EUROPIUM II AS A REPLACEMENT FOR CALCIUM II IN CONCANAVALIN A

A precipitation assay and magnetic circular dichroism study

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1. Introduction

The europium II ion has an ionic radius (1.1 Å) and chemical properties [1] which lie between those of calcium II (0.99 Å) and strontium II (1.13 Å) [2]. In the solubilities and structures of its salts it bears a closer resemblance to the group IIA cations (excluding magnesium II) than do the trivalent lanthanide ions which are commonly used as spectroscopically active probes for calcium II in biological systems [2,3]. We report here the first example of the replacement of calcium II by europium II in a protein, concanavalin A. This leads to a functioning system in the glycogan assay with an activity between that produced by strontium II and calcium II. Measurements of the magnetic circular dichroism (MCD) spectra of the europium II ion reveals a kinetic process in the presence of concanavalin A leading to a spectrum which is quite different to that observed for the aquo and EDTA complexes.

Apo-concanavalin A requires manganese II or another divalent transition metal ion bound to a site designated S1 before binding calcium II at an adjacent site (S2) and becoming fully active in complexing carbohydrates [4]. In the context of this paper it is noteworthy that the trivalent lanthanide ions do not substitute for either manganese II or calcium II but bind instead to another carboxylate site, S3 [5,6]. There have been a number of recent observations of kinetic phenomena accompanying the interaction of metal ions with concanavalin A under conditions where it is probable that site S2 is involved. On adding calcium II to manganese-activated apo-concanavalin A, time-dependent changes were observed in the protein difference spectra [7] and the proton relaxation enhancement [8,9]. The evidence presented here links a kinetic process directly with site S2.

2. Experimental

Concanavalin A (Sigma) was demetallised by the method in [4] with dialyses against 0.2 M sodium chloride and finally pH 5.25 buffer (0.05 M sodium acetate, 0.2 M sodium chloride). During each dialysis nitrogen, freed of oxygen by passage through chromium II solutions, was passed through the dialysate to remove oxygen. Transfers of dialysis sacks and manipulation of solutions were carried out in a glove box under nitrogen. Protein concentration was determined spectrophotometrically [10]. Manganese II was determined by ESR after acidification and calcium by atomic absorption, both metals were present to less than 0.06 g.atom/mol protein in all solutions containing apo-concanavalin A.

Europium III oxide (99.9%, Rare Earth Products Ltd.) was dissolved in perchloric acid and reduced electrolytically by a potential of 2.5 V between a mercury cathode and platinum anode. All manipulations of the resulting europium II perchlorate solutions were carried out under purified nitrogen. The europium II concentration was determined by oxidation with permanganate followed by back titration with iron II.

Absorbance measurements in the glycogen (Sigma) precipitation reaction were carried out on a Cecil 272 at 420 nm, spectra were run on a Cary 14. MCD spectra were recorded at room temperature with a

Cary 61 dichrograph using a superconducting solenoid generating 5.1 T.

3. Results and discussion

The glycogen precipitation reaction, monitored at 420 nm, provides a convenient assay of concanavalin A activity [11] which is unaffected by the absorption spectrum of the europium II ion. The time required for an absorbance of 0.5 to be reached has been used recently to assess activation by metal ions [12]. The relative activating effects of several metal ions at approx. 10^{-3} M are compared in fig.1 from which the following observations can be made. In the absence of a potential occupant for S2 there is very little activity, whereas in the presence of calcium II precipitation occurs within a few minutes. Since europium III can arise from the oxidation of europium II it was important to establish that this ion was not an effective activator, confirmation of this is obtained from the data in fig.1 and is in agreement with the failure of europium III to bind at S2 [5]. Europium II can be seen to activate concanavalin A with an efficiency between that of strontium II and calcium II, whereas barium II shows no activation.

The efficiency of activation in the glycogen precipitation assay follows the ionic radius rather closely when the series Ca^{2+} (0.99 Å), Eu^{2+} (1.1 Å), Sr^{2+} (1.13 Å) is considered, Ba^{2+} (1.35 Å) is too large and is known not to bind to S2 [13]. The smallest ion known to bind to S2 is, cadmium II (0.97 Å) and it is likely that strontium II (1.13 Å) is the largest as lead II (1.21 Å) binds to S3 and elsewhere [5,14]. Thus it appears that the S2 site discriminates metal ions on the basis of size and change, not binding the trivalent lanthanide ions [5,6], but does not discriminate between the 'a-type' or 'hard' ions Ca^{2+}, Eu^{2+} and the 'b-type' or 'soft' ion Cd^{2+}. It is probable that the extra charge of a trivalent ion cannot be accommodated at S2 because the increased electrostatic repulsion, due to its proximity to the divalent ion at S1, is not sufficiently attenuated by the overall negative charge of the ligands (−4) [14] and the small degree of electron delocalisation on the 'type a' metals at both S1 and S2.

The absorption spectrum of europium II in water, pH 7.8, (fig.2) shows two major bands at 248 nm (ϵ 1920) and 320 nm (ϵ 680). These bands are attrib-

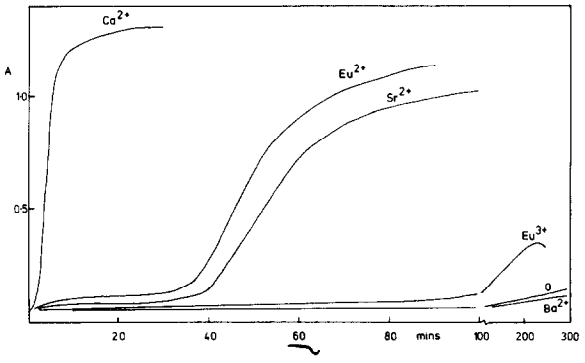

Fig.1. Glycogen precipitation assay showing A_{420} as a function of time after the addition of metal ions to a solution containing glycogen (320 μg/ml), apo-concanavalin A (3.9×10^{-5} M) manganese II (2×10^{-4} M) in acetate buffer, pH 5.25, 25°C. Final metal ion concentrations were 10^{-3} M except for europium II (8.8×10^{-4} M).

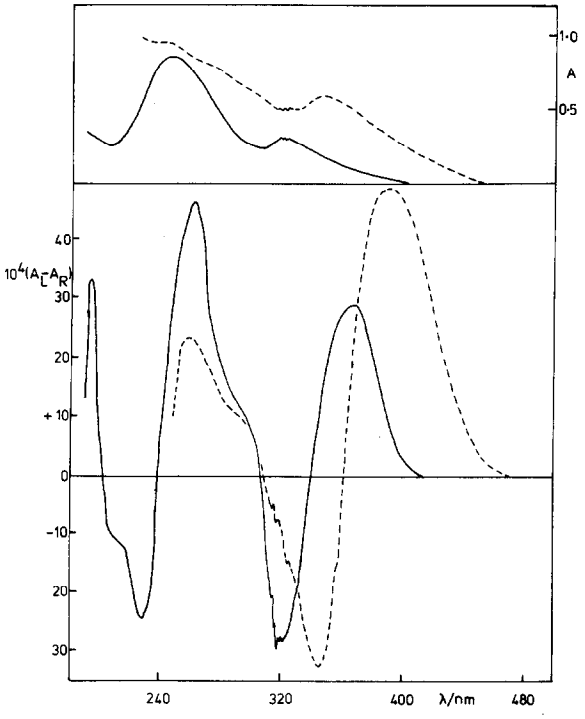

Fig.2. Absorption (upper cuves) and MCD spectra of europium II perchlorate in 0.5 cm cells. Solid line: europium II (8.84×10^{-4} M) in water pH 7.8. Broken line: europium II (1.69×10^{-3} M), EDTA (9.3×10^{-2} M), pH 11.8.

uted to transitions from the $4f^7$, ^8S, ground state to an excited state arising from the $4f^65d^1$ configuration [15,16]. Their intensity can be contrasted with that of the Laporte forbidden f → f transitions which are just discernable superimposed on the 320 nm band. The sensitivity of the europium II spectra to ligands is demonstrated by the effect of EDTA at high pH (fig.2); like the calcium and unlike the trivalent lanthanide ions mM concentrations of europium II do not precipitate at high pH even in the absence of a complexing agent. In the presence of EDTA the lower energy band undergoes a red shift to 350 nm whilst the higher energy maximum shifts to the blue where it is overlayed by ligand absorption, shoulders also develop at 270 nm and 243 nm. Again it is interesting to contrast this behaviour of the f → d bands with that of the f → f transitions which remained unchanged at 320 nm. The d orbital of the excited europous ion is much more responsive to the changing ligand field than the buried f orbitals.

In a cubic or octahedral site the d orbitals will split into two energy levels giving rise to the two bands observed in the spectrum, in lower symmetries more bands could be expected. The separation of the excited levels will depend on the nature of the ligands whilst the ground state remains essentially unaffected, thus the separations of the absorption bands or the shift of either gives a measure of the crystal field splitting and hence the nature of the ligands. As expected EDTA complexation increased the splitting, analogous effects have been produced by changes in the lattice parameters of host crystals [17]. A much more rigorous consideration of the many electron problems gives rise to two sets of transitions separated by a crystal field-dependent energy [16]; this justifies in some measure the simplified picture given above.

Strong magnetic circular dichroism (MCD) spectra were obtained from the europium II ion, fig.2. On addition of EDTA the spectrum underwent a comparable shift to that of the absorption spectrum, again the f → f transitions, which are now more clearly seen at 320 nm, are relatively unaffected by the ligand. The only previous report of the MCD of the europium II ion appears to be a low temperature measurement on doped calcium fluoride [18] in which only the low energy band was studied, a marked temperature dependence of the MCD is to be expected [19] but the spectrum has similarities with the

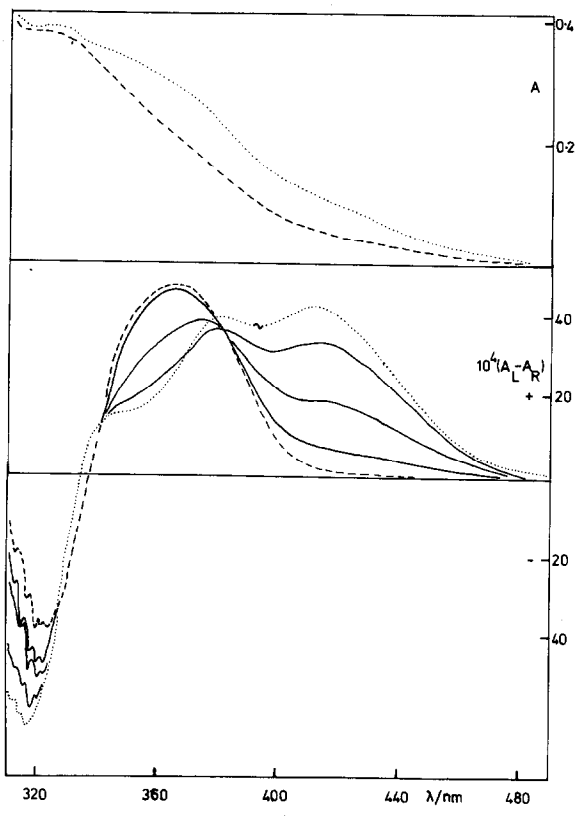

Fig.3. Absorption (upper curves) and MCD spectra of europium II in the presence of concanavalin A at pH 5.25. Broken line: calcium II and manganese II (5.6×10^{-3} M), europium II (3.7×10^{-3} M), apo-concanavalin A (5.7×10^{-4} M), 0.5 cm cell, 5 h after mixing. Solid and dotted lines: no calcium, manganese II (5.6×10^{-3} M), europium II (1.87×10^{-3} M), apo-concanavalin A (6.9×10^{-4} M), 1.0 cm cell, after 0.25 h, 1.25 h, 3.25 h and (dotted line) 6 h, respectively.

apparent A term shape depicted in fig.2.

Europium II added to apo-concanavalin A which had been activated with manganese II and calcium II under anaerobic conditions gave MCD spectrum indistinguishable from that in the absence of concanavalin A and its form was unchanged after 5 h, (fig.3). If calcium II was omitted the spectrum underwent time-dependent changes over a period of several hours with the growth of a new long wavelength positive maximum at 413 nm and other features which are evident in the final spectrum of fig.3, the small biphasic feature at 393 nm is due to europium III

formed by oxidation. The absorption spectrum also underwent changes with shoulders developing at 370 nm and 415 nm, closely related to the new MCD bands.

We interpret the time dependence as a reflection of the evolution of a stronger europium II binding site resulting from a slow confirmational change in the protein which is triggered by the binding of europium II. The site involved is clearly S2 as no changes in the spectrum are observed in the presence of competing calcium. The sugar binding site is also affected as a time dependent binding of 4-methylumbelliferyl α-D-mannopyranoside [20] occurs on the addition of calcium II to manganese II-activated concanavalin A (R.B.H. and B.D.M., unpublished). We consider the conformational change to be the same process as that inferred from NMR measurements [9] over a similar time scale and attributed to the *cis–trans* isomerisation of a proline amide bond. The splitting of the long wavelength MCD band may reflect the reduction of symmetry experienced by europium II on bonding to the fully evolved, seven coordinate calcium site defined by X-ray crystallography [14].

This work demonstrates that europium II is a useful probe for calcium binding sites including those which cannot be investigated by using the lanthanide III ions. In addition to the techniques employed here, fluorescence, ESR and Mössbauer spectra can be measured [2], it has also been shown that europium II can be used in proton relaxation studies [21]. Further work is required to characterise its spectra in different environments. The chief difficulty is in the powerful reducing character of the ion making it necessary to work anaerobically and choose systems with care.

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