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pH-Sensitive Binding of Cytochrome *c* to the Inner Mitochondrial Membrane. Implications for the Participation of the Protein in Cell Respiration and Apoptosis[†]

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ABSTRACT: Cytochrome *c* exhibits two positively charged sites: site A containing lysine residues with high pK_a values and site L containing ionizable groups with pK_{aobs} values around 7.0. This protein feature implies that cytochrome *c* can participate in the fusion of mitochondria and have its detachment from the inner membrane regulated by cell acidosis and alkalosis. In this study, we demonstrated that both horse and tuna cytochrome *c* exhibited two types of binding to inner mitochondrial membranes that contributed to respiration: a high-affinity and low-efficiency pH-independent binding (microscopic dissociation constant K_{sapp2}, ~10 nM) and a low-affinity and high-efficiency pH-dependent binding that for horse cytochrome *c* had a pK_a of ~6.7. For tuna cytochrome *c* (Lys22 and His33 replaced with Asn and Trp, respectively), the effect of pH on K_{sapp1} was less striking than for the horse heme protein, and both tuna and horse cytochrome *c* had closed K_{sapp1} values at pH 7.2 and 6.2, respectively. Recombinant mutated cytochrome *c* H26N and H33N also restored the respiration of the cytochrome *c*-depleted mitoplast in a pH-dependent manner. Consistently, the detachment of cytochrome *c* from nondepleted mitoplasts was favored by alkalinization, suggesting that site L ionization influences the participation of cytochrome *c* in the respiratory chain and apoptosis.

Mitochondria play a fundamental role in the cell apoptosis since they contain apoptogenic proteins procaspases (-2, -3, and -9), the apoptosis-inducing factor (AIF),[1] and cytochrome *c* (cyt *c*) that are released to the cytosol to participate in the degradation phase of apoptosis (*1*, *2*). The release of apoptogenic proteins occurs in response to different stimuli [cytokine withdrawal (*3*, *4*)] or ceramide treatment (*1*), each of which promotes the deregulation of intracellular pH as part of the trigger for cell death. In eukaryotic cells, cytosolic pH is controlled by proton pumps, proton channels, and ion transporters that drive H^+ or H^+ equivalents and HCO_3^- ions into and out of the cell.

The detachment of cyt *c* from the inner mitochondrial membrane to trigger events in the cytosol leading to apoptosis (*6*−*11*) implicates the existence of a reversible interaction of the protein with the lipid bilayer. Studies conducted with model systems have indicated the existence of two different cyt *c* sites, A and C, which are responsible for the association with lipid bilayers (*12*−*16*). Site A is an electrostatically interacting site made up of basic residues in cyt *c*, probably Lys72 and Lys73. Site C is another cyt *c* lipid-binding site with a high affinity for protonated acidic phospholipids via hydrogen bonds with the invariant Asn52 (*15*, *16*). However, on the external surface of the inner mitochondrial membrane, the clusters of positively charged amino acid side chains of site A are believed to be important for the recognition and binding of cyt *c* reductase and oxidase (*17*, *18*). Recently, we demonstrated the existence of one more sites for the electrostatic interaction of cyt *c* with acidic phospholipids (site L) involving amino acid residues Lys22, -25, and -27 and His26 and -33. Site L controls a pH-dependent binding of horse cyt *c* to membranes and has a pK_a of ~7.0 (*19*). These results led to the postulation that the interaction of cyt *c* with the inner mitochondrial membrane could be modulated by the mitochondrial transmembrane potential. The enhancement of pH in the intermembrane space resulting from the loss of the transmembrane potential might lead to the deprotonation of site

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[1]Abbreviations: cyt *c*, cytochrome *c*; AIF, apoptosis-inducing factor; MP, mitoplasts; MPc, cytochrome *c*-supplied mitoplast; DEPC, diethyl pyrocarbonate; PyPC, 2-[10-(1-pyrene)decanoyl]phosphatidylcholine; K_{sapp}, microscopic dissociation constant; $\Delta\Psi$, transmembrane potential; AA, antimycin A; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; RFI, relative fluorescence intensity; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; PG, phosphatidylglycerol; RLU, relative luminescence units.

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L and contribute to the detachment of cyt *c* from the mitochondrial membrane to trigger apoptosis in cytosol. Considering that site L was characterized in liposome models, a realistic model was rationalized by probing cyt *c* binding to the inner mitochondrial membrane of cyt *c*-depleted mitoplasts (MP) over a pH range (6.2−7.4) compatible with respiring and coupled mitochondria or in the presence of respiratory inhibitors or uncoupling compounds (*20*, *21*). The model used previously differs significantly from the inner mitochondrial membrane due to the absence of the large amount of associated proteins, including complexes III and IV, which also interact with cyt *c*. Thus, further research was quite necessary to determine whether the pH dependence observed for the binding of cyt *c* to the PCPECL liposomes was a peculiarity of this system or also occurs in the biological system, i.e., the mitochondria.

MATERIALS AND METHODS

Chemicals. Horse heart cyt *c* (type III), HEPES, sodium phosphate, diethyl pyrocarbonate, bovine serum albumin, sucrose, succinate, rotenone, ADP, EGTA [ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], K_2HPO_4, KOH Tris-HCl, α-cyano-4-hydroxycinnamic acid, NH_2OH, acetic acid, acetonitrile, perchloric acid, trifluoroacetic acid, benzamidine, *N*-ethylmaleimide, and phenylmethanesulfonyl fluoride were acquired from Sigma Chemical Co. (St. Louis, MO). Avanti Polar Lipids, Inc. (Alabaster, AL), provided 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine, and heart cardiolipin. Molecular Probes, Inc. (Eugene, OR), supplied 2-[10-(1-pyrene)decanoyl]phosphatidylcholine (PyPC). EDTA (ethylenediaminetetraacetic acid) was acquired from Merck S.A. (Rio de Janeiro, Brazil); ammonium hydroxide was acquired from QEEL Indústrias Químicas S.A. (Sao Paulo, Brazil). The ATP Monitoring Reagent kit was obtained from BioOrbit, and CM-32 and filter paper no. 50 were obtained from Whatman International Ltd. (Maidstone, England). Tuna cyt *c* was extracted from tuna heart according to the method described by Brautigan et al. (*22*) with some modifications. Briefly, 50 g of tuna heart muscle was homogenized in "Potter-Elvejhem" at high speed for 4 min at 4 °C with a 100 mL ice-cold solution of 0.3% $Al_2(SO_4)_3 \cdot 18H_2O$, 6.2 mM benzamidine, 5.5 mM *N*-ethylmaleimide, 0.1 mM phenylmethanesulfonyl fluoride, and 5 mM EDTA. The pH was set to 4.5 with 2 M acetic acid or 2 M NH_2OH, and the mixture was incubated for 30 min at 4 °C. The solution was spun at 8000*g* for 20 min at 4 °C, and the pH of the supernatant was set at 8.5. Then, it was filtered using Whatman no. 50 filter paper. The filtrate was dialyzed against deionized water in Spectrapor tubing (6000−8000 molecular weight cutoff) with two changes of 20 L of water per liter of filtrate per day. The cyt *c* was isolated via ion exchange chromatography. Approximately 10 g of CM-32 (carboxymethylcellulose) resin pre-equilibrated with 20 mM sodium phosphate (pH 8.0) was added to the dialyzed solution and maintained for 30 min with occasional stirring at 4 °C. The solution was spun at 580*g* for 5 min at 4 °C, and the supernatant was discarded. The resin was washed with 3 column volume equivalents of phosphate buffer; then, the cyt *c* was displaced from the resin by 500 mM NaCl in the same buffer. Fractions containing cyt *c* were dialyzed against deionized water in Spectrapor tubing (6000−8000 molecular weight cutoff) with two changes of 4 L of water. Diethyl pyrocarbonate-modified cyt *c* was prepared using samples of 100 μM cyt *c* in 10 mM HEPES, 10 mM sodium acetate, and 10 mM ammonium hydroxide buffer (pH 6.5 or 7.5), which were made to react with a 5-fold molar excess of DEPC at room temperature for 1 min. The samples were then dialyzed for 2 h before being subjected to MALDI-ToF mass spectrometry analysis.

MALDI-ToF Mass Analysis. All linear, reflectron, and post-source decay (PSD) spectra were recorded on an Ettan MALDI-ToF Pro mass spectrometer that can operate in the linear or reflectron mode using a harmonic reflectron that increases resolution and sharpens time focusing. The accelerating voltage was set at 20 kV. The samples were mixed with a 5-fold volume of a saturated solution (10 mg/mL) of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 0.5% trifluoroacetic acid, and 0.5 μL was loaded onto the stainless steel MALDI slides for analysis. The data were analyzed using Ettan MALDI-ToF Pro.

Preparation of Rat Liver Mitochondria. Mitochondria were isolated by conventional differential centrifugation from the livers of adult Wistar rats. The homogenate was prepared in 250 mM sucrose, 1.0 mM EGTA, and 5.0 mM HEPES buffer (pH 7.2). The mitochondrial suspension was washed twice in the same medium containing 0.1 mM EGTA, and the final pellet was diluted in 250 mM sucrose to a protein concentration of 80−100 mg/mL.

Preparation of Mitoplasts. Mitoplasts (mitochondria devoid of the outer membrane) were prepared exactly as described by Pedersen et al. (*23*, *24*). For respiratory assays, mitochondria (2.0 mg of protein) were incubated at 30 °C with 5 mM succinate and 2.5 μM rotenone in a standard incubation medium containing 125 mM sucrose, 65 mM KCl, 10 mM HEPES-KOH, 0.5 mM EGTA, and 10 mM K_2HPO_4 over a pH range of 6.0−7.5 (final volume of 1.6 mL).

Oxygen Consumption Rate Assay. The oxygen consumption rate was determined using an oxygraph equipped with a Clark-type electrode (Gilson Medical Eletronics) and thermostatic cell. The reactions were performed in a glass cuvette containing cyt *c* (0.01−0.5 nmol/mg of protein). The oxygen consumption rate was directly measured in an oxygraph per unit of time. The kinetic parameters for the interaction of cyt *c* with mitoplasts were determined using a nonlinear regression software system (Grafit version 3.0, Erithacus Software Ltd.). The data of the respiration rate in the function of cyt *c* concentration were analyzed on the basis of eq 1 that predicts the two forms of interaction of cyt *c* with mitoplasts (*25*) as follows:

$$v = \frac{V_{\max 1}[\text{cyt } c]}{K_{\text{sapp1}} + [\text{cyt } c]} + \frac{V_{\max 2}[\text{cyt } c]}{K_{\text{sapp2}} + [\text{cyt } c]} \quad (1)$$

where V is the oxygen consumption rate, [cyt *c*] is the cyt *c* concentration, $V_{\max 1}$ is the maximum oxygen consumption rate in a pH-dependent manner, $V_{\max 2}$ is the maximum oxygen consumption rate in a pH-independent manner, K_{sapp1} is the microscopic dissociation constant of cyt *c* by mitoplasts in a manner dependent on pH, and K_{sapp2} is the microscopic dissociation constant of cyt *c* by mitoplasts in a manner independent of pH.

The data from the influence of pH upon $1/K_{\text{sapp}}$ were analyzed on the basis of eq 2 (*26*) as follows:

$$\frac{1}{K_{\text{sapp}}} = \frac{\lim_1 + \lim_2 \times 10^{\text{pH} - \text{p}K_a}}{10^{\text{pH} - \text{p}K_a} + 1} \quad (2)$$

Equation 2 fits data when the pH−activity profile depends upon one ionizable group and does not assume that the activity is zero at high pH values. Lim_1 represents the limit of $1/K_{\text{sapp}}$ at low pH values, while lim_2 represents the limit of $1/K_{\text{sapp1}}$ at higher pH

values. K_{sapp1} is the microscopic dissociation constant for dissociation of cyt *c* by mitoplasts in a pH-dependent manner.

ATP Dosage. All samples were quenched with 334 μL of ice-cold 2 N perchloric acid for a final concentration of 0.5 N. Samples were spun at 2000*g* for 10 min at 4 °C. One hundred microliters of supernatants was removed and neutralized with 70 μL of 2 M KOH and 830 μL of 100 mM Tris-HCl and 2 mM EDTA (pH 7.75). Precipitates were spun down, and 100 μL of supernatant from each sample was used for ATP measurements using a luciferin-based ATP Monitoring Reagent kit (BioOrbit). The assay was conducted using a Berthold (Bad Wilbad, Germany) tube luminometer (EG&G Berthold AutoLumat LB 953) according to the manufacturer's instructions using ATP standards at final concentrations between 1 nM and 1 μM.

Preparation and Analysis of PyPC-Containing Liposomes. Lipids were first dissolved in chloroform, which was evaporated with N_2 gas. The lipid residue was kept under reduced pressure for at least 2 h, after which it was hydrated via addition of cold 10 mM HEPES buffer and 0.1 mM EDTA. Unilamellar liposomes were also obtained by extrusion of hydrated lipid dispersions in an Avanti Mini-extruder acquired from Avanti Polar Lipids, Inc. Samples were subjected to 11 passes through two polycarbonate filters (100 nm pore size, Nucleopore, Pleasanton, CA) installed in tandem. For fluorescence measurements, small unilamellar POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine)/DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine)/CL (heart cardiolipin) (49/30/20 molar ratio) vesicles containing PyPC 1% mol were used, and minimal exposure of the lipids to light was ensured during the procedure. Fluorescence emission spectra were recorded at 30 °C using a F-2500 Hitachi fluorescence spectrometer using excitation at 344 nm and emission at 398 nm with excitation and emission slits of 10 and 5 nm, respectively.

Quantitative Detection of Release of cyt c via an Enzyme-Linked Immunoadsorbed Assay (*ELISA*). The amount of cyt *c* released from mitoplasts incubated at different pH values was determined by an enzyme immunoassay technique using an Immunoassay Kit (Biosource International, Inc.). Succinate-energized mitoplast aliquots (0.4 mg of protein/mL) were incubated at pH 6.4, 6.8, 7.2, 7.4, 7.8, and 8.0 for 10 min at 30 °C and then centrifuged at 16000*g* for 10 min at 4 °C. The supernatant (50 μL) was added to wells and incubated with a biotin-conjugated monoclonal anti-cytochrome *c* for 2 h at 25 °C. After five washes, a streptavidin/HRP solution was added, and the plate was incubated for 30 min. The reaction was stopped by the addition of acid, and the optical density was determined at 450 nm using a BioTek ELX800 microplate reader (BioTek Instruments, Inc.). Sample concentrations were determined on the basis of a standard curve within a concentration range of 0.08−2.5 ng/mL ($\varepsilon = 0.595$ ng^{-1} mL).

Site-Directed Mutagenesis, Expression, and Purification of Recombinant Mutated cyt c. The recombinant mutated cyt *c* was obtained using the plasmid pJRhrsN constructed by Rumbley, Hoand, and Englander (*27*). These pUC-derived plasmids encode horse heart cyt *c* carrying the two substitutions H26N and H33N, and yeast heme lyase confers ampicillin resistance by the β-lactamase gene. The pJRhrsN2-derived plasmid was transformed into *Escherichia coli* strain BL21 Star (DE3) (Invitrogen) for protein expression. Recombinant mutated proteins were expressed and purified according to ref *27* with minor modifications (*28*). Twenty-five milliliters of starter culture grown in LB medium and 100 μg/mL ampicillin were inoculated in 1 L of Terrific broth with the antibiotic. The culture was grown with vigorous shaking (220 rpm) until the A_{550} reached 0.8. Maximal expression was obtained after incubation for 72 h under this condition. Expression of cyt *c* mutants was induced with 0.8 mM IPTG for 50 h at 303 K. The protein was purified using a CM-Sepharose fast-flow column (Amersham Pharmacia Biotechnology). Fractions with a 410 nm/280 nm absorbance ratio of > 4.0 were considered pure as supported by SDS−PAGE.

RESULTS

Effect of pH on the Respiration of cyt c-Depleted Mitoplasts Supplemented with Exogenous cyt c. The binding of cyt *c* to membranes of rat liver MP was accompanied by the onset of the organelle respiration over the pH range of 6.2−7.4 and the functionality of MP at acidic pH values by the capacity of ATP production after ADP loading. The assignment of site L protonation as the limiting step for cyt *c* binding to the mitoplast membranes was probed by comparing the affinity of horse heart cyt *c* (site L composition, Lys22, Lys25, His26, Lys27, and His33) to that of mitoplast membranes and tuna cyt *c* (site L composition, Asn22, Lys25, His26, Lys27, and Trp33) and by chemical blockage of this site by DEPC (diethyl pyrocarbonate).

The addition of horse cyt *c* to mitoplasts at concentrations of > 0.04 nmol/mg of protein restored the organelle respiration in a pH-dependent manner (Figure 1A, filled symbols). For tuna cyt *c*, with Lys22 and His33 replaced with Asn and Trp, respectively, concentrations above 0.075 nmol/mg of protein also restored the respiration in a pH-dependent manner (Figure 1A, empty symbols). However, for horse cyt *c*, the pH decrease from 7.2 to 6.2 led to a 5.3-fold K_{sapp1} decrease (from 109 ± 14 to 20.5 ± 2 nM), while for tuna cyt *c*, the K_{sapp1} decreased 3.9-fold (from 353 ± 14 to 90 ± 3 nM) in response to the pH decrease. Thus, at pH 7.2, a value at which site L amino acids should be deprotonated, horse cyt *c* exhibited an affinity for mitoplast membranes close to that of tuna cyt *c* at pH 6.2. Figure 1B shows the double-reciprocal plot of the cyt *c*-supplied mitoplast (MPc) respiration rate as a function of the concentration of horse (filled symbols) and tuna (empty symbols) cyt *c* added to the medium at pH 7.2 and 6.2 (squares and circles, respectively). In cyt *c*-saturated mitoplasts, the respiration rate (V_{max1}) extracted from the intercept at the ordinate axis was influenced by the pH of the medium that is responsible for the magnitude of $\Delta\Psi$ and not by the cyt *c* type. For horse and tuna cyt *c*, two binding types were observed: a very high-affinity ($K_{sapp2} \sim 10$ nM) and low-efficiency pH-independent binding and a pH-dependent binding. The pH-independent binding probably involves some type of hydrophobic protein−membrane interaction, and this is in agreement with literature data reporting that residual cyt *c* remained bound to the organelle membrane at high ionic strengths (*12*, *15*, *24*). The pH-independent cyt *c* binding to MP contributed to organelle respiration since AA (antimycin A) completely inhibited oxygen consumption under this condition (not shown). At pH 7.2, the uncoupling of MP by 1.0 μM CCCP (carbonyl cyanide 3-chlorophenylhydrazone) (Figure 1C,D) did not significantly affect the affinity of cyt *c* for the inner mitochondrial membranes ($K_{sapp1} = 116 \pm 7.8$), but as expected, it increased the maximal respiration rate from 256 to 408 nmol of O/min. At pH 6.2, the same CCCP concentration did not increase the respiration rate, probably because at the acidic external pH this amount of uncoupler was not enough to decrease the internal pH to a value

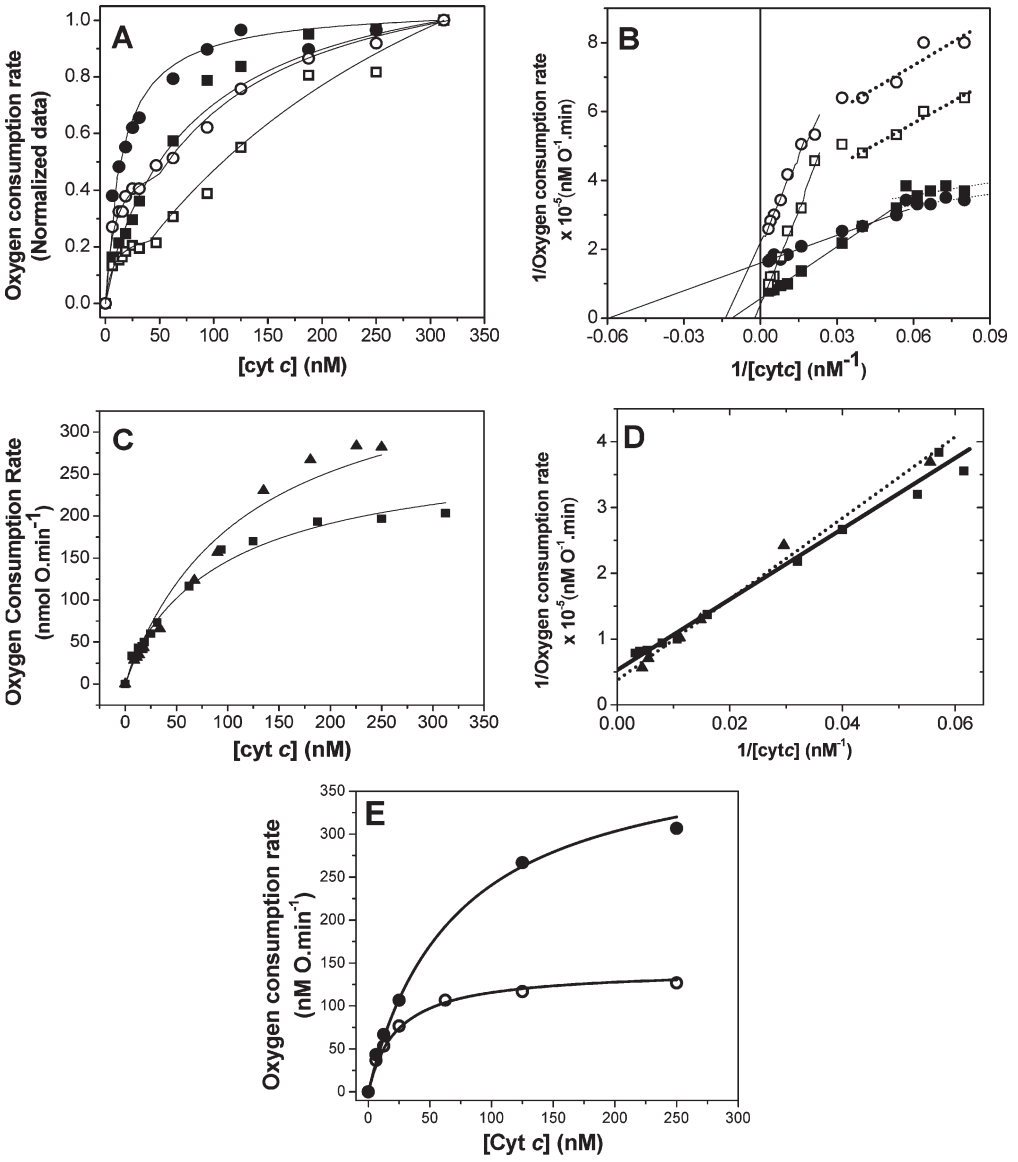


FIGURE 1: Effect of the concentration of cyt *c* added to the medium on the state 2 respiration rate of cyt *c*-depleted mitoplasts. (A) Normalized respiration rate of cyt *c*-depleted mitoplasts as a function of cyt *c* concentration supplied for mitoplasts. (B) Corresponding double-reciprocal plot of the cyt *c*-depleted mitoplast respiration rate as a function of cyt *c* concentration supplied for mitoplasts. Different concentrations of horse (filled symbols) and tuna (empty symbols) cyt *c* were added to the medium at pH 7.2 and 6.2 (squares and circles, respectively). (C) Respiration rate of cyt *c*-depleted mitoplasts as a function of cyt *c* concentration supplied for mitoplasts at pH 7.2 in the absence (filled squares) and presence of CCCP (filled triangles). (D) Corresponding double-reciprocal plots. (E) Respiration rate of cyt *c*-depleted mitoplasts as a function of recombinant mutated horse cyt *c* (H26N and H33N) concentration supplied for mitoplasts at pH 7.2 (filled circles) and pH 6.2 (empty circles). The experiments were conducted by using an organelle suspension containing 2 mg of protein at 30 °C at the indicated pH in the presence of 5 mM succinate.

able to disrupt the $\Delta\Psi$. Higher CCCP concentrations could not be used because they caused respiration inhibition. Recombinant mutated cyt *c* H26N and H33N also restored the respiration of cyt *c*-depleted mitoplasts in a pH-dependent manner; concentrations of >0.04 nmol/mg of protein (Figure 1E) and pH 6.2 exhibited slightly lower affinity for mitoplasts in comparison with the native form ($K_{sappl} = 23.3 \pm 1.7$ nM). However, the substitution of His26 and -33 with asparagine residues increased the affinity of cyt *c* for the inner mitochondrial membrane at pH 7.2 ($K_{sappl} = 70.35 \pm 3.6$ nM).

The affinity of native horse cyt *c* for the inner mitochondrial membrane (K_{sappl}) was also determined at five pH values [6.4, 6.8, 7.0, 7.4, and 7.5 (not shown)], and the $1/K_{sappl}$ values were plotted as a function of pH (Figure 2). The fitting of the data to eq 2 revealed that the cyt *c*-established respiration pK_{aobs} was 6.79 ± 0.028, a value identical to that observed by probing the binding to PC (phosphatidylcholine)/PE (phosphatidylethanolamine)/CL (cardiolipin) liposomes obtained for cyt *c* modified by DEPC at different pH values (*19*). Carbethoxylation by DEPC blocks selectively ionizable amino acid lateral chains of cyt *c* whose pK_a values are lower than the pH of the medium (*19*). A carbethoxylated cyt *c* sample previously characterized by MALDI-TOF mass spectrometry analysis as owning chemically blocked site L (*19*) was inefficient in restoring cyt *c*-depleted mitoplast activity (not shown).

Effect of pH on cyt c Binding to an Inner Mitochondrial Membrane Model. The effect of pH on the affinity of cyt *c* for the inner mitochondrial membranes was directly accessed by the quenching of 2-[10-(1-pyrene)decanoyl]phosphatidylcholine present in PCPE/CL vesicles, which were mixed with unlabeled vesicles, and by the detachment of cyt *c* from non-cyt *c*-depleted mitoplasts incubated at different pH values. Figure 3 shows the

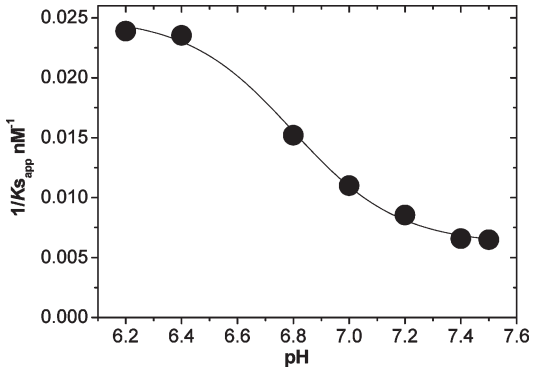


FIGURE 2: Effect of pH on the affinity of cyt *c* for inner mitochondrial membranes. Filled circles correspond to the affinity of native cytochrome for the inner mitochondrial membrane ($1/K_{sapp1}$) at different pH values obtained from double-reciprocal plots of respiration rates as a function of the concentration of cyt *c* added to the medium. The data were fitted by using eq 2 (—). The experiments were conducted using an organelle suspension containing 2 mg of protein/mL at 30 °C and pH 6.2 in the presence of 5 mM succinate.

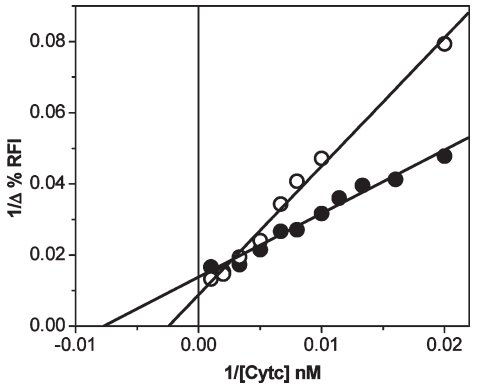


FIGURE 3: Effect of pH on the affinity of cyt *c* for the inner mitochondrial membrane model (PC/PE/CL liposomes) using a PyPC probe. Double-reciprocal plot of POPC/PyPC/DOPE/CL (49/1/30/20 molar ratio) liposome fluorescence as a function of native horse cyt *c* concentration at pH 7.4 (empty circles) and pH 6.2 (filled circles). The experiments were conducted using 25 μM liposomes in 10 mM HEPES buffer and 0.1 mM EDTA at 30 °C with excitation at 344 nm and emission at 398 nm.

double-reciprocal plot of RFI (relative fluorescence intensity) as a function of the concentration of horse cyt *c* at pH 6.2 (filled symbol) and 7.4 (empty symbol). Accordingly, the efficiency of the quenching of pyrene fluorescence due to cyt *c* bound to membranes was augmented at acidic pH, and the K_{sapp1} values were determined to be 408.6 and 129.1 nM at pH 7.4 and 6.2, respectively.

Effect of pH on the Detachment of Endogenous cyt c from Mitoplasts. Figure 4 shows the effect of pH on the detachment of cyt *c* from mitoplasts that were not depleted of cyt *c*. Coinciding with previously published turbidymetry data (*19*) and with the respiration data presented here, the detachment of cyt *c* from the inner mitochondrial membrane as a function of pH exhibits a sigmoid curve with a pK_{aapp} of ~7.0.

Figure 5A shows the effect of pH on the state 2 respiration rate of MPc. The decrease in the state 2 respiration rate exhibited by MPc with the decrease in pH could be explained by the larger ΔpH produced between mitochondrial matrix and external medium leading to a mimic of mitoplast coupling. Furthermore, the mitochondrial respiration rate is expected to decrease with the increase in the affinity of cyt *c* for the inner mitochondrial

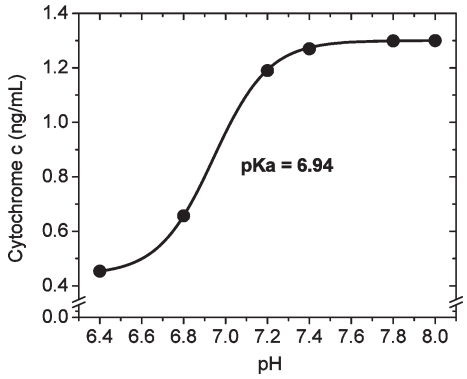


FIGURE 4: Effect of pH on the detachment of cyt *c* from nondepleted mitoplasts. The amount of cyt *c* released from mitoplasts incubated at different pH values was determined by an enzyme immunoassay technique using an Immunoassay Kit (Biosource International, Inc.) as described in Materials and Methods. The concentration of cyt *c* released was determined by running the samples at 450 nm using a BioTek ELX800 microplate reader. Sample concentrations were determined on the basis of a standard curve within a concentration range of 0.08−2.5 ng/mL (ε = 0.595 ng^{-1} mL).

membrane. Due to the absence of an external membrane, proton pumping from the matrix to the external side should promote a significant increase in the matrix pH but not a significant decrease in the pH at the external side, a very large environment in comparison with the virtual intermembrane space. This phenomenon signaled the uncoupling of the respiratory chain at higher pH values leading to the increase in the respiration rate. Furthermore, the decrease in the affinity of cyt *c* for the inner mitochondrial membrane is expected to increase the electron transport and respiration rate. The dosage of ATP (relative luminescence units) produced by MPc saturated with cyt *c* and assayed at different pH values (Figure 5B) is according to the ΔpH increase the cause of the MPc respiration rate decrease in response to the increase in pH. The ATP production of MPc was dependent on the respiratory chain electron transport since it was completely impaired by AA, an inhibitor of respiratory chain site II (Figure 5B). Thus, at pH > 7.0, the decrease in the rate of ATP production was not related to cyt *c* binding but due to the incapacity of MPc to generate the required ΔΨ. However, at pH < 7.0, the impairment of cyt *c* binding by DEPC carbethoxylation hampered ATP production (Figure 5B) since for MPc, the respiratory chain activity was fundamental to alkalinization of the matrix that, otherwise, should be acidified at the first phosphorylation round leading to ΔΨ disruption.

DISCUSSION

The first evidence pointing out the existence of site L in cyt *c* was obtained by probing the capacity of cyt *c* to fuse liposomes with a composition that mimicked the mitochondrial lipid fraction (*19*). To promote vesicle fusion, the protein should exhibit two spatially opposite sites owned by positively charged amino acids. In fact, although cyt *c* was able to bind in PC/PE/CL and PC/PE/PG liposomes over a wide pH range, the capacity to fuse the mentioned vesicles was observed only at the acidic pH range with the turning point around 7.0 (*19*). In our study, the role played by site L in the affinity of cyt *c* by the inner mitochondrial membrane was corroborated in a biological model: rat liver mitoplasts.

Site L Lysine Residues Are Responsible for the pH-Dependent Binding of cyt c to the Inner Mitochondrial

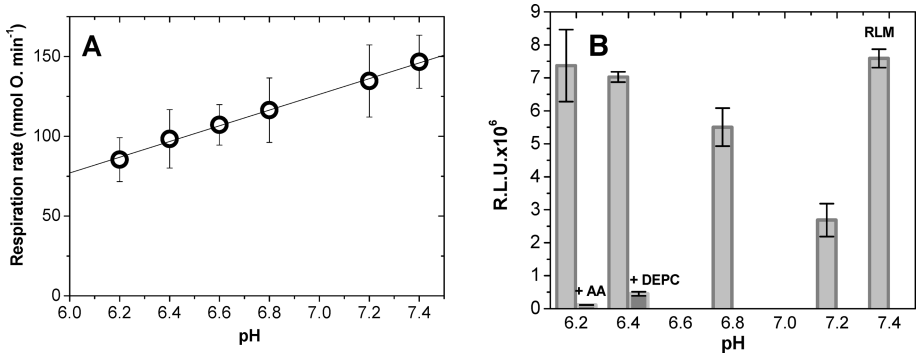


FIGURE 5: Effect of pH on the mitochondria and mitoplast function under different conditions. (A) Maximal respiration rate (nanomoles of O per milligram per minute) of mitoplasts supplied with horse cyt *c* (MPc) at different pH values, the fit of maximal respiration rate data using eq 2. (B) The bars represent the corresponding ATP production of MPc at different pH values in the absence and presence of antimycin A (where indicated) of cyt *c*-depleted mitoplasts loaded with DEPC-carbethoxylated cyt *c* at pH 6.5 and of coupled rat liver mitochondria at pH 7.4. The experiments were conducted using an organelle suspension containing 1 mg of protein/mL at 30 °C at the indicated pH in the presence of 5 mM succinate and 270 μM ADP. The antimycin A (AA) concentration was 1.0 μM, when present. The concentration of cyt *c* added to the medium was 0.4 nmol/mg of protein. ATP production was quantified by using a luciferin-based ATP Monitoring Reagent kit (BioOrbit) and was proportional to the relative luminescence units (RLU) of the system.

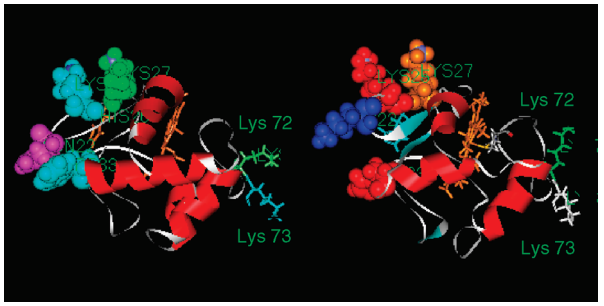


FIGURE 6: Tridimensional cytochrome structures. These structures were provided by crystallographic data (*29*, *30*) showing amino acid residues present at the L site. The horse cyt *c* structure is shown in the right panel, and the tuna cyt *c* structure is shown in the left panel.

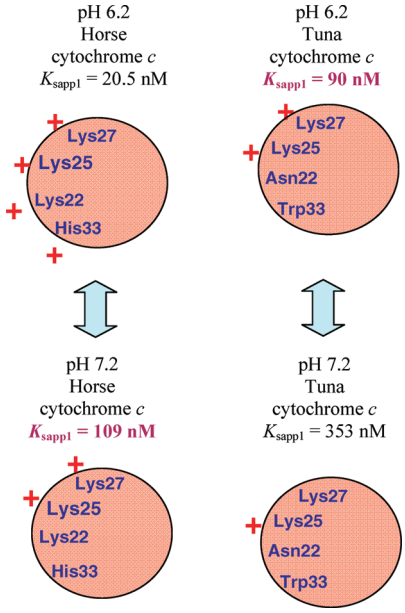
Membranes. The results obtained by comparing horse and tuna cyt *c* suggest that in horse cyt *c*, Lys22 and His33 lateral chains are the ionizable groups involved in cyt *c* binding to the inner mitochondrial membrane. Considering that even in tuna cyt *c*, the affinity for mitoplast membranes decreased 3.9-fold at pH 7.2, it is probable that at least one basic amino acid residue present at the L site of this protein has a quite lower pK_a value compared with that of horse cyt *c* and significantly contributes to the binding to the inner mitochondrial membrane. These proposals are summarized in Scheme 1. It is important to note that tuna cyt *c* was used in this study as a "natural site L mutant" with the advantage of having, according to crystallographic data (*29*, *30*), a tridimensional structure very similar to that exhibited by horse cyt *c* (Figure 6). The alignment of BLAST sequences of horse and tuna cyt *c* reveals that from the nine differences involving charged amino acid residues, five correspond to the replacement of negatively charged residues of glutamate in horse cyt *c* with uncharged or apolar amino acid residues. These differences certainly should not be the cause of the lower affinity for the inner mitochondrial membrane. Four differences correspond to basic positively charged amino acids: histidine and lysine. Histidine 33 belongs to site L as well as lysine 22. Therefore, it is necessary to consider lysine 60 and lysine 100. None of these lysine residues was modified by the treatment with DEPC at low pH values, suggesting that they do not exhibit low pK_a values. In fact, lysine 60 is followed in the sequence of horse cyt *c* by two glutamate residues, and therefore, it is expected that this residue exhibits a high pK_a value. Lysine 100 is the neighbor of a lysine residue at position 99; however, in the cyt *c* structure, contrary to what is observed for the lysine residues of site L, these lysine residues are not convergent. Thus, the use of tuna cyt *c* to probe the participation of site L is a very elegant and confident probe as compared to the use of cyt *c* obtained by site-directed mutagenesis. Although the lowering of pK_a values of clustered lysine residues at site L is expected, the exclusion of the histidine residues as the exclusive ionizable groups responding for the pK_a of respiration restoration as determined by exogenous cyt *c* was corroborated by site-directed mutagenesis. Recombinant mutated horse cyt *c* H26N and H33N also restored the respiration of cyt *c*-depleted mitoplasts in a pH-dependent manner and further exhibited at pH 6.2 a K_{sapp1} value close to that exhibited by wild-type horse cyt *c*. In addition, the substitution of His26 and -33 with Asn residues increased the affinity for the inner mitochondrial membranes at pH 7.2, probably due to the capacity of Asn to establish hydrogen bonds with phospholipids and/or van der Waals interactions with cyt *c* reductase (see below).

It is important to note that the far-UV CD spectra of the carbethoxylated and mutated cyt *c* revealed no significant changes in the α-helix content, and the near-UV CD did not indicate any significant change in the tertiary structure (not shown). In addition, the electronic absorption spectra of carbethoxylated and mutated cyt *c* revealed the presence of the charge transfer band at 695 nm, indicating that methionine 80 remained as the heme iron sixth ligand (*31*).

Considering the results obtained with mutant cyt *c*, it is not surprising that tuna cyt *c* exhibits pH-dependent binding qualitatively comparable with that of wild-type and mutant cyt *c* since three ionizable site L amino acids are preserved. In this regard, lysine 27 is an invariable residue, while lysine 25 and histidine 26 are highly conserved ones.

Cytochrome c Site L Could Interact with Phospholipids and Cytochrome c Reductase. With regard to the fraction of inner mitochondrial membrane that interacts with site L, results from previous studies identify the cyt *c* site A as the site interacting with cyt *c* oxidase and reductase (*17*, *18*). However, recently, the structure of isoform 1 cyt *c* bound to the cytochrome bc_1 complex at 1.9 Å resolution in a reduced state was reported (*32*). In this study, by comparison with the novel structure

Scheme 1: Microscopic Dissociation Constants (K_{sapp1}) of Horse and Tuna cyt *c* Dictated by the pH-Dependent Positive Charges of Site L



of the complex with bound isoform 2 cyt *c*, the authors identified a minimal core within the hydrophobic interface. Four core interactions encircle the heme groups surrounded by variable interactions. The crystal structure shows that heme c_1 and heme *c* are in close contact at the center of the interface and exhibit a 4.1 Å distance between two carbon atoms in the respective thioether-bonded substituents of the tetrapyrrol rings. The minimal core is composed of four contacts: the Phe230/Arg19 pair that contributes a strong π−cation interaction, the Ala103/Ala87 pair that participates in hydrophobic interactions, and the Phe230/Thr18 and Ala168/Val34 pairs that participate in van der Waals contacts. In iso-1-cytochrome *c* and iso-2-cytochrome *c*, the corresponding position 22 of site L is occupied by Gln and Thr, respectively. Interestingly, the Gln170/Gln22 pair forms van der Waals contacts within the variable interactions of the cytochrome bc_1−iso-1-cytochrome *c* complex, and the Phe230/Thr22 pair forms van der Waals interactions within the core interface of the cytochrome bc_1−iso-2-cytochrome *c* complex (*32*). Furthermore, another site L amino acid, residue 33, appears to participate in the interaction between cyt *c* and the bc_1 complex. The crystal structure of the iso-1-cytochrome *c* bound to the cytochrome bc_1 complex shows that the Gln170/Lys33 pair of the cyt c_1−cyt *c* complex forms van der Waals contacts within the variable interactions. Considering that in yeast cyt *c* isoforms position 22 of site L is not occupied by an ionizable group, for this residue, the interaction with the reductase in the yeast complex is not electrostatic. However, the structure of the yeast isoforms of cyt *c* differs significantly from that of superior eukaryotes. Differing from yeast cyt *c*, horse cyt *c* does not exhibit amino acid residues 22, 33, and 34 on the same face as amino acid residue 87. Further, different amino acids occupy the cited positions in the yeast and superior eukaryote cyt *c* (not shown). It is important to note that the binding of cyt *c* to cardiolipin occurs in the presence and absence of the reductase and oxidase and involves an unequivocally electrostatic interaction. The pH dependence observed for both the interaction of cyt *c* with liposomes and the inner mitochondrial membranes suggests that the interaction with lipids is significant in this phenomenon. A good estimative of the role played by respiration complexes can be realized by the comparison of the pH-dependent K_{sapp} values (affinity) obtained for mitoplasts (109 nM at pH 7.4 and 20 nM at pH 6.2) and liposomes (353 nM at pH 7.4 and 90 nM at pH 6.2). The significantly highest affinity of cyt *c* for the inner mitochondrial membrane attests to the fact that the respiratory complexes contribute to cyt *c* binding. Thus, besides the lipid fraction being the probable site L-interacting fraction of the inner mitochondrial membrane, the interactions of site L (electrostatic or not) with cyt *c* reductase and oxidase cannot be discarded, and only a crystal structure of superior eukaryote complexes could definitively elucidate this question.

Cytochrome c Site L Could Play an Important Role in the Apoptosis Promoted by Oxidative Stress and Alkalosis. With regard to the role of a pH-dependent positively charged site in the cyt *c* structure, the occurrence of apoptosis in response to a lack of $\Delta\Psi$ promoted by conditions of oxidative stress, such as those expected to occur during Ca^{2+}-induced mitochondrial permeability transition and photodynamic therapy and other factors that promote the deregulation of intracellular pH (*1*−*4*, *33*−*41*), suggest that the $\Delta\Psi$ disruption and cytosol alkalinization should contribute to the detachment of cyt *c* from the inner mitochondrial membrane for participation in caspase activation in the cytosol.

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