Ionic Strength of the Intermembrane Space of Intact Mitochondria as Estimated with Fluorescein-BSA Delivered by Low pH Fusion

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Abstract. The electrostatic interactions of cytochrome c with its redox partners and membrane lipids, as well as other protein interactions and biochemical reactions, may be modulated by the ionic strength of the intermembrane space of the mitochondrion. FITC-BSA was used to determine the relative value of the mitochondrial intermembrane ionic strength with respect to bulk medium external to the mitochondrial outer membrane. FITC-BSA exhibited an ionic strength–dependent fluorescence change with an affinity in the mM range as opposed to its pH sensitivity in the μM range. A controlled, low pH–induced membrane fusion procedure was developed to transfer FITC-BSA encapsulated in asolectin liposomes, to the intermembrane space of intact mitochondria. The fusion procedure did not significantly affect mitochondrial ultrastructure, electron transport, or respiratory control ratios. The extent of fusion of liposomes with the mitochondrial outer membrane was monitored by fluorescence dequenching assays using a membrane fluorescent probe (octadecylrhodamine B) and the soluble FITC-BSA fluorescent probe, which report membrane and contents mixing, respectively. Assays were consistent with a rapid, low pH–induced vesicle-outer membrane fusion and delivery of FITC-BSA into the intermembrane space. Similar affinities for the ionic strength–dependent change in fluorescence were found for bulk medium, soluble (9.8 $\pm$ 0.8 mM) and intermembrane space–entrapped FITC-BSA (10.2 $\pm$ 0.6 mM). FITC-BSA consistently reported an ionic strength in the intermembrane space of the functionally and structurally intact mitochondria within $\pm$20% of the external bulk solution. These findings reveal that the intermembrane ionic strength changes as does the external ionic strength and suggest that cytochrome c interactions, as well as other protein interactions and biochemical reactions, proceed in the intermembrane space of mitochondria in the intact cell at physiological ionic strength, i.e., 100–150 mM.

In spite of the central importance of ionic strength in the catalysis of electron transport by cytochrome c (Gupte et al., 1984; Pettigrew and Moore, 1987; Gupte and Hackenbrock, 1988*a*,*b*), the ionic strength in the intermembrane space of intact mitochondria, relative to the cytosol, is unknown. Two opposite views have been offered to deal with this question: (*a*) the intermembrane ionic strength should be similar to and reflect the ionic concentration and composition of the cytosol (Wojtczak and Sottocasa, 1972), given the high permeability of the outer membrane to ions and molecules smaller than 12 kD (Pfaff et al., 1968); or (*b*) the intermembrane ionic strength should be lower than the cytosolic ionic strength, because the ions should be excluded by the proximity of the highly charged, closely opposed inner and outer membranes (Nicholls, 1974; Ferguson-Miller et al., 1979). No direct measurements have been reported to support either possibility. However, using mitoplasts (outer membrane–free mitochondria) it has been shown that both the diffusion and electron transport rates of cytochrome c increase as ionic strength is increased from 0 to 150 mM, and a parallel ionic strength–induced increase in electron transport rate occurs in intact mitochondria (Gupte and Hackenbrock, 1988*a*,*b*).

Recently we have investigated the possible use of fluorescent probes to directly determine the ionic strength of the intermembrane space of the structurally and functionally intact mitochondrion. Small, soluble fluorescent probes which freely permeate the outer membrane failed to distinguish between fluorescence located in the intermembrane space and in the bulk, external solution. In addition, we found that fluorescent lipid probes incorporated into mitochondrial membranes reported effects occurring only in the proximity of the membranes, and essentially showed ionic strength–independent behavior. Thus, we reasoned that a soluble protein, labeled with an appropriate fluorophore, could be used to probe the ionic strength experienced by cytochrome c in the intermediate space of intact mitochondria. Of course, to deliver such a soluble fluorescent protein probe into the intermembrane space to reliably report ionic strength requires passing the protein probe through the protein-impermeable barrier of the outer mitochondrial membrane without disrupting mitochondrial structure and function. This paper

presents successful experiments in which a soluble ionic strength–reporting, fluorescent protein probe, FITC-BSA, was encapsulated into lipid vesicles and subsequently delivered into the intermembrane space of intact mitochondria by a low pH–induced membrane fusion method. Mitochondria subjected to this fusion protocol remained structurally and functionally intact. The intermembrane space–entrapped FITC-BSA proved to be a reliable soluble probe of intermembrane ionic strength in isolated, structurally and functionally intact rat liver mitochondria and consistently reported an ionic strength within ±20% of the ionic strength of the bulk environment external to the mitochondrial outer membrane. Thus, for mitochondria in the intact cell, cytochrome c interactions as well as other protein interactions and biochemical reactions, most likely occur in the intermembrane space at physiological ionic strength, i.e., 100–150 mM.

Materials and Methods

Preparation of Mitochondria

Liver mitochondria were isolated from male Sprague-Dawney rats according to Schnaitman and Greenwalt (1968), and then resuspended in H_{300} medium. Oxygen consumption was measured at 25°C using a Clark oxygen electrode. Respiratory control ratios (RCRs)[1] were determined using data acquisition-analysis software (Spectrofuge, Durham, NC).

Procedure for Encapsulation and Fusion

FITC-BSA was encapsulated in asolectin phospholipid vesicles by sonication in a Branson Sonifier Model W-185 (Branson Sonic Power Co., Danbury, CN). Asolectin (600 mg) was hydrated in 1.5 ml of H_{300} medium (without BSA) for at least 2 h at 0°C, and then mixed with an equal volume of a solution of FITC-BSA (25 mg FITC-BSA in 1.5 ml of 7.5-times diluted H_{300} medium). Samples were sonicated in three cycles at 40 W with a microtip probe (10 min at 0°C), adjusting pH to 7.4 with KOH between cycles to produce vesicles containing 8.33 mg FITC-BSA/ml vesicle suspension (Schneider et al., 1980 *a*,*b*). 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 FITC-BSA–containing asolectin vesicles (300 μg mitochondrial protein/mg asolectin) and incubated for 1 h at 15°C to induce adsorption of the vesicles to mitochondrial outer membranes. The pH was then decreased to 6.5 for 5 min at 15°C to induce fusion which delivers the FITC-BSA into the intermembrane space, and the FITC-BSA–containing mitochondria were returned to pH 7.4. After fusion, the FITC-BSA remaining in solution and nonfused vesicles containing FITC-BSA were removed by pelleting the mitochondria and subsequently resuspending them in FITC-BSA–free H_{300} medium. This process was repeated several times (i.e., performing a serial dilution up to 35,000-fold) until the contribution of any free FITC-BSA and any FITC-BSA-containing vesicles falls below 5% of the total measured fluorescence. After removal of free FITC-BSA, FITC-BSA–containing mitochondria were resuspended at a final concentration of 75–100 mg/ml of H_{300} medium, and assayed within 2–3 h.

Membrane Fusion Assays

Both content-mixing and membrane-mixing assays were used to detect membrane fusion and FITC-BSA delivery. Content mixing during fusion was measured by assaying for the relief of FITC-BSA self-quenching. Amounts of FITC-BSA that showed linear self-quenching relief were encapsulated into asolectin vesicles, and the resulting vesicles were fused with unlabeled mitochondria in a spectrofluorometer cuvette. When FITC-BSA is delivered into the intermembrane space of intact mitochondria, an increase in its fluorescence at 520 nm (excited at 468 nm) occurs associated with its dilution. Before fusion, free and vesicle-encapsulated FITC-BSA were separated by G-200 gel filtration chromatography, and the remaining fluorescence coming from FITC-BSA in solution was quenched with an excess of antifluorescein antibody added to the cuvette. The FITC-BSA–containing vesicles (25 mg per ml of vesicle suspension) prepared by gel filtration were incubated at 15°C with mitochondria (330 μg mitochondrial protein/mg asolectin) for 1 h in the presence of a 1:30 dilution of the antibody, followed by low pH–induced fusion. We determined that a 1:90 dilution was sufficient to maximally quench all fluorescence in the sample. A second aliquot of the antifluorescein antibody (1:30 dilution) was added at the end of the experiment to insure that any soluble FITC-BSA present was quenched. The use of this antibody showed that all fluorescence detected originated from FITC-BSA contained in the vesicles or IMS-FITC-BSA, and fluorescence increases represented relief of self-quenching of vesicle-encapsulated FITC-BSA that was delivered into the mitochondrial intermembrane space. The antibody (a gift of Dr. Edward W. Voss, Jr.) is monospecific for FITC and does not react with mitochondria or asolectin vesicles. A 1:150 dilution of this antibody is capable of completely quenching a solution containing 1 μg/ml of FITC-BSA.

Membrane mixing during fusion of the asolectin vesicles and mitochondrial outer membranes was measured by assaying the concentration-dependent relief of octadecyl-rhodamine B (R_{18}) self-quenching (Hoekstra et al., 1984, 1985). R_{18} was incorporated into the bilayer of asolectin vesicles during sonication, at a concentration that causes self-quenching of its fluorescence at 590 nm (excited at 560 nm), and where there is a linear relation between self-quenching and bilayer concentration of R_{18} (i.e., 0.5–1.0% for the mitochondrion-asolectin vesicle system). R_{18} concentrations higher than 2% may induce probe migration between membranes during experiments (see Hoekstra et al., 1984). R_{18}-labeled liposomes were fused with mitochondria, and mitochondrial outer and inner membranes separated by digitonin treatment (Schnaitman and Greenwalt, 1968). A careful quantitation of the fluorescence per mg of mitochondrial protein shows that at least 80% of the label is concentrated in the outer membrane after 1 h incubation, and the transfer to the mitochondrial membrane is negligible. To eliminate a contribution of fluorescence from free R_{18}, we routinely separate free R_{18} from vesicle-incorporated R_{18} by G-25 gel filtration chromatography, and use the R_{18}-labeled vesicle preparation immediately. The R_{18}-labeled vesicles were incubated at 15°C with freshly isolated mitochondria (330 μg mitochondrial protein per mg asolectin) for 1 h in a spectrofluorometer cuvette, followed by pH-induced fusion. Upon fusion of R_{18}-labeled vesicles with mitochondrial outer membranes, dilutions of self-quenched R_{18} molecules present in asolectin vesicle bilayers occurs as R_{18} diffuses into unlabeled mitochondrial outer membranes, indicated by an increase in R_{18} fluorescence at 590 nm. The maximal relief of self-quenching, i.e., the fluorescence value measured by adding Triton X-100 (0.1% final concentration) for R_{18} membrane-mixing and FITC-BSA content-mixing assays, is taken as 100% (infinite dilution or F_∞).

Fluorescence Measurements

The intensity of fluorescein emission at 520 nm was monitored digitally using a Perkin-Elmer fluorescence spectrophotometer 650-40 (Perkin-Elmer, Norwalk, CT) in the ratio mode at 4°C. The excitation wavelength was either 490, 450, or 468 nm (the latter to minimize error associated with light scattering of mitochondria), using slit widths of 5 nm. No spectral shifts occurred with change in ionic strength[2]. 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 (1981). The fluorescence ratio and energy modes of operation gave identical quantitative results. Statistical tests and nonlinear regression fitting of fluorescence data were performed using routines from SYSTAT (SYSTAT, Inc., Evanston, IL). For analysis, data were expressed as relative fluorescence (a quotient of two fluorescence emission measurements obtained with the same emission and excitation wavelengths) or fluorescence excitation ratios (a quotient

1. *Abbreviations used in this paper*: H_{300} medium, 300 mOsm solution composed of 220 mM mannitol, 70 mM sucrose, 2 mM Hepes buffer (pH 7.4), and 0.5 mg/ml BSA; IMS-FITC-BSA, FITC-BSA 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; RCR, respiratory control ratio, defined as the ratio between oxygen consumption of mitochondria in respiratory state 3 (in the presence of substrate and ADP) and respiratory state 4 (in the presence of substrate alone); R_{18}, octadecylrhodamine B.

2. Ionic strength is an ionic concentration I, defined as: $I = (\Sigma c_i z_i^2)/2$, where c_i is the 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.

of two fluorescence emission measurements obtained with the same emission wavelength at two different excitation wavelengths).

Lifetime Measurements

Fluorescence lifetime measurements were made on an SLM 48000 spectrofluorometer (SLM Instruments Inc., Urbana, IL) equipped with a modified, 3-position, multitemperature cuvette holder (Barrow and Lentz, 1985) and a 150-W Xe lamp mounted horizontally (Photon Technology International, Princeton, NJ). The 468- or 490-nm wavelengths were used to excite FITC-BSA or 6-carboxyfluorescein for lifetime measurements, while emission at 520 nm was monitored through a 3-mm high band pass filter (KV 500; Schott Optical Glass & Scientific Products, Duryea, PA). Multifrequency acquisition (15–110 MHz) was used for heterogeneity analysis and separation of lifetime components.

Fluorescence and Electron Microscopy

Fluorescence microscopy was performed with a Zeiss Axiophot microscope (Zeiss Instruments, West Germany), equipped with a 100× oil-immersion objective (Plan-NEOFLUAR, Ph.3, N.A. 1.3; Zeiss Instruments, West Germany), filters for fluorescein fluorescence, and automatic exposure control. Mitochondria loaded with FITC-BSA were attached to polylysine-coated coverslips (treated overnight with 0.25 mg/ml poly-L-lysine in 0.1 M Na-borate, pH 8.4), and photographed with 3200 ASA Kodax TMax film. Transmission EM was carried out as described previously (Hackenbrock, 1972; Hackenbrock and Miller, 1975). Thin sections were photographed with a JEOL 200CX electron microscope operated at 60 kV.

Materials

BSA (fatty acid-free BSA), equine muscle Na-ADP (grade IX), D-mannitol, Na-succinic acid, sucrose, and Triton X-100 were purchased from Sigma Chemical Co. (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. Sephadex G-25 and G-200 were obtained from Pharmacia Ltd. (Uppsala, Sweden). 6-Carboxyfluorescein (isomer-free), fluorescein-5-isothiocyanate (FITC; isomer I), and octadecylrhodamine B (R_{18}; chloride salt) were purchased from Molecular Probes, Inc. (Eugene, Oregon). FITC-BSA was prepared according to Zavortink et al. (1983), Wojcieszyn et al. (1983), or provided commercially (Molecular Probes, Inc.). All three preparations gave the same results with FITC-BSA dye/protein ratios between 0.5 and 6.5.

Results

Fusion of FITC-BSA–containing Asolectin Vesicles with the Mitochondrial Outer Membrane Does Not Affect Mitochondrial Structure or Function

The low pH–induced fusion method used to deliver FITC-BSA to the intermembrane space was optimized to maintain the native structure and function of the mitochondria: (*a*) adsorption incubation of mitochondria with FITC-BSA–containing vesicles was carried out for 1 h at 15°C (pH 7.4) preceding low pH–induced fusion to enhance delivery of FITC-BSA into the mitochondrial intermembrane space (shorter adsorption intervals gave less FITC-BSA incorporation), prefusion adsorption is thought to facilitate fusion events (Nir et al., 1986), and most likely diminishes mitochondrial self-fusion in the mitochondrion-asolectin vesicle system since fusion between mitochondria was not observed; (*b*) the pH was lowered only to 6.5 during fusion (mitochondrial aggregation occurs at lower pHs; Hackenbrock and Chazotte, 1986), and the time of exposure to low pH was limited to 5 min at most; (*c*) the adsorption and fusion incubation temperature was kept at 15°C (higher temperatures decreased RCRs by 20% or more after 1 h, and at 37°C RCRs were completely lost in less than 10 min); and (*d*) FITC-BSA concentration in vesicles was kept below 25 mg protein per ml of sonicated vesicles (higher concentrations lowered RCRs after fusion).

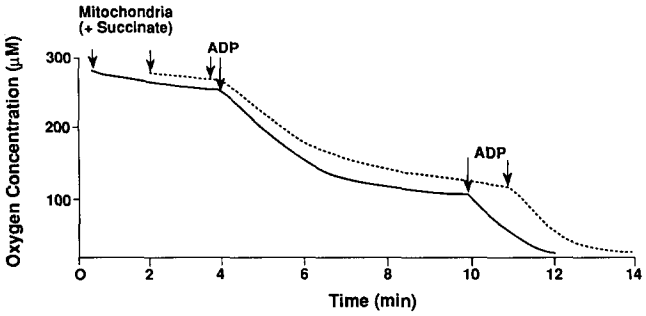

Figure 1. Oxygen consumption of freshly isolated, control mitochondria (———) and mitochondria after membrane fusion and delivery of FITC-BSA into the intermembrane space (– – –). Reaction medium: 220 mM mannitol, 70 mM sucrose, 2.5 mM HK_2PO_4, 2.5 mM $MgCl_2$, 0.5 mM EDTA, 2 mM Hepes (pH 7.4), 5 mM succinate as substrate, 0.2 mM ADP, and 1 mg mitochondrial protein/ml.

We determined that the fusion procedure delivered FITC-BSA into the intermembrane space of intact mitochondria without adversely affecting mitochondrial structure or function. The RCRs of freshly isolated, control mitochondria were 5.5–8.0, while the RCRs of mitochondria after fusion and delivery of FITC-BSA were, on average, 80–95% of the RCRs of the controls (Fig. 1). Fusion carried out with liposomes lacking FITC-BSA similarly decreases the RCRs (not shown). Transmission EM showed no detectable effect on the typically condensed configuration (Hackenbrock, 1966), indicative of freshly isolated mitochondria after incorporation of FITC-BSA (Fig. 2, *a* and *b*). It should be noted that the fusion procedure enriched the mitochondrial outer membranes with asolectin by as little as 8% (see Discussion), which did not change the appearance of the outer membrane determined by EM, or its permeability to FITC-BSA entrapped in the intermembrane space (IMS-FITC-BSA) determined by assays presented below. The intensity of the fluorescence of FITC-BSA in the mitochondrial intermembrane space was readily observed in the fluorescence microscope (Fig. 2 *c*). This fluorescence was essentially IMS-FITC-BSA fluorescence. When a standard preparation of FITC-BSA–containing mitochondria was compared with a preparation of mitochondria fused with vesicles lacking FITC-BSA and resuspended in FITC-BSA at standard concentration (i.e., a "mock" fusion experiment), it was found that after the sequential washes with FITC-BSA–free medium the fluorescence of the mock preparation was only (4.27 ± 0.07)% (mean ± SD; $n = 3$) of the fluorescence of FITC-BSA–containing mitochondria loaded by the standard procedure.

Demonstration of Membrane Fusion and FITC-BSA Delivery Into the Mitochondrial Intermembrane Space

Two fluorometric assays measuring membrane mixing and content mixing were used to demonstrate the extent of membrane fusion coupled with FITC-BSA delivery into the mitochondrial intermembrane space (Fig. 3). Both assays are necessary since a positive result in only one is not sufficient to demonstrate fusion and delivery (Loyter et al., 1988; Wil-

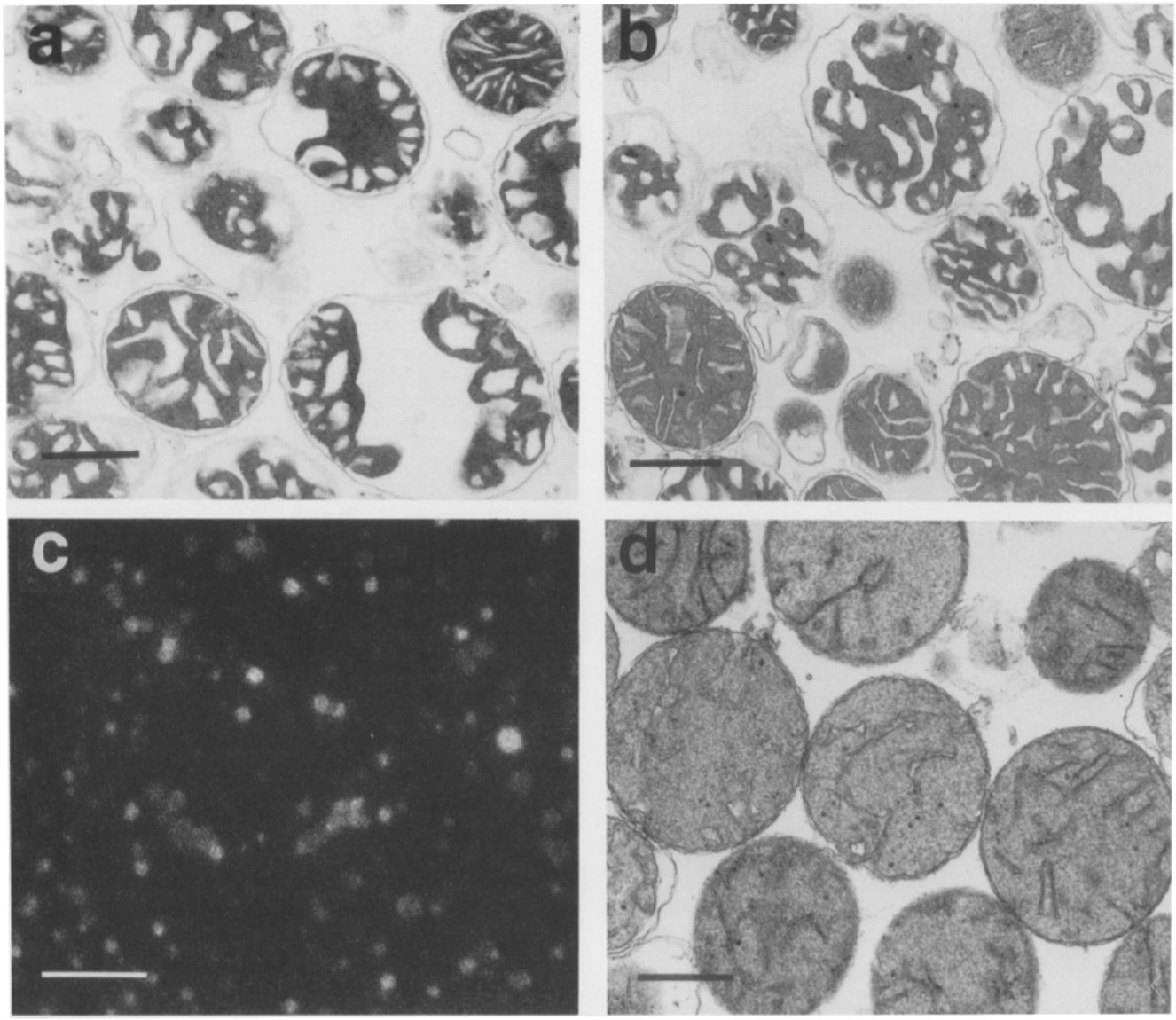

Figure 2. Ultrastructural and fluorescence microscopy of isolated mitochondria. (*a*) Ultrastructure of control mitochondria in 300 mOsm medium shows the typical condensed configuration with enlarged intermembrane space. (*b*) Ultrastructure of mitochondria after delivery of FITC-BSA into the intermembrane space and resuspension in 300 mOsm medium; the typical condensed configuration is maintained. (*c*) Fluorescence microscopy of mitochondria after delivery of FITC-BSA into the intermembrane space. (*d*) Ultrastructure of mitochondria after delivery of FITC-BSA into the intermembrane space and resuspended in 150 mOsm medium shows the typical orthodox configuration with restricted intermembrane space. Bars: (*a*, *b*, and *d*) 0.6 μm; (*c*) 5 μm.

schut and Hoekstra, 1986). The membrane-mixing assay showed that during the adsorption step preceding the low pH fusion, a very gradual, small increase in R_{18} fluorescence at 590 nm occurred indicating a low level of membrane mixing. Lowering the pH to 6.5 resulted in a substantial fluorescence increase, indicative of a high degree of rapid membrane mixing and fusion (Fig. 3 *A*). Using this fluorescence increment, and the infinite dilution fluorescence (F_∞), it was calculated that 38% of the asolectin vesicles adsorbed to mitochondria underwent fusion at low pH.

The content-mixing assay showed an increase in fluorescence at 520 nm, indicative of the dilution of vesicle-encapsulated FITC-BSA as the probe was delivered into the intermembrane space (Fig. 3 *B*). Fluorescence from any free FITC-BSA was eliminated at the beginning of the experiment by adding an excess amount of a specific antifluorescein antibody (Fig. 3 *B*, *left Ab arrow*). A direct estimate of the percentage of fluorescence quenching obtained during the time-course experiments is difficult. Controls with FITC-BSA–containing vesicles alone and antifluorescein antibody permitted us to calculate an appropriate excess of antibody to quench all fluorescence originating from FITC-BSA in solution. The content mixing of vesicles with the intermembrane space was detected before and after the pH was lowered to 6.5 (Fig. 3 *B*, *H^+ arrow*). However, the low pH–induced fluorescence increase was significantly much greater when the data was normalized relative to a common baseline (Fig. 3 *C*); which revealed that as much as 90% of the content mixing occurred with lowering the pH. A final aliquot of antifluorescein antibody did not have an effect on FITC-

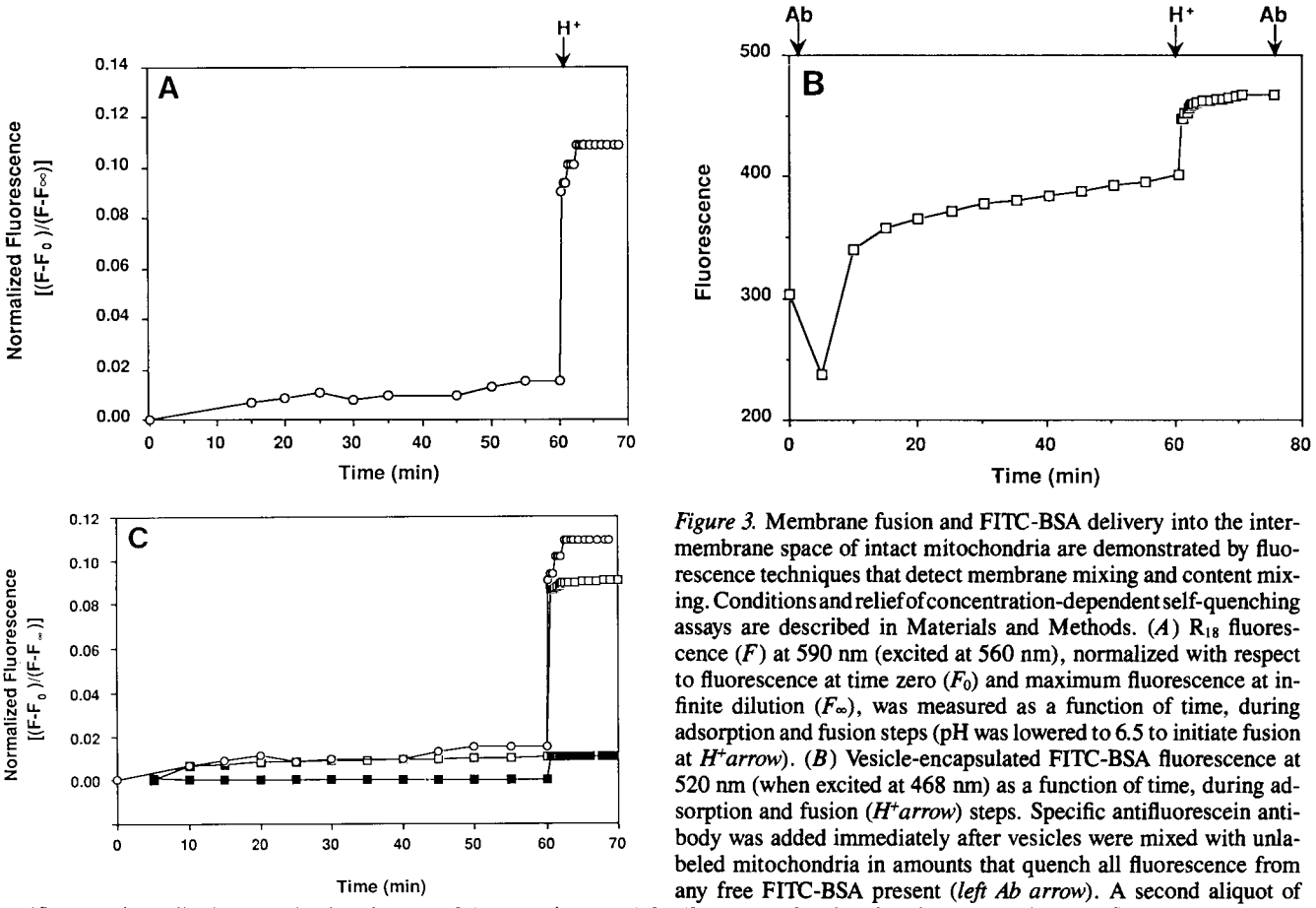

Figure 3. Membrane fusion and FITC-BSA delivery into the intermembrane space of intact mitochondria are demonstrated by fluorescence techniques that detect membrane mixing and content mixing. Conditions and relief of concentration-dependent self-quenching assays are described in Materials and Methods. (*A*) R_{18} fluorescence (*F*) at 590 nm (excited at 560 nm), normalized with respect to fluorescence at time zero (F_0) and maximum fluorescence at infinite dilution (F_∞), was measured as a function of time, during adsorption and fusion steps (pH was lowered to 6.5 to initiate fusion at *H⁺arrow*). (*B*) Vesicle-encapsulated FITC-BSA fluorescence at 520 nm (when excited at 468 nm) as a function of time, during adsorption and fusion (*H⁺arrow*) steps. Specific antifluorescein antibody was added immediately after vesicles were mixed with unlabeled mitochondria in amounts that quench all fluorescence from any free FITC-BSA present (*left Ab arrow*). A second aliquot of antifluorescein antibody was added at the end of the experiment (*right Ab arrow*), showing that the low pH-induced fluorescence did not originate from free FITC-BSA released in the medium during fusion. (*C*) Comparison of the R_{18} (○) and FITC-BSA (□) relief of self-quenching data shown in *A* and *B*, and self-fusion of asolectin vesicles containing FITC-BSA subjected to low pH (■). Experiments were normalized with respect to maximal fluorescence (F_∞) for each system, considering that FITC-BSA is initially delivered to an empty compartment and concentrates there during the experiment.

BSA fluorescence, showing that the fluorescence increase induced by low pH was not due to a release of free FITC-BSA into the external medium during fusion (Fig. 3 *B*, *right Ab arrow*). A contribution of vesicle-vesicle fusion was also considered. Small unilamellar vesicles such as the FITC-BSA-containing asolectin vesicles present during the time-course of the experiment could self-fuse, thereby diluting their FITC-BSA content as the volume of the fused vesicles increases. A control using FITC-BSA-containing vesicles was subjected to the same protocol as experiments using both vesicles and mitochondria (Fig. 3 *B*), and only a minor vesicle self-fusion was found, as detected with the content-mixing assay (Fig. 3 *C*).

In Fig. 3 *C*, membrane-mixing (R_{18}) and content-mixing (FITC-BSA) assays were normalized and compared on the same scale, using their respective infinite dilution fluorescence values (F_∞). Both assays showed parallel time courses for the mixing of asolectin vesicle membranes with outer mitochondrial membranes and the mixing of vesicle contents that occurred when FITC-BSA was delivered to the intermembrane space. Fusion of membranes and delivery of the FITC-BSA were completed within 1–2 min at pH 6.5. The results shown in Fig. 3, taken together, demonstrate significant (90%) membrane fusion and FITC-BSA delivery at pH 6.5, with some fusion-delivery occurring during the adsorption step at pH 7.4.

Demonstration of the Entrapment of FITC-BSA by Manipulation of the Volume of the Intermembrane Space

The content-mixing assay shown in Fig. 3 *B* indicates delivery of FITC-BSA to a FITC-BSA-free compartment, i.e., the intermembrane space of mitochondria. Further evidence of entrapment of FITC-BSA in the intermembrane space (IMS-FITC-BSA) of intact mitochondria was obtained by osmotically changing the volume of this compartment (Fig. 4). Mitochondria in H_{300} medium (300 mOsm) showed the typical condensed configuration (Hackenbrock, 1966), with a large intermembrane volume both before and after delivery of FITC-BSA (Fig. 2, *a* and *b*). Equilibration with media of lower osmolarity increases the matrix volume (Tedeschi and Harris, 1955; Stoner and Sirak, 1969), thus, decreasing the intermembrane space. At 150 mOsm the volume of the intermembrane space is minimum, characteristic of the orthodox configuration (Hackenbrock, 1966; Fig. 2 *d*). It was determined that a sequential, osmotically induced decrease in the intermembrane space occurred with a parallel self-quenching of fluorescence indicative of an increase in the concentration of IMS-FITC-BSA (Fig. 4). The effect of decreasing osmolarity on IMS-FITC-BSA fluorescence was approximately linear from ~240 to ~100 mOsm. Osmolarity in itself was found to have no effect on the fluorescence of FITC-BSA in

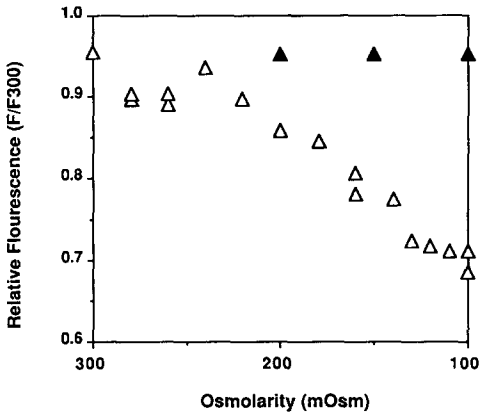

Figure 4. The fluorescence of FITC-BSA entrapped in the intermembrane space changes via self-quenching or relief thereof as the mitochondria intermembrane space is changed osmotically. Fluorescence of IMS-FITC-BSA at 520 nm (excited at 468 nm) was measured in the range of 100–300 mOsm. The results are expressed as relative fluorescence, dividing fluorescence at a given osmolarity (F) by fluorescence in H_{300} medium (F_{300}; △). Mitochondria containing IMS-FITC-BSA at 300 mOsm show the usual condensed configuration containing a large intermembrane space (Fig. 2 *b*), while decreases in osmolarity result in decreases in the volume of the intermembrane space as in the orthodox configuration (Fig. 2 *d*). The decrease of fluorescence of IMS-FITC-BSA at 100, 150 or 200 mOsm is reversed by restoring the osmolarity to 300 mOsm which returns the mitochondria to the condensed configuration, thereby increasing the volume of the intermembrane space (▲).

solution. Significantly, self-quenching (decrease in fluorescence) of the IMS-FITC-BSA was readily reversed by osmotically transforming orthodox to condensed mitochondria, thus, increasing the volume of the intermembrane space and reducing the concentration of the entrapped FITC-BSA (Fig. 4, *solid symbols*). These experiments reveal that after the encapsulation and fusion procedures, FITC-BSA is confined to the intermembrane space of structurally and functionally intact mitochondria.

FITC-BSA Fluorescence Is Ionic Strength Dependent

It was determined that the fluorescence of FITC-BSA in solution increased with increasing ionic strength (Fig. 5). This effect occurred equally with different monovalent ions (e.g., KCl and NaCl). Ionic strength also affects the free fluorophore itself, i.e., 6-carboxylfluorescein (not shown). A simple testable hypothesis for the ionic strength effect on FITC-BSA fluorescence is that the ionic strength alters the fluorescein quantum yield; however, this was found not to be the case. 6-carboxylfluorescein showed a single ionic strength–independent lifetime (τ = 3.96 ns) at I = 0 mM and at I = 150 mM. For FITC-BSA, there were two ionic strength–independent lifetime components: at I = 0 mM, τ_1 = 0.72 ns (23.4% of the total), and τ_2 = 3.48 ns (76.5%); at I = 150 mM, τ_1 = 0.94 ns (25.6%), and τ_2 = 3.88 ns (74.4%). Thus, neither the lifetimes nor the percentages of fluorophore molecules that exhibit a particular lifetime change with ionic strength.

It was found that the FITC-BSA fluorescence excitation ratios, (Ex_2/Ex_1) 490/450 nm (Fig. 6 *A*), commonly used for monitoring pH (Heiple and Taylor, 1980, 1982; Murphy et al., 1984), and 468/450 nm (Fig. 6 *B*), which we found better suited for monitoring ionic strength in mitochondrial samples because of light scattering, were sensitive to both ionic strength and pH. The excitation ratios of 490/450 nm and 468/450 nm revealed that the pK of BSA-bound fluorescein decreased as the ionic strength increased (Fig. 6). A parallel experiment using free 6-carboxylfluorescein showed smaller pK decreases. Clearly, the effects of pH and ionic strength on fluorescence have different characteristics (see Figs. 5 and 6). Ionic strength sensitivity followed a hyperbolic saturation curve in the mM range (K_1 of FITC-BSA is 10–15 mM; see Figs. 5 and 7), while the pH sensitivity followed a sigmoidally shaped curve in the μM range (pK of 6-carboxyfluorescein is 5.96 and pK of FITC-BSA is 7.63). The shape of the pH curves were maintained at different ionic strengths, supporting the use of fluorescein as a pH-sensitive probe (Thomas et al., 1979; Heiple and Taylor, 1980, 1982; Murphy et al., 1984; Bright et al., 1987). From these experiments, changes in the quantum yield to explain the ionic strength effect on fluorescein fluorescence can be ruled out. Our data is consistent with a static quenching mechanism to explain the ionic strength dependence of this fluorophore. We believe that as the ionic strength increases, the activity coefficients of the ionic forms of the fluorescein molecule (given by Debye-Hückel theory or its modified forms; Lewis and Randall, 1961; Stell and Joslin, 1986) are affected, and the multiple equilibria shifts toward more fluorescent mixtures of fluorophores (i.e., the pK decreases).

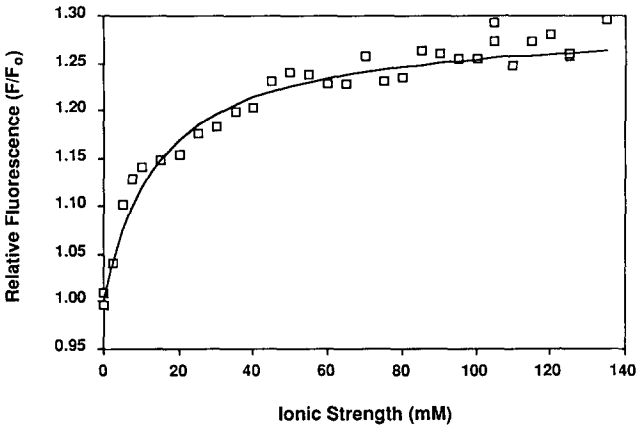

Figure 5. Fluorescence of FITC-BSA in solution (excitation, 468 nm; emission, 520 nm) increases with ionic strength. Relative fluorescence, i.e., fluorescence at a given ionic strength (F) divided by fluorescence at zero ionic strength F_0), is measured for FITC-BSA (3 μg/ml; dye/protein ratio 5.3) as a function of KCl-driven ionic strength. Data were fitted to a hyperbolic, single-component saturation curve by nonlinear regression to calculate the maximal relative increase fluorescence ($(F/F_0)_{max}$), and the ionic strength which gives 50% of this maximum (apparent affinity or K_I). The equation used was: $F/F_0 = A + (B \cdot I/(C + I))$, where A is ~1.0, B is the maximum curve span (i.e., $[(F/F_0)_{max} - 1]$), and C is K_I. The maximal relative increase in the FITC-BSA fluorescence at 520 nm ($(F/F_0)_{max}$) was 1.291 ± 0.009 (A = 1.006 ± 0.009; B = 0.291 ± 0.009), and the apparent affinity (K_I) was 14.5 ± 2.1 mM (minimal squares sum or SS_{min} = 0.007; N = 34). The same values were obtained with FITC-BSA in solution with dye/protein ratios between 0.5 and 6.5 (not shown).

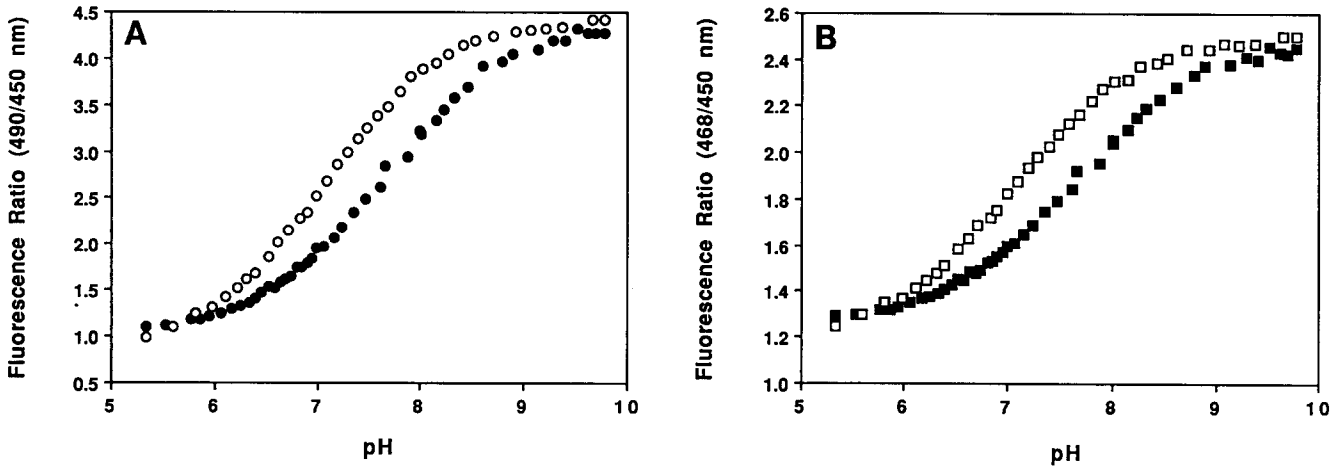

Figure 6. Effects of pH and KCl-driven ionic strength on fluorescence excitation ratios of FITC-BSA in solution. Fluorescence excitation ratios (Ex_2/Ex_1) 490/450 nm (*A*) and 468/450 nm (*B*) for FITC-BSA emission at 520 nm were determined as a function of pH and I. The p*K*s of FITC-BSA were calculated using these ratios, and the equation: $(Ex_2/Ex_1) = A + B/(1 + 10^{pK\text{-}pH})$, where *A* and *B* are constants that define the span of fluorescence intensities (Slavík, 1989). (*A*) For fluorescence excitation ratio 490/450 nm: at 0 mM (●), p*K*(I = 0 mM) = 7.63 ± 0.03, with *A* = 1.25 ± 0.03 and *B* = 2.95 ± 0.04 (SS_{min} = 0.432, *N* = 45); at 150 mM (○), p*K*(*I* = 150 mM) = 7.13 ± 0.03, with *A* = 1.14 ± 0.04 and *B* = 3.17 ± 0.04 (SS_{min} = 0.235, *N* = 35). (*B*) For fluorescence excitation ratio 468/450 nm: at 0 mM (■), p*K*(*I* = 0 mM) = 7.63 ± 0.03, with *A* = 1.35 ± 0.01 and *B* = 1.06 ± 0.01 (SS_{min} = 0.061, *N* = 45); at 150 mM (□), p*K*(*I* = 150 mM) = 7.10 ± 0.06, with *A* = 1.30 ± 0.02 and *B* = 1.18 ± 0.01 (SS_{min} = 0.227, *N* = 35). For 6-carboxyfluorescein (data not shown), fluorescence excitation ratio 490/450 nm gave for 0 mM, p*K*(*I* = 0 mM) = 5.96 ± 0.08, *A* = 0.92 ± 0.03, *B* = 1.25 ± 0.04 (SS_{min} = 0.379, *N* = 45), and for 150 mM, p*K*(*I* = 150 mM) = 5.60 ± 0.08, *A* = 0.87 ± 0.03, *B* = 1.27 ± 0.04 (SS_{min} = 0.517, *N* = 45).

Comparison of the Ionic Strength Response of FITC-BSA in Solution and Entrapped in the Intermembrane Space

The IMS-FITC-BSA saturation curve in response to ionic strength was normalized using concentration-independent fluorescence ratios (468/450 nm), and found to consistently give a simple, one-component saturation curve with affinities similar to FITC-BSA in solution (Fig. 7). Using fluorescence ratioing, the effects of ionic strength on soluble FITC-BSA and IMS-FITC-BSA were compared. The saturation curve for FITC-BSA in solution showed an affinity of 10.2 ± 0.6 mM (Fig. 7, *solid line*), and for IMS-FITC-BSA of 9.8 ± 0.8 mM (Fig. 7, *dashed line*). The maximal values of fluorescence change ($[F/F_o]_{max}$) were somewhat different: 1.211 ± 0.002 for FITC-BSA in solution and 1.172 ± 0.003 for IMS-FITC-BSA. This is most likely related to a small difference in the estimation of the intrinsic fluorescence (F_o) at 468 and 450 nm, which affects the entire curve. For 6-carboxyfluorescein (not shown), the maximal change $(F/F_o)_{max}$ was 1.24 ± 0.04, and the apparent affinity (K_1) was 9.2 ± 3.9 mM (SS_{min} = 0.001). The effect of ionic strength on the fluorescence did not originate from fluorescence from soluble FITC-BSA, from free FITC-BSA–containing vesicles, or from nonfused FITC-BSA–containing vesicles attached to the mitochondrial outer membranes. Any FITC-BSA in solution or free FITC-BSA–containing vesicles were washed out by sequencing pelleting and resuspension in FITC-BSA-free H_{300} medium (see Materials and Methods). Furthermore, vesicle-encapsulated FITC-BSA alone showed that an increase of ionic strength from 0 to 75 mM (at 300 mOsm) increased fluorescence only 0.88 ± 0.01% (mean ± SD; *n* = 3).

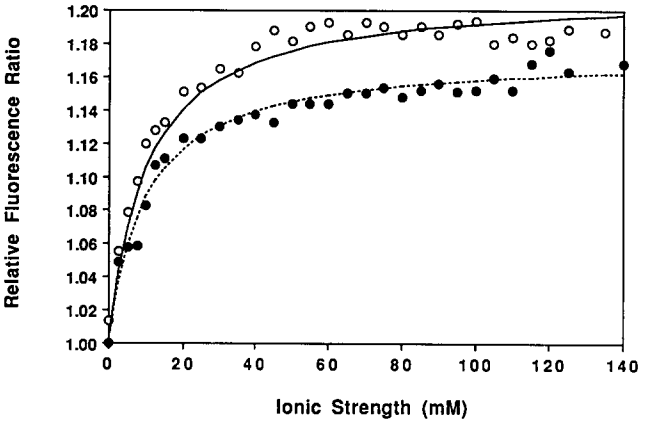

Figure 7. Comparison of the effect of ionic strength on the fluorescence of FITC-BSA in solution and IMS-FITC-BSA. The fluorescence excitation ratio 468/450 nm (with emission at 520 nm) was determined for FITC-BSA in solution (○) and IMS-FITC-BSA (●) at different KCl-driven ionic strengths. For soluble FITC-BSA, $(F/F_0)_{max}$ was 1.211 ± 0.002 and K_1 was 10.2 ± 0.6 mM (SS_{min} = 0.002; *N* = 31); for IMS-FITC-BSA, $(F/F_0)_{max}$ was 1.173 ± 0.003 and K_1 was 9.8 ± 0.6 mM (SS_{min} = 0.001; *N* = 34).

These data clearly reveal that changes in bulk ionic strength resulted in changes in intermembrane space ionic strength. Quantitatively, FITC-BSA fluorescence consistently reported an ionic strength in the mitochondrial intermembrane space within ±20% of the ionic strength in the bulk media external to the outer membrane. Using the affinities obtained, K_1(FITC-BSA in solution)/K_1(IMS-FITC-BSA), the ratio between mitochondrial intermembrane space ionic strength and the ionic strength of the bulk solution is 1.04 ± 0.20 (mean ± 2 SD). Therefore, for an external, bulk ionic strength of 150 mM, the probability of an intermembrane ionic strength

lower than 130 mM (mean-2 SD) is 2.5%. From these data, it is suggested that in the intact cell the mitochondrial intermembrane ionic strength is well within the range of cytosolic ionic strength, i.e., 100–150 mM.

Discussion

We present here a novel method of encapsulation and fusion that allows the delivery of fluorescent protein probes into the intermembrane space of isolated mitochondria without disrupting structure or function. The procedure is based on a controlled low pH–induced fusion of asolectin vesicles with the outer membranes of intact mitochondria under conditions that limit the lipid enrichment of the outer membrane by as little as 8%. Delivery of an ionic strength–reporting protein probe, FITC-BSA, into the mitochondrial intermembrane space, was routinely carried out while preserving mitochondrial ultrastructure and 95% of the native respiratory control.

We have provided evidence for the delivery of FITC-BSA into the intermembrane space of mitochondria by using two fuorescence assays: (*a*) a membrane-mixing assay based on the relief of fluorescence self-quenching of octadecylrhodamine (R_{18}), incorporated into the bilayer of asolectin vesicles, when the vesicles fuse with mitochondrial outer membranes; and (*b*) a vesicle contents assay based on the relief of fluorescence self-quenching of FITC-BSA, concentrated in asolectin vesicles, when the vesicles fuse with mitochondrial outer membranes. The assay of membrane mixing alone does not rule out the possibility of direct transfer of R_{18} from labeled to unlabeled membranes, whereas the content-mixing assay may be affected by possible content leakage (Bentz et al., 1985). We minimized the direct transfer of R_{18} by decreasing its concentration in the vesicle bilayer (Hoekstra et al., 1984). The lack of content leakage was determined by using a specific antifluorescein antibody in amounts that would quench, and therefore identify, any fluorescence coming from free, soluble FITC-BSA. The combination of both optimized assays provides a sensitive detection system for membrane fusion.

The data reveal that the majority of the fusion with delivery of FITC-BSA molecules into the intermembrane space, occurs when the pH is reduced to 6.5. Both assays showed ~10% of the total fusion to occur during the adsorption step before the pH is lowered. This is surprising, given that asolectin vesicles (Miller and Racker, 1976) and mitochondrial outer membranes (Hackenbrock and Miller, 1975; Daum, 1985) have a high density of negative surface charges. In any case, without the adsorption step, the level of low pH–induced incorporation of FITC-BSA decreases below the detectable level, i.e., the number of successful fusion events decreases. Fusion is not driven by the presence of Ca^{2+}, a known fusogen, or serum albumin since chelation of Ca^{2+} with EGTA/EDTA and/or elimination of BSA from the media did not reduce binding of asolectin vesicles to mitochondria or modify the extent of incorporation of FITC-BSA into the intermembrane space.

Calculations of the efficiency of delivery of FITC-BSA into the intermembrane space were performed based on comparisons of the degree of fluorescence of FITC-BSA solutions of known concentration with the fluorescence of FITC–BSA-loaded mitochondria, the number of mitochondria per mg of mitochondrial protein, mitochondrial surface area, and size of small unilamellar vesicles.[3] The calculations indicate that: (*a*) 800 (130–1,600) molecules of FITC-BSA are delivered into the intermembrane space per mitochondrion; (*b*) 2,170 (360–4,400) asolectin vesicles fuse with each mitochondrion; (*c*) 39.8% (6.6–79.8%) of the surface area of a mitochondrial outer membrane is coated with asolectin vesicles that successfully fuse after decreasing the pH; and (*d*) asolectin lipids constitute as little as 8% (mean, 15%; range, 8–50%) of the outer membrane phospholipid after fusion. These estimates indicate that substantial amounts of FITC-BSA can be delivered to the intermembrane space of intact mitochondria through the procedure described here without disrupting mitochondrial structure or function. By increasing FITC-BSA concentration in the liposome preparation, the upper limit of 1,600 FITC-BSA molecules delivered per mitochondrion can be reached without excessive lipid enrichment of the outer membrane.

We have shown that FITC-BSA can be used as a reliable probe of ionic strength in the intermembrane space of the intact mitochondrion. The affinity of the ionic strength sensitivity of the fluorescence was determined to be in the mM range, as opposed to the pH sensitivity of the fluorescein in the μM range. This effect of ions on fluorescein fluorescence is usually obscured by the use of buffers of moderate ionic strength, performing measurements on cells (which contain a high ionic strength), or by working in a pH-range that suppresses the response to ionic strength (Fig. 5). The maximal effect of ionic strength on fluorescence was greater for FITC-BSA than for free 6-carboxyfluorescein, but nonetheless did affect the free fluorophore. The increase in fluorescence of both FITC-BSA in solution and IMS-FITC-BSA (Figs. 4 and 7), taken together with our lifetime measurements, indicate a mechanism in which ionic strength affects the ground state of fluorescein (static quenching) through changes on the activity coefficients of the various ionic forms of the fluorophore. The constancy of the lifetime for both soluble and protein-linked fluorescein suggests that the excited state, and therefore the quantum yield, is not affected by ionic strength. We used KCl-driven ionic strength since this salt is responsible for the majority of the ionic strength as well as osmolarity of the

3. These theoretical estimates of membrane fusion and delivery of FITC-BSA into the intermembrane space are based on the general understanding that smaller unilamellar vesicles preferentially fuse with mitochondrial membranes (Schneider et al., 1980*a*). Routinely we sonicate liposome preparations extensively and use them immediately to maximize the number of small vesicles. Values of (4.3–9.0) 10^9 mitochondria per mg of mitochondrial protein (Bahr and Zeitler, 1962; Gear and Bednarek, 1972; Schwerzmann et al., 1986), a surface area of 170 cm^2 per mg of mitochondrial protein (Schwerzmann et al., 1986), and a 210 Å average diameter for small unilamellar vesicles involved in fusion (Yeagle, 1987) were used in these calculations. Experimental measurements of fluorescence in control FITC-BSA solutions and the fluorescence from 4 mg of FITC-BSA-loaded mitochondria give the number of molecules of FITC-BSA delivered into the intermembrane space of a mitochondrion. With 8.33 [3–16.7] mg/ml FITC-BSA in the vesicle preparation (i.e., $7.6 \cdot 10^{16}$ molecules of FITC-BSA per ml), we calculated the number of vesicles fused and the number of molecules of FITC-BSA delivered into the intermembrane space. The percentage of surface area of the mitochondrial outer membrane was then obtained from the ratio between the surface areas of a mitochondrion and a single vesicle. The ratio of mitochondrial outer membrane lipid volume (area per mitochondrion × membrane thickness) to the lipid volume of the minimal number of vesicles that fuse with mitochondrial outer membranes gives the theoretical minimum enrichment of 8%. It should be noted that these calculations are approximate averages.

cytoplasm. No ion-specific effects on fluorescence were observed.

We found that the fluorescence of FITC-BSA in solution increased as a single component, hyperbolic saturation curve, whether we used relative increases in fluorescence or fluorescence ratios (see Figs. 5 and 7). For IMS-FITC-BSA, we normalized the intermembrane space concentration of FITC-BSA by using fluorescence ratios (Fig. 7). Using IMS-FITC-BSA we found that the ionic strength of the intermembrane space is within 10–20% of the value for bulk ionic strength external to the outer membrane, and that changes in the intermembrane space ionic strength parallel changes in the bulk ionic strength. This finding suggests that the normal, physiological ionic strength in the intermembrane space of mitochondria in situ is close, if not equal, to the cytosolic ionic strength (I = 100–150 mM). Significant to the true ionic strength of the intermembrane space, the binding of cytochrome c to each of its redox partners is thought to be a complex, electrostatic interaction composed of multiple binding affinities. However, binding studies have been traditionally carried out under conditions of low ionic strength (Pettigrew and Moore, 1987). Since our results indicate that the intermembrane ionic strength is normally 100–150 mM, the interactions of cytochrome c with its major redox partners, cytochrome c_1 and cytochrome c oxidase, is likely to be less complex. It is known, e.g., that some cytochrome c binding sites detected at low ionic strength (Ferguson-Miller et al., 1976; Stonehuerner et al., 1979) disappear at moderately high ionic strengths (Ferguson-Miller et al., 1976, 1979; Mauk et al., 1982). Also, an increase in ionic strength appears to release cytochrome c into the intermembrane space of intact mitochondria (Matlib and O'Brien, 1976). Using inner membrane preparations, it has been determined that an increase in ionic strength results in an increase in the lateral diffusion of cytochrome c on the inner membrane, which parallels an increase in the rate of cytochrome c-mediated electron transfer (Gupte and Hackenbrock, 1988*a*,*b*). Also, at an ionic strength of 100–150 mM, it was found that the diffusion rate of cytochrome c was highest, its affinity for the inner membrane was lowest, its diffusion mode was three dimensional, its collision efficiency with its redox partners was highest, and its electron transport rate was highest. Such findings, together with our present result showing the ionic strength of the intermembrane space to be within ±20% of the bulk, external medium, strongly indicate that cytochrome c functions as a three-dimensional redox diffusant in the intermembrane space of intact mitochondria at cellular physiological ionic strength, i.e., 100–150 mM. The direct determination of the ionic strength of the intermembrane space should prove to be important in studying other protein interactions and biochemical reactions as well, in the intermembrane space of intact mitochondria.

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