrix of one proton per electron transferred to yield water (scalar reaction) and (B) redox-linked proton translocation from the matrix to the aqueous intermembrane space (vectorial reaction), which has been repeatedly demonstrated in mitochondria [3,4] and in artificial phospholipid vesicles [5,6] although the H^+/e^- ratio is still a matter of debate [7].

The value of the H^+/e^- stoichiometry to be calculated from redox and pH measurements de-

* To whom correspondence should be sent at the present address: the Institute of Chemistry, Faculty of Medicine, University of Rome 'La Sapienza', Piazzale Aldo Moro 5, 00185 Rome, Italy.

Abbreviations: TAME, *N*-α-tosyl-L-arginyl-*O*-methyl ester; CCCP, carbonyl cyanide m-chlorophenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

pends on at least two experimental factors, both leading potentially to critical changes in the apparent stoichiometry. The first is the rate of proton back diffusion into the vesicle interior, which may dramatically decrease the apparent stoichiometry leading to an underestimate. We have already pointed out [6] that the use of the stopped-flow (which allows measurements of the vectorial protons on a time scale less than 1 s) minimizes the effect of the back leak. A second important parameter is the correct value of the buffer power of the system. In the absence of protonophores, the total buffer power is accounted for by the outer phospholipid leaflet and the aqueous medium external to the vesicle (where the buffer power is kept low for obvious reasons); on the other hand, when the proton and ion permeability is artificially increased (i.e., in the presence of saturating amounts of ionophores), the total buffer power is expected to increase because of the contribution of the internal vesicle medium and the internal phospholipid leaflet of the bilayer to the total buffer capacity.

Knowledge of the absolute buffer capacity of the system and its time dependence under specific conditions is therefore necessary in order to analyze correctly the time-course of the observed H^+/e^- stoichiometry for the aliquot of protons which have partitioned in between the various buffers present. This paper deals with a stopped-flow calibration method which makes use of the pH indicator phenol red to monitor the trypsin-catalysed release of protons via the hydrolysis of *N*-α-tosyl-L-arginyl-*O*-methyl ester (TAME). This method has been tested in the presence and absence of artificial phospholipid vesicles, either containing or non-containing asymmetrically oriented cytochrome oxidase. The buffer capacity of the external and internal vesicle compartment has been determined in the stopped-flow by modulating the membrane proton permeability with appropriate ionophores, such as valinomycin, nigericin and CCCP. It is shown that, under our conditions of pH and ionic strength, the buffer power is contributed largely by the phospholipids, and that the time course of the total buffer capacity increase depends on the type and concentration of the protonophores and the speed of the proton pulse induced by the hydrolysis of TAME.

Materials and Methods

Enzymes and chemicals

Cytochrome *c* oxidase was prepared according to Yonetani [8]. The final suspending buffer contained 0.2% sodium cholate instead of Emasol 4130. Trypsin (EC 3.4.21.4), asolectins (Type II-S L-α-phosphatidylcholine from soybean), valinomycin, nigericin and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), were from Sigma Chem. Co. Stock solutions of the three ionophores were in 95% ethanol. *N*-α-tosyl-L-arginine methyl ester (TAME, from Merck Co.) was dissolved to 2 mM in bidistilled water and stored at 4°C; its concentration was determined spectrophotometrically using an average extinction coefficient of 335 at 229 nm for a 1% (w/v) solution. Phenol red was dissolved in alkaline water to 48 mM and was stored at 4°C. Its concentration was measured using an extinction coefficient of 58 $mM^{-1} \cdot cm^{-1}$ at 556.6 nm and pH ⩾ 10 at 20°C. All other chemicals were of analytical grade.

Phospholipid vesicles preparation

Liposomes or cytochrome oxidase-containing vesicles were prepared according to Casey et al. [5]. Additionally, the dialysed vesicles (typically 10 ml) were passed through a G25 Sephadex column (2.5 × 20 cm), previously equilibrated with 50 μM potassium/Hepes, containing 43.6 mM KCl/46.1 mM sucrose (pH 7.3), which is isotonic with the internal buffer of the vesicles (0.1 M potassium/Hepes, pH 7.3) and used for the experiments within 2–3 h. The final phospholipid concentration was 12.5 mg/ml.

Stopped-flow measurements

Potassium/Hepes buffers at concentrations from 0.2 to 3.8 mM, containing 60 μM Phenol red and TAME (from 0 to 100 μM) were mixed, at 20°C, in a Durrum Gibson thermostated stopped-flow apparatus (2 cm light path), with a solution of trypsin (6 μM) in 0.2 potassium/Hepes (pH 7.3). Similar experiments were carried out either with cytochrome oxidase-containing vesicle or with liposomes, eluted from the G25 Sephadex column (see above); in this case trypsin (6 μM) was added to the vesicle-containing syringe. Stock trypsin solutions (25 mg/ml) were prepared in doubly

distilled water. Particular care was taken to bring all solutions to pH 7.3 with KOH just before the experiment. Valinomycin, nigericin and CCCP were added to the vesicle-containing syringe to concentrations described in the text and figure legends. Unless expressly stated, all concentrations are after mixing.

Results

The hydrolysis of TAME

$$RCOOCH_3 + 2\,H_2O \rightarrow RCOO^- + CH_3OH + H_3O^+$$

(where R is a tosyl-arginyl radical) is virtually irreversible and, at pH 7.3, one proton per molecule is released [9]. The reaction is catalysed by trypsin, and thus can be made to occur over a suitable time range (e.g., $t_{1/2} \approx 0.5$ s), by choosing an appropriate concentration of trypsin. This allows to modify the velocity of production of the pulse of protons to be used in the calibration. The time course of hydrolysis can be followed in a lightly buffered solution by means of a pH indicator (Phenol red, $pK_a = 7.8$). The dependence of the amplitude of the phenol red acidification signal was followed at 556.6 nm, as a function of TAME concentration (from 0 to 50 μM after mixing). These same experiments were performed in the presence of (i) different concentrations of Hepes buffer (from 0.2 to 2 mM after mixing), (ii) and either cytochrome oxidase-containing vesicles or liposomes; in the latter cases the experiments were carried out both in the presence and absence of valinomycin, CCCP and nigericin, in order to evaluate the contribution of the external and internal phospholipid leaflet of the vesicles to the total buffer capacity.

Fig. 1 shows the Phenol red absorbance increase as a function of substrate concentration in the absence of vesicles at different Hepes concentrations. As expected, the amplitude of the acidification signal is inversely related to the buffer capacity, the straight lines through the data points were obtained by linear regression analysis and correlated to 98%.

The buffer capacity β is defined as the amount of acid (base), in mol/l, added to obtain a unitary pH change at that point on the titration curve (in our case at pH 7.3) is given by [10]:

$$\beta = \frac{d[\text{titrant}]}{d\,\text{pH}} = 2.303 \cdot \left(\frac{Kc[H^+]}{K + [H^+]} + [H^+] + \frac{K_w}{[H^+]} \right)$$

where K is the acid dissociation constant, c is the concentration of the buffer, $[H^+]$ is the concentration of protons and K_w is the ion product constant of water. This quantity can be calculated from Fig. 1, since β is equal to the reciprocal of the slope of the titration curve. The dependence of the observed absorbance change at 556.6 nm on the buffer capacity β is shown in the inset to Fig. 1.

Fig. 2 shows the results of similar experiments

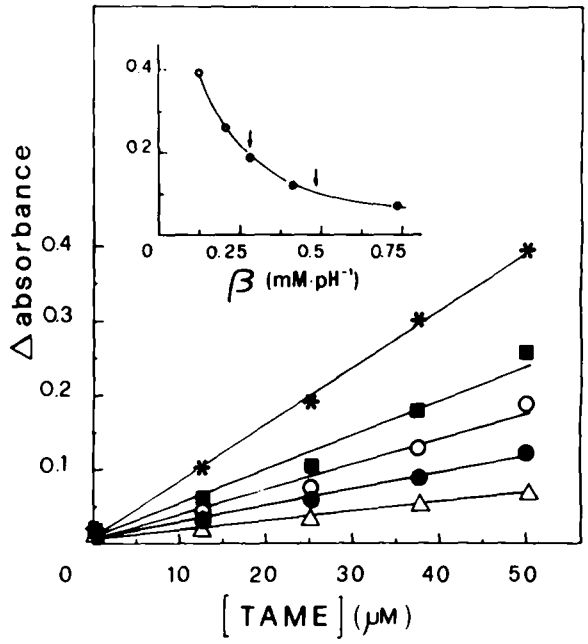

Fig. 1. Phenol red absorbance changes as a function of hydrolyzed TAME at different Hepes concentrations. Hepes buffers containing 60 μM phenol red and TAME, at different concentrations, were mixed in the stopped-flow apparatus with a solution of trypsin (6 μM) in 200 μM potassium Hepes (pH 7.3). For details, see Materials and Methods. Hepes concentrations after mixing were: 0.2 mM (*); 0.4 mM (■); 0.6 mM (⊙); 1 mM (●); 2 mM (△). Straight lines were obtained by linear regression analysis. Inset: Phenol red absorbance changes obtained from Fig. 1 were plotted as a function of the buffer capacity β, calculated as the reciprocal of the slope of the titration curve (see text). The arrows indicate the buffer capacity of ion-tight (left arrow) and ion-leaky (right arrow) liposomes and/or cytochrome oxidase-containing vesicles as determined in Fig. 2.

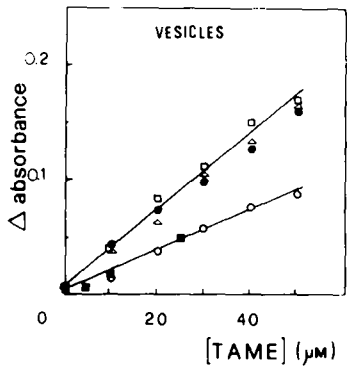

Fig. 2. Effect of ionophores on the phenol red absorbance changes as a function of hydrolyzed TAME in the presence of vesicles. Liposomes or cytochrome oxidase-containing vesicles, eluted from the G25 Sephadex column were diluted to a lipid concentration of 12.5 mg/ml in 50 μM Hepes, 43.6 mM KCl, 46.1 mM sucrose (pH 7.3); ionophores were added to the vesicle-containing syringe at the concentrations indicated below. Trypsin was added to a concentration of 6 μM. These solutions were mixed with TAME and phenol red in the above buffer. For details, see Materials and Methods. Liposomes (●); liposomes + 5 or 10 μM CCCP (□); liposomes + 5 μM valinomycin (△); liposomes + 0.5 μM nigericin (■); liposomes + 5 μM valinomycin + 10 μM CCCP (⊙).

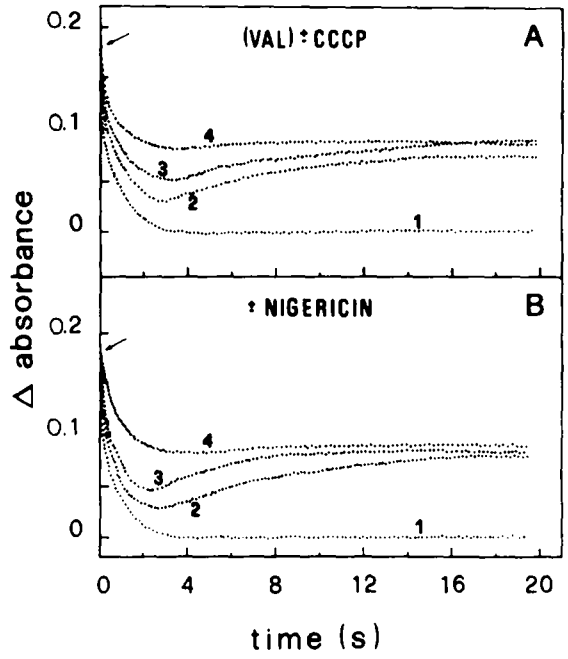

Fig. 3. Time course of the phenol red absorbance change following hydrolysis of TAME in the presence of liposomes and ionophores. (A) To the liposome-containing syringe, in the presence of 5 μM valinomycin, the following amounts of CCCP were added: (1) 0 μM; (2) 0.05 μM; (3) 0.1 μM; (4) 1–5 μM. (B) To the liposome-containing syringe the following amounts of nigericin were added: (1) 0 μM; (2) 0.05 μM; (3) 0.1 μM; (4) 0.4 μM. All other conditions as in Fig. 2. Arrows indicate total absorbance recovery.

carried out with liposomes or cytochrome oxidase-containing vesicles; the concentration of Hepes in the external aqueous phase was kept constant at 50 μM. In the absence of ionophores, the slope of titration curve corresponds to a system with $\beta = 0.26$ mM · pH^{-1} (buffer capacity normalized to the phospholipid concentration at pH 7.3 = 41.6 nmol · pH^{-1} · mg^{-1}) (see also inset to Fig. 1); this value is consistent with the buffer power of liposomes or cytochrome oxidase-containing vesicles determined potentiometrically under the same conditions (not shown). The presence of cytochrome oxidase, as well as the presence of saturating amounts of CCCP or valinomycin, added independently, do not change the buffer capacity of the system. On the contrary, addition of valinomycin and CCCP together, or nigericin either alone or in the presence of valinomycin, reduces the Phenol red signal to about 50% of its value ($\beta = 0.48$ mM · pH^{-1}). This result is clearly shown also in Fig. 3 which depicts the time courses of the acidification signal (i.e., the buffer capacity changes) at different concentrations of CCCP, in the presence of 2.5 μM valinomycin (0.44 μg/mg of lipids) (Fig. 3A), or in the presence of nigericin alone (Fig. 3B). Under these conditions, 0.5 μM CCCP (0.016 μg/mg of lipids) or 0.2 μM nigericin (0.023 μg/mg of lipids) exert approximately their maximal protonophoric activity by making the two separate buffer compartments (i.e., the external and internal aqueous phases separated by the phospholipid bilayer) readily accessible to protons in the time scale of the experiment ($t_{1/2}$, approx. 0.5 s).

Discussion

The irreversible stoichiometric proton release associated to the hydrolysis of TAME in the presence of trypsin is a well-characterized reaction [9]. The system appears therefore very convenient to determine the buffer power of the system of interest directly in the stopped-flow apparatus. Moreover, the acidification pulse can be made to occur

on a time scale faster than passive proton diffusion into the vesicle interior, but not too fast to be conveniently followed in the stopped-flow apparatus (as it would occur by mixing with an acid or a base). Since the buffer power is dependent only on the pK and concentration of the species (at a given pH and temperature), the calibration curve relating ΔA to the total concentration of TAME (Fig. 1) can be used to assess the buffer power of cytochrome oxidase-containing vesicle and liposomes under our standard conditions.

When liposomes or cytochrome oxidase-containing vesicle were suspended in 50 μM Hepes and 60 μM phenol red in the absence of ionophores, β(out) was found to be 0.26 $mM \cdot pH^{-1}$ (Fig. 2). The contribution of cytochrome oxidase to β is shown to be insignificant, consistent with its low concentration. Under these conditions, only the external aqueous phase, containing Hepes and phenol red, and the outer layer of the artificial membrane contribute to β; the former contributions, however, only account for 10% or less of the total value of β(out). Therefore β(out) is predominantly expressed by phospholipid headgroups of the outer leaflet. On the other hand, when the titration is conducted in the presence of valinomycin plus CCCP or nigericin alone (Figs. 2 and 3), i.e., when the membrane proton permeability has been sufficiently increased, the buffer capacity doubles, $\beta(tot) = 0.48\ mM \cdot pH^{-1}$ because of the contribution of the inner phospholipid layer and the internal vesicle medium (0.1 M Hepes); $\beta(in) = 0.48 - 0.26 = 0.22\ mM \cdot pH^{-1}$, is contributed to about 30% by 0.1 M Hepes, whose actual concentration was normalized for the ratio between the total volume of the vesicle suspension and their internal volume, as determined independently [11]. The buffer capacity of the internal phospholipid leaflet is therefore approx. 60–70% of that of the outer leaflet. This observation can be tentatively justified on a 4-fold basis: (i) according to the geometry of the vesicles (30 nm diameter on average), the inner leaflet surface is approx. 2-times smaller than the outer leaflet, and therefore it contains a smaller number of phosholipid molecules [11]; (ii) phospholipids relevant to β may be asymmetrically distributed between the two layers [12]; (iii) the packing of the phospholipids in each layer, i.e., the limiting area per phospholipid molecule, is different in the two layers [13]; and (iv) the dielectric properties of the inner aqueous medium are/may be different from those of bulk water, thus shifting the equilibrium constants of the relevant phospholipid headgroups.

The data reported in Fig. 3 also show that a rather low concentration of CCCP, in the presence of valinomycin, or nigericin alone is able to 'switch-on' the second buffer reservoir. On the contrary CCCP alone, even at high concentration (0.16 μg/mg of lipids) seems to be poorly effective in carrying protons across the membrane (see Fig. 2), since its function is not counterbalanced by an antiport of charges. Thus only in the presence of valinomycin, i.e., under conditions of electroneutrality [14], CCCP can exert its protonophoric function rapidly enough, as already indicated by measurements with conventional methods [14,15].

On the other hand nigericin, which is an electroneutral H^+/K^+ antiporter [2], efficiently makes protons freely available to the two (in, out) buffer compartments. It should be noticed that the protein, under the experimental conditions used, does not provide additional buffering capacity; moreover, it is unable to make the internal compartment readily accessible to protons (see Fig. 2), which confirms independently the impermeability of cytochrome oxidase-containing vesicles to protons, over a short time.

References

1 Mitchell, P. (1966) Biol. Rev. 41, 445–502
2 Nicholls, D.G. (1982) Bioenergetics, Ch. 4, Academic Press, New York
3 Wikström, M. (1977) Nature (London) 266, 271–273
4 Sigel, E. and Carafoli, E. (1978) Eur. J. Biochem. 89, 119–123
5 Casey, R.P., Chappell, J.B. and Azzi, A. (1979) Biochem. J. 182, 149–156
6 Sarti, P., Jones, M.G., Antonini, G., Malatesta, F., Colosimo, A., Wilson, M.T. and Brunori, M. (1985) Proc. Natl. Acad. Sci. USA, in the press
7 Reynafarje, B., Alexandre, A., Davies, P. and Lehninger, A.L. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 7218–7222
8 Yonetani, T. (1961) J. Biol. Chem. 236, 1680–1688
9 Hummel, B.C.V. (1959) Can. J. Biochem. Physiol. 37, 1393–1399
10 Guenther, W.B. (1968) in Quantitative Chemistry, pp. 66, Addison Wesley Publishing Co., Reading, MA
11 Sarti, P., Colosimo, A., Brunori, M., Wilson, M.T., and Antonini, E. (1983) Biochem. J. 209, 81–89
12 Litman, B.J. (1974) Biochemistry 13, 2844–2848
13 Seimiya, T. and Ohki, S. (1973) Biochim. Biophys. Acta 298, 546–561
14 Terada, H. (1981) Biochim. Biophys. Acta 689, 225–242
15 McLaughlin, S.C.A. and Dilger, J.P. (1980) Physiol. Rev. 60, 825–861