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The ionic strength of the intermembrane space of intact mitochondria is not affected by the pH or volume of the intermembrane space

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Ionic strength affects the electron transport activity of cytochrome *c* through its electrostatic interactions with redox partners and membrane lipids. We previously reported (Cortese, J.D., Voglino, A.L. and Hackenbrock, C.R. (1991) J. Cell Biol. 113, 1331–1340) that the ionic strength (*I*) of the intermembrane space (IMS-*I*) in isolated, intact condensed mitochondria is similar to the external, bulk *I*, over a wide range of bulk *I*. We now consider the possible effects of IMS-pH and IMS-volume, both variable parameters of mitochondrial function in situ, on IMS-*I*. IMS-pH and IMS-*I* were measured with pH- and *I*-sensitive fluorescent probes (highly fluorescent FITC-dextran for IMS-pH and FITC-BSA for IMS-*I*). These probes were delivered into the IMS of intact mitochondria via probe encapsulation into asolectin vesicles, followed by low pH-induced fusion of the vesicles with the outer membranes of intact mitochondria. IMS-pH was found to be 0.4–0.5 units lower than bulk pH over the pH range 6.0–8.5 for mitochondria with a large IMS-volume separating the two mitochondrial membranes (condensed configuration), and 0–0.2 units lower for mitochondria with a small IMS-volume and membranes closely opposed (orthodox configuration). This small pH difference between IMS-pM and bulk pH did not influence the similarity between IMS-*I* and bulk *I*. When the IMS-volume was osmotically decreased, bringing the two mitochondrial membranes in close proximity as in the orthodox configuration, IMS-*I* followed the bulk *I* above 10 mM but did not respond to changes in bulk *I* below 10 mM. The lack of response of the IMS-*I* below 10 mM indicates that the close proximity of the two mitochondrial membranes excludes ions only at low, nonphysiological *I*. Since the similarity of IMS-*I* and bulk *I* is unaffected by either IMS pH or IMS-volume above a bulk *I* of 10 mM, at cytosolic physiological *I* (i.e., 100–150 mM) cytochrome *c* can be expected to be a free, three-dimensional diffusant in the IMS irrespective of the pH or volume of the IMS.

Abbreviations: FITC-BSA, bovine serum albumin labelled with fluorescein isothiocyanate; FITC-dextran, dextran labelled with fluorescein isothiocyanate; H_{300} medium, 300 mOsm solution consisting of 220 mM mannitol, 70 mM sucrose, 2 mM Hepes buffer (pH 7.4), and 0.5 mg/ml BSA; *I*, ionic strength, defined as an ionic concentration: $I = (\Sigma c_i z_i^2)/2$ where c_i is the millimolar concentration of the *i*-th ion and z_i is the charge of the *i*-th ion. For salts formed with monovalent cations and anions (e.g., KCl or NaCl), ionic strength is equivalent to salt concentration; IMS, intermembrane space of mitochondria; IMS-FITC-BSA, FITC-BSA entrapped in the intermembrane space of intact mitochondria; IMS-FITC-dextran, FITC-dextran entrapped in the intermembrane space of intact mitochondria; K_I, apparent affinity of ionic strength-dependent changes in FITC-BSA fluorescence, defined as the ionic strength that gives 50% of the maximal increase in fluorescence; pK_{eff}, effective overall pK resulting from the contributions of the various fluorophore groups that exhibit pH-dependent fluorescence changes as a result of fluorophore ionization.

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Introduction

Studies of mitoplasts (outer membrane-free mitochondria) have shown that both the diffusion and electron transport rates of cytochrome *c* increase as ionic strength (*I*) is increased from 0 to 150 mM, and that a parallel ionic strength-induced increase occurs in electron transport in isolated, intact mitochondria [1,2]. These findings are consistent with the view that cytochrome *c* is a three-dimensional diffusant in the intermembrane space (IMS) of intact mitochondria [3,4], and that in the IMS of intact mitochondria in situ, cytochrome *c* is immersed in an aqueous environment with an *I* similar to the bulk *I* of cytosol. That the *I* of the IMS (IMS-*I*) is similar to, and reflects the ion composition of cytosolic *I*, is consistent with the permeability of the mitochondrial outer membrane to ions and small molecules [5,6]. Alternatively, it has been suggested that the IMS-*I* could be very low, given

that highly charged and closely opposed inner and outer membranes might exclude ions from the IMS [7,8]. However, recent experimental support for the similarity between the IMS-I of intact mitochondria and external, bulk I has been presented [9]. In that study, a soluble ionic strength-reporting, fluorescent protein probe (fluorescein-bovine serum albumin or FITC-BSA), was encapsulated into asolectin vesicles and delivered into the IMS of intact mitochondria by low pH-induced fusion of the liposomes with the outer membranes of mitochondria. It was found that the IMS-I of intact mitochondria was within $\pm 20\%$ of the external, bulk I, over a wide range of bulk I.

Since the ionic environment of cytochrome c under physiological conditions might be affected by such physiological variables as IMS-pH and IMS volume, we now consider the possible effects of both variables on IMS-I. IMS-pH and IMS-I were measured with pH- and I-sensitive fluorescent probes (FITC-dextran for IMS-pH and FITC-BSA for IMS-I). These probes were delivered to the IMS of intact mitochondria via probe encapsulation in liposomes followed by the low pH-induced fusion of the liposomes with the outer membranes of mitochondria [9]. The effects of altering external bulk pH and I on the fluorescence of IMS-entrapped probes were followed as I-dependent or pH-dependent fluorescence changes. Osmotically and metabolically induced transitions between condensed and orthodox configurations of mitochondria [10] occur with substantial changes in IMS-volume and proximity of the two mitochondrial membranes, and were used to study the effects of membrane proximity on IMS-I.

It was determined that a small pH difference exists between IMS-pH and bulk pH but that this does not influence the similarity of IMS-I and bulk I. Also, when the IMS-volume was maximally decreased, as in the orthodox configuration, the IMS-I followed changes in the bulk I, except when the bulk I was below 10 mM. This finding indicates that the close proximity of the two mitochondrial membranes can exclude ions only at very low, nonphysiological I. Thus the similarity of IMS-I and bulk I is unaffected by either IMS-pH or IMS-volume above a bulk I of 10 mM, and at cytosolic, physiological I (i.e., 100–150 mM), cytochrome c can be expected to be a free, three-dimensional diffusant in the IMS irrespective of the pH or volume of the IMS.

Materials and Methods

Preparation of mitochondria

Liver mitochondria were isolated from male Sprague-Dawley rats according to Schnaitman and Greenwalt [11], and then resuspended in H_{300} medium. Oxygen consumption was measured at 25°C using a Clark oxygen electrode. Respiratory control ratios (RCR), defined as the ratio between oxygen consumption of mitochondria in respiratory state 3 (in the presence of succinate as the respiratory substrate and ADP) and respiratory state 4 (in the presence of substrate alone) were determined using data acquisition-analysis software (Spectrofuge, Durham, NC). RCRs were in the range 5.5–7.0 for all mitochondrial preparations used.

Delivery of fluorescence probes into the intermembrane space of intact mitochondria

The encapsulation and membrane fusion protocol developed by Cortese et al. [9] was used to deliver fluorescent probes (FITC-BSA and FITC-dextran) into the intermembrane space (IMS) of intact mitochondria. Briefly, FITC-BSA and FITC-dextrans were first encapsulated in phospholipid vesicles by sonication. Asolectin (200 mg) was hydrated in 1.5 ml of H_{300} medium (without BSA) for at least 2 h at 0°C, then mixed with an equal volume of a solution of a fluorescent probe (25 mg in 1.5 ml of 7.5-times diluted H_{300} medium). The resulting sample was sonicated at 0°C in three cycles (10 min each) at 40 W with a microtip probe. Freshly isolated, intact mitochondria (200 mg protein in 27 ml of H_{300} medium without BSA; pH 7.4) were mixed with 3 ml of the probe-containing asolectin vesicles (to give a ratio of 330 μg mitochondrial protein per mg asolectin) and incubated for 1 h at 15°C to induce adsorption of the probe-containing vesicles to mitochondrial outer membranes. To accomplish membrane fusion and delivery of the fluorescent probes into the IMS, the pH was decreased to 6.5 for 5 min at 15°C. The probe-containing mitochondria were then returned to pH 7.4, and washed to remove any fluorescent probe and non-fused vesicles by repeated pelleting and resuspension at 4°C in a large volume of probe-free H_{300} medium [9]. Mitochondria loaded with fluorescent probes were resuspended at a final concentration of 75–100 mg mitochondrial protein per ml in H_{300} medium, and assayed within 2–3 h.

Fluorescence measurements

The intensity of fluorescein emission at 520 nm was monitored digitally using a Perkin-Elmer 650-40 fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT) in the ratio mode a 4°C. The excitation wavelength was either 450 nm or 468 nm, using excitation and emission slit widhts of 5 nm. No spectral shifts occured with change in ionic strength. For each measurement, the fluorescence intensity was signal averaged for 10 s. Quinine sulfate in 0.1 M H_2SO_4 was used as a control for stability of fluorescence intensity before and after each experiment. Controls for turbidity were included, and inner filter effect corrections were those of Geren and Millet [12]. For analysis, data were expressed as relative fluorescence (the quotient of two fluorescence emission measurements at 520 nm obtained with the

same excitation wavelength (468 nm)) or fluorescence excitation ratios (the quotient of two fluorescence emission measurements at 520 nm obtained at two different excitation wavelengths (450 and 468 nm)).

Relative fluorescences obtained with FITC-BSA in solution and IMS-FITC-BSA at different ionic strengths I were fitted to a hyperbolic curve using the equation:

$$F_I/F_0 = 1 + \{[(F_{max}/F_0) - 1]/(1 + K_I/I)\} \quad (1)$$

where F_I is the fluorescence at a given I, F_0 is the fluorescence at $I = 0$, F_{max} is the maximal fluorescence obtainable by increasing I, and K_I is the apparent affinity of ionic strength-dependent changes in FITC-BSA fluorescence, defined as the ionic strength that gives 50% of the maximal increase in fluorescence [9]. Fluorescence excitation ratios obtained with FITC-dextran in solution and IMS-FITC-dextran at different pH were fitted to a sigmoidal equation that relates fluorescence at a given pH with the effective pK of the mixture of fluorescent species [13]. In its simplest form, the equation is:

$$R_{pH} = A + [B/(10^{pK_{eff} - pH} + 1)] \quad (2)$$

where R_{pH} is the value of fluorescence excitation ratio obtained at a given pH, pK_{eff} is the effective overall pK resulting from the contributions of the various fluorophore groups that exhibit pH-dependent fluorescence changes as a result of fluorophore ionization, and A and B are constants that define the span of fluorescence data. A and B are only sensitive to the degree of self-quenching exhibited by the probe; therefore, pK_{eff} is not affected by their values. For purposes of comparison in the same scale (Figs. 2 and 3), fluorescence excitation ratios are expressed as normalized fluorescence excitation ratios (NF_{pH}):

$$NF_{pH} = (R_{pH} - A)/B \quad (3)$$

Statistical tests and non-linear regression fitting of fluorescence data were performed using routines from SYSTAT software (SYSTAT, Evanston, IL). Tests performed verified a low degree of correlation between parameters fitted, and the presence of random deviations from theoretical curves.

Materials

Fatty acid free bovine serum albumin (BSA), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), equine muscle Na-ADP (grade IX), D-mannitol, sodium palmitate, sodium succinic acid, and sucrose were purchased from Sigma Chemicals (St. Louis, MO). Asolectin was purchased from Associated Concentrates (Woodside, L.I., NY), and Hepes was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). All other chemicals were of the highest purity available commercially. FITC-BSA (about 6.0 FITC/protein molar ratio) and FITC-dextran (molecular mass = 40 and 70 kDa; 6.3 FITC/dextran molar ratio) were purchased from Molecular Probes (Eugene, OR).

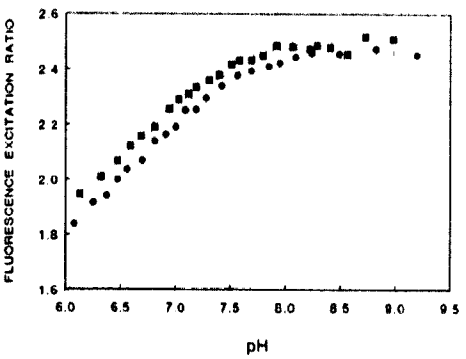


Fig. 1. Sensitivity of the fluorescence of FITC-dextrans in solution to pH and ionic strength (I). Fluorescence excitation ratios (emission: 520 nm; excitation: 468 nm or 450 nm) were measured at 21°C for FITC-dextrans (M_r = 40000) in a 300 mOsm solution containing 300 mM sucrose, 2 mM Hepes (pH 7.4) and 0 (○), 20 (●), 100 (□), or 150 mM (■) KCl. Inner-filter effects were eliminated by keeping FITC-dextrans absorbances (at 450, 468, and 520 nm) below 0.005. These fluorescence excitation ratios were found to be sensitive to both pH and I.

Results

Similarity between IMS-pH and bulk pH

Fluorescence excitation ratios of FITC-dextran in solution were found to be more sensitive to pH than to I (Fig. 1). For any given I, a sigmoidal curve for FITC-dextran fluorescence as a function of pH was obtained. This pH-sensitivity of the soluble FITC-dextran probe can be used to explore IMS-pH when the probe is entrapped in the IMS. Curves of fluorescence excitation ratio as a function of pH were constructed for solutions of FITC-dextran (reference) and IMS-FITC-dextran (sample). Each curve will have a measurable effective pK (pK_{eff}; see Materials and Methods), and a comparison of pK_{eff}s for soluble and IMS-entrapped dextran gives a $\Delta pK_{eff} \approx \Delta$pH. If the IMS-pH is below the bulk pH, the IMS-FITC-dextran curve will be right-shifted with respect to the curve obtained for FITC-dextran in the bulk solution, since the IMS-FITC-dextrans will experience a lower pH, thus emitting less fluorescence; therefore, the pK_{eff} will be higher and $\Delta pK_{eff} = (pK_{FITC\text{-}dextran} - pK_{IMS\text{-}FITC\text{-}dextran}) \approx \Delta pH < 0$. A $\Delta pH > 0$ indicates that IMS-pH is higher than bulk pH. FITC-dextrans with different molecular mass gave similar results on IMS-pH estimation; results are only presented for the 40 kDa form of FITC-dextran.

IMS-pH was found to be 0.4–0.5 units lower than bulk pH over the pH range 6.0–8.5 for mitochondria with a large IMS-volume separating the two mitochondrial membranes (condensed configuration) in 300 mOsm medium (bulk $I \approx 0$; Fig. 2A, Table I), and 0–0.2 units lower for mitochondria with a small IMS-volume and membranes closely opposed (orthodox configuration) in 120 mOsm medium (bulk $I \approx 0$; cf. Fig. 2A and B). These results show that the IMS-pH equals the bulk pH when the two mitochondrial membranes are in close proximity at low I. It was further found, however, that the IMS-pH of the orthodox configuration could be decreased by an increase in bulk I (120 mOsm; $I = 20$ mM; Fig. 2C). This finding is consistent with proton sequestration by the negatively charged mitochondrial membranes in close proximity that is overcomed by competition with ions at higher I. A similar effect of membrane proximity on IMS-I is observed when mitochondria in the rotenone/antimycin A-inhibited respiratory state (condensed configuration; Fig. 3A) are compared with mitochondria undergoing active respiration in the presence of succinate (orthodox configuration; Fig. 3B, see Table I).

It should be noted that the effects of the closely opposed, negatively charged membranes present in the orthodox configuration on the concentration of IMS protons (i.e., IMS-pH) suggested here are probably superimposed on the presence of IMS protons produced by mitochondrial respiration. Some indication of this comes from experiments where IMS protons and membrane charge were manipulated. Uncoupling of condensed mitochondria with carbonylcyanide *m*-chlorophenylhydrazone (CCCP) collapses the small ΔpH between the IMS and bulk medium (Table I). Further, a brief treatment with sodium palmitate, a molecule that incorporates spontaneously into mitochondrial membranes, increasing their fixed negative charge and reversibly uncoupling mitochondria [14,15], produced mitochondria with a measurable ΔpH (about −0.3) between the IMS and the bulk medium (Table I). This experiment indicates that some excess of IMS-protons can be mantained by interactions with fixed negative membrane charges even for uncoupled mitochondria.

IMS-pH does not affect the similarity between IMS-I and bulk I

Even though the measured difference between bulk pH and IMS-pH is small (0–0.5 pH units), it might affect IMS-I measurements. Thus, the extent of effect of pH differences on I measurements was determined for FITC-BSA in solution (Fig. 4, Table II). The apparent affinities for I or K_I obtained were found to be similar through an order of magnitude change in proton concentration (pH 7.4 ± 0.5). The fluorescence span between $I = 0$ (F_0) and maximal fluorescence (F_{max}), measured either as $[F_{max} - F_0]$ or $[F_{max}/F_0]$, did not

TABLE I

Effective pKs of soluble and IMS-entrapped FITC-dextran [a]

Experiment	Effective pK (soluble FITC-dextran)	Effective pK (IMS-FITC-dextran)	ΔpH [b]
300 mOsm sucrose [c]	6.763 ± 0.009	7.277 ± 0.026	(−0.514) ± 0.035
	(0.0015; 23) [d]	(0.0075; 23)	
120 mOsm sucrose	6.679 ± 0.068	6.653 ± 0.052	0.026 ± 0.120
	(0.0055; 23)	(0.0238; 28)	
120 mOsm sucrose + 20 mM KCl	6.705 ± 0.011	7.088 ± 0.027	(−0.383) ± 0.038
	(0.035; 23)	(0.028; 26)	
Active respiration [e] (succinate)	6.583 ± 0.033	6.803 ± 0.027	(−0.220) ± 0.060
	(0.0051; 23)	(0.025; 23)	
Inhibited respiration (succinate + antimycin A)	6.443 ± 0.020	6.842 ± 0.029	(−0.399) ± 0.035
	(0.0117; 23)	(0.0096; 30)	
Uncoupled (CCCP) [f]	6.864 ± 0.023	7.127 ± 0.058	(−0.263) ± 0.081
	(0.0116; 31)	(0.0041; 21)	
Palmitate-treated [g] (300 mOsm sucrose)	6.709 ± 0.018	6.674 ± 0.044	0.035 ± 0.062
	(0.0045; 31)	(0.0011; 31)	

[a] Data were analyzed by nonlinear regression using Eqns. 2 and 3 in text.

[b] The difference $(pK_{FITC\text{-}dextran} - pK_{IMS\text{-}FITC\text{-}dextran}) = \Delta pK_{eff} \approx \Delta pH$ (i.e., the pH difference between IMS and bulk).

[c] Measurements for mitochondria in 300 mOsm sucrose, 120 mOsm sucrose, and 120 mOsm sucrose plus $I = 20$ mM medium were carried out as in Fig. 2.

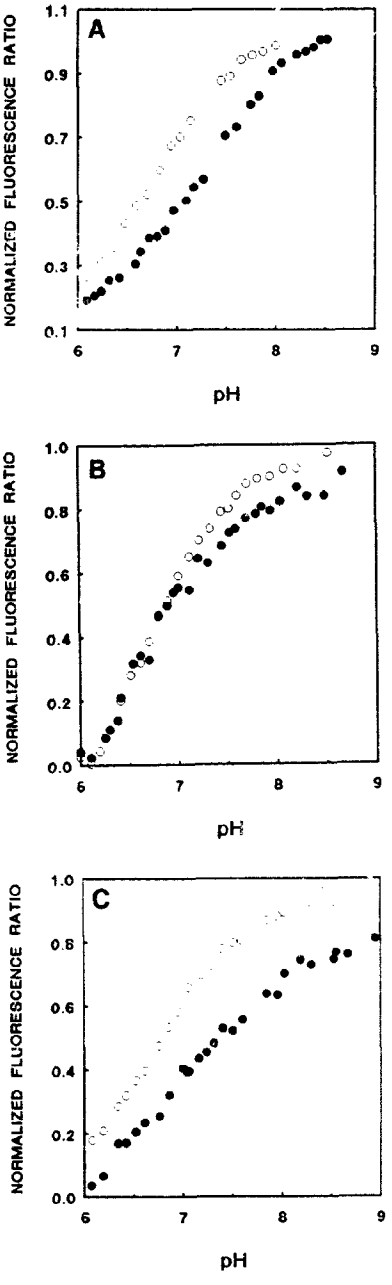
[d] SS_{min} is the minimal sum of squares; N is the number of experimental data points fitted with Eqn. 2.

[e] Measurements for mitochondria with 5 mM sodium succinate (active respiration) and with rotenone/antimycin (inhibited respiration) were carried out as in Fig. 3.

[f] Measurements for mitochondria suspended in 300 mOsm sucrose medium, and uncoupled by the addition of 1 μM CCCP, were carried out as in Fig. 2.

[g] Measurements for mitochondria briefly exposed to 80 μM palmitate (10 min at 15°C) were carried out as in Fig. 2.

change substantially in the same pH range (pH 6.9–7.9). These results suggest a negligible pH-dependent difference between K_1 measurements for FITC-BSA in solution and IMS-FITC-BSA (Fig. 5; see also Ref. 9), and no correction of the published K_1 values obtained with IMS-FITC-BSA is needed, even for the largest ΔpH between IMS and bulk medium measured (i.e., ≈ 0.5; Table I).



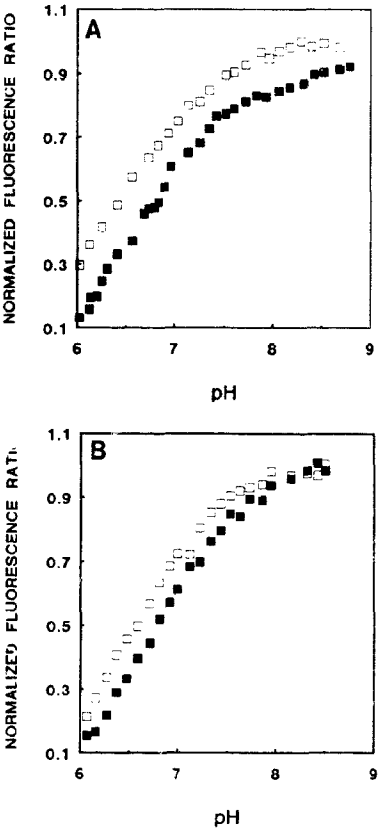


Fig. 3. Effect of respiratory state-induced changes of IMS-volume (membrane proximity) on IMS-pH estimated with FITC-dextrans entrapped in the IMS (IMS-FITC-dextrans) of intact mitochondria. Effects of IMS-volume during mitochondrial function were determined as in Fig. 2 by comparing bulk pH effects on the normalized fluorescence excitation ratios of FITC-dextrans in solution (—) and IMS-FITC-dextrans (■) for: (A) mitochondria during inhibited respiration (5 mM sodium succinate as substrate and kept in the condensed configuration with 1 μg/ml rotenone and 1 μg/ml antimycin A); and (B) mitochondria in active respiratory state (5 mM succinate as substrate results in transition to orthodox configuration during 10 min at 15°C). Measurements of fluorescence excitation ratios were performed at 15°C. Results are summarized in Table I.

Fig. 2. Effect of IMS-volume (membrane proximity) on IMS-pH estimated with FITC-dextrans entrapped in the IMS (IMS-FITC-dextrans) of intact mitochondria. IMS-pH was estimated by comparing changes in normalized fluorescence excitation ratios (emission: 520 nm; excitation: 468 nm or 450 nm) of FITC-dextrans in solution (○) and IMS-FITC-dextrans (●) as a response to changes of bulk pH. (A) Mitochondria maintained in the condensed configuration with large IMS-volume at a medium osmolarity of 300 mOsm (bulk $I \approx 0$). IMS-pH is ≈ 0.5 units lower than bulk pH. (B) IMS-volume is decreased by lowering the medium osmolarity to 120 mOsm giving the orthodox configuration (bulk $I \approx 0$). IMS-pH increased approaching the value for FITC-dextrans in solution (ΔpH ≈ 0). (C) IMS-pH for orthodox mitochondria was decreased by increasing bulk I (120 mOsm; $I = 20$ mM). Measurements of normalized fluorescence excitation ratios were performed at 4°C. Results are summarized in Table I.

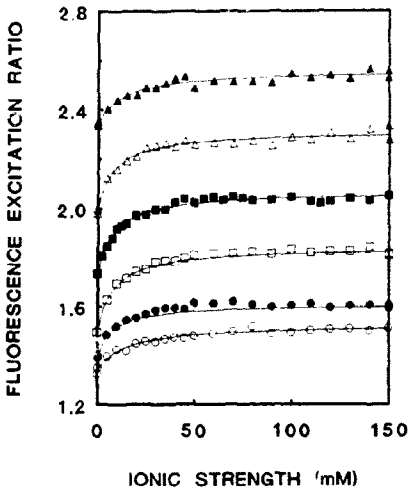


Fig. 4. Effect of pH on the ionic strength sensitivity of the fluorescence of FITC-BSA in solution. The effect of pH on I-measurements obtained from relative fluorescence was determined at 21°C for FITC-BSA in 300 mOsm solutions of sucrose at pH 6.0 (○), 6.4 (●), 6.9 (□), 7.4 (■), 7.9 (△), and 8.4 (▲). The apparent affinities of the change induced by I (or K_I) obtained and the fluorescence spans (emission: 520 nm; excitation: 468 nm) between fluorescence at $I = 0$ (F_0) and maximal fluorescence (F_{max}) – measured as $[F_{max} - F_0]$ or (F_{max}/F_0) – are summarized in Table II. The small pH-dependent difference between K_Is for FITC-BSA in solution in the range of pH 7.4 ± 0.5 indicates that no corrections of K_I values of Fig. 5 are needed. Apparent affinity of ionic strength-dependent changes in FITC-BSA fluorescence (K_I) is defined as the ionic strength that gives 50% of the maximal increase in fluorescence.

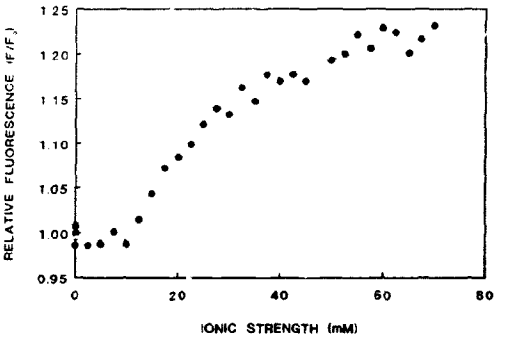


Fig. 5. Effect of osmolarity induced configurational change (condensed-orthodox) on IMS-I estimated with FITC-BSA entrapped in the IMS (IMS-FITC-BSA) of intact mitochondria. The effects of I on the relative fluorescence of IMS-FITC-BSA (emission: 520 nm; excitation: 468 nm) were compared for: (A) mitochondria osmotically maintained in the condensed configuration at 300 mOsm (○), and (B) for mitochondria maintained in the orthodox configuration at 150 mOsm (●). Relative fluorescence was determined at 15°C as F (fluorescence at a given I) divided by F_0 (fluorescence at $I = 0$). Measurements of relative fluorescence were performed at 4°C. The experiment shown was carried out at pH 7.4, but similar curves are obtained at pH 6.0 and 8.0 (not shown). For FITC-BSA in solution, K_I was (9.61 ± 1.09) mM and the maximal relative fluorescence obtainable by increasing I $[(F_{max}/F_0)]$ was 1.171 ± 0.012 (minimal sum of squares or $SS_{min} = 0.00098$; number of experimental points fitted or $N = 20$). For IMS-FITC-BSA and for the rising part of the curve, K_I was (10.95 ± 2.96) mM and (F_{max}/F_0) was 1.220 ± 0.010 ($SS_{min} = 0.00221$; $N = 27$).

IMS-volume affects IMS-I only at low, nonphysiological I

An effect of IMS-volume on IMS-I compatible with ion sequestration was found when I is changed at constant bulk pH (Fig. 5). The fluorescence of both IMS-FITC-BSA and FITC-BSA in solution is affected by I [9]. This effect of I on IMS-FITC-BSA was compared for mitochondria osmotically mantained in the condensed (300 mOsm) and orthodox (150 mOsm) configuration (Fig. 5). For mitochondria in the condensed configuration (the two membranes are separated), the response of IMS-FITC-BSA fluorescence to changes of bulk I is saturable (i.e., hyperbolic). For mitochondria in the orthodox configuration (membranes close), bulk I below 10 mM does not affect IMS-FITC-BSA fluorescence. Above 10 mM, IMS-FITC-BSA fluorescence increases with apparent affinities (≈ 10 mM) similar to FITC-BSA in solution, approaching the same maximal fluorescence at high I (Fig. 5). This result indicates that the fluorescent probe

TABLE II

Effects of pH on ionic strength-dependent changes of FITC-BSA fluorescence [a]

pH	$F_{(0)}$ [b]	F_{max} [c]	$[F_{max} - F_{(0)}]$	$[F_{max}/F_{(0)}]$	Affinity (K_I) [d] (mM)	SS_{min} (N) [e]
6.0	1.342 ± 0.007	1.526 ± 0.009	0.184 ± 0.006	1.137 ± 0.011	14.93 ± 2.50	0.001605 (23)
6.4	1.367 ± 0.008	1.585 ± 0.012	0.218 ± 0.009	1.159 ± 0.014	7.49 ± 1.53	0.002078 (24)
6.9	1.495 ± 0.005	1.840 ± 0.008	0.345 ± 0.006	1.231 ± 0.009	8.11 ± 0.52	0.001628 (24)
7.4	1.730 ± 0.013	2.068 ± 0.013	0.338 ± 0.014	1.195 ± 0.016	8.36 ± 0.58	0.003536 (27)
7.9	1.994 ± 0.009	2.312 ± 0.014	0.318 ± 0.011	1.159 ± 0.013	8.35 ± 1.25	0.005465 (24)
8.4	2.343 ± 0.008	2.558 ± 0.013	0.215 ± 0.010	1.092 ± 0.010	13.02 ± 2.43	0.004205 (24)

[a] Data were taken from Fig. 4 and analyzed by nonlinear regression using Eqn. 1 in text.
[b] Fluorescence at zero ionic strength.
[c] Maximal fluorescence obtainable by increasing ionic strength.
[d] Apparent affinity of I-dependent changes in FITC-BSA fluorescence, calculated as the I that gives 50% of maximal fluorescence.
[e] SS_{min} is the minimal sum of squares; N is the number of experimental data points fitted with Eqn. 1.

does not detect any increases of IMS-*I* for bulk $I < 10$ mM, but it detects increases of IMS-*I* for bulk *I* higher than 10 mM whether it is in solution or entrapped in the IMS. This observation resembles the effects of IMS-volume and IMS-*I* on IMS-pH (Figs. 2 and 3, Table I). For the orthodox mitochondrial configuration at low *I*, proximity of the closely opposed, negatively charged inner and outer mitochondrial membranes can sequester protons (i.e., the pH increases) or K^+ ions from KCl (i.e., the *I* does not increase). With an *I* higher than 10 mM, the ionic excess could contribute to IMS-*I* (*I* increases). At physiological *I* (i.e., 100–150 mM), IMS-*I* and bulk *I* are similar for mitochondria in the orthodox configuration, as was previously reported for the condensed configuration [9].

Discussion

Previous hypotheses have ascribed either a cytosolic *I* of 100–150 mM [5,14] or a very low *I* [7,8] to the IMS. More recently, the IMS-*I* has been measured for isolated, intact rat liver mitochondria in the condensed configuration, and found to be similar to the external, bulk *I*, over a wide range of *I* [9]. However, since the inner and outer mitochondrial membranes have a net

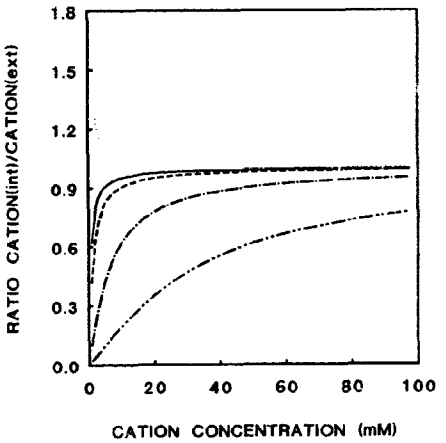


Fig. 6. Donnan equilibrium ion exclusion ratios at various values for the excess charge from nonpermeable ions. Calculations of the Donnan ion exclusion ratio were performed for a situation where nonpermeable ions contained in a semipermeable compartment equilibrate with a solution of permeable monovalent ions. Here (int) refers to the compartment that contains the nonpermeable ions, and is analogous to the IMS that contains proteins and lipids with nonzero charge that cannot diffuse into the bulk medium (ext). Eqn. 2 from the footnote on this page was solved for excess charges of −1 mM (·····), +1 mM (———), +2 mM (— — —), +10 mM (—·—·—), and +50 mM (·····), and the exclusion ratio (i.e., $[\text{cation}_{int}]/[\text{cation}_{ext}]$) found for various values of the external cation concentration in the range 0–100 mM. The exclusion ratio for the system studied here (mitochondria exposed to solutions of different KCl concentration) will be $[K^+]_{IMS}/[K^+]_{bulk}$. Monovalent cation concentration is identical to *I*.

negative surface charge [16], the close apposition of the two membranes in the orthodox configuration could in principle exclude free ions from the IMS compartment through a Donnan equilibrium [8]. In addition, the presence of a large pH difference between the IMS and the bulk medium could affect our previously reported measurements of IMS-*I*.

The data presented here show that the volume of the IMS, thus the proximity of the two membranes, does not affect the similarity between IMS-*I* and external, bulk *I* higher than 10 mM. Only at very low, nonphysiological bulk *I*, and only for mitochondria in the orthodox configuration, is the IMS-*I* different from the bulk *I*. Thus, there is a defined range for bulk *I* (0–10 mM) where the IMS-*I* does not equilibrate with the bulk *I*, indicating that ion titration of a relatively constant number of membrane negative surface charges is responsible for the effects observed at low *I*. Any excess of cations in the bulk *I* over this small fixed number of membrane charges will contribute to the IMS-*I*. When the IMS-volume is very large as in the condensed configuration, even at very low *I*, there are sufficient cations in this compartment to titrate all membrane surface charges. Our results are consistent with ion sequestration operating in the IMS of intact mitochondria at low, nonphysiological IMS-*I*, and only when the membranes are in close apposition. An alternative explanation is that membrane proximity causes ion exclusion via a Donnan equilibrium *. Ion exclusion would be gradually eliminated through a displacement of this equilibrium by higher bulk concentrations of ions, and a difference between IMS-*I* and bulk *I*

* A typical Donnan equilibrium [17] is established across a semipermeable membrane that separates two compartments I and II, both containing permeable ions (cation M^+ and anion X^-) initially at a concentration C^1, but only one of them (here compartment II) containing a nonpermeable ion (cation N^{n+}) at a concentration $[N^{n+}]$. When ion activities equilibrate in both compartments, this ionic distribution originates an equilibrium characterized by the equation:

$$C^2 = [M^+]_{II} \cdot (\{M^+\}_{II} + n \cdot [N^{n+}]_{II}) \quad (1)$$

For a mixture of positively ($[N^{n+}]_{II}$) and negatively ($[A^{a-}]_{II}$) charged ions confined in compartment II, a modified equation is obtained:

$$C^2 = [M^+]_{II} \cdot \{[M^+]_{II} + (n \cdot [N^{n+}]_{II} - a \cdot [A^{a-}]_{II})\} \quad (2)$$

The term $(n \cdot [N^{n+}]_{II} - a \cdot [A^{a-}]_{II})$ is the net fixed charge of compartment II (cationic and anionic charges involved could be membrane-bound or present as ions that cannot cross the outer membrane boundary). The solution of this quadratic equation for the cation concentration $[M^+]_{II}$ is plotted in Fig. 6 (as the Donnan exclusion ratios $([M^+]_{II}/[M^+]_I)$ that correspond to the ratio of concentrations of monovalent cation in the IMS with respect to bulk cation concentration) for different values of the net fixed charge (i.e., $n \cdot [N^{n+}]_{II} - a \cdot [A^{a-}]_{II}$).

would be apparent for the entire range of I, not just below a bulk I of 10 mM. Under a narrow range of conditions, a Donnan equilibrium can produce ion exclusion phenomena with similar characteristics to ion sequestration at low I (Fig. 6). Depending on the net fixed charge of the IMS (total charge from IMS-membrane lipids plus IMS-proteins), cations can be excluded for $I < 20$ mM. The net fixed charge should be between +1 to +2 mM (Fig. 6). This net fixed charge is probably unreasonable for mitochondria, considering that a balance inclined toward a net negative charge could be expected by the large number of negative surface charges on mitochondrial membranes [16], and the tendency to avoid significant interactions between soluble, positively charged IMS-proteins and negatively charged IMS-membrane lipids. An additional prediction for a Donnan equilibrium is that some dependence on IMS-volume or IMS-pH occurs. The net fixed charge concentration should change with IMS-volume; if the net fixed charge is negative and its concentration increases when the IMS-volume decreases, curves for fluorescence of IMS-FITC-dextran such as those in Figs. 2 and 3 should depart more from those of FITC-dextran in solution for the orthodox configuration. Some dependence on IMS-pH would also be expected, as charges associated with IMS-protein change with pH; the 'lag' on equilibration of bulk I with IMS-I should change and fluorescence curves for IMS-FITC-dextran at different pH should approach those of FITC-dextran in solution at low pH. None of these effects have been detected experimentally. Therefore, a Donnan equilibrium affecting IMS-ions at low I seems to be a less likely explanation than ion sequestration to account for the effect of IMS-volume on IMS-I.

Our method of delivery of pH-sensitive molecules to the IMS now allows the direct probing of IMS-pH and the evaluation of the possible effect of pH on IMS-I under different metabolic and osmotic conditions (Figs. 2 and 3, Table I). Previous measurements of the pH difference between mitochondrial matrix and suspending medium showed a ΔpH of 0.3–0.5 units (matrix alkaline) with respect to the bulk medium [18–23]. These studies assumed that the ΔpH between the IMS and bulk medium was zero. However, our data reveals that the IMS-pH can be lower than bulk pH by about 0.2–0.5 units, depending on conditions, and that it can be made similar to bulk pH either by bringing mitochondria to the orthodox configuration osmotically (Fig. 2B) or by collapse of the proton gradient with an uncoupler (Table I). Changes of IMS-volume affect IMS-pH (Figs. 2 and 3), and the underlying mechanism seems to be the same as for the effect of IMS-volume on IMS-I. IMS-protons are sequestered when the IMS-volume is decreased as in the orthodox configuration, and this effect is overcome by an increase in bulk I (KCl concentration > 10 mM). This finding also indicates that IMS-pH at physiological I is approximately constant, and at most 0.4 units lower than the bulk, external pH, when mitochondria are in the orthodox configuration. This difference is not large enough to require corrections in our previous report revealing the similarity between bulk I and IMS-I, as indicated by similar values of the apparent affinity for I or K_I found for pH 6.9–7.0 (see Fig. 4 and Table II), but suggests that the IMS is capable of sustaining a pH that differs from cytosolic pH. This would provide initial support for the idea of a barrier to proton diffusion between the inner mitochondrial membrane and the bulk cytosol medium [24]. It should be noted that I-induced pH changes have been also detected in chloroplasts [25,26]. Also, our measurements of IMS-pH are not influenced by the use of FITC-dextran as a fluorescent probe. At the low concentrations of IMS-FITC-dextran in our experiments (5–6 μM calculated as in Ref. 9), and the low negative net charge of the FITC-dextran (originating from fluorescein dissociation), closing of the mitochondrial outer membrane channels as was reported using dextran sulfate [27] or various polyanions [27,28] is unlikely to occur.

Overall, our results support an IMS-I that is similar to the bulk, external I in both extreme configurational states of rat liver mitochondria, for bulk I higher than 10 mM. Although we cannot conclude directly from this similarity between IMS-I and bulk I that cytochrome c diffuses in three dimensions in the IMS of intact mitochondria (given the complex nature of the interactions of cytochrome c with its redox partners and membrane lipids), our present data and previous studies [1,2] strongly support such a diffusional mode. Our studies suggest that ionic strength-dependent interactions in the IMS compartment, which could occur at nonphysiological I, will be quenched considerably, and in some cases totally, by an IMS-I in the range of 100–150 mM. The findings of many previous studies (reviewed in Ref. 29), which showed nonlinear Scatchard plots for the binding of cytochrome c to its reductase and oxidase at low I indicative of the presence of multiple binding sites [8,30–32], are most likely irrelevant at physiological conditions consistent for in situ mitochondria (i.e., IMS-I of 100–150 mM), where binding will be characterized by linear Scatchard plots indicating a single binding site, and lower affinities between cytochrome c and redox partners. Thus, many ionic strength-dependent protein–protein and lipid–protein interactions of cytochrome c which occur at nonphysiological I will be minimized in intact mitochondria at a physiological IMS-I equal to cytosolic I, permitting cytochrome c to diffuse in three dimensions to collide with its redox partners at random. To approach this question more directly, our research in progress is addressing the dynamics and function of

exogenous cytochrome *c* delivered through the encapsulation-fusion protocol into the IMS of functionally intact rat liver mitochondria [33].

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