J-Aggregate Formation of a Carbocyanine as a Quantitative Fluorescent Indicator of Membrane Potential†

Martin Reers,*,‡ Thomas W. Smith,‡ and Lan Bo Chen§

Cardiovascular Division, Brigham and Women's Hospital, and Dana Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115

Received August 27, 1990; Revised Manuscript Received January 18, 1991

ABSTRACT: The spectral properties of a novel membrane potential sensitive probe (JC-1) were characterized in aqueous buffers and in isolated cardiac mitochondria. JC-1 is a carbocyanine with a delocalized positive charge. It formed under favorable conditions a concentration-dependent fluorescent nematic phase consisting of J-aggregates. When excited at 490 nm, the monomers exhibited an emission maximum at 527 nm and J-aggregates at 590 nm. Increasing concentrations of JC-1 above a certain concentration caused a linear rise in the J-aggregate fluorescence, while the monomer fluorescence remained constant. The membrane potential of energized mitochondria (negative inside) promoted a directional uptake of JC-1 into the matrix, also with subsequent formation of J-aggregates. The J-aggregate fluorescence was sensitive to transient membrane potential changes induced by ADP and to metabolic inhibitors of oxidative phosphorylation. The J-aggregate fluorescence was found to be pH independent within the physiological pH range of 7.15–8.0 and could be linearly calibrated with valinomycin-induced K^+ diffusion potentials. The advantage of JC-1 over rhodamines and other carbocyanines is that its color altered reversibly from green to red with increasing membrane potentials. This can be exploited for imaging live mitochondria on the stage of a microscope.

In the last two decades, many indicators for monitoring membrane potential have been described with increasing emphasis toward fluorescent probes. In photography, many of these compounds are used as photosensitizers covering a wide spectral range. The most common membrane potential sensitive compounds are rhodamines and carbocyanines (James, 1977). The successful use of these membrane potential probes in living cells is to a great extent based on their nondestructive detection and the lack of immediate toxicity. Recent reviews by Waggoner (1988), Freedman and Novak (1988), Loew (1982), and Chen (1988) summarize in detail the mechanisms by which membrane potentiometric probes behave in cells, organelles, and vesicles.

Fluorescent probes can be categorized into two groups: (1) fast dyes with a response time to a change in the membrane potential of less than milliseconds and (2) slow dyes with a response time of seconds. Fast dyes were designed to be immobilized within the electric field of a cell membrane and to respond to a change in membrane potential with a change in intensity (hypo- or hyperchromic) (Freedman & Novak, 1983) or with a spectral shift (electrochromic) (Gross et al., 1986). Electrochromic dyes bear the potential to apply the desirable ratio technique to monitor membrane potential on the stage of a microscope independent of the dye concentration (Montana et al., 1989). However, electrochromic dyes have been hampered by their small dynamic range when subjected to a membrane potential change (0.1% ΔF/mV).

Slow dyes, in contrast, have found a wide field of application. Thus, carbocyanines and rhodamines have been systematically screened and synthesized. These molecules redistribute between compartments according to the membrane potential across the separating membranes. The transmembrane electric field (negative inside) and the chemical gradient of the dye are ideally the only forces that determine the equilibrium of the dye distribution. However, nonspecific partitioning into the hydrophobic bilayer leads to a deviation from strictly Nernstian behavior, complicating accurate measurements. In addition, many of the slow dyes at high concentrations exhibit a decline in fluorescence. This has been explained by the formation of dimers or multimers with a lower quantum yield, often referred to as a quenching effect. The quenching of the fluorescence signal of rhodamine 123 has been linearly correlated to membrane potential changes (Emaus et al., 1986). In other cases, mathematical equations have been developed for individual cationic dyes to predict the nonlinear quenching as a function of dye concentration in order to quantify mitochondrial membrane potential (Tomov, 1986; Bunting et al., 1989).

A special type of multimer of carbocyanines that was first identified by Jelley (1937) and hence called J-aggregates was noticed by Sims and co-workers (Sims et al., 1974) for 3,3′-dipentylthiodicarbocyanine [DiS-C_3-(5)][1] in aqueous solution. They observed a broad emission peak above 700 nm at high dye concentrations. The change in magnitude of this peak was looked upon as a complicating factor for the interpretation of the quenched signals of hyper- and depolarization of red blood cell membranes. Recently, the J-aggregate formation has been exploited to visualize mitochondria in a variety of cells (Chen, 1989). The most suitable dye thus far identified has been 5,5′,6,6′-tetrachloro-1,1,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (denoted JC-1). It displayed a fairly narrow red peak that was sensitive to a variety of mitochondrial membrane potential modulating agents.

The purpose of this investigation was to characterize JC-1 and its J-aggregates and correlate its fluorescence with mem-

†Supported by NIH Grants HL19259 (M.R. and T.W.S.) and GM38318 (L.B.C.).

* Address correspondence to this author at Behringwerke AG, P.O. Box 1140, W-3550 Marburg, Germany.

‡ Brigham and Women's Hospital and Harvard Medical School.

§ Dana Farber Cancer Institute and Harvard Medical School.

[1] Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CJC, critical J-aggregate concentration; CMC, critical micellar concentration; DiS-C_3-(5), 3,3′-dipentylthiodicarbocyanine, Mops, 4-morpholinepropanesulfonic acid; RFI, relative fluorescence intensity.

0006-2960/91/0430-4480$02.50/0

brane potentials in isolated cardiac mitochondria. We found that the formation of J-aggregates of JC-1 faithfully reported membrane potential changes in isolated cardiac mitochondria that could be calibrated with a K^+ diffusion potential.

Experimental Procedures

Mitochondrial Preparation. Heart mitochondria were prepared from Sprague-Dawley rats weighing approximately 225 g. All animals had been provided with chow and water ad libitum before they were decapitated. A subpopulation of mitochondria, the interfibrillar mitochondria (Palmer et al., 1977), was isolated as described previously (Reers et al., 1989). The final mitochondrial pellet was suspended in buffer I consisting of 210 mM sucrose, 70 mM mannitol, and 3 mM K-Mops, pH 7.2, at a concentration of approximately 60 mg/mL, as determined by the biuret assay (Gornall et al., 1949) in the presence of 1% (w/v) deoxycholate.

Determination of the Respiration Control Ratio. Oxygen consumption was recorded with a Clark electrode in a 1.8-mL thermostated glass vessel. Mitochondria (0.25 mg/mL) were incubated in buffer consisting of 110 mM KCl, 20 mM Mops, 10 mM glutamate, 1 mM malate, and 1 mM EGTA, pH 7.0 at 25 °C. State 3 was induced by addition of 0.15 mM ADP. Only mitochondrial preparations that demonstrated a respiration control ratio (RCR) above 10 were used.

Fluorescence and Absorbance Measurements. All measurements were performed with a SPEX Fluorolog-2 instrument (SPEX, Edison, NJ) equipped with a magnetic stirrer and a thermostated cuvette mount. The cuvette content was continuously stirred with a glass-coated stir bar. The temperature was held at 37 °C. Emission scans were recorded over the range of 490–620 nm with a bandwidth of 7.2 nm. The excitation wavelength was 490 ± 1.8 nm. Synchronous scans were recorded with both monochromators scanning with a difference of 15 nm. The wavelengths of the emission monochromator were plotted on the abscissa. The slit widths for emission and excitation were the same as with the emission scans. The emission light intensity was corrected for the lamp spectrum with a rhodamine standard and then expressed as the relative fluorescence intensity (RFI). Fluorescence in experiments without mitochondria was recorded in the rectangle mode and with mitochondria in the front-face mode.

The extinction coefficient of JC-1 was determined in DMSO with a Hewlett-Packard 8150A diode array spectrophotometer and was found to be 128 700 M^{-1} cm^{-1} at 510 nm.

Estimation of pK_a and CJC[1] for JC-1. The pK_a for JC-1 was estimated in buffer II consisting of 110 mM KCl, 10 mM MgATP, 1 mM K-EGTA, 20 mM K-Mops,[1] 10 mM succinate, 1 μM rotenone, and sufficient sucrose/mannitol (3:1) to achieve 300 mOsm. A JC-1 solution (46 nM) at 37 °C in the 3-mL quartz cuvette was titrated with 6 M HCl and 5 M KOH to cover a range from pH 5 to 9. For each pH value, a synchronous scan and an emission scan were acquired. The RFI of the maxima (bandwidth of 7 nm) were plotted versus pH and used for the estimation of the pK_a.

The critical J-aggregate concentration (CJC) for JC-1 was determined in 3 mL of buffer II at 37 °C at varying pHs and increasing concentrations of JC-1. The solvent DMSO was kept at a constant concentration of 0.16% (v/v). After 10-min incubation, synchronous and emission scans were obtained. Similarly, an apparent CJC in mitochondria (CJC_{mito}) was determined, defined as the added concentration of JC-1 to a suspension of energized mitochondria that formed J-aggregates. Mitochondria were continuously stirred with a magnetic glass stirrer in an open quartz cuvette, providing a constant supply of oxygen.

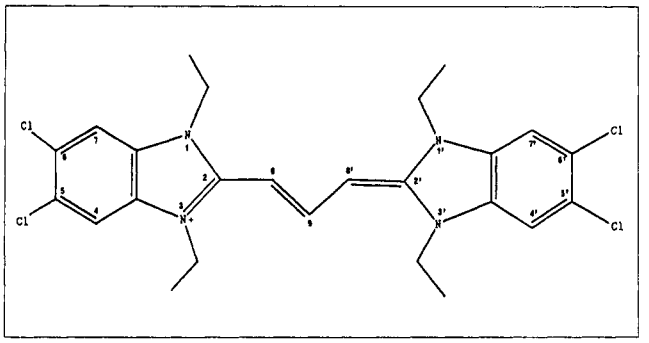

FIGURE 1: Chemical structure of JC-1. The chemical name is 5,5′,6,6′-tetrachloro-1,1,3,3′-tetraethylbenzimidazolylcarbocyanine iodide. The abbreviated name JC-1 stands for the first J-aggregate-forming cationic dye that has been found to be membrane potential sensitive.

Calibration of JC-1 in Cardiac Mitochondria. Mitochondria (0.25 mg/mL) were suspended in buffer III consisting of 200 mM sucrose, 20 mM mannitol, 1 mM NaEDTA, and 20 mM NaMops, pH 6.5, 7.15, or 8.0 at 37 °C. Subsequent additions of aliquots of a 1 M KCl solution, $[K]_{out}$, in the presence of 20 nM valinomycin created a potassium diffusion potential. The matrix potassium concentration, $[K]_{in}$, was taken as 120 mM (Rossi & Azzone, 1969). The membrane potential ($\Delta\psi$ in millivolts) was calculated by using the Nernst equation: $\Delta\psi = -60 \log ([K]_{in}/[K]_{out})$.

Results

Fluorescence Spectra of JC-1. JC-1 (Figure 1) exists in aqueous solution as a monomer with an excitation and emission peak in the green range (green peak). The Stokes shift, which is the difference between the excitation and emission maxima, was found to be 10 nm (Smiley et al., 1991). Under favorable conditions (see below), an additional peak (red peak) appeared, 63-nm red-shifted from the green peak. The appearance of the red peak was consistent with the formation of J-aggregates (Jelley, 1937). In contrast to the spectral properties of the monomer, the excitation and emission maxima of the J-aggregates were found to be identical (no Stokes shift). Figure 2 shows emission scans of JC-1 at varying excitation wavelengths in buffer II with the pH adjusted to 8.0. The fluorescence intensities of both peaks increased when the excitation wavelength was discontinuously increased. Maximal fluorescence intensity at any given wavelength was achieved by scanning a sample in the "synchronous mode" (Figure 2); emission and excitation monochromators scan continuously and simultaneously with an offset that is limited by the slit widths of the light beam. We used an offset of 15 nm with a slit width corresponding to 3.6 nm. The emission maxima in the synchronous mode were found to be at 527 and 590 nm for the monomers and J-aggregates, respectively. If not stated otherwise, the following fluorescence spectra were obtained by using the synchronous acquisition mode.

Formation of J-Aggregates in Physiological Buffer above the Critical J-Aggregate Concentration (CJC). The formation of J-aggregates depends strongly on the chemical environment. The critical parameters are the concentration of the dye, pH, ionic strength, and temperature. We investigated the J-aggregate formation in buffer attempting to mimic cytosolic and mitochondrial conditions. JC-1 spectra at increasing concentrations in buffer II at pH 8.0 were recorded. The intensity of the green peak (527 nm) increased initially linearly with increasing amounts of JC-1. At high concentrations, the fluorescence signal reached a plateau. This suggested that the monomer concentration reached a level that remained un-

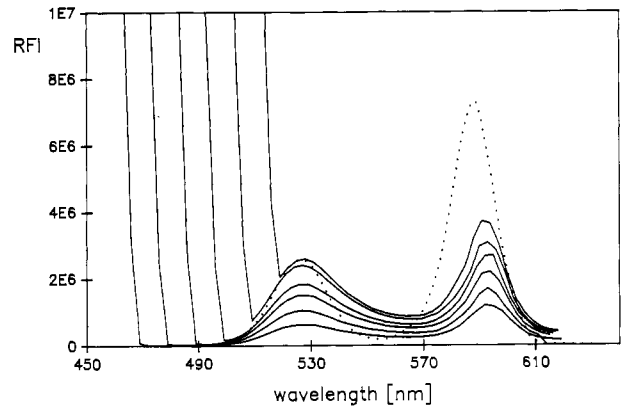

FIGURE 2: Emission and synchronous scans of JC-1. Emission scans of JC-1 (1.8 μM) in buffer II [110 mM KCl, 20 mM Mops, 10 mM MgATP, 10 mM succinate, 1 μM rotenone, 1 mM Mg^{2+}, 1 mM P_i, 1 mM EGTA, and mannitol/sucrose (3:1), pH 8.0 at 37 °C] were recorded with excitation wavelengths from 460 to 510 nm (solid lines). In the synchronous scanning mode, the excitation wavelength was continuously changed simultaneously with the emission wavelength (dotted line). The offset was 15 nm. The slit widths of all monochromators were set to 1 mm, corresponding to 3.6-nm bandwidth. The emission wavelengths were plotted on the abscissa. The fluorescence of the synchronous scan was normalized to the fluorescence at 525 nm of the emission scan (510-nm excitation).

Table I: Critical J-Aggregate Concentration of JC-1[a]

	CJC (μM)			
	monomer		J-aggregate	
pH	sync[b]	emission	sync	emission
6.5	3.61	3.41	0.99	0.84
7.15	1.31	1.52	0.29	0.26
8.0	0.25	0.25	0.11	0.11
mito[c]	0.16[d]	0.22[d]	0.16	0.15

[a] The fluorescence at varying concentrations of JC-1 in buffer II at 37 °C at indicated pH values covering the range of pH 6.5–8.0 was recorded and the CJC determined. [b] Data were obtained from maxima of the synchronous and emission scans. [c] Mitochondria were energized with 10 mM succinate in the presence of 1 μM rotenone at 37 °C in buffer II [110 mM KCl, 10 mM MgATP, 1 mM Mg^{2+}, 1 mM P_i, 10 mM succinate, 1 mM EGTA, and mannitol/sucrose (3:1), pH 7.15]; values for CJC refer to the minimal concentration of JC-1 that forms J-aggregates added to buffer II or a mitochondrial suspension of 0.25 mg of protein/mL (CJC_{mito}). [d] Estimated values from Figure 6.

changed upon further additions of JC-1. However, a red peak (590 nm) became apparent consistent with J-aggregate formation. The formation of water-soluble aggregates from monomers above a certain concentration is analogous to the formation of lipid micelles from lipid monomers; a characteristic parameter describing this phenomenon is the critical micellar concentration (CMC) (Tanford, 1973). In analogy, we defined a critical J-aggregate concentration (CJC) as the nominal JC-1 concentration that formed J-aggregates, depicted by the appearance of the red peak (see Discussion).

Figure 3 shows the fluorescence intensity of the green and the red peaks at their maxima. From the green peak, a CJC of 250 nM was determined and from the red peak 110 nM. When these experiments were performed at different pH values, the CJC changed. Table I summarizes the observed CJC values at pH 6.5, 7.15, and 8.0. The CJC increased by at least 1 order of magnitude with a pH fall of 1.5 units. This suggested to us that the formation of J-aggregates is pH dependent, that the protonated dye is nonfluorescent,[2] or both.

[2] S. T. Smiley, M. Reers, C. Hartshorn, A. Chen, T. W. Smith, G. D. Steele, Jr., and L. B. Chen, personal communication.

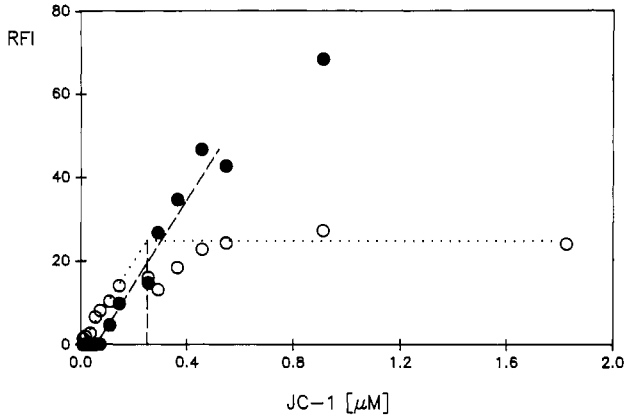

FIGURE 3: Critical J-aggregate concentration of JC-1 at pH 8.0. Increasing amounts of JC-1 (9.1 nM–1.8 μM) were added to buffer II (see Figure 2) at pH 8.0 at 37 °C. The scanning settings in the synchronous mode were described in Figure 2. The maxima from the green (at 527 nm, open circles) and red (at 590 nm, closed circles) peaks were plotted versus the nominal JC-1 concentration. In analogy to the formation of micelles from lipids above the critical micellar concentration (CMC), a critical J-aggregate concentration (CJC) was defined as the minimal concentration of JC-1 that formed J-aggregates. The intersection of the initial rise and the asymptotic fluorescence of the monomers (dotted lines) upon increasing [JC-1] determines the CJC derived from the monomers. The intersections of the dashed lines with the abscissa are the CJC derived from the signals of the monomers and the J-aggregates, respectively.

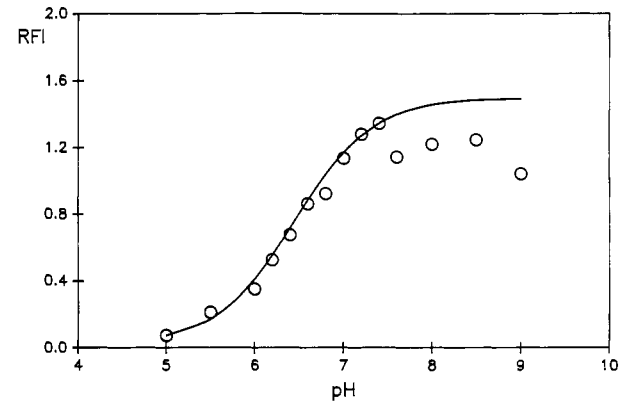

FIGURE 4: Determination of the pK_a of JC-1 monomers. JC-1 (46 nM) was added to buffer II (see Figure 3) at 37 °C. The pH was adjusted with 6 M HCl and 10 M KOH. The dilution was negligible. The average fluorescence of the monomers (527 ± 3 nM) from synchronous scans was plotted versus pH. No J-aggregates were detected at 590 nm. The pK_a was determined from this and three other experiments and was found to be 6.45 ± 0.06 (SD) (4).

Estimation of the pK_a of the JC-1 Monomer in Physiologic Buffer. We determined the pK_a of JC-1 in our buffer system at 37 °C (Figure 4). The dye exists in two forms in solution dependent on the concentration: as monomers and as J-aggregates. In order to determine the pK_a of the monomer, we had to choose a JC-1 concentration that, over the range of pH from 5 to 9, would not form J-aggregates, because this would lead to a lower fluorescence maximum at high pH. The titration of JC-1 was performed with 46 nM JC-1 starting at pH 5.0. Synchronous and emission scans were obtained. This allowed us to observe any J-aggregate formation. In a representative graph (Figure 4), the fluorescence values averaged over seven wavelengths from 524 to 530 nm were plotted as a function of pH. By use of this curve, the pK_a was estimated to be 6.45 ± 0.06 (SD) (4).

Formation of J-Aggregates in the Matrix of Energized Cardiac Mitochondria. Isolated cardiac mitochondria were incubated in buffer II, pH 7.15, at a protein concentration of

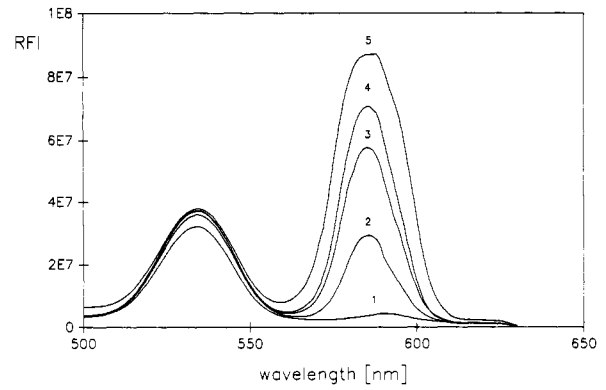

FIGURE 5: J-aggregate formation in energized cardiac mitochondria. Isolated cardiac mitochondria (0.25 mg/mL) were energized with 10 mM succinate and 1 μM rotenone in Buffer II, pH 7.15 at 37 °C. Different amounts of JC-1 were added: 180 nM (1), 270 nM (2), 310 nM (3), 360 nM (4), and 550 nM (5). After 10-min incubation of the dye, synchronous and emission scans were recorded. Then the mitochondrial suspensions were centrifuged for 30 s; the spectra of the supernatants were recorded and subsequently subtracted from initial scans, resulting in spectra of mitochondria-associated dye.

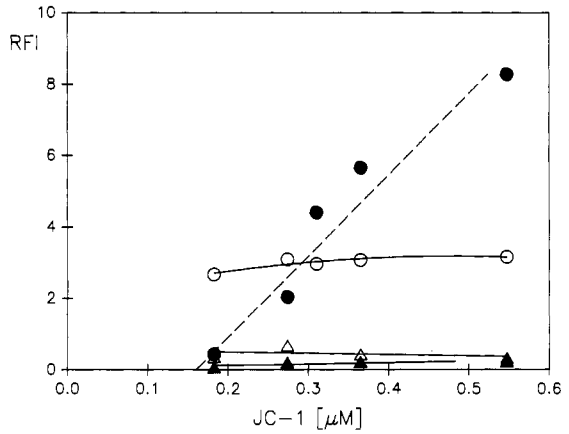

FIGURE 6: Apparent critical J-aggregate concentration in cardiac mitochondria. Experimental conditions were the same as in Figure 5. J-aggregate formation was also found to be linear in mitochondria with additions of JC-1 (closed circles). The monomer fluorescence (open circles), mostly associated with the mitochondria, was largely independent of the JC-1 concentration. The apparent critical J-aggregate concentration (CJC_{mito}), defined by the minimal [JC-1] that was needed to form J-aggregates at a mitochondrial protein concentration of 0.25 mg/mL, was found to be 160 nM at 37 °C (dashed line). After centrifugation of the mitochondrial suspensions, the fluorescence of the monomers (open circles) and the J-aggregates (closed triangles) of the supernatants was found to be negligible.

0.25 mg/mL at 37 °C. Mitochondria were energized with succinate/rotenone prior to additions of JC-1. After 10 min of incubation with the dye, synchronous and emission scans were recorded. Following a rapid centrifugation step of the mitochondrial suspension, synchronous and emission scans were obtained from the supernatant. Figure 5 shows synchronous scans at different JC-1 concentrations of only mitochondria-associated dye from which the corresponding supernatant spectra were subtracted. An apparent critical J-aggregate concentration in mitochondria (CJC_{mito}) can be defined as the minimal concentration of JC-1 added to a suspension of mitochondria that forms J-aggregates. The value of the CJC_{mito} depends on the concentration of mitochondria in a suspension. The CJC_{mito} derived from values of the red peak was determined to be 160 nM (Table I) at 0.25 mg/mL protein.

Response of the J-Aggregates in Cardiac Mitochondria to CCCP, Antimycin, or ADP. The inner mitochondrial membrane is known to possess a membrane potential, negative on

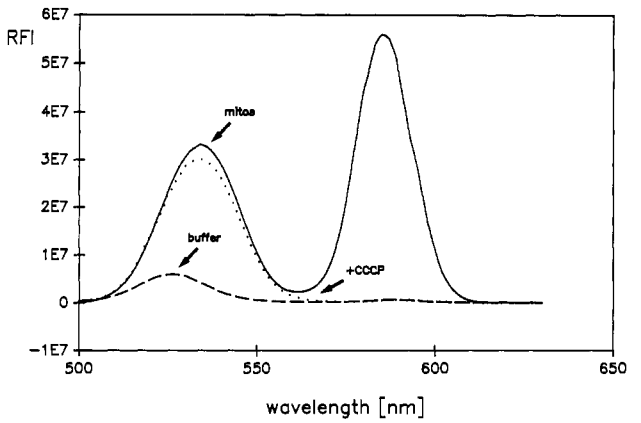

FIGURE 7: Synchronous spectra of JC-1 in coupled and uncoupled mitochondria. Isolated cardiac mitochondria (0.25 mg/mL) were incubated with JC-1 (310 nM) in buffer II, pH 7.15 at 37 °C (solid line). Addition of the uncoupler CCCP reversed J-aggregate formation (dotted line). A red shift of the mitochondria-associated monomers occurred when compared to JC-1 (310 nM) in buffer alone (dashed line).

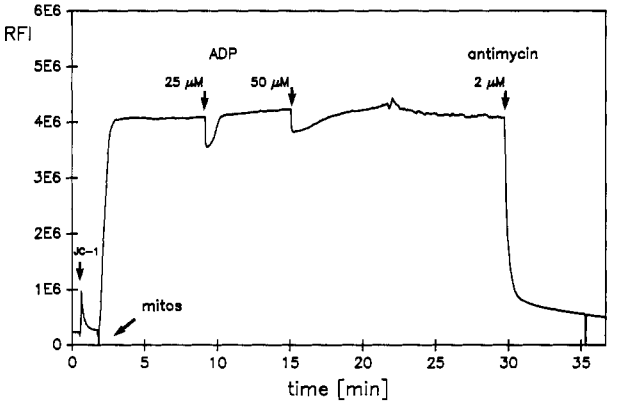

FIGURE 8: Fluorescence response of J-aggregates to ADP and antimycin. Fluorescence was monitored front-face at 590 ± 3.6 nm [(575 ± 3.6)-nm excitation] in a mitochondrial suspension containing succinate and rotenone. Additions at the arrows were 310 nM JC-1, 0.25 mg/mL mitochondria, 0.025 and 0.050 mM NaADP, and 2 μM antimycin.

the matrix side. The force encountered by a positive delocalized charge of a molecule on the surface of the outside membrane is directional and leads to an accumulation of this compound in the matrix as a direct function of the membrane potential. When [JC-1] exceeds the CJC in the matrix, J-aggregates would be expected to form. Figure 7 shows a synchronous scan of a suspension of coupled mitochondria in the presence of 310 nM JC-1 in buffer II, pH 7.15 at 37 °C. At this concentration, J-aggregates were formed. Upon addition of the uncoupler CCCP, the red peak disappeared. As a control, 310 nM JC-1 in buffer II was scanned. The green peak of JC-1 with mitochondria present shifted about 10 nm to the red, but the intensity of the monomer fluorescence within the mitochondria was at least 10-fold higher. Even after deenergization with CCCP the intensity of the green peak remained high, suggesting that the monomers were still associated with the mitochondria and were not completely released into the buffer.

In a time-based experiment, the red peak of JC-1 in succinate-energized mitochondria was continuously monitored. Addition of mitochondria to buffer II that contained JC-1 caused within 30 s a rapid increase of the signal, indicating J-aggregate formation (Figure 8). Additions of ADP transiently reduced the signal, with a complete decline with antimycin present. The amount of ADP did not affect the decline

Table II: Effect of JC-1 on the Mitochondrial Respiration Control Ratio (RCR)[a]

	RCR at JC-1 (μM) of					
	0	0.18	0.27	0.31	0.37	0.55
mean	11.2	10.0	9.5	10.8	8.9	7.9
SD	1.5	3.1	1.8	3.0	1.2	2.3
n	4	4	4	4	4	4

[a] Mitochondria (0.25 mg/mL) were incubated in buffer consisting of 110 mM KCl, 20 mM Mops, 10 mM glutamate, 1 mM malate, and 1 mM EGTA, pH 7.0 at 25 °C; JC-1 was added after 2 min; state 3 was induced with 0.15 mM ADP; the respiration control ratio was calculated after at least 5-min incubation of JC-1. [b] RCR was not statistically different by one-way ANOVA: $P = 0.469$, $F = 0.98$.

of membrane potential but did affect the duration, suggesting saturation of the adenine nucleotide translocator.

Effect of JC-1 on Mitochondrial Respiration and Respiration Control. To determine whether the dye JC-1 had an effect on respiration of isolated cardiac mitochondria, mitochondria isolated from the myofibrils of heart muscle were incubated with JC-1 in the presence of glutamate/malate in state 4. Respiration of state 3, initiated with 0.15 mM ADP, followed by state 4 was recorded in the presence of different concentrations of JC-1. The respiration at states 3 and 4 and consequently the respiration control ratios (RCR) were not affected by JC-1 over the range 0–550 nM (Table II).

Calibration of J-Aggregate Fluorescence in Cardiac Mitochondria Using Potassium Diffusion Potentials. JC-1 exhibited under favorable conditions two emission wavelengths (green and red) that could potentially be used to monitor membrane potential. Covering the full range of $\Delta\psi$ in mitochondria (approximately 180 mV), a concentration of JC-1 of approximately 2–3 times below the CJC_{mito} must be used in order to employ the green peak as an indicator. However, the signal-to-noise ratio would be low under these circumstances. Above the CJC_{mito}, the intense red peak could be used at a [JC-1] of 2–3 times the CJC_{mito}. In the following experiments, 310 nM JC-1 was used in a mitochondrial suspension of 0.25 mg/mL. After incubation of the mitochondria with dye in buffer III containing 20 nM valinomycin and different amounts of KCl, the fluorescence was recorded. Assuming 120 mM K^+ in the matrix, the diffusion potential of K^+ was calculated by employing the Nernst equation. The red peak responded linearly to the K^+ diffusion potential at all the pH values tested (Figure 9), although at pH 6.5 the slope was found to be shallower.

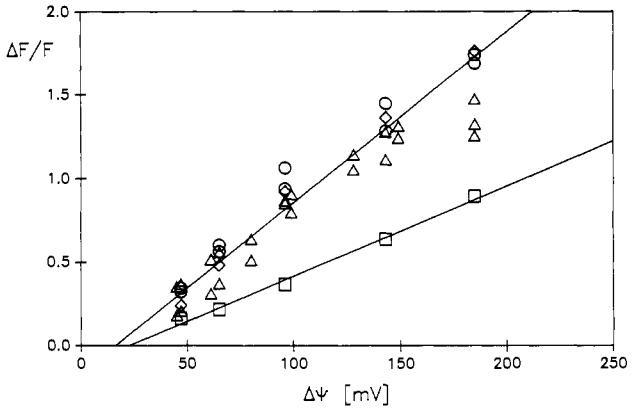

FIGURE 9: Calibration of the fluorescence of J-aggregates in cardiac mitochondria with K^+ diffusion potentials. Isolated cardiac mitochondria were incubated in buffer III consisting of 200 mM sucrose, 20 mM mannitol, 20 mM NaMops, 1 mM NaEDTA, 1 μM rotenone, 2.5 μM oligomycin, 310 nM JC-1, and 20 nM valinomycin at different pHs: 6.5 (squares), 7.15 (triangles), 7.7 (diamonds), and 8.0 (circles) at 37 °C. The concentration of matrix KCl was taken to be 120 mM. The KCl concentration in the extramitochondrial space varied from 0.1 to 20 mM. ΔF is the difference of the fluorescence at the individual K^+ diffusion potentials and the fluorescence F obtained after the addition of 2 μM CCCP. The membrane potential was calculated by using the Nernst equation. The emission light at 590 ± 3.6 nm (excitation at 490 ± 1.8 nm) was continuously monitored in the front-face mode. Each calibration curve consists of two to four experiments.

Discussion

The need for nontoxic fluorescent probes that monitor membrane potential has resulted in an extensive library of candidates. The majority of such compounds fall into the category of slow redistributive dyes. Out of this category, most often two chemically distinct groups of compounds have been used as fluorescent probes: rhodamines (Johnson et al., 1980) and carbocyanines (Sims et al., 1974). The dye characterized here belongs to the group of singly positively charged carbocyanines. It contains a highly symmetrical polymethine-linked fluorophore, consisting of two heterocycles with two hydrophobic ethyl substituents each. In addition to these common features of symmetrical carbocyanine dyes, both heterocycles contain two electron-drawing chlorine substituents, accounting for the low pK_a of the imidazole nitrogen (see below). The compound is planar (sp^2 hybridization). The positive charge delocalized over the heterocycles confers sensitivity to membrane potential. With the chemical name 5,5′,6,6′-tetrachloro-1,1,3,3′-tetraethylbenzimidazolylcarbocyanine iodide and the often used nomenclature, the dye would be abbreviated CBI-C_2-(3). Another nomenclature suggested by Chen (1989) addresses a subgroup of the carbocyanine dyes that form J-aggregates suitable for tracking membrane potentials. Hence, the first *J*-aggregate-forming, cationic dye found was denoted JC-1.

The formation of nematic phases, such as J-aggregates, has been extensively described in the photography literature (James, 1977). However, the structure–aggregation relationship is still unclear. When carbocyanine dyes are prepared in aqueous solutions, increasing their concentration produces aggregation (nematic phase) of the dye molecules, and most cyanine dyes exhibit a spectrum with new maxima at shorter wavelengths (H-bands) than the monomer maximum. However, some planar carbocyanines form a nematic phase exhibiting a maximum at a longer wavelength. This maximum appeared to result from interactions of large numbers of molecules in a parallel orientation (Jelley, 1937). In general, constituents in a solution with regularly spaced negative charges favor J-aggregate formation by stabilizing the cation–cation arrangement of the carbocyanines (Kay et al, 1964a,b). Therefore, nucleic acid strands and proteins (e.g., cytochrome *c* at a pH above its p*I*) lower the concentration required for J-aggregate formation. In the work reported here, J-aggregate formation displayed a behavior with similarities to micelle formation of amphipathic detergent-like compounds. Below the CMC, amphipathic molecules exist as monomers while above the CMC the monomer concentration remains constant and micellar aggregates form. Analogously, above a defined JC-1 concentration (critical J-aggregate formation concentration, CJC), the fluorescent spectra suggest that also two phases existed, the monomer phase and the nematic phase (J-aggregate) (Figures 2 and 5).

JC-1 behaved in a similar manner, both physically and spectrally, in cardiac mitochondria as it did in solution. The membrane potential of energized and coupled mitochondria caused uptake of JC-1 monomers into the matrix. If a sufficient quantity of JC-1 was added, the CJC was exceeded in

the matrix, and the red peak appeared in addition to the monomer peak (Figures 5 and 8). The hyperchromic red shift of the monomer peak was consistent with the spectral characteristics of other carbocyanines and rhodamines. The red peak was found to be sensitive to the protonophore CCCP and to the inhibitor antimycin, both of which cause ultimately a deenergization of the mitochondria (Figures 7 and 8). In addition, small membrane potential changes (e.g., as induced by addition of ADP) could be detected (Figure 8). The monomer peak was almost completely insensitive to membrane potential changes. The rate-limiting step for J-aggregate formation would presumably be the diffusion of the monomer across the mitochondrial membranes into the matrix, suggesting that the actual J-aggregate formation is much faster. Importantly, the J-aggregate formation was found to be fully reversible, excluding the possibility of a metabolic covalent conversion of JC-1 to a red dye.

JC-1 fluorescence was found to be pH-dependent (Figures 4 and 9). The protonated form lost its fluorescence completely due to interruption of the conjugation at the polymethine link (James, 1977). This was exploited to determine the pK_a of JC-1 by observing the monomer fluorescence in aqueous medium. The JC-1 concentration must be below the CJC at the highest pH in order to avoid J-aggregate formation, decreasing the maximal signal at high pH and leading to an artificially lowered pK_a. Data in Figure 4 were obtained from JC-1 spectra covering the range from 500 to 630 nm. Despite the fact that no J-aggregate formation was detected at high pH, the monomer signal decreased, suggesting that other, yet unknown factors were responsible. Within the physiological pH range of 7–8, however, the monomer concentration is considered as not being altered altered significantly by pH changes. Therefore, we derived the conclusion that the J-aggregate concentration and the resulting fluorescence signal would not be affected through pH-dependent withdrawal of monomers. However, the J-aggregate and its fluorescence could be sensitive to pH. The fluorescence response of the J-aggregates in mitochondria to a K^+ diffusion potential was found to be linear, with essentially the same slopes between pH 7.15 and 8. Under the same conditions at pH 6.5, the red peak intensity also responded linearly but with a shallower slope. This reduced dynamic range is possibly due to a direct pH effect on J-aggregates or to an indirect effect by alteration of negative charges of J-aggregate supportive components in the matrix such as proteins.

In order to quantify the pH influence on the apparent membrane potentials reported by the red peak in energized mitochondria, four assumptions were made: (1) only the singly positively charged monomer concentration influences the J-aggregate formation; (2) the extramitochondrial monomer concentration is constant and below the CJC, and the intramitochondrial monomer concentration is kept constant, buffered by the J-aggregates; (3) only the matrix pH (pH_m) is changing. Upon acidification of matrix pH from 8.15 to 7.15, approximately 13% of the monomers must be replenished at the expense of the J-aggregates. Given a constant membrane potential of 180 mV (negative inside) and conformity with the Nernst equation, the membrane potential reported by the red peak would be 176 mV, a 2% error (Figure 10). If, in addition, the assumption is included that (4) a decline in the pH gradient is counteracted by an equivalent increase in membrane potential ($\Delta\mu_{H^+} = \Delta pH + \Delta\psi = 240$ mV), the membrane potential should increase linearly with increasing acidification. Only at pH values below the pK_a did the apparent membrane potential deviate significantly from linearity.

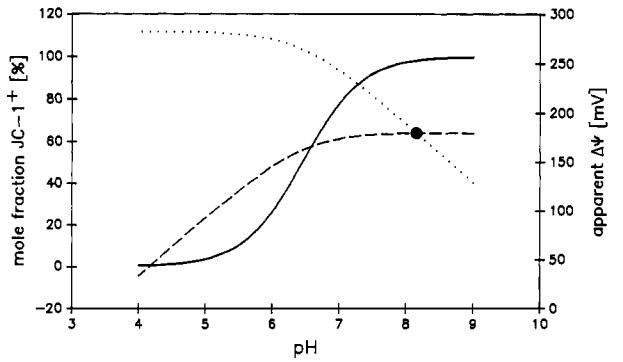

FIGURE 10: Predicted pH sensitivity of the membrane potential reported by JC-1. The singly positively charged monomer and therefore its fluorescence decrease with a decline of the buffer pH (solid line). One can assume the pH-sensitive reaction to be $H\text{-}JC\text{-}1^{2+} \leftrightarrow H^+ + JC\text{-}1^+$ in which the amount of singly charged monomer can be calculated by mass action. As total JC-1 concentration ($[JC\text{-}1_{tot}]$) = $H\text{-}JC\text{-}1^{2+} + JC\text{-}1^+$, the mole fraction, x, of $JC\text{-}1^+$ can be calculated as $x = [JC\text{-}1^+]/[JC\text{-}1_{tot}] = K_a/([H^+] + K_a)$. The pK_a was taken to be 6.45. The dashed curve represents the pH effect expressed as the apparent membrane potential reported by JC-1. The dotted line is the predicted dye response with the assumption that $\Delta\mu_{H^+} = \Delta pH + \Delta\psi = 240$ mV. For further details, see text.

JC-1 has the advantage that it changes its color dependent on the magnitude of the membrane potential. At low membrane potentials, mitochondria exposed to metabolic inhibitors appeared green and at high $\Delta\psi$ greenish orange (data not shown). This allowed us to gather additional spatial information when mitochondria were placed on the stage of a fluorescence microscope. Archiving was performed with an inexpensive 35-mm camera and a fast film. However, inherently of this recording method, only semiquantitative information could be obtained.

The question arises whether it is possible to apply quantitative ratio methodology to this dye. Ratioing of two wavelengths would make membrane potential measurements independent of light path length and volume changes of the compartments involved. In principle, JC-1 could be employed as a dual-emission indicator by ratioing the fluorescence of the red peak over that of the green peak. The value the red peak represents is the membrane potential sensitive numerator, while the value of the green peak would represent the membrane potential insensitive denominator. The fluorescence intensities of both peaks are volume-dependent; assuming that mitochondria swell by a factor of 2 without a change in membrane potential, the intensities of both signals would double since the ratio of monomer molecules to J-aggregates at a given membrane potential would be constant. Therefore, under ideal conditions (that include that the monomer and the J-aggregates are not membrane-associated), JC-1 could be employed in the ratio mode. This potential application of JC-1 is currently under investigation in our laboratory.

In summary, JC-1 is a member of the carbocyanine family; its fluorescent J-band was sensitive to membrane potential in isolated liver (Smiley et al., 1991) and cardiac mitochondria. JC-1 did not exhibit any concentration-dependent quenching effects. Its J-aggregate fluorescence intensity increased linearly with increasing membrane potential over the range of 30–180 mV. An advantage of JC-1 and possibly other dyes that form J-aggregates is that the spectral characteristics can be studied in aqueous buffer solutions without intact membranes present. JC-1 could potentially be used in the ratio mode as a dual-emission probe for membrane potential. Perhaps most interestingly, JC-1 can be used to monitor mitochondrial $\Delta\psi$ in isolation or within cells. Mitochondrial populations with

differing membrane potentials would be inherently color-coded at the properly selected concentration of JC-1: green at low and red-orange at high membrane potentials. Within the category of slow membrane potential dyes, fluorescent J-aggregate-forming indicators could find a wide range of applications, including videomicroscopy with the emerging development of the color CCD camera.

ACKNOWLEDGMENTS

We thank Joseph Arruda for technical assistance and Michelle Somers for typing the manuscript.

Registry No. JC-1, 21527-78-6; K, 7440-09-7.

REFERENCES

Bunting, J. R., Phan, T. V., Kamali, E., & Dowben, R. M. (1989) *Biophys. J. 56*, 979-993.
Chen, L. B. (1988) *Annu. Rev. Cell Biol. 4*, 155-181.
Chen, L. B. (1989) *Int. Conf. Videomicrosc.* (Abstract).
Emaus, R. K., Grunwald, R., & Lemasters, J. J. (1986) *Biochim. Biophys. Acta 850*, 436-448.
Freedman, J. C., & Novak, T. S. (1983) *J. Membr. Biol. 72*, 59-74.
Freedman, J. C., & Novak, T. S. (1989) *Methods Enzymol. 172*, 102-122.
Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751-766.
Gross, D., Loew, L. M., & Webb, W. W. (1986) *Biophys. J. 50*, 339-348.
Jelley, E. E. (1937) *Nature 139*, 631-632.
Johnson, L. V., Walsh, M. L., & Chen, L. B. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 990-994.
Kay, R. E., Walwick, E. R., & Gifford, C. K. (1964a) *J. Phys. Chem. 68*, 1896-1906.
Kay, R. E., Walwick, E. R., & Gifford, C. K. (1964b) *J. Phys. Chem. 68*, 1907-1916.
Loew, L. M. (1982) *J. Biochem. Biophys. Methods 6*, 243-260.
Montana, V., Farkas, D., & Loew, L. M. (1989) *Biochemistry 28*, 4536-4539.
Palmer, J. W., Tandler, B., & Hoppel, C. L. (1977) *J. Biol. Chem. 252*, 8731-8739.
Reers, M., Kelly, R. A., & Smith, T. W. (1989) *Biochem. J. 257*, 131-142.
Rossi, E., & Azzone, G. F. (1969) *Eur. J. Biochem. 7*, 418-426.
Sims, P. J., Waggoner, A. S., Wang, C-H., & Hoffmann, J. F. (1974) *Biochemistry 13*, 3315-3330.
Smiley, S. T., Reers, M., Hartshorn, C., Chen, A., Smith, T. W., Steele, G. D., Jr., & Chen, L. B. (1990) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
Sturmer, D. M., & Heseltine, D. W. (1977) in *The theory of the photographic process* (James, T. H., Ed.) pp 194-235, Macmillan Publishing Co., Inc., New York.
Tanford, C. (1973) in *The hydrophobic effect: Formation of Micelles and Biological Membranes* (Tanford, C., Ed.) pp 47-49, John Wiley & Sons, New York.
Tomov, T. T. (1986) *J. Biochem. Biophys. Methods 13*, 29-38.
Waggoner, A. S. (1988) *Gen. Soc. Physiol. Ser. 43*, 209-215.

Electron Spin Echo Envelope Modulation Studies of Lectins: Evidence for A Conserved Mn^{2+}-Binding Site†

John McCracken, Jack Peisach,* Lokesh Bhattacharyya, and Fred Brewer*

Department of Molecular Pharmacology, Microbiology and Immunology, and Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10461

Received November 14, 1990; Revised Manuscript Received January 24, 1991

ABSTRACT: Electron spin echo envelope modulation (ESEEM) experiments have been used to investigate the Mn^{2+}-binding site in a series of lectins including concanavalin A, pea lectin (*Pisum sativum*), isolectin A from lentil (*Lens culinaris*), soybean agglutinin (*Glycine max*), *Erythrina indica* lectin, and *Lotus tetragonolobus* isolectin A. Together with model studies, the results provide direct evidence for a single nitrogen atom of a conserved residue bonded directly to Mn^{2+} in all of them. ESEEM measurements of the lectins exchanged with deuterium oxide, together with model studies, provide evidence for the presence of two water molecules coordinated to the Mn^{2+} in all of the proteins. In contrast to concanavalin A, the absence of solvent exchange at the Mn^{2+} site in the pea and lentil lectins demonstrated by nuclear magnetic relaxation dispersion measurements [Bhattacharyya, L., Brewer, C. F., Brown, R. D., III, & Koenig, S. H. (1985) *Biochemistry 24*, 4985-4990] must therefore be due to slow exchange of the water ligands of the bound Mn^{2+}. Binding of saccharides was observed to have little effect on the structural features of the Mn^{2+} site in the lectins as determined by ESEEM.

Lectins are cell-agglutinating proteins of nonimmune origin that bind to specific carbohydrate determinants without chemically modifying them. They are found in animal tissues and invertebrates, although the majority of the best studied lectins are from plants. It is this latter class that has been widely used to explore the membrane properties of both normal and transformed cells (Brown & Hunt, 1978; Lis & Sharon, 1981).

Legume lectins have been extensively studied for their carbohydrate-binding properties. They include those from Jack bean (concanavalin A) (Con A),[1] pea (PSA), lentil (LcH),

† This work was supported by National Institutes of Health Grant CA-16054 (C.F.B.), Core Grant P30 CA-13330 (C.F.B. and J.P.), Grant GM-40168 (J.P.) and RR-02583 (J.P.).

* To whom correspondence should be addressed.

0006-2960/91/0430-4486$02.50/0