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# BINDING STOICHIOMETRY OF GADOLINIUM TO ACTIN: ITS EFFECT ON THE ACTIN-BOUND DIVALENT CATION

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Summary: Using $^{45}Ca^{2+}$ and $^{153}Gd^{3+}$ we studied the effects of binding the lanthanide ion, gadolinium, to skeletal muscle G-actin. $Gd^{3+}$ can specifically displace 6-7 $Ca^{2+}$ from their binding sites on actin. Furthermore, a total of 6-7 $Gd^{3+}$ can be shown to bind to actin, and this result is not affected by the subsequent addition of polymerizing quantities of KCl. We conclude that $Gd^{3+}$ binds only to the $Ca^{2+}$-binding sites of actin. The number of these $Gd^{3+}$ sites closely corresponds to the known number of high and low affinity sites for divalent cations such as $Ca^{2+}$.

## INTRODUCTION

In a series of recent reports (1-3) we have demonstrated that gadolinium ($Gd^{3+}$) and other ions of the lanthanide series can cause the formation of highly-ordered aggregates of actin subunits. These aggregates have been shown to be quite different from either F-actin or aggregates of F-actin (paracrystals) and their structure is at present being analysed. Implicit in these reports was the fact that the lanthanide ions do indeed bind to actin, for how else could they affect the viscosity of actin in the ways we have demonstrated? However, these studies raise two important questions. Do the lanthanide ions bind to actin? And, if so, do they displace the native divalent cations from the high and low affinity sites on this protein? (4-6) This report resolves these questions.

## Materials and Methods

Actin was prepared as described previously (1). The buffer used throughout these experiments was piperazine-N, N'-bis (2-ethanesulphonic acid) (PIPES). The G-actin solution contained: 0.15mM ATP; 0.5mM β-mercaptoethanol; 2mM $NaN_3$; 2.0mM PIPES

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pH 6.9. This actin (35-50 µg) is observed to migrate essentially as a single band on SDS (sodium dodecyl sulphate) gel electrophoresis (7) and is apparently free of other known contractile proteins. Protein concentration was determined by the absorbance at 290 nm using an extinction coefficient for G-actin of 0.63 mg $cm^{-2}$ (8). Lanthanide ion concentration was determined from stock solutions as described previously (1) and accurate dilutions were made just before an experimental solution was required. Electron paramagnetic resonance (EPR) binding measurements were performed using 160 µM (6.7 mg/ml) actin in quartz cells at 20°C in a Varian E4 EPR spectrometer. EPR spectra peak height was shown to be a linear function of $Gd^{3+}$ concentration over the range 3-6 x $10^{-3}$M. Samples of actin containing $^{153}Gd^{3+}$ or $^{45}Ca^{2+}$ were counted on a Nuclear-Chicago 1105 spectro/shield butane/helium gas flow counter using a window in the Geiger operational mode. All results were corrected to allow for the instrument's electronic counting dead time of 120 µs.

## RESULTS

### Binding of $Gd^{3+}$ to Actin. $^{153}Gd^{3+}$ Experiments:

These experiments were performed as follows: (i) 50 µM G-actin (2.09 mg/ml) was incubated with $Ca^{2+}$ (100 µM) in the G-actin solution for 60 min at 0°C; (ii) a slurry of Dowex 50 ($H^+$) equal to 1/14th the total volume was added, mixed and sedimented after 90 sec; (iii) in the presence of 150 µM ATP, G-actin was incubated with the stated molar ratios of $^{153}Gd$/Gd for 30 min. at 20°C; (iv) actin was polymerized in 0.1M KCl for 60 min. at 20°C and the $^{153}Gd^{3+}$ content determined; (v) the actin was then sedimented (80,000 xg for 180 min.); (vi) both the pellet (treated with 2 N HCl to release the $^{153}Gd$/Gd) and the supernatant were counted at least in quadruplicate. The results of three separate experiments are shown in Fig. 1. These data establish that the first 3 moles of $Gd^{3+}$ added to the G-actin/ATP solution remain in the supernatant, i.e. no $Gd^{3+}$ is apparently bound to actin. With the addition of 3.0 to approximately 9.5 moles of $Gd^{3+}$ there is a linear increase in the number of moles of $Gd^{3+}$ which bind to actin. Addition of more $Gd^{3+}$ resulted in no further binding. We found good numerical

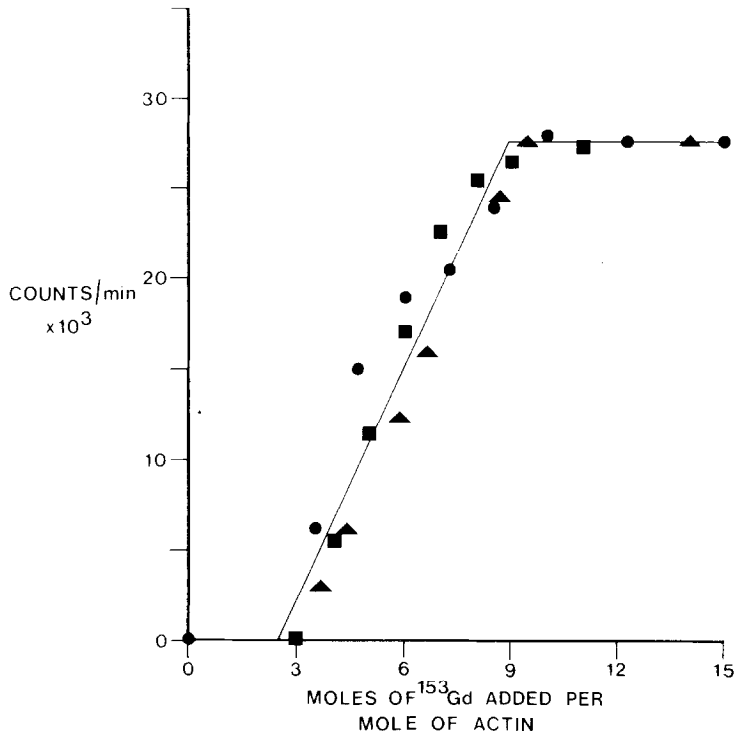


Figure 1. The effect of adding $^{153}GdCl_3$ to 50 μm G-actin (in 0.15mM ATP; 0.5mM β-mercaptoethanol; 2mM PIPES, pH6.9; 2mM $NaN_3$) prior to addition of 0.1M KCl followed by sedimentation. In three separate experiments, β emission was counted and found not to increase in the pellet until the concentration of $^{153}Gd^{3+}$ had exceeded the free ATP concentration. The addition of 6.5 ± 0.3 (mean ± S.E.M.) moles of $^{153}Gd^{3+}$ per actin (in excess of free ATP concentration) resulted in a linear increase in activity from the pellets (correlation coefficient, r = 0.963). There was a corresponding decrease in the counts from the supernatant. Any further addition of $^{153}Gd^{3+}$ had no effect on the total number of counts from the pellets. Each data point is the mean of 4 separate measurements and was obtained from separate samples.

equivalence between the total counts of $^{153}Gd^{3+}$ (i.e. the counts from the G-actin plus $^{153}Gd^{3+}$ in solution) and the sum of the counts from the supernatant and actin pellets. The relationship between bound and added $Gd^{3+}$ is well fitted (correlation coefficient, r = 0.963) by the linear least squares relationship (solid line). Thus an average of 6.5 ± 0.3 (mean ± S.E.M.) moles of $Gd^{3+}$ bind to actin. The binding of $Gd^{3+}$ to actin

commences after all of the available ATP molecules in solution have formed a ligand with $Gd^{3+}$ (1).

EPR Experiments. $Gd^{3+}$ is the only lanthanide ion to exhibit an electron paramagnetic resonance spectrum at room temperature. The spectrum is centred at G = 2 and is broad (3500 ± 1000 gauss). The peak is broadened 8-fold when $Gd^{3+}$ binds to ATP and for practical purposes the spectrum is unmeasurable. Furthermore, when $Gd^{3+}$ binds to macromolecules such as G-actin, the spectrum is undetectable. The appearance of an EPR spectrum demonstrates free $Gd^{3+}$ in solution. By extrapolating the curve relating EPR peak height to the moles of $Gd^{3+}$ added per mole of G-actin, we can determine the number of moles of $Gd^{3+}$ bound to actin, both in the presence and absence of 0.1M KCl.

Figure 2a illustrates the results obtained when increasing concentrations of $Gd^{3+}$ are added to 160 μm G-actin (6.7 mg/ml) in a solution containing 150 μm ATP. The data show that 6.5 moles of $Gd^{3+}$ are bound but this must be reduced by approximately 1 to 5.5 to account for the known ATP binding (see Fig. 1).

Figure 2b summarizes data obtained when $Gd^{3+}$ is bound to actin after which KCl is added to a final 0.1 M. The EPR peak height indicates that a net 6.0 moles of $Gd^{3+}$ remain bound to actin in the presence of 0.1 M KCl, after allowing for ATP binding.

In the presence of 11 ATP's per actin in solution, 6.7 moles of $Gd^{3+}$ were found to bind to actin. The average of these data (6.1 ± 0.3 moles of $Gd^{3+}$ per actin) is in reasonable agreement with the $^{153}Gd^{3+}$ experiments reported above.

Displacement of $^{45}Ca^{2+}$ from Actin by $Gd^{3+}$. 50 μm G-actin was incubated with 100 μm $^{45}Ca/Ca$ (60 min, 0°C). Any unbound $Ca^{2+}$ was removed by a brief (90 sec) exposure to Dowex 50 ($H^+$). This procedure specifically labelled the high affinity divalent

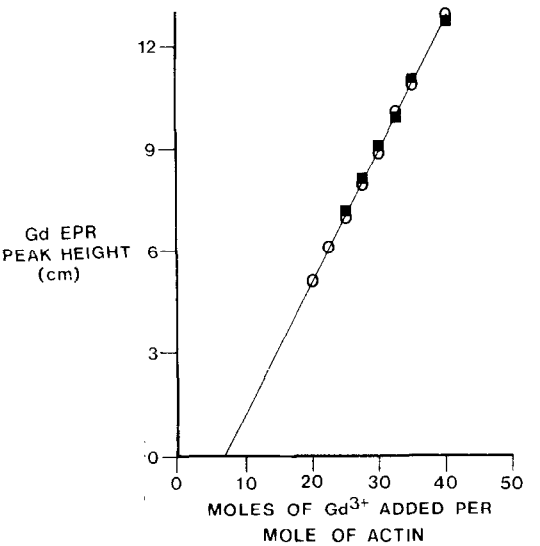


Figure 2. At a machine gain of 6.3 X $10^3$, 3-6 mM $GdCl_3$ was added to 0.2 ml samples of 160 µm G-actin (for reaction conditions see Fig. 1 legend). In the presence of 150 µM free ATP, data was obtained from samples: (a) in the absence of added KCl (■); and (b) in the presence of 0.1M KCl (O). The intercept of the linear regression with the abscissa was corrected by the concentration of ATP in solution (see reference 1). We thereby obtained a measure of the total number of $Gd^{3+}$ bound to actin. The correlation coefficient of the linear regression fitted to each set of data is 0.999. These data indicated that the average number of $Gd^{3+}$ bound to G-actin is 5.8 ± 0.2 moles.

cation site of G-actin with stoichiometric (1:1) amounts of $^{45}Ca^{2+}$. To this labelled G-actin, varying amounts of $Gd^{3+}$ were added (30 min, 0°C) and then KCl was added to a final concentration of 0.1 M. The protein was then sedimented and the supernatant was counted as previously described. Each data point is an average of 4 samples. Figure 3 illustrates the percentage of $^{45}Ca^{2+}$ released from actin by varying amounts of added $Gd^{3+}$. After adding 3 moles of $Gd^{3+}$ per mole of actin $^{45}Ca^{2+}$ is progressively displaced from actin. After addition of 9-10 moles of $Gd^{3+}$ there is no further release of $^{45}Ca^{2+}$. We know that the actin solution contains 3 moles of ATP per actin, and since $^{45}Ca^{2+}$ displacement is detected after the addition of only 3

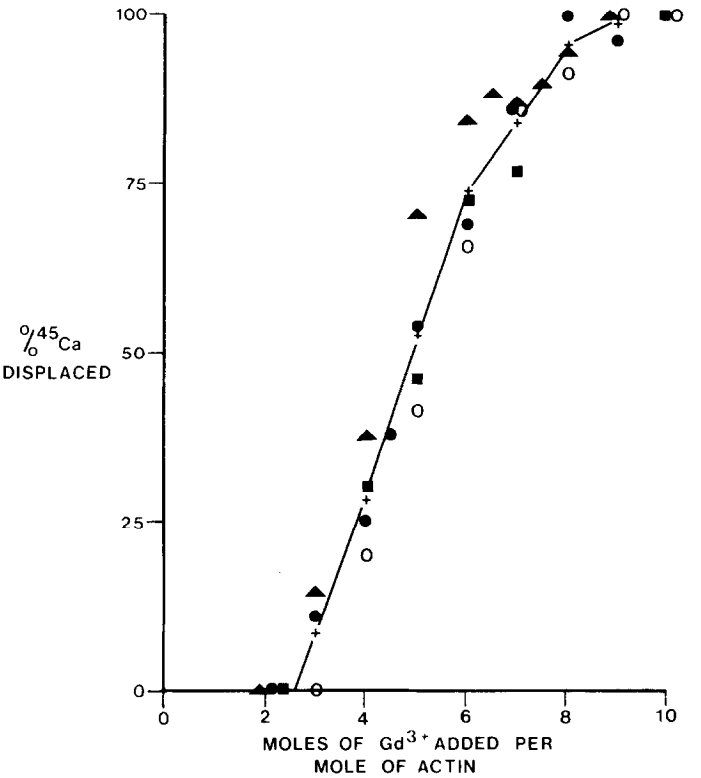


Figure 3. $GdCl_3$ was added to samples of 50 μM $^{45}$Ca-G-actin (for reaction conditions see Fig. 1 legend) in the presence of 150 μM free ATP. The proportion of $^{45}Ca^{2+}$ released from actin by $Gd^{3+}$ was measured in 4 separate experiments, with each data point being the mean of 4 separate determinations. The means of the data are shown by (+). The solid line joins the means. Approximately 6.5 moles of $Gd^{3+}$ per actin (in excess of the free ATP) are required to displace 100% of the $^{45}Ca^{2+}$ from the binding sites.

moles of $Gd^{3+}$ per actin, we conclude that $Gd^{3+}$ begins to displace $Ca^{2+}$ from the single high affinity site on actin when all of the ATP molecules in solution have formed a ligand with $Gd^{3+}$. These data confirm the earlier observations i.e. actin contains a total of 6-7 Ca-binding sites. $Gd^{3+}$ can apparently displace all labelled $Ca^{2+}$.

## DISCUSSION

We have reported elsewhere (9-11) that $Gd^{3+}$ can interact with Ca-binding sites and, only by assuming some binding of $Gd^{3+}$ to

G-actin, could our previous data (1-3) be sensibly interpreted. The data presented here unequivocally demonstrate that $Gd^{3+}$ binds to G-actin. The maximum number of moles of $Gd^{3+}$ which bind is apparently between 6.1 and 6.5. From the $^{45}Ca^{2+}$ experiments we can deduce that the high affinity Ca-binding site of actin (i.e. the one site which retains $^{45}Ca^{2+}$ after treatment with Dowex 50) has a higher affinity for $Gd^{3+}$. 6-7 moles of $Gd^{3+}$ are required to displace all the bound $Ca^{2+}$. There is therefore a close correspondence between the number of moles of bound $Gd^{3+}$ and the number required to displace $Ca^{2+}$. The EPR data indicate that the number of moles of $Gd^{3+}$ bound is not affected by 0.1M KCl. This interpretation assumes that $Gd^{3+}$ displaces $Ca^{2+}$ from its high affinity site first and then from the 5-7 known (4-6) low affinity sites on actin. Thus, we can conclude that the number of $Ca^{2+}$ and $Gd^{3+}$ binding sites are equal and that the sites are probably identical.

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