Magnesium dependent complexation of tri-anionic calix[4]arene detergents by the nucleotide binding domain 1 (NBD1) of multidrug resistance protein MRP1†

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The tri-carboxylatomethylene-mono-alkoxy calix[4]arenes have been shown by fluorescence spectroscopy to bind to the NBD1 domain of MRP1 protein in a magnesium dependent manner. The observed associations constants are of the same order as that observed for ATP–Mg, the natural substrate for this protein.

The calix[*n*]arenes are amongst the most widely studied organic macrocyclic host systems in supramolecular chemistry.[1] Their ease of selective chemical modification at either the *para*-aromatic position or at the phenolic lower rim combined with relative procedures for region selective functionalisation is the major key to their applications ranging from gas storage[2] through ion complexation,[3] biopharmaceutical applications,[4] to protein complexation,[5] diagnostics[6] and cell transfection.[7]

Work by Hamilton *et al.* has shown how coupling peptide loops at the *para*-position of calix[4]arene leads to protein recognition,[8] Zadmard and Schrader have demonstrated nanomolar protein complexation at interfaces,[9] Oshima *et al.* have shown the capacity of calix[*n*]arenes to transport cytochrome C.[10] The interaction of phosphonatocalix[4]arenes with calf intestine alkaline phosphatase has been demonstrated by Kalchenko *et al.*[11] We have previously demonstrated complexation between serum albumins and the water soluble *p*-sulfonatocalix[*n*]arenes by Electrospray Mass Spectrometry,[12] and for the water soluble *O*-alkylsulfonato-*p*-acylcalix[8]arenes by Dynamic Light Scattering.[13] The interaction of resorcinarene based Solid Lipid Nanoparticles with Bovine Serum Albumin showed formation of a protein monolayer around the SLN surface.[14] In the case of the anti-coagulant activity of mono-O-substituted-*p*-sulfonatocalix[*n*]arenes, the activity was shown to be dependent on the glycosylaminoglycan recognising protein, Heparin Cofactor II.[15] By both Western Blot methodology and ELISA techniques, interaction between the *p*-sulfonatocalix[*n*]arenes and the pathological form of the prion protein PrP has been demonstrated to lead to enhancement of the interaction with suitable antibodies.[6,16] Recent work has shown that certain phosphonatocalix[4]arenes can inhibit tyrosine kinases.[17]

The complementarity in the chemistry of alkyl-mono-substitution at the phenolic face of calix[4]arene and the fact that the quinone methide substitution at the *para*-aromatic position can only occur on non-*O*-substituted rings have allowed the rational construction of cone shaped amphiphilic calix[4]arene derivatives having detergent properties.[18]

The tri-carboxylatomethylene-mono-alkoxycalix[4]arenes were designed for the extraction and solubilisation of membrane proteins such as the ATP-binding cassette (ABC) superfamily of membrane transporters,[19] implicated in multi-drug resistance (MDR) phenotype.[20] However the initial extraction of the ABC transporter BmrA, a bacterial drug transporter,[21] yielded partially deactivated proteins, this deactivation probably occurs *via* an interaction with the Nucleotide Binding Domain of BmrA, as the domain is both essential for activity and is a magnesium dependent binding region of anionic nucleotides. In order to understand and resolve the inactivation process, we have focused on interactions of tri-carboxylatomethylene-mono-alkoxycalix[4]arenes with the NBD1 region of the ABC transporters.

Multidrug Resistance Protein 1 (MRP1), a member of the ABC superfamily, is known to transport a wide range of substrates including organic anions, many of which are conjugated to either glutathione (GSH), sulfate or glucuronate. Physiological substrates of MRP1 include GSH and leukotriene C_4 (LTC_4).[22] The transport is powered by ATP hydrolysis, involving two different cytosolic nucleotide binding domains, NBD1 and NBD2.[22] NBD1 performs a regulatory role whereas NBD2 performs the catalytic role. Binding of ATP

DSIERRPVKDGGGTNSITVRNATFTWARSDPPTLNGITFSIPEGALVAVVGQVGCGK
SSLLSALLAEMDKVEGHAIKGSVAYVPQQAWIQNDSLRENILFGCQLEEPYYRSVI
QACALLPDLEILPSGDRTEIGEKGVNLSGGQKQRVSLARAVYSNADIYLFDDPLSA
VDAHVGKHIFENVIGPKGMLKNKTRILVTHSMSYLPQVDVIIVMSGGKISEMGSYQ
ELLARDGAFAEFLRTYASTEQEQDAEEN

Scheme 1 The amino acid sequence of NBD1 of MPR1, in blue tryptophan residues, in red Walker A and Walker B sequences,[25] in green the Signature S sequence of ABC transporters, in yellow one putative binding site for tri-carboxylatomethylene-mono-alkoxycalix[4]arenes.

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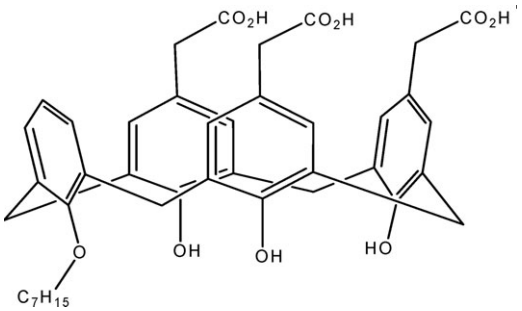
† Electronic supplementary information (ESI) available: The ESI gives details of the experimental work, the fluorescence measurements with a figure showing quenching by the calixarenes in the absence of salts, and the preparation and purification of the NBD1. See DOI: 10.1039/c0nj00323a



Scheme 2 Molecular structure of tri-carboxylatomethylene-mono-heptoxycalix[4]arene.

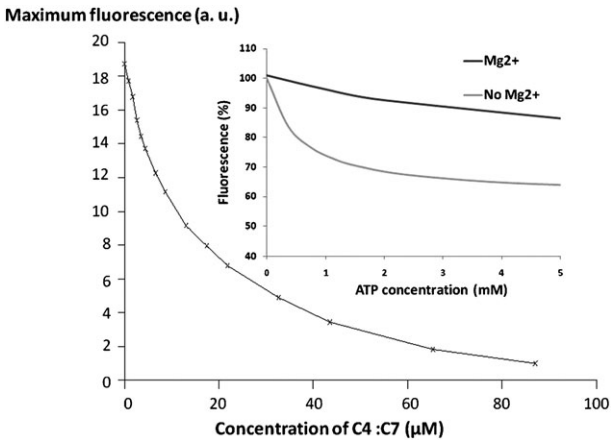


Fig. 1 Fluorescence titration curve for the maximum fluorescence emission of NBD1 as a function of the concentration of tri-carboxylatomethylene-mono-heptoxycalix[4]arene. The inset shows titration curves for ATP in the absence and presence of 20 mM Mg^{2+}.

to NBD1 is required for nucleotide binding at NBD2, while NBD1 binds ATP even when NBD2 is inactivated.[23,24]

The amino acid sequence of NBD1 is given in Scheme 1.

Putative binding sites for tri-carboxylatomethylene-mono-alkoxycalix[4]arenes will contain two or more cationic amino acid residues and it is interesting to note that one such site is present adjacent to Walker B.

It is thus of interest to probe the interaction of the tri-carboxylatomethylene-mono-alkoxycalix[4]arenes, see Scheme 2 for the molecular structure, with NBD1 as this domain is a highly probable binding site for these molecules to interact with proteins of the ABC transporter family.

The interactions between the tri-carboxylatomethylene-mono-alkoxycalix[4]arenes and the full NBD1 domain of MRP1 (Asp^{628} to Asn^{881}), see ESI†, were studied by measurement of the fluorescence quenching of the tryptophan as a function of the concentration of the tri-carboxylatomethylene-mono-alkoxycalix[4]arenes. A typical series of spectra are given in the ESI† and in Fig. 1 is given the intensity of the fluorescence maximum at 342 nm as a function of tri-carboxylatomethylene-mono-heptoxycalix[4]arene concentration; similar titration curves for ATP binding to NBD1 are given in the inset. Curve-fitting and determination of dissociation constants were performed with the GraFit program,[26] and converted to association constants. The measured values are global association constants and do not give any information on the stoichiometry of the complex.

For the tri-anionic-mono-alkoxycalix[4]arenes, Fig. 2, values of K_{ass} between 1.72×10^4 M^{-1} and 20×10^4 M^{-1} are observed. For *O*-alkyl chain lengths of C8 and below, the observed association constants are in the range of 1.72×10^4 M^{-1} to 2.2×10^4 M^{-1}; there is then a clear increase in the observed values rising to 15×10^4 M^{-1} and 20×10^4 M^{-1} for chain lengths of ten and twelve carbon atoms respectively. For longer chain lengths the values decrease to $3–4 \times 10^4$ M^{-1}. Interestingly there is a similar effect for the extraction of membrane proteins with short chain lengths tending to desolubilise the proteins and the molecules with C10 and C12 chains extracting the proteins into suspension.[18]

In comparison, β-cyclodextrin, which is known to bind to tryptophan in solution or to the amino-acid when it is exposed on a protein surface, had no effect on the fluorescence implying

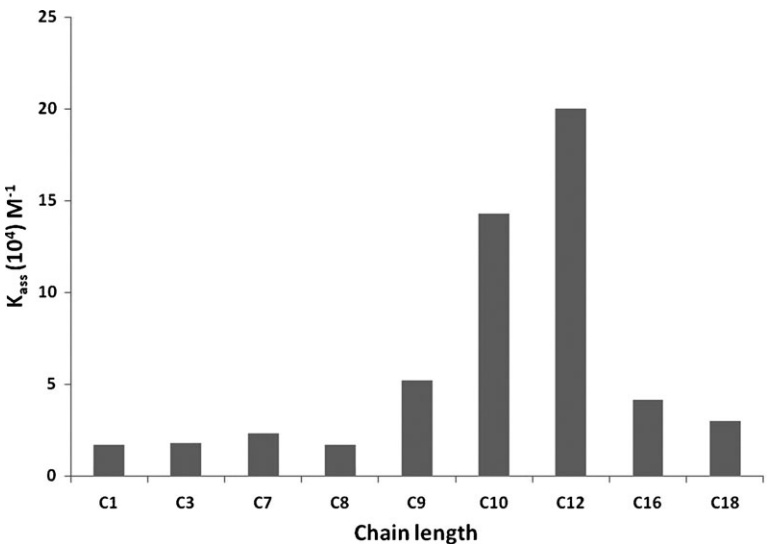


Fig. 2 Variation of the association constants for the binding of the tri-anionic-mono-alkoxycalix[4]arenes to the cytosolic NBD1 domain of the MRP1 membrane protein.

that the tryptophan residues are in the interior of the protein structure. Tri-(amino-methylene)-calix[4]arene-mono-propyl ether, the cationic analog of the currently studied molecules, shows an association constant of 0.4×10^4 M^{-1} and *p*-sulfonatocalix[4]arene and *p*-sulfonatocalix[6]arene have K_{ass} values of 1.0×10^4 M^{-1} showing that the interaction between NBD1 and tri-anionic-mono-alkoxycalix[4]arenes is not purely an electrostatic interaction. For the binding of ATP to the NBD1 domain in the absence of cations an association constant of 0.0143×10^4 M^{-1} is measured. Thus the natural non-cyclic substrate binds very weakly to NBD1 while the cyclic tri-anionic-mono-alkoxycalix[4]arenes bind more strongly.

However ATP binding to NBD1 is known to be cation dependent as shown by the K_{ass} value of 0.19×10^4 M^{-1} (calculated from the data shown in the inset of Fig. 1), and so the influence of Na^+, K^+, Mg^{2+} and Ca^{2+}, being the major cations present in physiological media, was observed *via* the effect on the fluorescence spectra. All suspensions were prepared from a single mother stock at pH 7, the buffer is given in the ESI† and is the same as used for NBD1–ATP binding experiments, and with an ionic strength of 0.17 M in sodium so as to approximate physiological conditions.

Table 1 Binding constants of tri-carboxylatomethylene-mono-heptoxycalix[4]arene to NBD1 in the presence of cations at their physiological concentrations

Salt	Ref.	Na^+	K^+	Mg^{2+}	Ca^{2+}
Concentration/mM	0	250	10	5	10
K_{ass} (10^4 M^{-1})	2.2	2.0	2.2	3.3	1.8

While the presence of the cations Na^+, K^+ and Ca^{2+} at their extracellular physiological concentrations has no effect on the binding of tri-carboxylatomethylene-mono-heptoxycalix[4]arene to NBD1 (Table 1), there is a strong concentration dependent effect of Mg^{2+} on the binding, with the observed association constant increasing from 2.2×10^4 M^{-1} at zero concentration of Mg^{2+} to a maximum of 5.6×10^4 M^{-1} at a concentration of 7.5 mM in Mg^{2+}, as shown in Fig. 3 and Table 2. In comparison, the binding of ATP to NBD1 increases from 0.0143×10^4 M^{-1} in the absence of Mg^{2+} to 0.19×10^4 M^{-1} at a concentration of 20 mM in Mg^{2+}. Thus for tri-carboxylatomethylene-mono-heptoxycalix[4]arene the binding increases by 2.5 in the presence of Mg^{2+}, for the natural ligand ATP a tenfold increase in the binding is observed.

In contrast to the binding either in the absence or in the presence of other cations, in the case of the Mg^{2+} dependent binding of tri-carboxylatomethylene-mono-heptoxycalix[4]-arene to NBD1 there exists a clear blue shift in the maximum of the fluorescence emission, which decreases from 342 nm to 330 nm, indicative of a more hydrophobic environment for tryptophan residues.

The interaction of tri-carboxylatomethylene-mono-alkoxycalix[4]arenes with the NBD1 domain of the MRP1 membrane protein has been demonstrated. This interaction

Table 2 Variation in the determined K_{ass} for tri-carboxylatomethylene-mono-heptoxycalix[4]arene binding to NBD1 as a function of Mg^{2+}

Concentration Mg^{2+}/mM	0	1	5	7	7.5	10
K_{ass} (10^4 M^{-1})	2.2	2.5	3.3	4.3	5.6	5.2

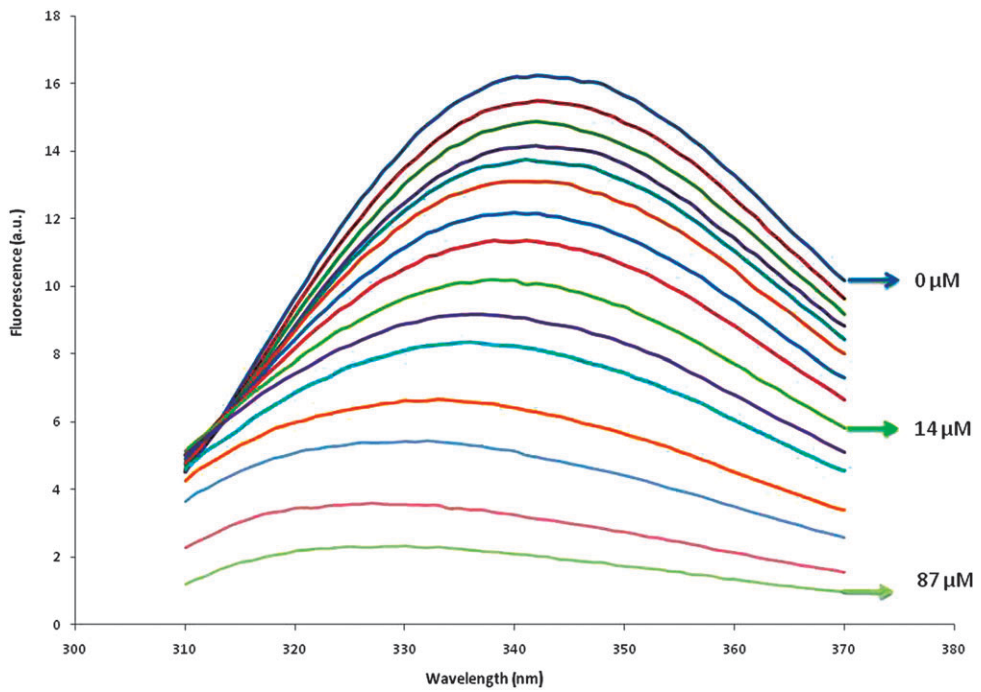


Fig. 3 Variation of the intensity of the fluorescence emission of NBD1 as a function of the concentration of tri-carboxylatomethylene-mono-heptoxycalix[4]arene in the presence of Mg^{2+} at a concentration of 7 mM. The concentration of tri-carboxylatomethylene-mono-heptoxycalix[4]-arene increases from top to bottom of the figure.

may be involved in the reversible partial deactivation of the ABC transporter proteins by these ligands. The interaction is selectively magnesium dependent as is the case for ATP binding to the NBD1 domain.

Experimental

Full details of the experimental procedures are given in the ESI.† Calixarenes were synthesized following literature methods.[1] NBD1 was purified as described in ESI.† Fluorescence study was carried out by measuring the fluorescence of the several ratios of calixarene : NBD1 : salt in the buffer solution. The excitation wavelength was 295 nm and the emission spectrum was recorded from 310 nm to 370 nm. All measurements are expressed as arbitrary units, a.u.

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