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THE EFFECT OF pH ON RATE CONSTANTS, ION SELECTIVITY AND THERMODYNAMIC PROPERTIES OF FLUORESCENT CALCIUM AND MAGNESIUM INDICATORS

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Summary Fluorescent calcium indicators fluo-3, fura-2 and indo-1, and fluorescent magnesium indicators mag-fura-2 (FURAPTRA) and mag-indo-1 were evaluated for the effects of pH on their association and dissociation rates, ion selectivity and thermodynamic properties. Calcium indicator affinities for Ca and Mg were reduced and the discrimination between Ca and Mg decreased in fura-2 and indo-1 at acidic pH. Alterations in apparent dissociation constants were caused primarily by reduced association rates. Magnesium indicators did not show these changes. The enthalphies of the calcium indicators' Ca complex were 1-3 kcal/mole and magnesium indicators' Mg complex were 7-9 kcal/mole. The potential effects of a biexponential dissociation rate of fluo-3 and of Ca interactions with magnesium indicators were examined. © 1991 Academic Press, Inc.

Fluorescent ion indicators have become important tools in determining intracellular cation activities (1). Although widely used, there are a number of difficulties associated with the use of these indicators, some of which stem from changes in the equilibrium binding or kinetic properties of the indicators as they interact with intracellular components or non-standard environments (2-4). It was recently found that the calcium indicators have a reduction in binding affinity at acidic pH (4). To determine whether these changes were caused by alterations in either the association or dissociation rates of these indicators, the K_ds and dissociation rates for both calcium and magnesium were determined for the calcium indicators fura-2, indo-1 and fluo-3 and the magnesium indicators mag-fura-2 (FURAPTRA (5)) and mag-indo-1. This information was used to calculate the association rates, the energies of activation, enthalpies, and entropies that describe the formation of these indicator-cation complexes (6). Some preliminary results have been reported (7).

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Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis-(b-aminoethyl ether)N,N,N',N'-tetracetic acid: K_d apparent equilibrium dissociation constant.

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Methods

Preparation of buffers, indicators and cation standards

Water was obtained from a Nanopure still (Barnstead, Dubuque, IA) with initial resistance of >16 Mn. Acrylic cuvettes were used as reaction vessels and their contents mixed with stirbars. Solutions composed of 0.1M KCL, 30 mM KOH with either 40 mM HEPES or 40 mM MES were then backtitrated with HCL to adjust pH to 7.40 or 7.00 (HEPES) or pH 6.50, 6.00 or 5.50 (MES). Total adventitious calcium levels were <0.5 μM as determined by atomic absorption and fluo-3 measurements.

Calcium standards were prepared from calcium carbonate and HCL : dilutions were compared to NBS traceable standards (Orion, Cambridge, MA) with atomic absorption spectroscopy and arsenazo III absorbance measurements. Magnesium standards were prepared from magnesium sulfate and standards were adjusted for pH before use. The indicators fluo-3, fura-2, indo-1, mag-indo-1 and mag-fura-2 were obtained from Molecular Probes (Eugene, OR) as the acetoxymethyl esters, dissolved in dry DMSO and stored at -20 ^{o}C until used. Aliquots of dye were thawed and saponified with 0.1 N KOH to form the K^+ salt, which was diluted 1/1000 for use.

Determination of K_d

Fluorescence measurements were made with a spectrofluorometer (Aminco-Bowman, Silver Springs, MD) with a stirred, water-jacketed cuvette chamber with temperature maintained by a refrigerated water bath (Brinkmann Instruments, Westbury, NY). Excitation slit width was 5.5nm and emission slit width was 11 nm. Excitation and emission wavelengths in nm were excitation 340 and 380 and emission 500 (fura-2 and mag-fura-2); excitation 350 and emission 410 and 490 (indo-1 and mag-indo-1); excitation 506 and emission 530 (fluo-3).

Measurements of indicator calcium and magnesium K_ds were performed as described in (4) with modifications. Initial measurements of buffer autofluorescence were made and then fluorescent indicators were added at 125 nM final concentration. Terminology and calculations for the determination of the K_ds were as described in (8). R_{min}, the ratio of the fluorescence of the calcium-free indicator as measured at wavelength 1 and 2 and F_{min}, the fluorescence of the calcium-free indicator were measured after EDTA (for calcium determinations) or EGTA (for magnesium determinations) was added. The appropriate cation was then titrated for 6-8 additions. R_{max}, the ratio of the fluorescence of the calcium-bound indicator as measured at wavelength 1 and 2 and F_{max}, the fluorescence of the calcium-bound indicator were measured in the same cuvette by the addition of excess calcium or magnesium. EDTA and EGTA buffers were prepared and their values calculated as in (4).

Since the magnesium complex of fluo-3 and indo-1 is only weakly fluorescent, the magnesium K_ds for these indicators were found by first forming the calcium complex and then displacing calcium by adding magnesium (9). For these experiments the calcium concentration was 10 μM with 750 nM indicator. The K_ds for calcium binding were determined as described above or obtained from (4).

Stopped-flow measurements.

Stopped flow experiments were performed with a Hi-Tech PQ/SF-53 fluorescence spectrophotometer (Hi-Tech, Salisbury, UK) equipped with a dual grating monochromator between the 75 watt xenon light source and the reaction cuvette excitation window. The light emitted by the sample was filtered at the appropriate wavelength by narrow or high band pass filters and was measured by a photomultiplier tube. The quartz sample cuvette as well as the syringes containing the reactants were maintained at the desired temperature by a circulating water bath. The pneumatic rams were driven by a nitrogen pressure of 6 bar, which resulted in an instrument dead time that was empirically determined to be <1 msec.

The photomultiplier output voltage was continuously monitored in real-time and was stored on VHS tape for latter analysis. Using the KCl-HEPES or MES buffer system

described above, 2-4 μM indicator in the presence of 0.25-10 mM calcium or 2 mM magnesium was combined with an equal volume of 20 mM EDTA. For measurements of magnesium kinetics, EGTA was present with the indicator to eliminate the contribution of calcium to the initial signal. To determine the reaction rate constants, the data was replayed, digitized at 50 μ sec/point and 8-12 replicate experiments were digitally averaged. The averaged data was then computer fit to single or double exponential decay equations using either sequential integration, or more commonly, Levenburg-Marquardt algorithms. In a few experiments each replicate was individually analyzed; in these cases, the rate constants among replicates differed by less than 5%.

Results

As shown in Figure 1.A., the K_ds for magnesium binding to fluo-3, fura-2 and indo-1 indicated a decline in affinity as the pH was reduced from 7.40 to 5.50, similar to the decline seen in calcium affinity (4). The magnesium:calcium selectivity ratio (K_d magnesium/K_d calcium) at pH 7.40 was 29,300 for indo-1, 52,800 for fura-2 and 24,900 for fluo-3. At pH 5.50, these values change to 8,700, 23,300 and 35,900, respectively. This corresponds to a 3.4 fold decrease for indo-1, a 2.3 fold decrease for fura-2 and a 1.4 fold increase for fluo-3. In contrast to the effects of pH on the calcium indicators, both the calcium and magnesium K_ds of mag-fura-2 and mag-indo-1 were relatively unaffected by an acidic environment (see Figure 1.B.). The selectivity ratio at pH 7.40 was 92 for mag-fura-2 and 91 for mag-indo-1; at pH 5.50, these ratios changed to 95 and 119 respectively. This amounts to a 1.03 and 1.3 fold increase, respectively.

Table 1 displays the K_d, dissociation rate and calculated association rate for the indicators at pH 7.40 and 5.50. Measurements of either the fluorescence ratios or individual wavelengths, the latter most frequently used, yielded identical values for dissociation rates. Association rates were calculated by dividing the dissociation rate by the K_d for the specific indicator and pH. This information was used to calculate the energy of activation and the entropy for both association and dissociation processes for these compounds (6). These values are shown in Table 2.

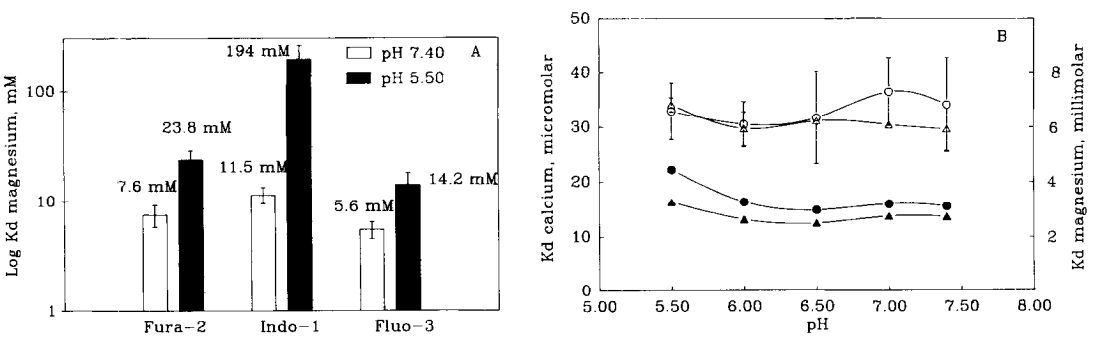

Figure 1. A. The effect of pH on magnesium binding to fluo-3, fura-2 and indo-1. Using buffers described in Methods, the K_ds of the indicators for magnesium at 22°C were measured at pH 7.40 and 5.50. B. The effects of pH on magnesium and calcium binding to mag-fura-2 and mag-indo-1. The magnesium K_ds of mag-fura-2 (▲) and mag-indo-1 (●) and the calcium K_ds of mag-fura-2 (△) and mag-indo-1 (○) were measured at 22°C. Each point is a mean ± s.d. of 3-6 individual titrations.

Table 1
The K_ds, dissociation (k_{off}) and association (k_{on})
rates of calcium and magnesium to fluorescent indicators

Indicator	Cation	T,°C	pH	k_{on} 1/M 1/sec	k_{off} 1/sec	K_d
Fluo-3	Calcium	22	7.40	$9.2x10^8$	424±18	462±15 nM
		37		$1.5x10^9$	605±4	407±24 nM
		22	5.50	$5.5x10^7$	296±12	5415±252 nM
		37		$1.6x10^8$	785±5	4773 nM[a]
Fura-2	Calcium	22	7.40	$7.6x10^8$	109±6	144±5 nM
		37		$1.5x10^9$	196±3	130±7 nM
		22	5.50	$1.6x10^8$	161±4	1022±16 nM
		37		$3.0x10^8$	282±5	935 nM[a]
Indo-1	Calcium	22	7.40	$9.4x10^8$	180±15	191±5 nM
		37		$1.7x10^9$	298±3	179±2 nM
		22	5.50	$1.5x10^8$	242±15	1628±101 nM
		37		$3.1x10^8$	412±4	1330 nM[a]
Mag-fura-2	Magnesium	10	7.40	$9.9x10^4$	493	4.97±0.25mM
		22		$5.9x10^5$	1587	2.72±0.12mM
		37		-	*	1.66±0.07mM
		10	5.50	$2.2x10^4$	135	6.05±0.60mM
		22		$1.4x10^5$	448	3.25±0.11mM
Mag-indo-1	Magnesium	10	7.40	$1.4x10^5$	901	6.28±1.02mM
		22		-	*	3.10±0.05mM
		37		-	*	2.03±0.02mM
		10	5.50	$1.9x10^4$	150	7.88±0.93mM
		22		$2.3x10^5$	1010	4.44±0.17mM

The K_ds for calcium of fluo-3, fura-2 and indo-1 were obtained directly or by interpolation from (4); the latter values are designated by [a]. * too fast to measure.

Stopped flow measurements of the dissociation rates for calcium for fura-2, fluo-3 and indo-1 showed only about a 50% change compared to changes in K_ds of 7-11 fold over the pH range 7.40 to 5.50 (see Table 1). The decreased association rates are therefore the predominant cause for the changes in K_ds. The dissociation rates for calcium binding to magnesium indicators were too rapid to be measured with stopped flow; dissociation rates of related compounds are about 5000-13000/sec (10).

We confirmed the presence of biexponential dissociation in fluo-3 (11); all other indicators demonstrated a single dissociation constant. This biexponential decay was present when calcium levels in the solution containing fluo-3 were greater than ~25 micromolar prior to mixing with EDTA (see Figure 2). At levels of calcium below this, dissociation was monoexponential. In both cases of mono- and bi-exponential

Table 2
Thermodynamic functions for calcium and magnesium binding to fluorescent indicators

Indicator and ion	pH	Condition	Ea kcal/mol	δS cal/mol*K	▲H kcal/mol	▲S cal/mol*K
Fluo-3, calcium	7.40	Association	5.9	0.4	1.6	34.2
		Dissociation	4.3	-33.8		
	5.50	Association	12.9	18.7	1.1	38.0
		Dissociation	11.8	-19.3		
Fura-2, calcium	7.40	Association	8.3	8.2	1.2	35.6
		Dissociation	7.1	-27.4		
	5.50	Association	7.9	3.9	1.1	31.5
		Dissociation	6.8	-27.6		
Indo-1, calcium	7.40	Association	6.9	4.2	0.8	33.7
		Dissociation	6.1	-29.5		
	5.50	Association	9.0	7.4	2.5	35.0
		Dissociation	6.5	-27.6		
Mag-fura-2 magnesium	7.40	Association	24.6	49.3	8.4	40.2
		Dissociation	16.2	9.1		
	5.50	Association	25.7	50.2	9.1	42.3
		Dissociation	16.6	7.9		
Mag-fura-2 calcium	7.40				2.1	27.9
	5.50				2.9	30.1
Mag-indo-1 magnesium	7.40				7.2	35.7
	5.50				7.9	37.6
Mag-indo-1 calcium	7.40				3.4	31.8
	5.50				1.0	23.7

The energies of activation (E_a), partial entropies of association and dissociation (δS), were calculated as in (6). Enthalpies (▲H) and entropies (▲S) of the reaction were calculated from either $E_a$association-$E_a$dissociation or δS association-δS dissociation, respectively, or from van't Hoff plots.

dissociation, the rapid dissociation rate was identical within experimental error. The second dissociation constant of fluo-3 had a rate of 38/second and comprised about 15% of the amplitude of the first component at pH 7.4 and 22°C. The biexponential decay also occurred when Mn was used to displace calcium from fluo-3, in lieu of using EDTA to remove calcium. Increasing EDTA concentrations in the second syringe did not eliminate this biexponential dissociation. The presence of the second component declined over time and was reduced from 15% to 5% of the magnitude of the first component in 2 hours after saponification of the indicator. Association rates for fluo-3 were calculated using only the rapid dissociation constant.

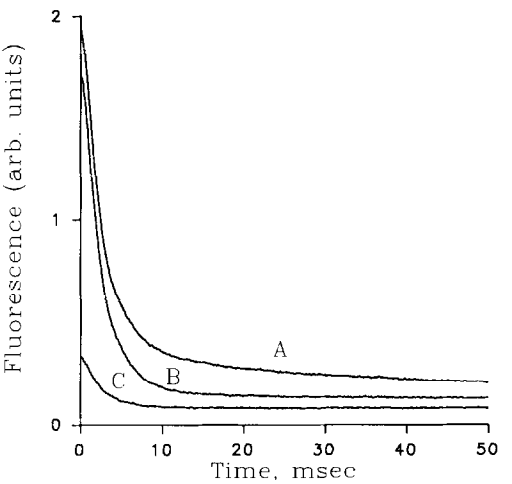

Figure 2. The biexponential dissociation of calcium from fluo-3 as measured by stopped flow. At calcium concentrations > 25μM prior to the addition of EDTA, fluo-3 exhibits a biexponential decay (trace A:calcium concentration initially 1mM). At lower initial calcium concentrations the dissociation is monoexponential (trace B and C:calcium concentration initially 10 μM and 400 nM, respectively).

The response of mag-fura-2 and mag-indo-1 to acidic buffers is quite different from the fluorescent calcium indicators. Neither the dissociation nor association rates to magnesium or to calcium changed appreciably when the pH was decreased to 5.50. Both mag-indo-1 and mag-fura-2 have K_ds for calcium that are in the 30 μM range (see Figure 1.B.), with calcium dissociation rates that were too fast to measure.

The R_{max} of mag-fura-2 is about 5 fold greater when it binds calcium instead of magnesium, a factor that will influence intracellular magnesium measurements at elevated calcium activities (see Discussion). Both mag-indo-1 and mag-fura-2 are also sensitive to the concentration of EGTA. After correcting for changes in magnesium activity due to EGTA binding, the presence of 1 mM EGTA yielded K_ds for magnesium of 2.72 mM$\pm$0.12mM for mag-fura-2 and 3.10$\pm$0.05 mM for mag-indo-1 when measured at 22^{o}C and pH 7.40 (n=5). Under the same conditions, these values increased to 3.47$\pm$0.06 mM and 3.76$\pm$0.23 mM, respectively, in the presence of 5 mM EGTA.

Discussion

Previous studies (4,6, 11-13) have demonstrated that these tetracarboxylic acid indicators normally have a bimolecular reaction with divalent ions and the association rate can be accurately determined through measurements of the K_d and the dissociation rate of the indicator-cation complex. The present study demonstrates that the decreased affinity of fluorescent calcium indicators in an acidic environment is primarily due to a decrease in the association rate of calcium, which is in competition with protons associated with the liganding carboxylic acid moieties. This occurs

because of the necessity of deprotonating the carboxylic acid moieties prior to the complexation of the divalent cation. This explanation should also be true for other cations, such as magnesium, that bind to these indicators and demonstrate the aforementioned effect. The apparent insensitivity of calcium and magnesium K_ds of mag-fura-2 and mag-indo-1 to acidification may explained by a more acidic pK_a of the liganding carboxylic acids (5). For calcium indicators such as indo-1 and fura-2, the effects of acidification reduce the ability of the chelator to discriminate between calcium and magnesium. This alteration, which could be manifested in conditions such as ischemia where intracellular magnesium is elevated in the presence of an intracellular acidification (14), could affect intracellular calcium activity measurements.

An anomalous biexponential fluo-3 dissociation rate was observed, similar to that seen in previous study (11). The second component in that study had a faster dissociation rate (~220/sec) and a greater amplitude (~33%) than that measured in the present study. These differences may be due in part to the curve fitting routines, acquisition time and rate, or the batch of free acid used in (11) rather than freshly saponified ester used in the present study. The present study demonstrates that the second component is present when Mn is used to displace calcium, eliminating an interaction of fluo-3 with EDTA as the cause of this effect. The biexponential decay was transformed into a single rapid dissociation by reducing the calcium concentration present with the indicator to below 25 μM prior to the stopped flow addition of EDTA. This suggests that the moiety present in our experiments has a much lower affinity for calcium than fluo-3 and should not be a problem in the measurement of calcium transients with a peak concentration less than 25 μM. The fact that this component declined within two hours suggests the presence of either a transient hydrolysis product of fluo-3 or of a contaminant might have caused this effect.

All the indicators have rate constants that increase with increasing temperature. The enthalpies at pH 7.40 and pH 5.50 range between 1-3 kcal/mole for the calcium indicators, indicating a slightly endothermic reaction. These values are much less than the 10 kcal/mole obtained for azo-1 (6) or the value of 9.5 kcal/mole for quin2 (12). The enthalpies of mag-fura-2 and mag-indo-1 for magnesium are 7-9 kcal/mole at pH 7.40 and 5.50. Binding is entropically favored for all indicators tested, since at $22^{o}C$ $-T\delta S$ is in the range of -6 to -12 kcal/mole which overcomes the endothermic complexation.

If both calcium and magnesium are present at elevated levels in the cytoplasm, it is possible that the signal from calcium bound to a magnesium indicator can artifactually increase the apparent intracellular magnesium activity. Murphy and her co-workers (15) mentioned that in the presence of an elevated calcium activity of 1 μM and a basal magnesium activity of 0.5 mM, the peak intracellular magnesium activity they saw would have been overestimated by 7%. A caveat to this comment is that if the experimenter was making a ratio measurement, the presence of a calcium*mag-fura-2 complex, which has an R_{max} 5 fold greater than the magnesium*mag-fura-2 complex, would generate an observed ratio measurement that would have to be corrected by considering differences in R_{max}, as well as the calcium K_d and amount of indicator

bound by calcium. This alteration in the observed fluorescence ratio is also the reason why calcium must be chelated prior to an R_{max} measurement of magnesium*mag-fura-2. These problems are less critical if mag-indo-1 is used, since the R_{min} and R_{max} for that indicator are very similar for the calcium and magnesium complexes at the excitation and emission wavelengths used in our experiments.

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References

1. Tsien, R.Y. Ann. Rev. Neurosci. (1989) 12: 227-53.
2. Blatter, L. A. and Wier, G. W. (1990) Biophysical J. 58: 1491-9.
3. Konishi, M., Olson, A., Hollingworth, S. and Baylor, S. M. (1988) Biophysical J. 54: 1089-1104.
4. Lattanzio, F. (1990) Biochem. Biophy. Res. Comm. 171: 102-8.
5. Raju, B., Murphy, E., Levy, L. A., Hall, R. D. and London, R. E. (1989). Am. J. Physiol. 256: C540-8.
6. Kao, J. P. Y. and Tsien, R. Y. (1988) Biophysical J. 53: 635-9.
7. Lattanzio, F. A. and Bartschat, D. K. (1991) Biophysical J. 59: 357a.
8. Grynkiewicz, G., Poenie, M and Tsien, R. Y. (1985) J. Biol. Chem. 260:3440-50, 1985.
9. Hesketh, T. R., Smith, G. A., Moore, J. P., Taylor, M. V. and Metcalfe, J. C. (1983) J. Biol. Chem. 258: 4876-82.
10. Levy, L. A., Murphy, E., Raju, B. and London, R. E. (1988) Biochemistry 27: 4041-48.
11. Eberhard, M. and Erne, P. (1989) Biochem. Biophy. Res. Comm. 163: 309-14.
12. Quast, U., Labhardt, A. M. and Doyle, V. M. (1984) Biochem. Biophys. Res. Comm. 123: 604-11.
13. Jackson, A. P., Timmerman, M. P., Bagshaw, C. R. and Ashley, C. C. (1987) FEBS Letters 216: 35-9.
14. Murphy, E., Steenbergen, C., Levy, L.A., Raju, B. and London, R.E. (1989) J. Biol. Chem. 260:5622-5627.
15. Murphy, E., Freudenrich, C. C., Levy, L. A., London, R. E. and Lieberman, M. (1989) Proc. Nat. Acad. Sci. 86: 2981-4.