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Exploring Calbindin-IMPase fusion proteins structure and activity

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ABSTRACT

Calbindin-D28k is a calcium binding protein that is highly expressed in the mammalian central nervous system. It has been reported that calbindin-D28k binds to and increases the activity of inositol Monophosphatase (IMPase). This is an enzyme that is involved in the homeostasis of the Inositol trisphosphate signalling cascade by catalysing the final dephosphorylation of inositol and has been implicated in the therapeutic mechanism of lithium treatment of bipolar disorder. Previously studies have shown that calbindin-D28k can increase IMPase activity by up to 250 hundred-fold. A preliminary in silico model was proposed for the interaction.

Here, we aimed at exploring the shape and properties of the calbindin-IMPase complex to gain new insights on this biologically important interaction. We created several fusion constructs of calbindin-D28k and IMPase, connected by flexible amino acid linkers of different lengths and orientations to fuse the termini of the two proteins together. The resulting fusion proteins have activities 200%–400% higher the isolated wild-type IMPase. The constructs were characterized by small angle X-ray scattering to gain information on the overall shape of the complexes and validate the previous model. The fusion proteins form a V-shaped, elongated and less compact complex as compared to the model. Our results shed new light into this protein-protein interaction.

1. Introduction

The interaction between the calcium binding protein calbindin and the IMPase enzyme is of particular interest due to the therapeutic significance of IMPase in the treatment of bipolar disorder and autophagy [1–3]. In the 1970's, a study demonstrated that lithium treatment reduces inositol levels in the brains of mammals by inhibition of IMPase [4]. This formed the basis of the inositol depletion hypothesis of lithium efficacy in the treatment of bipolar disorder [1,5,6]. More recently, it was shown that inhibition of IMPase leads to an increase in autophagy *in vitro*. It was thus proposed that this effect could be used therapeutically in the treatment of neurodegenerative disorders in which protein aggregation plays a role in disease pathogenesis [3,7]. IMPase is a homodimeric phosphodiesterase enzyme which is involved in the phosphatidylinositol signalling cascade. This cascade is initiated by the activation of G-protein-coupled receptors (GPCR) causing the production of Inositol trisphosphate (IP3) and diacylglycerol (DAG) via the activation of Phospholipase C (PLC). IP3 induces the release of calcium from intracellular stores and DAG activates Protein Kinase C (PKC). IMPase performs the final dephosphorylation of inositol, recycling this back to free inositol [8–11]. IMPase is extremely thermostable displaying a melting temperature (Tm) of 79°C and each monomer possesses an αβαβα topology [12]; Bone, Springer, and Atack, 1992).

Calbindin-D28k was initially identified as a calcium-binding protein in the absorptive epithelia of *gallus gallus domesticus* (Wasserman, Corradino, and Taylor, 1969). Calbindin-D28k is highly expressed in the human central nervous system (CNS), and binds to several other proteins indicating a role as a calcium sensor. Binding partner proteins include: Ran-binding-protein-M, caspase-3, ATPases and cyclic nucleotide phosphodiesterases (Bellido et al., 2000; Lutz et al., 2003; Morgan et al., 1986; Reisner, Christakos, and Vanaman, 1992). Calbindin-D28k is composed of six EF-hand domains that exhibit a typical helix-loop-helix topology. Four out of these EF-hands coordinate a calcium ion in the central loop (EF1, EF3, EF4 and EF5). The six domains form a globular structure with significant flexibility in the N-terminal region [13,14].

Binding between these two proteins was initially identified by affinity chromatography, using immobilized calbindin-D28k and phage-displayed peptides to explore the calcium sensing function of calbindin-D28k,The interaction was shown to increase the activity of IMPase by up to 250 fold [15]. and to occur with a K_d of 0.9 μM both when calbindin-D28k is bound to calcium and when it is in the apo form, demonstrating that it is calcium independent [15]. The

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calbindin-IMPase interaction has been demonstrated *in situ* in the spines and dendrites of cerebellar Purkinje neurons [16]. Mutagenesis studies, based on an in silico model of the protein complex, have demonstrated by biochemical assays that Asp24 and Asp26 of calbindin-D28k are critical for the activation of IMPase. Both these residues are involved in calcium coordination. These data were utilised to design peptide inhibitors [17]. The interest in this interaction is now of burning interest because of the therapeutic potential of IMPase in bipolar disorder. An understanding of how calbindin-D28k facilitates the increase of IMPase activity could reveal novel ways for inhibiting the enzyme.

Here, we describe how we employed small angle X-ray scattering (SAXS) to gain new insights into the shape and flexibility of the calbindin-D28k and IMPase complex. We fused the two proteins by Gly-rich flexible amino acid linkers to connect their termini and produce stable protein complexes. The use of flexible amino acid linkers has been widely exploited to study protein interactions [18]. Linking proteins together helps to stabilise the interaction by increasing the local concentration of the binding partner. The approach is often employed to study proteins that have relatively weak affinities or form only transient interaction. We found that the fusion proteins have activities 200%–400% higher than that of isolated IMPase. We used these constructs to perform SAXS and gain information on the overall shape of the complexes to validate the previous in silico model. The fusion proteins with IMPase form a V-shaped, more elongated and less compact complex as compared to the linear in silico model. Our results shed new light on this protein-protein interaction.

2. Methods

2.1. Fusion constructs and protein expression

Two sets of fusion proteins were developed. The "FP" group where calbindin-D28k was fused to the C-terminus of IMPase and the "Cal-FP" group where IMPase was fused to the C-terminus of calbindin-D28k (Fig. 1). The Gly-rich linkers were constituted of repeats of an 11 amino acid polypeptide linker (GGGSASGGGSG) that was designed based on previous studies [18]. Protein expression was performed in a pET-15b vector at the Nco1 and BamH1 sites in *E. coli* Rosetta 2 (DE3) cells (Novagen). A poly-histidine tag followed by a rhinovirus (HRV) 3C protease cleavage sequence was incorporated into the coding sequence of the N-terminus of the fusion proteins. The synthetic constructs were produced by GenScript. Transformed cells were plated on Luria broth (LB) agar containing 50 mg/ml ampicillin for selection. Colonies were harvested and grown in LB broth (50 ug mL^{-1} ampicillin) to an OD_{600} of 0.6 in 1 L cultures at 310 K, with shaking at 200 rpm. The cultures were then incubated on ice for 2 h before induction with 0.4 mM IPTG and incubation overnight at 293 K with shaking at 180 rpm.

2.2. Purification

Purification of calbindin-D28k was performed according to [14]. IMPase purification was performed as described in Kraft et al., 2018. Briefly, the cells were pelleted by centrifugation at 9500*g* for 20 min. The cell pellets were resuspended in a buffer of 150 mM NaCl, 1 mM MgCl, 20 mM Tris pH 7.8 and protease inhibitor cocktail (cOmplete™, EDTA-free, Roche). The sample was sonicated for 5 min with 5-sec intervals (5 sec on, 5 sec off) on ice. To ensure quantitative removal of DNA, 1 μL of non-specific endonuclease (Benzonase® Nuclease, sigma) was added and the sample incubated on ice for a further 10 min. The lysate was clarified at 30,00 g for 20 min. The supernatant was mixed with cobalt affinity resin (Talon metal-affinity resin) and incubated for 1 h at 277 K in buffer. The resin was then pelleted by centrifugation at 9500*g* for 20 min. Pellets were resuspended in a buffer of 150 mM NaCl, 10 mM imidazole and 20 mM Tris pH 7.8. The flow-through was discarded and the resin was washed 7 times with buffer. The fusion proteins were eluted using 5 washes of 250 mM imidazole. HRV C3 protease was added to cleave the His-Tag at a protease-to-target protein ratio of 1:100 (w/w) and incubated overnight at 277 K. The cleaved protein was purified by size exclusion chromatography on a Superdex G200 in 150 mM NaCl and 20 mM Tris pH 7.8. Typically, $>$ 8 mg of protein was obtained from each 1 L of culture.

2.3. SDS-page

1 μL of 1 mg/ml protein was mixed with 6 μL of water and 2.5 μL of NuPAGE™ LDS Sample Buffer. The samples were placed in a dry bath at 95 °C for 5 min and then loaded onto a NuPAGE Novex 4–12% bis-tris gel. The gel was run at 180 V for 40 min in NuPAGE™ MES SDS Running Buffer. A Mark12™ Unstained Standard was used as the standard protein ladder. (Thermo Fisher, Ladder: LC5677, Gel: NP0322BOX, Sample buffer: NP0007, running buffer: NP0002). Coomassie blue was used for gel staining (InstantBlue^{tm}, Expedeon).

2.4. Activity assay

A colorimetric phosphate quantification assay was used as an endpoint activity assay. Wild-type or fusion IMPase (15 nM) and calbindin-D28k (500 nM) were used in a final volume of 12 μL 3 μL of substrate was added to produce a final concentration of 0.125 mM Inositol 1-phosphate in a final reaction volume of 15 μL. Reactions were

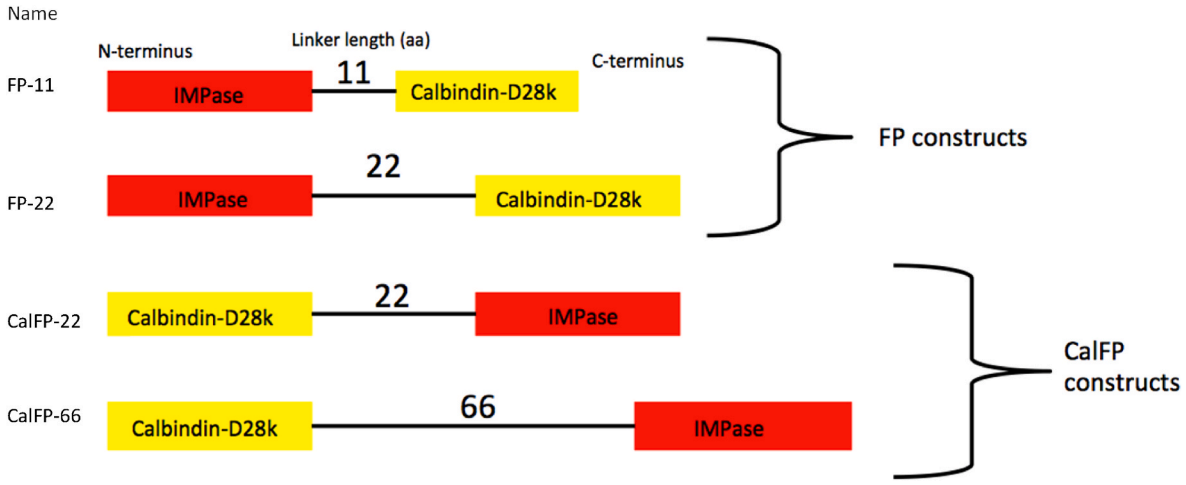

Fig. 1. Fusion protein constructs. Two different fusion directionalities were tested. Nomenclature used was FP (fusion protein) when IMPase was present at the N-terminus and CalFP when Calbindin-D28k was present at the N-terminus. Three different linker lengths were employed. The names of each construct are stated.

incubated at 37 °C for 35 min, with 80% humidity to prevent evaporation. The assay buffer was composed of 20 mM Tris (pH7.8), 150 mM NaCl and 3 mM MgCl. The addition of 30 μL of BIOMOL® green (Enzo) reagent was used to stop the reaction. Plates were incubated at room temperature for 30 min to allow the colour to fully develop. The assay was performed in Clear 384 well BRANDplates® pureGrade™ F-bottom plates (BRAND). Absorbance was measured at $\lambda = 630$ nm using a PHERAstar FS plate reader from BMG Labtech.

2.5. SAXS measurements

SEC-SAXS was performed at the B21 beamline (Diamond Light Source UK). Fusion proteins were dialyzed overnight using Pur-A-Lyzer™ mini dialysis kits (Sigma-Aldrich PURN60030-1 KT) in a buffer consisting of 20 mM Tris-HCl (pH7.8), 150 mM NaCl and 3 mM MgCl, at 277K. Using an HPLC system, 45 μL of the protein samples were loaded onto a Shodex KW-403 column at the concentrations of 12, 10, 10 and 15.1 mg/mL for FP, CAL-FP-66, CAL-FP-22 and FP-22 respectively. X-ray intensity data were collected as the eluent moved from the column to the beam at a flow rate of 0.16 mL/min. The intensity was plotted against its angular dependants q ($q = 4\pi\sin\theta/\lambda$). The system operated with an exposure time of 3 s at 12.4 keV (1 Å) using a PILATUS 2 M detector located at a distance of 4 m (Eiger M4 detector was used for FP-22). Data were analyzed using the SCÅTTER and ATSAS program suites (Franke et al., 2017; Franke and Svergun, 2009; Kozin and Svergun, 2001). The FoXS web server was employed to compute the theoretical scattering profile using a computational docking model of the calbindin-IMPase interaction [17] for comparison with the experimental data (Schneidman-Duhovny et al., 2013, 2016).

3. Results

3.1. Production of fusion proteins and assessment of catalytic activity

All four fusion proteins as well as the individual calbindin-D28k and IMPase were successfully purified to a high level of purity (Fig. 2). FP-11 showed a 200% increased relative activity, when compared to isolated wild-type IMPase. FP-22, Cal-FP-22 and Cal-FP-66 had an increased relative activity of approximately 400% that of the original enzyme. IMPase and calbindin-D28k together had a relative activity of 600%, compared to WT IMPase alone (Fig. 2). These results confirm previous data and demonstrate increased IMPase activity with the fusion proteins. The constructs were made to find the optimal size of linker chain. With a short linker with a length of 11aa that has less activity and a highly extended linker of 66aa to test for steric hindrance in comparison to shorter linkers. The 22aa linker has the same activity as the extended linker fusion protein.

3.2. Structural analysis of fusion proteins

We then used the constructs to collect SAXS data. This low resolution technique can provide unique information on the overall envelop of a molecule or a complex allowing reconstruction of the overall shape. One of the parameters provided is Dmax, which refers to the maximum particle size of the measured specimen. We observed that this parameter increased with the increase of the linker length, with values of 162, 176, 175 and 218 Å obtained for FP-11, FP-22, Cal-FP-22 and Cal-FP-66 respectively. The radius of gyration (Rg), that constitutes a function of the distance to the center of density, also increased with the linker length. The shortest, FP-11, displayed a Rg of 46 Å and the longest, Cal-FP-66, displayed a Rg of 57 Å. The Porod slope for all four fusion proteins was q^4 indicating that the proteins are not unfolded but possess a more globular structure. The Kratky plot, normalized to Rg, demonstrated that, as linker length increases, the protein becomes more flexible. The multiple peaks indicated a multi-domain characteristic of fusion proteins. At high q the graph approaches the baseline indicating a folded but partially flexible structure and this flexibility increases slightly with increasing linker length (Fig. 3B). The distance distribution function showed multiple peaks indicating the multi-domain characteristic of the fusion proteins and demonstrated the increase in Dmax where the distribution meets the X-axis (Fig. 3C).

Ab initio envelope construction from SAXS data revealed a V-shaped structure of the fusion proteins, compared to the *in-silico* docking model that displays a more linear confirmation. The experimental intensities deviated significantly from the calculated model intensities. This deviation is significant at both high and low q regions of the experimental intensities, reflecting differences in the shape and size of the fusion proteins (Fig. 4).

4. Discussion

The use of flexible linkers to stabilise protein-protein interactions has long been used to gain insights about the structure and function of protein complexes [18]. This method is used to stabilise transient, low affinity protein interactions, as, for example, TCR-peptide-MHC molecule complexes [18–20]. Here, we used this approach to study the interaction between apo calcium binding calbindin-D28k and the enzyme IMPase, a putative therapeutic target of lithium in bipolar disorder. This interaction has a relatively low affinity (0.9 μM) [15] when compared to other protein-protein interactions that often exhibit affinities in the low nanomolar and picomolar range with large protein interfaces. We reasoned that the production of fusion proteins of IMPase

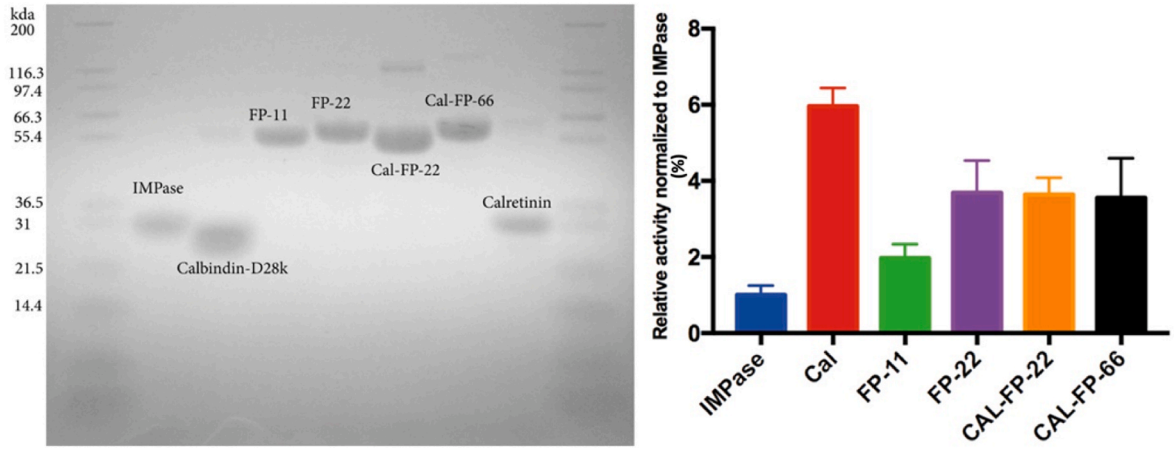

Fig. 2. Purification and enzymatic characterization of the fusion proteins as compared to isolated wild-type IMPase. A) SDS PAGE gel of proteins after size exclusion chromatography. B) bar graph of the results from the activity assay with standard deviations. Cal indicates a sample containing both IMPase and Calbindin-D28k.

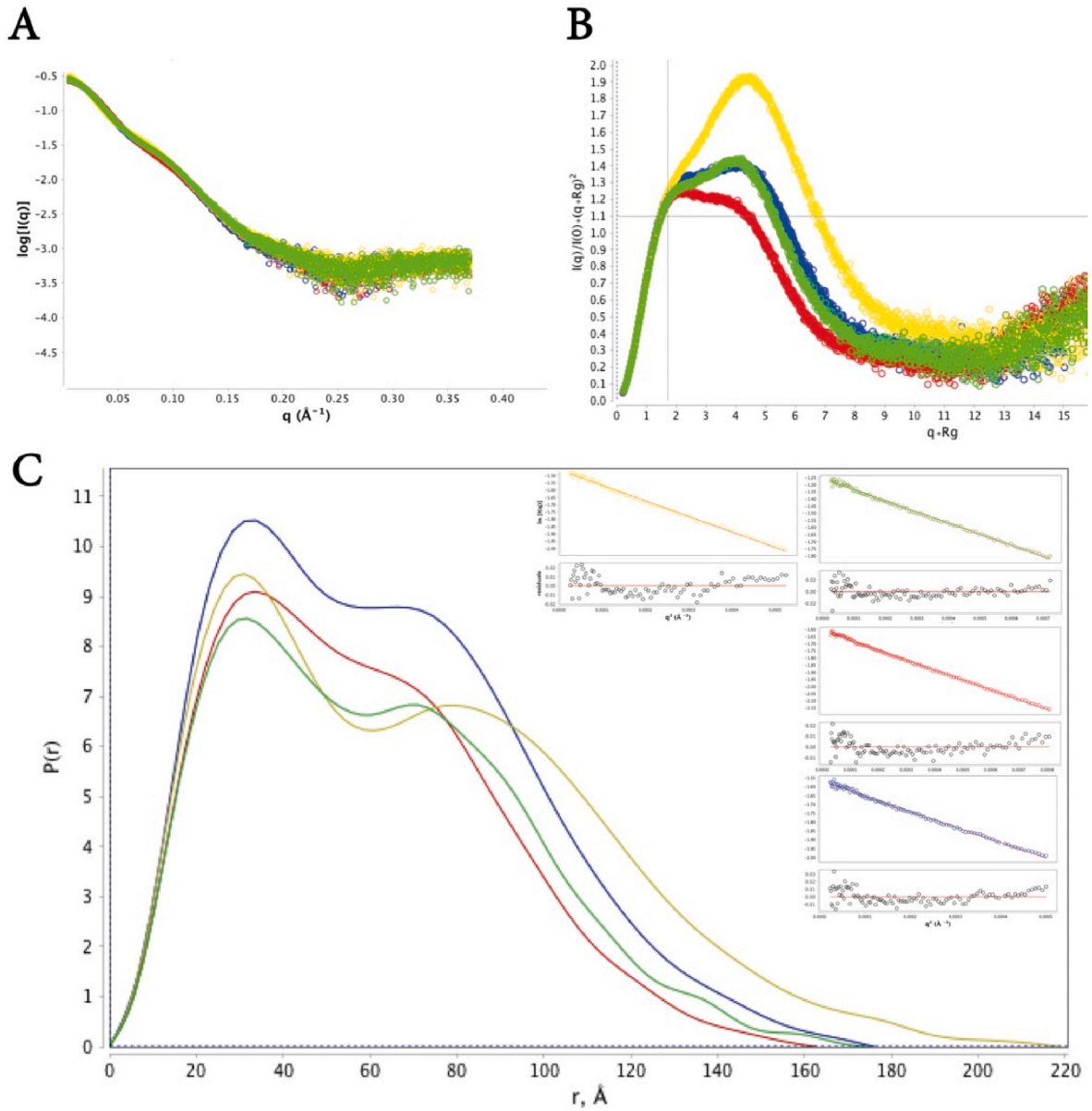

Fig. 3. A) Log10 SAXS intensity versus scattering vector, *q*. Plotted range represents the positive only data within the specified *q*-range. B) Dimensionless Kratky plot. Crosshair marks the Guinier-Kratky point (1.7, 1.1). C) Pair-distance, P(r), distribution function. Maximum dimension, *dmax*, is the largest non-negative value that supports a smooth distribution function. The Guinier fitting for all data sets are also shown. In all plots Red = FP-11, Blue = FP-22, Green = Cal-FP-22 Yellow = Cal-FP-66. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and calbindin-D28k exploiting the use of linkers, would result in a more stable complex and allow easier study of the interactions between these proteins.

IMPase has remained of significant therapeutic interest since the development of the inositol depletion hypothesis, and, more recently, with the discovery of the induction of autophagy [4,7,21]. IMPase is an extremely stable protein that is able to withstand high temperatures in excess of 80°C [12]. Despite the interest in IMPase as a therapeutic target, the discovery of specific inhibitors that could be effective *in vivo* has remained elusive. The finding that calbindin-D28k can allosterically increase the catalytic activity (Vmax) of IMPase indicates that the enzyme could have significant structural plasticity. Understanding this interaction and how it results in an increase in catalytic activity of IMPase may reveal novel therapeutic approaches to IMPase inhibition. Unlike a traditional calcium sensor Calbindin-D28k increases the activity both in the calcium loaded and un-loaded states, this is despite residues involved in the portion interaction are also involved in calcium binding [15].

Several novel fusion proteins were produced using different linker lengths and directionality. The linker length resulted in an effect on the activity of the fusion protein, with the 11 amino acid linker (FP-11) displaying 50% less catalytic activity than the other fusion proteins but possessing twice the catalytic activity of isolated wild-type IMPase. The fusion proteins with linker lengths of 22 amino acids or more displayed the same catalytic activity, with no observable effect from the directionality of the proteins in the constructs. All fusion proteins showed catalytic activity appreciably higher than that observed when the two wild type proteins were mixed together. The most catalytically active fusion proteins displayed a fourfold relative activity in comparison to the unlinked mixture of calbindin-D28k and IMPase, with a relative activity of sixfold when compared to the isolated wild-type IMPase. In the activity assay, a ratio of 1:33 IMPase to calbindin-D28k molecules was present as compared to the 1:1 ratio present in the fusion protein assay, this high ratio was used to test the maximum possible activation

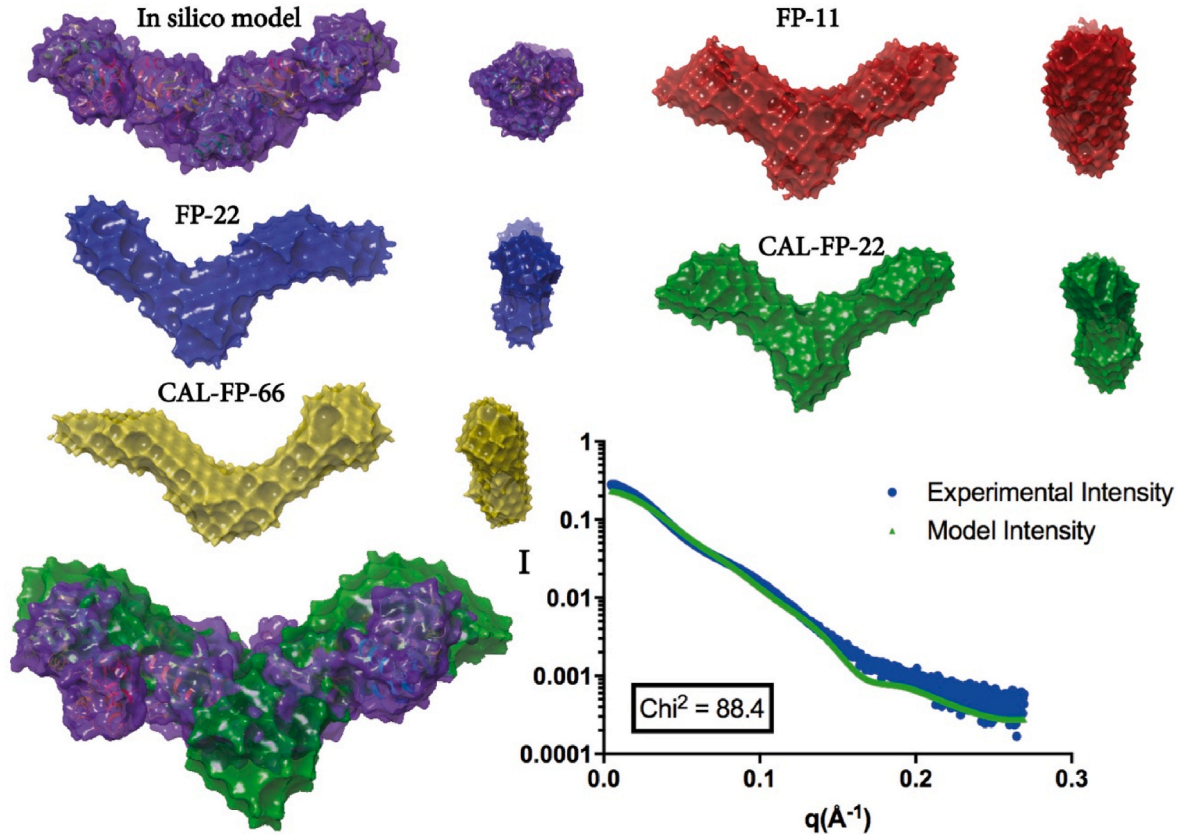

Fig. 4.). *Ab initio* envelope construction from the SAXS data of individual fusion proteins. Bottom right) A superimposition of the *in-silico* model and Cal-FP-22 fusion protein envelope. Bottom left) Log10 SAXS intensity versus scattering vector q (Blue), The calculated foXS scattering curve of the *in-silico* model (Green). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of IMPase. One hypothesis to explain this difference is that the flexible linker may cause some steric hindrance to the protein interaction or negatively affects the structural plasticity of the proteins compared to the isolated wild type forms, thus explaining why the fusion proteins lacked the full catalytic activity of the wild type complex. However, the activity of the longer fusion proteins is closer to the activity displayed by the native complex than that of isolated wild-type IMPase, rendering them a suitable model of the complex.

The SAXS data varied with varying linker length. The longer linker Cal-FP-66 displayed higher flexibility and possessed a structure distinct from the ideal globular system. All proteins exhibited the expected multidomain shape in the Kratky and Distance distribution function plots. Both Rg and Dmax increased with linker length. *Ab-initio* envelope reconstruction produced an identical structure for all fusion proteins irrespective of linker length or directionality. The envelopes retained a V-shape structure compared to the *in-silico* model of the complex. Comparison of the SAXS data to the calculated scattering of the *in-silico* model [17] revealed deviations at low and high q values. This may be due to a difference in the overall shape of the molecules from the more linear in silico model to the V-shaped structure.

5. Conclusion

The production of fusion proteins using flexible linkers was successfully employed in this work to study the calbindin-IMPase interaction. We observed that coupling of the two proteins via a flexible linker increases the catalytic activity of IMPase. Several fusion protein isoforms were analyzed by SAXS and all possessed a V-shape, in contrast to a previous *in-silico* model that predicted a linear conformation. These fusion protein constructs provide a model that could be exploited for detailed analysis of the calbindin-IMPase interaction in both crystallographic and mutagenic studies.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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