The 240-kDa subunit of human erythrocyte spectrin binds calmodulin at micromolar calcium concentrations

Åsa Berglund, Lars Backman and Vithaldas P. Shanbhag

Department of Biochemistry, University of Umeå, S-901 87 Umeå, Sweden

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The binding of the isolated α-subunit of human erythrocyte spectrin to calmodulin is demonstrated by partitioning in aqueous two-phase systems. The affinity of the α-subunit for calmodulin is slightly higher than that of the spectrin dimer, whereas the β-subunit interacts only very weakly. The binding is in all cases calcium-dependent and is abolished on addition of chlorpromazine. At an ionic strength close to physiological conditions, about 1 μM free calcium is required to induce maximum binding of calmodulin to spectrin dimer.

Calmodulin (Human) Spectrin Erythrocyte cytoskeleton Aqueous two-phase partitioning

1. INTRODUCTION

The red cell cytoskeleton is mainly composed of spectrin, a 100 nm long and flexible protein containing two antiparallel, non-identical subunits of 220 kDa (β) and 240 kDa (α), respectively [1–3]. Spectrin-like molecules, such as brain fodrin and intestinal TW 260/240, have been found in a variety of non-erythroid cells. These proteins show large structural similarities, share an actin-binding capacity and are immunologically related [4–6]. In spite of many characteristics in common with human spectrin, the proteins are not identical since there is rather poor immunological cross-reaction between fodrin and human erythrocyte spectrin [4–7]. In addition, there are differences between the peptide maps of mammalian erythrocyte spectrin and those of fodrin and avian spectrin-like proteins [7]. A difference also exists in the binding of calmodulin to different spectrins. While the binding to fodrin, TW 260/240 protein and avian spectrin is readily demonstrated [8,9], a weak interaction was only recently found between calmodulin and human erythrocyte spectrin [10–12]. The binding site for calmodulin on the avian spectrin-like molecules has in all cases been found on a common 240 kDa subunit [8]. A similar subunit present in mammalian fodrin has also been shown to contain a calmodulin-binding site [13,14]. Considering all the properties the spectrins have in common, it is reasonable to expect that the calmodulin-binding site in human erythrocyte spectrin is also located on the 240 kDa subunit. Available experimental evidence suggests, however, that the binding site is either on the β-subunit [15] or on both subunits [16]. Since the high urea concentration (6 M) used in these experiments might have exposed a binding site which is not physiologically relevant, we have reexamined the binding in the absence of urea, using partitioning in polymer two-phase systems. The discovery that calmodulin binds to spectrin and spectrin-like proteins gave rise to speculations concerning a possible role of calmodulin in the regulation of cytoskeletal organization [17], but no evidence for such a role has yet been presented. Before trying to judge the physiological significance of the spectrin-calmodulin binding in the human erythrocyte, it is necessary to consider the amount of calcium required for an interaction in relation to the amount of calcium present in the erythrocyte. The free calcium concentration in a resting red cell is thought to be very low, 10^{-8}–10^{-7} M [18], and not sufficient for activating calmodulin. However,

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shear stress has been shown to increase the influx of calcium [19] and this could cause large local changes in the calcium concentration, sufficient to activate calmodulin. Furthermore, there are differences in the literature concerning the amount of calcium required for calmodulin to activate enzymes or bind to other target proteins [20–22]. A possible explanation for this is that different target proteins might interact with different conformational states of calmodulin [23] and thus require different amounts of calcium.

Therefore, we have examined the amount of calcium required for the two proteins to interact, in order to determine whether the binding between calmodulin and human spectrin may occur at physiological calcium concentrations.

2. MATERIALS AND METHODS

Human spectrin dimer was purified from fresh blood by the method of Ohanian and Gratzer [24], using dissociation of cytoskeletons in Tris-HCl and gel filtration. After concentration of spectrin by ammonium sulphate precipitation and dialysis against 10 mM Tris-HCl (pH 7.5), the protein solution was warmed to 37°C for 15 min to remove any large aggregates of spectrin. The α- and β-subunits were obtained by dissociation of the spectrin dimer in urea followed by separation on an ion exchanger as described by Yoshino and Marchesi [25]. Calmodulin was prepared from bovine brain [26] and radiolabelled by reductive methylation [27] using [^{14}C]formaldehyde (New England Nuclear). Gel electrophoresis [28] was used to determine the purity of the protein preparations.

Stock solution of $CaCl_2$ was purchased from BDH. To control the calcium concentration in the phase systems, all buffers and polymers were freed from calcium by treatment with Chelex X-100 resin (Bio-Rad). The total amount of calcium in the final phase systems was determined by flame atomic absorption spectroscopy. The free calcium concentration was controlled by calcium-EGTA buffers and the amount of free Ca^{2+} was calculated using an association constant of 2.98×10^6 M^{-1} derived from stability constants given by Sillén and Martell [29].

The polymer two-phase systems [30] were prepared in the following way: a phase-mixture containing 14.7% (w/w) Ficoll 400, 8.0% (w/w) dextran T500 (both from Pharmacia, Sweden), 0.27% (w/w) polyethylene glycol 8000 (Union Carbide, USA), 133 mM KCl, 0.13 mM $MgCl_2$, 13.3 mM Pipes (1,4-piperazinediethanesulfonic acid), 1.33 mM EGTA and $CaCl_2$ ranging from 0 to 1.33 mM was prepared from stock solutions. The pH was adjusted to 7.0 in the buffer stock after the addition of $CaCl_2$ and EGTA. The phase mixture was continuously stirred while aliquots of 0.75 g were weighed into polystyrene tubes and 0.25 ml of sample was added. After 10 min incubation at room temperature with end-over-end rotation, the phases were separated by centrifugation at $2250 \times g$ for 10 min. The concentration of [^{14}C]calmodulin was determined by counting 0.1 ml upper and lower phase, respectively, in Lumagel scintillation fluid (Lumac, The Netherlands). The concentrations determined in the two phases were then used to calculate the partition coefficient. The partitioning of spectrin was determined by measuring the protein concentration in each phase by the method of Bradford [31].

3. RESULTS AND DISCUSSION

The partition coefficient of calmodulin in an aqueous polymer two-phase system is defined by

$$K_{CaM} = \frac{[CaM]_{upper}}{[CaM]_{lower}}$$

where $[CaM]_{upper}$ and $[CaM]_{lower}$ denote the concentrations in the upper and lower phase, respectively. In the presence of a calmodulin-binding protein, a certain fraction of calmodulin will be bound and this fraction will most probably partition differently from free calmodulin. Therefore it is more appropriate to describe the partition behaviour of calmodulin in terms of an apparent partition coefficient, defined as [32]

$$K^{app}_{CaM} = \frac{[CaM]^{total}_{upper}}{[CaM]^{total}_{lower}} = \frac{[CaM]^{free}_{upper} + [CaM]^{bound}_{upper}}{[CaM]^{free}_{lower} + [CaM]^{bound}_{lower}}$$

Thus, in the absence of binding protein, calmodulin partitions as the free protein and the apparent partition coefficient equals the partition coefficient of free calmodulin. However, at increasing concentrations of binding protein, an in-

creasing fraction of calmodulin will be bound and the apparent partition coefficient will approach that of the formed complex.

In the phase systems used here, both subunits, as well as spectrin dimer, were highly concentrated in the upper dextran-rich phase; the partition coefficients were in all three cases around 30. Calmodulin alone partitioned more equally between the two phases (K_{CaM} = 1.45), while in the presence of spectrin the apparent partition coefficient of calmodulin increased significantly (fig.1). This partition behaviour clearly demonstrates, as shown previously [10], that calmodulin interacts with spectrin. The α-chain affected the partitioning of calmodulin to a somewhat greater extent than the dimer, which means that the α-subunit is a little more effective in binding calmodulin. The weaker binding to the $\alpha\beta$-dimer could be due to steric hindrance caused by the β-chain, or a conformational change in the α-chain upon binding of the β-subunit. Since the influence of the β-chain on the partitioning of calmodulin was considerably less, this polypeptide interacts only very weakly with calmodulin. There are reports in the literature

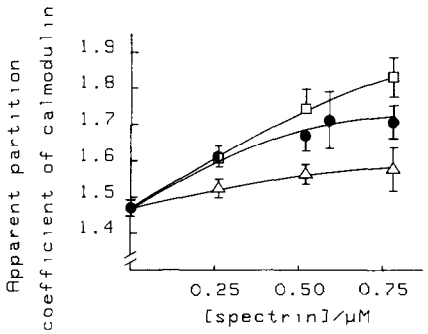

Fig.1. The effect of spectrin dimer (●), α-subunit (□) and β-subunit (△) on the partitioning of calmodulin. The free Ca^{2+} and calmodulin concentrations in the system were 10 μM and 130 nM, respectively. Spectrin dimer and the isolated subunits had partition coefficients around 30. (Note that the spectrin concentrations in the upper phase are almost twice as high as the total concentrations given in the figure.) Each point represents a mean value of 3–8 experiments and a 95% confidence interval is indicated. After addition of the sample, the composition of the phase system was: 11% Ficoll, 6% dextran, 0.2% polyethylene glycol, 0.1 M KCl, 10 mM Pipes, 0.1 mM $MgCl_2$, 1 mM EGTA and $CaCl_2$ varying from 0 to 1.0 mM. Phase volumes: 0.48 ml (upper phase), 0.44 ml (lower phase).

that the isolated subunits are unstable and prone to aggregation [25,33]. We have therefore experimentally confirmed that both the subunits are stable under the conditions used here. Analysis by polyacrylamide gel electrophoresis under non-denaturing conditions showed that the α- and β-subunits reassociated into a dimer when mixed after partitioning in the aqueous two-phase system (not shown). We can hence conclude that the 240 kDa subunit contains the binding site for calmodulin.

In the absence of calcium the partitioning of calmodulin did not significantly change upon addition of either spectrin dimer or the isolated subunits, thus showing that the binding is calcium-dependent. Since the calmodulin inhibitor chlorpromazine [34] completely abolished the increased partitioning caused by spectrin or the subunits (table 1), the interactions appear to be specific. Addition of chlorpromazine did not affect the partition coefficient of spectrin or that of the subunits. The binding of [^{14}C]calmodulin to spectrin was also inhibited by addition of excess unlabeled calmodulin (not shown), showing that the spectrin-binding activity is not caused by the labeling procedure.

In spite of the large differences found in peptide pattern between human spectrin and the other members of the spectrin family [7], many functions seem to be conserved. They are actin-binding proteins [5,35] and in the presence of protein 4.1

Table 1

Effect of chlorpromazine on the interaction between calmodulin (50 nM) and 0.8 μM spectrin (1.5 μM in the upper phase), at 10 μM free Ca^{2+}

Sample	Apparent partition coefficient of calmodulin	
	Without chlorpromazine	+ 80 μM chlorpromazine
CaM alone	1.471 ± 0.021 (15)	1.452 ± 0.062 (6)
+ spectrin dimer	1.707 ± 0.044 (8)	1.482 ± 0.024 (6)
+ α-spectrin	1.830 ± 0.054 (4)	1.512 ± 0.048 (4)
+ β-spectrin	1.577 ± 0.059 (3)	1.475 ± 0.036 (3)

The number of experiments are given in parentheses and error estimates are calculated on a 95% confidence level. The composition of the phase system was as in fig.1

ternary complexes are formed [36]. It has also been shown that brain fodrin and spectrin from mammalian sources can form hybrid molecules that are capable of binding to ankyrin [6]. Therefore, from a functional point of view, it is not surprising that the calmodulin-binding site is located on the common 240 kDa subunit as in other spectrins. However, the binding must differ in some respect since this association is weaker.

Fig.2 shows the partition behaviour of calmodulin alone and together with spectrin at different free calcium concentrations. It is evident that about 1 μM free calcium is required to obtain binding of calmodulin to spectrin. In three independent experimental series the concentration of calcium necessary to achieve maximum difference between the partitioning of calmodulin alone and in the presence of spectrin, i.e. to reach full binding strength, was 1.1, 1.3 and 1.5 μM, respectively. To calculate the binding strength from partitioning data it is necessary to know the partition coefficient of the complex formed. Therefore an experiment was done where excess calmodulin was added to the phase systems, in order to obtain as large a fraction as possible of bound spectrin molecules. From these experiments it was found that the apparent partition coefficient of spectrin

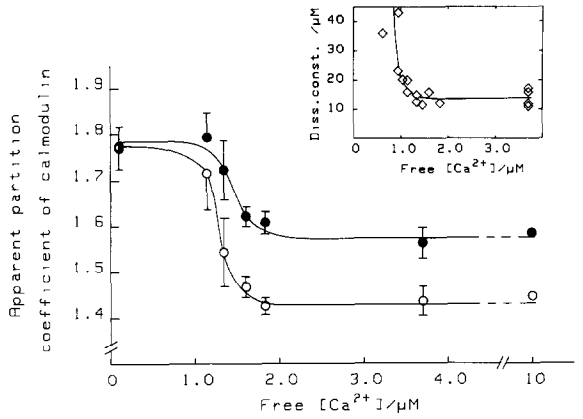

Fig.2. Partitioning of calmodulin at increasing concentrations of calcium, in the absence (○) or presence (●) of 0.8 μM spectrin dimer (1.5 μM in the upper phase). The calmodulin concentration in the system was 50 nM. Mean values of 4–6 experiments are shown, together with a 95% confidence interval. The composition of the phase system was as in fig.1. (Inset) The dissociation constant for the spectrin-calmodulin complex as a function of the calcium concentration.

was independent of whether calmodulin was present. Thus, the partitioning of the complex appears to be the same as that of free spectrin. The dissociation constants could then be calculated, assuming a 1:1 stoichiometry for the complex [12,37], and can be seen to change from infinity at very low calcium concentrations to around 13 μM at calcium concentrations exceeding 1 μM (fig.2, inset).

In addition, fig.2 shows that the partition coefficient of calmodulin decreases gradually when the calcium concentration is increased up to 1.8 μM, and that higher concentrations of calcium do not cause any further change in the partition. The amount of calcium added to the phase system is negligible compared to other ions present and does not affect the composition or alter the interface potential, which are the major determinants of the partition behaviour in the two-phase system. Therefore, the altered partitioning must be a consequence of a conformational change in calmodulin upon binding calcium. Accordingly, the main conformational change as monitored by the partitioning technique must have occurred at calcium concentrations below 1.8 μM in contrast to that observed by other methods [38].

Although numerous experiments have shown different effects of increased intracellular calcium on the erythrocyte properties such as shape and deformability [39,40], the role of calcium in the normal life of the cell is still unknown. As mentioned earlier, it was recently shown that physiological shear stress can increase the flux of calcium into the cell [19], and thereby produce a calcium signal. Since the binding and activation of the calcium pump by the calcium-calmodulin complex appear to be slow [20], the signal would exist for a finite time before calcium is removed by the activated pump. Thus, a free calcium concentration in the micromolar range is likely to be present at certain times in the red cell and this would be sufficient to produce maximal binding of calmodulin to spectrin. However, the significance of this binding remains to be clarified. Since the binding strength is rather weak (13 μM) one can argue that the interaction is of minor physiological importance and perhaps only a relic from ancestors of spectrin. On the other hand, it should be recognized that the concentrations of the two proteins in the red cell are high; calmodulin is pre-

sent at 2–3 μM [20,41] and the spectrin concentration is in the range of 3 μM [42]. Consequently, a calcium signal could cause about 10% of the spectrin in the cell to bind to calmodulin, in spite of the low affinity. In addition, the high concentration of calmodulin in erythrocytes also suggests that this protein has functions besides the activation of the calcium pump in the membrane, since a nanomolar concentration of calmodulin is sufficient for saturation of these high-affinity binding sites [43].

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