ARSENAZO III, A RESONANCE RAMAN INDICATOR WITH HIGH SELECTIVITY FOR Ca^{2+}

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1. Introduction

The accurate and rapid measurement of μM levels of free Ca^{2+} in cells is very important in view of the regulatory role of Ca^{2+}, and the existence of rapid changes in its concentration during processes like muscle contraction, photoreceptor cell response [1–3]. The metallochromic dyes murexide, antipyrylazo III and arsenazo III which exhibit spectral changes upon complexation of Ca^{2+} were employed as calcium indicators in [4,5]. Their advantage over atomic absorption and radio-isotope tracing techniques is that the indicators can be used in situ with no need for any sample separations, and they are faster than calcium-specific electrodes. Because of the sensitivity of arsenazo III to <1 μM of Ca^{2+}, it was used extensively in the study of calcium fluxes in many subcellular organelles and in whole cells [4,6–8].

One of the major drawbacks of this indicator is that its complex with Mg^{2+}, as well as with other divalent cations, exhibits similar spectral changes relative to the free dye as the Ca^{2+} complex [4,9]. Therefore, Mg^{2+} interferes strongly in the detection of Ca^{2+}, although the latter has a binding constant which is 1 or 2 orders of magnitude larger than that of Mg^{2+}, depending on the ionic strength of the solution [4,10]. The selectivity to Ca^{2+} can be enhanced, at the cost of lowered sensitivity, by using dual wavelength excitation and differential detection [11].

Here, I demonstrate a novel method with high selectivity for Ca^{2+} over Mg^{2+}, by measuring the resonance Raman (RR) spectrum of the indicator and monitoring changes in this spectrum as a function of binding of Ca^{2+}. Since the vibrational Raman spectrum is very sensitive to conformation [12], one can expect that any possible structural differences between the calcium and magnesium complexes would manifest themselves in the spectra. It is shown that by properly choosing the excitation and scattering wavelengths, high sensitivity to μM levels of Ca^{2+} along with selectivity over Mg^{2+} can be obtained. These results imply the potential usefulness of other RR probes for cations in biological systems.

2. Materials and methods

High purity, calcium-free arsenazo III and ethyleneglycol bis (β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) were purchased from Sigma. $CaCl_2$ and $MgCl_2$ were added from concentrated stock solutions. X-537-A was a gift from Dr A. Gafni.

Large unilamellar liposomes were prepared by the method of freezing and thawing of pre-sonicated liposomes [13]. Soybean lecithin (Sigma) (20 mg) or egg yolk lecithin (Calbiochem) (20 mg) in 1 ml 10 mM Tris buffer (pH 7.0) containing 1 mM $CaCl_2$, were sonicated to clarity, in a cylindrical bath sonicator. The suspension was frozen in liquid nitrogen and left to thaw at room temperature and then sonicated for 1 min. Liposomes with a trapping volume of 10 μl H_2O/mg phospholipid can be obtained by this method [14]. The extraliposomal Ca^{2+} was removed by cation-exchange resin (Dowex 50W-X8, 50-100 mesh, J. T. Baker Chemical Co.) pre-equilibrated with Tris buffer (pH 7.0) in microcolumns in pasteur pipettes [15].

The RR spectra were excited with an Ar^+ laser (Coherent Radiation), analyzed with a Spex-1403 double monochromator and detected with a cooled RCA C31034-02 photo-multiplier tube followed by an amplifier–discriminator (Pacific Instruments). The counted photons were fed into an Apple II microcomputer which also stepped the monochromator and was used for data reduction. This microcomputer-controlled spectrometer will be described elsewhere. Absorption spectra were measured on a Cary-17 spectrophotometer.

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3. Results and discussion

The absorption spectra of arsenazo III and of its complexes with Ca^{2+} and Mg^{2+} are shown in fig.1. As can be seen, both complexes exhibit similar spectral changes relative to the free dye, i.e., increased absorption above 560 nm, and a decrease between 450–560 nm. This is the reason for the need for dual wavelength absorption spectroscopy, at 675–685 nm, if selectivity toward Ca^{2+} is to be enhanced, in the currently used method [4].

The RR spectra of arsenazo III and of its complexes with Ca^{2+} and with Mg^{2+} are shown in fig.2. The spectra span the region where most of the vibrational bands appear. The most important observation is that big changes occur in the RR spectrum upon binding of Ca^{2+}, both in the location and intensity of bands, while the Mg^{2+}–dye complex gives a spectrum quite similar to that of the free dye. The exact assignment of the spectral bands of the dye is not necessary for its analytical use, but may be important for the evaluation of the structural changes occurring in the dye upon binding to a cation.

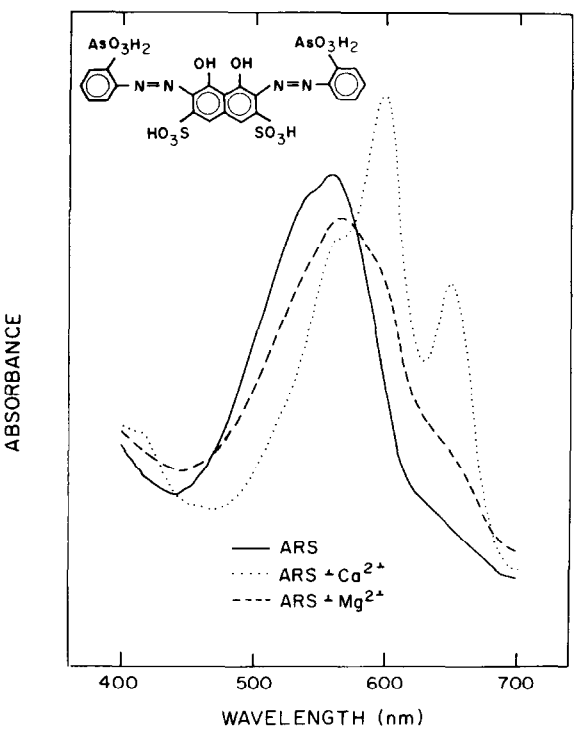

Fig.1. Absorption spectra of arsenazo III (50 μM); arsenazo III (50 μM) + $CaCl_2$ (250 μM); and arsenazo III (50 μM) + $MgCl_2$ (2 mM). All spectra measured in water (pH 7).

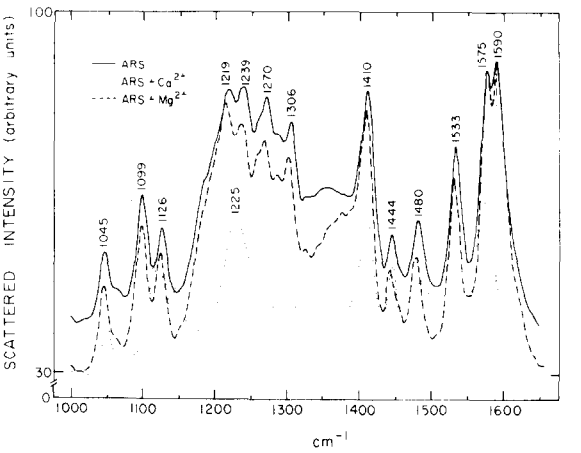

Fig.2. Resonance Raman spectra of arsenazo III, arsenazo III + $CaCl_2$, and arsenazo III + $MgCl_2$; concentrations as in fig.1. The spectra were excited with laser light at 496.5 nm, 100 mW, and detected with spectral resolution of 3 cm^{-1}.

The first point to be noticed is that the intensity of the whole spectrum of the Ca^{2+}–dye complex is much weaker than that of the free dye. This can be readily explained, since these spectra were excited at 496.5 nm, where binding of Ca^{2+} lowers the absorbance of the dye and therefore diminishes the resonance enhancement of the vibrational modes of the complex.

The major change in the region of the aromatic ring modes (1400–1600 cm^{-1}) is the drastic weakening of the 1590 cm^{-1} band and the down-shift of the 1575 cm^{-1} band. Since these bands were also found to be sensitive to deuteration, there could be a contribution from the azo group and from the structure $-\overset{+}{N}H=N-$ whose existence may be enhanced due to the *ortho* phenolic group [16], and whose absence in the complex may indicate the involvement of the azo group in the complexation, and a concomitant strain on the aromatic rings.

The spectral region 1000–1350 cm^{-1} contains aromatic bending modes, sulfonate vibration and a phenolic mode probably at 1219 cm^{-1} which is sensitive to deuteration and disappears in the Ca^{2+} complex, probably moved to 1147 cm^{-1} because of the involvement of the phenolic group in the complexation.

The Mg^{2+} complex hardly exhibits any spectral changes relative to the free dye, and this may be indicative of a difference in the structures of the Ca^{2+} and the Mg^{2+} complexes. Unlike Mg^{2+}, Ca^{2+} can form 2:1, 1:1 and 1:2 complexes with arsenazo III [17].

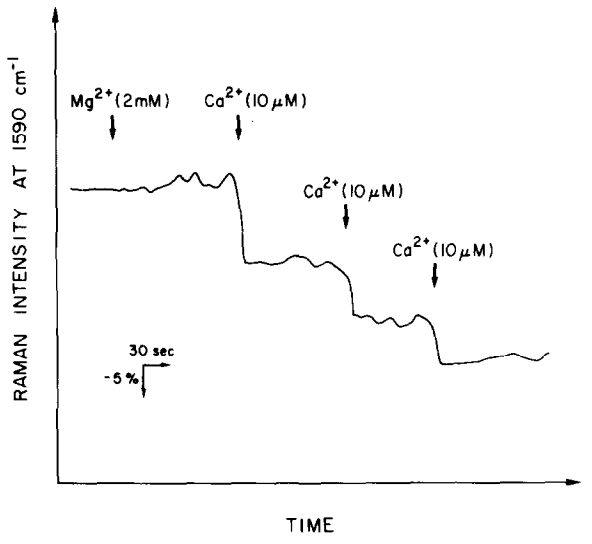

Fig.3. Changes in the resonance Raman scattering intensity of arsenazo III (50 μM) at 1590 cm^{-1} (excitation at 496.5 nm, 100 mW) upon the addition of $MgCl_2$ and $CaCl_2$.

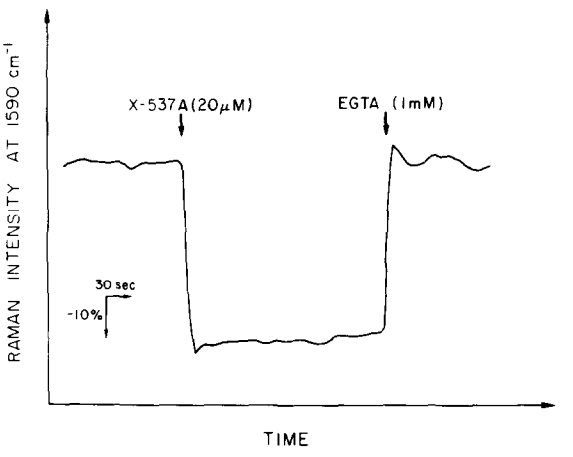

Fig.4. Resonance Raman scattering intensity of arsenazo III (50 μM) at 1590 cm^{-1} (excitation at 496.5 nm, 100 mW). The solution contains large unilamellar liposomes (20 mg/ml) loaded with 1 mM $CaCl_2$, and buffered inside and outside with 10 mM Tris buffer (pH 7). The arrows indicate the addition of X-537 A and EGTA.

Based on these findings, a decrease in the Raman intensity of arsenazo III at 1590 cm^{-1} should be a specific indication to Ca^{2+} with high selectivity over Mg^{2+}. This is observed in fig.3. As was explained, the decrease in intensity is a combination of the resonance enhancement factor and an intrinsic change in the Raman spectrum. The effect of other cations was also tested and it was found that Sr^{2+} and Ba^{2+} interfere slightly at >0.1 mM. K^+ and Na^+, as well as changes over pH 5–9 had no effect on the RR spectra.

The sensitivity of the Raman intensity of arsenazo III to Ca^{2+} is also demonstrated in fig.4. The Ca^{2+} which is trapped in large unilamellar vesicles is released into the calcium-free surrounding medium, by the ionophore X-537A. The efflux is monitored by the RR intensity at 1590 cm^{-1}. No penetration of the dye through the lipid bilayer was observed.

It should be noted that for the routine use of the indicator, one needs only an interference filter for a wavelength which is removed by ~1590 cm^{-1} from the laser wavelength which is being used, and a detecting device for the light passing through the filter.

The RR scattering intensity from the dye arsenazo III at 1550–1600 cm^{-1} is highly sensitive and selective to Ca^{2+}, giving a relative decrease of up to 60%. Thus, arsenazo III may be used as a RR probe for the detection of Ca^{2+} and for the study of its transport in biological systems. The possible use of other indicators as RR probes of various ions of biological interest is being pursued in this laboratory.

Acknowledgement

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