Quin 2: the dissociation constants of its Ca^{2+} and Mg^{2+} complexes and its use in a fluorimetric method for determining the dissociation of Ca^{2+} –protein complexes

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Fluorimetric or spectrophotometric titrations with the appropriate cations gave K_d values of $2.9 \pm 0.2 \text{ nM}$ and $89 \pm 5 \mu\text{M}$ respectively for the Ca^{2+} and Mg^{2+} complexes of quin 2 at pH 7.5. Mixtures of quin 2 and vitamin D-dependent Ca^{2+} -binding protein from pig duodenum were titrated fluorimetrically with Ca^{2+} in the absence or presence of Mg^{2+} . These measurements were used with the K_d values of the Ca^{2+} and Mg^{2+} complexes of quin 2 to obtain K_d or apparent K_d values for Ca^{2+} –protein complexes ranging from 5 nM to 5 μM with good accuracy.

Quin 2 is a tetracarboxylic acid derivative of quinoline with metal-chelating properties, designed and synthesized for use in the measurement of intracellular concentrations of free Ca^{2+} (Tsien, 1980; Tsien *et al.*, 1982). It has a high affinity for Ca^{2+} under physiological conditions, it fluoresces with an emission maximum at 492 nm which is well away from that of proteins, and its fluorescence is enhanced about 6-fold by formation of the Ca^{2+} complex. Its properties suggest that it might also be used to determine the K_d of Ca^{2+} bound to proteins, in a fluorimetric method which does not depend on measurements of the intrinsic fluorescence of the proteins. The method would be especially useful when it is uncertain that changes in the intrinsic fluorescence of a protein during a titration with Ca^{2+} are proportional to the amount of Ca^{2+} bound. Such a problem arises in the case of proteins like those in the troponin-C superfamily, which contain at least two Ca^{2+} -binding sites, tyrosine residues situated asymmetrically relative to the binding sites, and no tryptophan (Barker & Dayhoff, 1979).

The proposed method involves the titration of a mixture of quin 2 and protein with Ca^{2+} and the measurement of changes in quin 2 fluorescence, from which, with the known K_d and stoichiometry of Ca^{2+} -binding to quin 2, concentrations of free and protein-bound Ca^{2+} may be calculated. The method was tested with the vitamin D-dependent Ca^{2+} -binding protein from pig duodenum, a protein in the troponin-C superfamily which consists mainly of two Ca^{2+} -binding sites and which contains only one tyrosine residue (Hof-

mann *et al.*, 1979). It binds Ca^{2+} with K_d values of 4.7 nM and 6.3 nM (Bryant & Andrews, 1984), but addition of Mg^{2+} , which binds at the same sites, produced a range of apparent K_d values for Ca^{2+} up to 5 μM .

Materials and methods

Quin 2 (free acid) was obtained from Lancaster Synthesis, Morecambe, Lancs., U.K. Its absorption spectra in the presence of EDTA and in the presence of excess Ca^{2+} were similar to those shown by Tsien (1980). Analysis with an X-ray spectrometer attached to an electron microscope showed that it was free from Ca^{2+} , although traces of Cu^{2+} and K^+ were detected.

Vitamin D-dependent Ca^{2+} -binding protein was isolated from pig duodenal mucosa (Bryant & Andrews, 1983). The Ca^{2+} -free form was prepared by treatment with Amberlite CG-50 ion-exchange resin (BDH Chemicals, Poole, Dorset, U.K.) and its concentration in solution determined spectrophotometrically (Bryant & Andrews, 1984).

Tris, HCl and KCl were AristaR grade (BDH Chemicals); other chemicals were AnalaR grade. Double-distilled water was used.

Fluorescence titration

Fluorescence measurements were obtained with an Hitachi–Perkin–Elmer model MPF-3L fluorescence spectrometer (Perkin–Elmer, Beaconsfield, Bucks., U.K.). Quin 2 fluorescence was excited at 339 nm, this being the wavelength at which the Ca^{2+} -free form and the Ca^{2+} complex have the

same absorbance, and measured at 492 nm (Tsien, 1980). Monochromator slits were 3.5 nm for excitation and 11 nm for emission. Quin 2 and protein were dissolved in 2 mM-Tris/HCl buffer, pH 7.5, and titrations were performed in a 1 cm-square cell containing 1.5 ml of solution at 25°C. Additions were made in portions of 4 μ l.

Difference spectroscopy

Spectral measurements were made on solutions (2 ml) of quin 2 in 2 mM-Tris/HCl buffer, pH 7.5, in 1 cm-square cells at 25°C with a Perkin-Elmer model 552 double-beam spectrophotometer. Quin 2 concentration was the same in both cells and Mg^{2+} in buffer solution was added in portions (usually 4 μ l) to one cell and equal volumes of buffer were added to the other. Difference spectra were recorded from 350 nm to 210 nm at a scan rate of 2 nm/s, response time 0.5 s and band-pass 2 nm, with absorbance scale settings of -0.3 to $+0.4$.

Results

K_d values for the Ca^{2+} and Mg^{2+} complexes of quin 2

Tsien (1980) and Hesketh *et al.* (1983) reported that quin 2 bound Ca^{2+} and Mg^{2+} with K_d values of 80–90 nM and 2 mM respectively, but these were apparent K_d values, obtained in the presence of KCl concentrations chosen to simulate physiological conditions. Since the effect of K^+ on the

apparent affinities for Ca^{2+} and Mg^{2+} was not determined, K_d values for Ca^{2+} and Mg^{2+} in the absence of a competing cation could not be calculated.

In the absence of competing cations, quin 2 bound Ca^{2+} too tightly to obtain $K_d^{Ca^{2+}}$ by direct titration. However, quin 2 solutions were standardized in this way for Ca^{2+} -binding capacity and $\Delta F_{max.}$, the fluorescence change when saturated with Ca^{2+} . When the molarity of quin 2 solutions was determined spectrophotometrically in the presence of excess Ca^{2+} with ϵ_{245} 3.6×10^4 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ (Tsien, 1980), the material used bound 0.87 mol of Ca^{2+} /mol. Formation of the Ca^{2+} complex increased the fluorescence by a factor of 6.2.

Values for apparent $K_d^{Ca^{2+}}$ were obtained by titrating quin 2 with Ca^{2+} in the presence of different concentrations of Mg^{2+} , which was bound competitively (Fig. 1a). The K_d for Ca^{2+} was therefore related to apparent $K_d^{Ca^{2+}}$ in the presence of Mg^{2+} by:

$$\text{apparent } K_d^{Ca^{2+}} = K_d^{Ca^{2+}} \left(1 + \frac{[Mg^{2+}]}{K_d^{Mg^{2+}}} \right) \quad (1)$$

where $[Mg^{2+}]$ is the concentration of free Mg^{2+} and $K_d^{Mg^{2+}}$ is the dissociation constant of the Mg^{2+} complex of quin 2. The Mg^{2+} concentrations used in the experiments were high enough to use total Mg^{2+} concentration in eqn. (1) instead of free Mg^{2+} concentration without appreciable error. The graph of apparent $K_d^{Ca^{2+}}$ against total Mg^{2+}

Fig. 1. Fluorimetric determination of the binding of Ca^{2+} to quin 2 in the presence of Mg^{2+} . (a) Quin 2 (17 μ M) was titrated with Ca^{2+} in the presence of different concentrations of Mg^{2+} and its fluorescence measured. Experimental points, Mg^{2+} concentrations, and values for apparent $K_d^{Ca^{2+}}$ used to calculate the corresponding curves are respectively: O, 50 mM, 2.0 μ M; ●, 200 mM, 7.1 μ M. The apparent $K_d^{Ca^{2+}}$ values were obtained from Scatchard plots of the results. (b) Graph of values for apparent $K_d^{Ca^{2+}}$ obtained from fluorimetric titrations against total Mg^{2+} concentration.

concentration was linear (Fig. 1b), in agreement with eqn. (1). The ordinate intercept of the graph ($0.26 \pm 0.21 \mu\text{M}$) was $K_d^{\text{Ca}^{2+}}$, but it was not determined with sufficient accuracy to give a reliable value. The slope of the graph [$(3.33 \pm 0.02) \times 10^{-5}$], which was determined more accurately, was $K_d^{\text{Ca}^{2+}}/K_d^{\text{Mg}^{2+}}$.

$K_d^{\text{Mg}^{2+}}$ was obtained from the absorbance changes which occurred when quin 2 was titrated with Mg^{2+} , since formation of the Mg^{2+} complex affected the absorption spectrum of quin 2 more than its fluorescence (Tsien, 1980; Hesketh *et al.*, 1983). The difference spectrum had a positive peak at 263 nm and a negative peak at 241 nm, and the absorbance difference between these two wavelengths was used. Three determinations gave $K_d^{\text{Mg}^{2+}} 89 \pm 5 \mu\text{M}$ for quin 2.

The slope of the graph in Fig. 1(b), combined with $K_d^{\text{Mg}^{2+}}$, then gave $K_d^{\text{Ca}^{2+}} 2.9 \pm 0.2 \text{ nM}$ for quin 2.

Determination of K_d or apparent K_d values for Ca^{2+} -protein complexes

Fluorimetric titration curves for the addition of Ca^{2+} to quin 2 alone and to a mixture of quin 2 and vitamin D-dependent Ca^{2+} -binding protein are shown in Fig. 2(a). The relative amounts of quin 2 and binding protein in the mixture were chosen to give a roughly even distribution of bound Ca^{2+} between the two during the titration, but similar K_d

values were obtained with different ratios of quin 2 and protein. The effect of adding protein is clearly evident. By assuming that the fluorescence changes of the mixture at 492 nm were proportional to the amounts of Ca^{2+} bound to quin 2, free Ca^{2+} concentrations at points on the titration curve were calculated from fluorescence measurements, $K_d^{\text{Ca}^{2+}} 2.9 \text{ nM}$ for quin 2, and the known Ca^{2+} -binding capacity of quin 2 in the mixture. The corresponding concentrations of protein-bound Ca^{2+} were then obtained by difference. The values for free and protein-bound Ca^{2+} concentration fitted a linear Scatchard plot, and a binding curve calculated with a single K_d value (Fig. 2b). The mean value for $K_d^{\text{Ca}^{2+}}$ for the binding protein (Table 1) showed good agreement with $K_d^{\text{Ca}^{2+}}$ obtained by a different method (Bryant & Andrews, 1984), and the concentration of protein-bound Ca^{2+} at saturation confirmed that the protein bound 2 mol of Ca^{2+} /mol.

A range of values for apparent $K_d^{\text{Ca}^{2+}}$ for the Ca^{2+} -protein complex was obtained by adding Mg^{2+} (Table 1). Fluorimetric titrations of the resultant mixtures with Ca^{2+} and measurement of the quin 2 fluorescence showed that the binding of Ca^{2+} was apparently weaker than in the absence of Mg^{2+} (Fig. 2a). Since quin 2 bound Mg^{2+} , the appropriate values for apparent $K_d^{\text{Ca}^{2+}}$ for quin 2 were used in the calculations instead of $K_d^{\text{Ca}^{2+}}$. The results at each Mg^{2+} concentration fitted linear

Fig. 2. Determination of K_d and apparent K_d values for Ca^{2+} -protein complexes by fluorimetric titration with Ca^{2+} in the presence of quin 2

(a) Fluorimetric titrations. Quin 2 ($28 \mu\text{M}$) was titrated with Ca^{2+} : ○, in the absence of both Ca^{2+} -binding protein and Mg^{2+} ; ●, in the presence of $20 \mu\text{M}$ - Ca^{2+} -binding protein; △, in the presence of $10 \mu\text{M}$ - Ca^{2+} -binding protein and 100 mM - Mg^{2+} . (b) Theoretical curves fitted to results which were obtained from (a) as described in the text. ○, Protein-bound Ca^{2+} in the absence of Mg^{2+} and the binding curve calculated with $K_d^{\text{Ca}^{2+}} 4.9 \text{ nM}$; ●, protein-bound Ca^{2+} in the presence of 100 mM - Mg^{2+} and the binding curve calculated with apparent $K_d^{\text{Ca}^{2+}} 5.0 \mu\text{M}$.

Table 1. Determination of K_d or apparent K_d values for Ca^{2+} bound to vitamin D-dependent Ca^{2+} -binding protein in the absence or presence of Mg^{2+} by fluorimetric titration in the presence of quin 2

Results (a) are each the mean of three determinations. Values for apparent $K_d^{\text{Ca}^{2+}}$ in the presence of Mg^{2+} were calculated with eqn. (1) in the text and either (b) $K_d^{\text{Ca}^{2+}}$ 2.9 nM and $K_d^{\text{Mg}^{2+}}$ 89 μM for quin 2 (the present paper) or (c) $K_d^{\text{Ca}^{2+}}$ 5.5 nM and $K_d^{\text{Mg}^{2+}}$ 100 μM for the Ca^{2+} -binding protein (Bryant & Andrews, 1984).

$K_d^{\text{Ca}^{2+}}$ or apparent $K_d^{\text{Ca}^{2+}}$ (nM)			
[Mg^{2+}] (mM)	Quin 2 (calculated)	Vitamin D-dependent Ca^{2+} -binding protein	
		Found	Calculated
0	2.9	3.7 ± 1.1 (a)	4.7, 6.3
1	35 (b)	48 ± 12 (a)	56 (c)
10	330 (b)	540 ± 100 (a)	510 (c)
100	3250 (b)	5000 ± 100 (a)	5000 (c)

Scatchard plots and binding curves calculated with single K_d values (Fig. 2b), and the mean values for apparent $K_d^{\text{Ca}^{2+}}$ for the protein showed good agreement with ones calculated from other data (Table 1).

Discussion

Quin 2 binds Ca^{2+} with very high affinity, and this makes it especially useful, in the method described above, for determining K_d values for Ca^{2+} -protein complexes in the nanomolar range. Even in this range, contamination of buffers with Ca^{2+} was not a problem, possibly because the quin 2 provided sufficient Ca^{2+} -buffering capacity to deal with it. If the protein has a much lower affinity for Ca^{2+} than has quin 2, titration of quin 2-protein mixtures with no competitive cation present may not give satisfactory results, whatever the relative amounts of quin 2 and protein, because of the uneven distribution of bound Ca^{2+} between the two during the titration. Calculations with eqn. (1) and possible values for the K_d of protein-bound Ca^{2+} and Mg^{2+} indicate that, in such cases, addition of Mg^{2+} may well result in a more even distribution of bound Ca^{2+} . The calculation of apparent $K_d^{\text{Ca}^{2+}}$ values for the protein in the presence of Mg^{2+} should then enable the determination of $K_d^{\text{Ca}^{2+}}$ by extrapolation of the results to zero Mg^{2+} concentration.

In order to avoid problems due to contamination with Ca^{2+} , methods for determining K_d values for high-affinity Ca^{2+} binding depend, like the proposed method with quin 2, on competitive binding of the Ca^{2+} . The results therefore depend on the

accuracy of K_d values for other binding equilibria which are used in the calculations. The precision of the method with quin 2 is indicated by the standard errors on the results in Table 1, and the precision of other methods may be assessed from published results. For example, titration of the vitamin D-dependent Ca^{2+} -binding protein with Ca^{2+} in the presence of Mn^{2+} , and a separate determination of K_d values for Mn^{2+} , gave $K_d^{\text{Ca}^{2+}}$ 4.7 ± 0.8 nM and 6.3 ± 1.8 nM for the two sites (Bryant & Andrews, 1984). Ca^{2+} buffers containing EGTA are widely used in Ca^{2+} -binding studies to control free Ca^{2+} concentrations, and K_d values for Ca^{2+} -EGTA are usually calculated from the metal-chelation data of Schwarzenbach *et al.* (1957). On this basis, titrations of α -lactalbumin in the presence of EGTA and measurement of the changes in protein fluorescence gave values for $K_d^{\text{Ca}^{2+}}$ in the range 1.5–3.0 nM (Permyakov *et al.*, 1981). However, a different K_d for Ca^{2+} -EGTA may be required for conditions used in experiments with proteins (Kim & Padilla, 1980), and on this basis a fluorimetric titration with Ca^{2+} in the presence of EGTA gave $K_d^{\text{Ca}^{2+}}$ 0.2 nM for α -lactalbumin (Murakami *et al.*, 1982). The method with quin 2 has the advantage over methods with EGTA that the affinity of quin 2 for Ca^{2+} in different experimental conditions is easily determined, and the results indicate that its precision is as good as that of other titrimetric methods for determining K_d values for high-affinity binding of Ca^{2+} to protein.

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