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Generation and characterization of calmodulin-DHFR sandwich fusion protein

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Abstract : A calmodulin-dihydrofolate reductase (DHFR) sandwich fusion protein was generated by insertion of calmodulin into the β-bulge region of DHFR to observe the effects of structurally constraining the calmodulin structure. The calcium binding properties of the sandwich protein were almost identical to calmodulin. Similar to calmodulin (10.7 μM), the sandwich protein bound four equivalents of calcium, with half saturation ($K_{0.5}$) observed at a [Ca^{2+}] of 8 μM. However, nicotinamide adenine dinucleotide (NAD) kinase activation property of the sandwich protein was lower than that of calmodulin. The sandwich protein activated NAD kinase, but to only half of the level obtained with calmodulin. The $K_{0.5}$ for both calmodulin and the sandwich protein were approximately the same (1-2 nM). Methylation analyses of the sandwich protein show that insertion of calmodulin into DHFR results in a large decrease in methylation. The V_{max} observed with the sandwich protein (95 nmole/min/ml) was only 22% of the value observed with calmodulin (436 nmol/min/ml) in the presence of calcium. Addition of trimethoprim to the reaction significantly inhibited the observed methylation rate. Overall, the data suggest that the insertion of calmodulin into the DHFR structure has little effect on calcium binding by the individual lobes of calmodulin, but may constrain the lobes in a manner that results in altered interaction with the calmodulin-dependent proteins, and severely perturbed the methyltransferase recognition site.

Keywords : Calmodulin, Calmodulin-DHFR sandwich fusion protein, Calmodulin methyltransferase, Dihydrofolate reductase (DHFR), methylation

Introduction

Dihydrofolate reductase (DHFR) catalyzes the NADPH dependent reduction of 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. DHFR has a β-bulge region between two consecutive β-type hydrogen bonds which includes positions 136 and 137 on one strand and a position 155 on the other strand [1, 5]. The role of β-bulges is not clear, however they might facilitate insertions or deletions in â-strands or position crucial residues by accentuating the local twist of the strands [20, 24, 25]. In the case of DHFR, insertions are tolerated in the β-bulge region, for example, residues 160-167 of human DHFR form a seven-residue disruption of the β-sheet hydrogen bonding, and make β-bulge, so called "β-blow-out" [27]. The fact suggests that the possibility of the tolerance by insertion of sequences into the β-bulge region of bacterial DHFR. In this study, we took advantage of this property of DHFR to generate a sandwich protein in which calmodulin was inserted into the β-bulge region of *E. coli* DHFR and transformants were selected by region of *E. coli* DHFR and transformants were selected by growth on trimethoprim. The sandwich protein was purified and its abilities to bind calcium, to activate calmodulin-dependent enzymes, and to serve as a substrate for the methyltransferase were evaluated.

Materials and Methods

Sandwich protein gene construction and expression

To insert the calmodulin gene into the β-bulge region of the chromosomal DHFR gene of *E. coli*, the calmodulin construct was mutagenized to: 1. remove the stop codon; 2. introduce an eight amino acid hydrophilic linker region at the C-terminal; and 3.

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A.

Cassette I:
  HpaI
5' AAC TAC GAA GAG TTC GTT CAG GTT ATG ATG GCT AAG GCT ACC GAT
3' TTG ATG CTT CTC AAG CAA GTC CAA TAC TAC CGA TTC CGA TGG CTA
    N   Y   E   E   F   V   N   V   M   M   A   K   A   T   D

                        BglII   SmaI   BamHI
   GAT AAG GAT AAG AAG ATC TTC CCG G        3'
   CTA TTC CTA TTC TTC TAG AAG GGC CCTAG    5'
    D   K   D   K   K   I

Cassette II:    5'  AATTAGATCTCT       3'
                3'      TCTAGAGACTAG   5'
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B.

1 70
MISLIAALAVDRVIGMENAMPWNLPADLAWFKRNTLNKPVIMGRHTWSIGRPLPGRKNIILSSQPGTDD
71 140
RVTWVKSVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQKLYLTHIDAEVEGDTHFPDYEPDDWEI***SDQL***
141 *210*
TDEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLNLMAR
211 *280*
KMKDTDSEEELKEAFRVFDKDGNGFISAAELRHVMTNLGEKLTDEEVDEMIREADVDGDGQVNYEEFVQV
281 *319*
MMAK-ATDDKDKK-ILFSEFHDADAQNSHSYSFEILERR
linker

Fig. 1. Sandwich protein construction and sequence. A. Cassette formation. Cassette I was inserted into *HpaI* and *BamHI* cleaved calmodelin/pUC18 vector (Roberts *et al.* [22]). The resulting plasmid was cleaved with *EcoRI* and *BclI* and the cassette II was inserted into the vector to introduce a *BglII* site (shown in bold) in the 5' end of the gene. Both the 5' and 3' ends of the calmodulin gene were digested with *BglII*, and inserted into the *BglII* site of dihydrofolate reductase (DHFR) gene in the pD136 plasmid. B. Amino acid sequence of sandwich protein. The sequence of calmodulin is shown in italics and bold. The calcium-binding domains of calmodulin are underlined. The eight residue linker region separating the C-terminus of calmodulin and the C-terminus of DHFR is indicated.

introduce *BglII* restriction sites at the ends of the gene to facilitate cloning into the DHFR gene.

Two steps of cassette mutagenesis were performed to achieve these goals. First, a 73 bp cassette I was constructed with a blunt end corresponding to the *HpaI* site of the calmodulin gene [21] and with an extension which contained *BglII*, *SmaI*, and *BamHI* restriction sites at the 3' end (Fig. 1). The cassette was inserted into *HpaI* and *BamHI* cleaved calmodulin/pUC18 vector and was cloned into *E. coli* strain UT481 by using similar approaches to those previously described [22]. The resulting plasmid was cleaved with *EcoRI* and *BclI* and the cassette II was inserted into the vector to introduce a *BglII* site in the 5' end of the calmodulin gene (Fig. 1). The calmodulin gene was removed as a single fragment by digestion with *BglII*, and was inserted into the *BglII* site of DHFR gene in pD136 plasmid. Transformation of *E. coli* JM109 and plasmid preparation were performed as descrived above. The correct orientation of calmodulin gene was determined by *HindIII* digestion.

The sandwich protein was prepared by the following procedure. Cells were grown at 37°C in 6 *l* of modified version of terrific broth [26] containing 200 μg/ml of TMP for 48-52 h. Cells were centrifuged at 6,000 × g for 10 min, were resuspended in 10 mM Tris-HCl, pH 7.0, 1 mM EDTA (1 × TE buffer), and were lysed by sonication. The cell lysate was centrifuged at 10,000 × g for 30 min. Supernatants were adjusted to 40% saturation by the slow addition of ammonium sulfate with stirring. After an additional 40 min of stirring, the sample was centrifuged at 10,000 × g for 20 min. The

resulting supernatant was adjusted to 90% saturation with solid ammonium sulfate, was stirred for 40 min, and was centrifuged at 10,000 × g for 20 min. The pellet obtained from the 40 to 90% saturation precipitation step was resuspended in 10 ml of 50 mM potassium phosphate, pH 6.0, 1 mM EDTA (1 × PE buffer), was dialyzed against the same buffer overnight at 4°C, and was applied to a methotrexate column equilibrated with 1 × PE buffer. The column was washed with 50 ml of 1 × PE, pH 6.0, 1 M KCl, with 50 ml of 1 × PE, pH 8.0, 1 M KCl, and then with 50 ml of 1 M sodium borate, pH 9.0, 1 M KCl. The sandwich protein was eluted with 1 M sodium borate, pH 9.0, 1 M KCl, 1 mM folate. Fractions with the greatest DHFR activity were pooled and dialyzed overnight against 1 × TE buffer. The dialyzed sample was applied to a DEAE-Sephacel column. The column was washed with 200 ml of 1 × TE buffer and was eluted with a linear salt gradient from 0 to 0.5 M KCl in 1 × TE buffer. Sandwich protein was purified from the fractions which gave DHFR activity by phenyl-Sepharose as described above. Fractions containing high activity of DHFR were pooled, and were precipitated and stored as a slurry in 40% ammonium sulfate at 4°C until use. Before use, the sample was centrifuged, resuspended in 1 ml of 1 × TE buffer, and dialyzed against the same buffer for 4 h.

Purification of nicotinamide adenine dinucleotide (NAD) kinase

Pea NAD kinase was purified through the polyethyleneglycol (PEG) by using the procedure of Roberts *et al.* [21]. After this step, calmodulin was removed by a modified DEAE-ion exchange chromatography. After PEG precipitation, the enzyme was resuspended in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 3 mM $MgCl_2$, 0.2 mM $CaCl_2$, and was centrifuged for 15 min at 27,000 × g at 4°C to remove insoluble material. The supernatant was applied to a DEAE-Sephadex A-50 column (2.5 × 6 cm), and the column was washed with 50 mM Tris-HCl, pH 7.0, 100 mM KCl, 3 mM $MgCl_2$, 10% (v/v) glycerol, 0.1 mM $CaCl_2$, until the A_{280} was less than 0.02. Calmodulin-dependent NAD kinase was eluted with 50mM Tris-HCl (pH 7.0), 100 mM KCl, 3 mM $MgCl_2$, 10% (v/v) glycerol, 1mM EGTA. Active fractions were pooled and stored at –80°C.

Enzyme Assays

Methyltransferase activity was assayed by a modification of the general protocol of Rowe *et al.* [23] as described by Oh and Roberts [17] and Han *et al.* [10]. The standard reaction mixture (100 μl) contained calmodulin (1.67 μg) and 12.0 μM [^{3}H]AdoMet (1.5 μCi) in 0.1 M glycylglycine-NaOH, pH 8.0, 0.15 M KCl, 2 mM $MgCl_2$, 5 mM DTT, 0.01% (w/v) Triton X-100, 0.1 mM $CaCl_2$. The reaction was initiated by the addition of enzyme and incubation was typically done for 20 min at 37°C. The reactions were microfuged for 6 min and the supernatants were combined with 200 μl of a 1 : 1 phenyl-Sepharose slurry equilibrated in 50 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 0.1 mM $CaCl_2$. The mixture was vortexed and microfuged. The collected resin was washed with 2 ml of same buffer. [^{3}H]methyl-labeled calmodulin was eluted with 0.1 M NH_4HCO_3, pH 8.0, 2 mM EDTA and radioactivity was measured by liquid scintillation counter (Beckman LS 3801; Beckman Instruments, USA).

A modified protocol was used for the assay of the calmodulin methyltransferase in the presence of calmodulin binding peptides. Assays were performed under the standard calmodulin methyltransferase assay conditions in the presence of 0.1 mM $CaCl_2$ and 1 μM calmodulin. After termination of the reaction, 10 μl of 10 mM EGTA was added and the peptide was removed by adsorption to 40 μl of phosphocellulose. The unabsorbed fraction, which contains calmodulin, was then analyzed by the standard phenyl-Sepharose method as described above.

The analysis of kinetic data and determination of kinetic parameters for the methyltransferase were done by using the form of the Michaelis-Menten equation for sequential bireactant systems. Pea NAD kinase activity was assayed as described by Roberts *et al.* [23]. Assays for the calcium dependence of NAD kinase activation were done in 50 mM Hepes-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM EGTA, 3 mM ATP, 2 mM NAD, 0.1 μM calmodulin, and various $CaCl_2$ concentrations that yielded a free Ca^{2+} ranging from 10^{-8} to 10^{-3} M as determined by the approach described above.

Calcium Binding

All buffers were decalcified by treatment with Chelex 100 resin (BioRad, USA) as described previously [4]. Free calcium concentrations in solution was quantified with a Variand AA-1475 atomic

absorption spectrophotometer by using a hollow calcium lamp (Fisher Scientific, USA). Thermal settings were: dry at 120°C for 30 sec, char at 1,100°C for 20 sec, and atomize at 2,500°C for 5 sec. Standard curves were made by reading serial dilutions of an atomic absorption calcium standard. Both samples and standards were diluted with decalcified deionized water.

Prior to calcium binding analyses, calcium was removed from calmodulin by precipitaion with trichloroacetic acid as described by Haiech *et al.* [8]. After the last precipitation step in this protocol, the pellet was briefly washed with Chelex-treated water, and was resuspended in 1 ml of 100 mM Hepes-NaOH, pH 7.5, 0.1 M KCl. Calcium stock solutions were standardized by atomic absorption spectrophotometry as described above.

Calcium-binding studies were done by the flow dialysis approach of Colowick and Womack [2] as adapted for calmodulin [8, 19]. All experiments were performed with a Macrodialyzer apparatus (Spectra, USA), with a dialysis membrane (Mr cutoff = 3,500, Spectra/Por* 6; Spectra, USA) separating the sample (upper) and perfusion (lower) chambers.

Results

The function of calmodulin is linked to the ability of the two lobes to 'clasp' a hydrophobic, basic target structure [18]. A novel approach to studying calmodulin / protein structure and function was developed by making a 'sandwich protein' which was constructed by insertion of calmodulin into the β-bulge region of bacterial chromosomal DHFR (Fig. 2).

The calcium binding properties of the sandwich protein were analyzed by flow dialysis (Fig. 3). Similar to calmodulin, the sandwich protein bound four equivalents of calcium, with half saturation ($K_{0.5}$) observed at a [Ca^{2+}] of 8 M. This value is slightly higher than that of calmodulin (10.7 μM). Macroscopic binding constants obtained by fitting the data to the Adair equation were calculated and yielded values similar to calmodulin including a high affinity pair (K_1 and K_2) and lower affinity pair (K_3 and K_4) of binding sites (Table 1). This result suggests that the insertion of calmodulin into the β-bulge of calmodulin does not adversely affect its ability to associate with calcium.

The ability of the sandwich protein to activate a calmodulin-dependent enzyme, NAD kinase (Fig. 4) was evaluated. The sandwich protein activated NAD kinase, but to only half of the level obtained with calmodulin. The $K_{0.5}$ for both calmodulin and the sandwich protein were approximately the same (1-2 nM). The addition of trimethoprim (1 μM) to the assay did not drastically affect the activation profile suggesting that DHFR portion of the sandwich protein is folded in the assay.

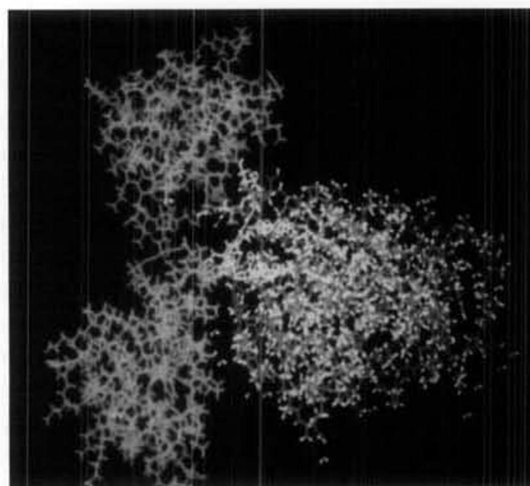

Fig. 2. Constructed model structure of calmodulin-dihydrofolate reductase sandwich protein. The model was constructed by insertion of apo-form of calnodulin (blue color, Kuboniwa *et al.* [12]) into the β-bulge region (position 134 and 135) of bacterial chromosomal DHFR (red color, Filman *et al.* [5]).

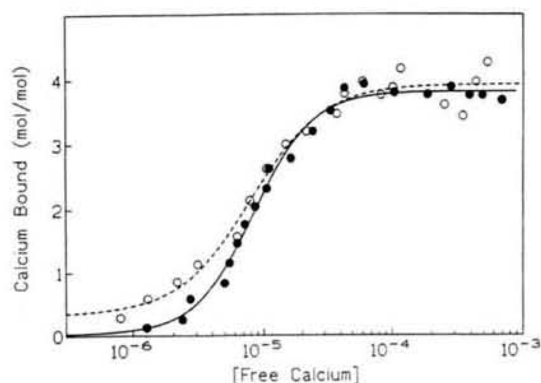

Fig. 3. Calcium binding isotherms of calmodulin-dihydrofolate reductase sandwich protein. Calcium binding curves of calmodulin (●) and sandwich protein (○) were generated by flow dialysis as described in the Materials and Methods. Protein concentrations were 30 μM in 50 mM Hepes-NaOH buffer, pH 7.5, 0.1 M KCl, 2 mM $MgCl_2$.

Table 1. Calcium binding parameters for sandwich protein

Protein	K_1^*	K_2	K_3	K_4 (μM^{-1})
Calmodulin	0.152 (0.049)	0.187 (0.084)	0.022 (0.013)	0.065 (0.031)
Sandwich Protein	0.158 (0.092)	0.252 (0.186)	0.036 (0.024)	0.040 (0.020)

*K_1, K_2, K_3 and K_4 are the apparent macroscopic binding constants calculated by fitting the data in Fig. 3 to the Adair-Klotz equation as described in Materials and Methods. The R^2 for the fit was 0.9340 for the sandwich protein and 0.9979 for calmodulin. The numbers in parentheses indicate standard deviations of values obtained from fitted binding data.

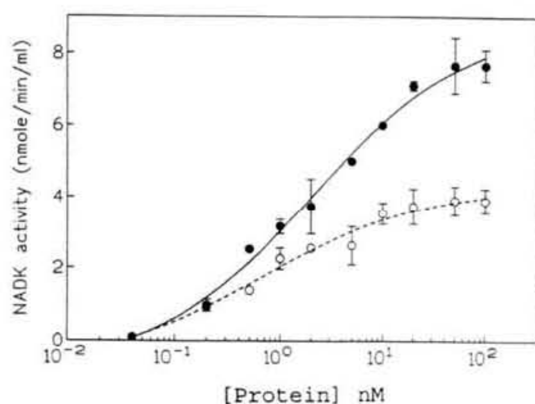

Fig. 4. Nicotinamide adenine dinucleotide (NAD) kinase activation by calmodulin- dihydrofolate reductase (DHFR) sandwich protein. NAD kinase activation of calmodulin (●) or calmodulin-DHFR sandwich protein (○) were measured at various concentrations in the presence of 0.1 mM $CaCl_2$ as described in the Materials and Methods. Error bars show the standard error of the mean.

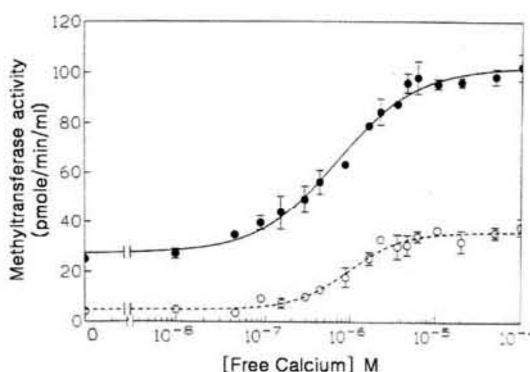

Fig. 5. Calcium dependence of the methylation of calmodulin-dihydrofolate reductase (DHFR) sandwich protein. Methyltransferase activity was measured over the range of calcium concentrations with 1 μM calmodulin (●) or 1 μM calmodulin-DHFR sandwich protein (○). Free calcium concentrations were controlled by using EGTA/Ca^{+2} buffering system as described in the Materials and Methods. The other assay components were: 12 μM S-adenosylmethionine, 2 mM $MgCl_2$, 0.15 M KCl, 0.1 M glycylglycine, pH 8.0. Error bars show the standard error of the mean.

Methylation analyses of the sandwich protein show that insertion of calmodulin into DHFR results in a large decrease in methylation (Figs. 5-7). The trend of the residual methylation is same, with calcium stimulating the rate of methylation of the sandwich protein (Fig. 5), with barely detectable methylation observed in the absence of calcium (Figs. 5 and 6). Similar $K_{0.5}$ values were observed for two proteins (0.796 μM for calmodulin and 0.970 μM for sandwich protein), which is consistent with the similar calcium binding properties of the two protein based on calcium binding measurements (Fig. 3).

Methyltransferase activities were measured at a high, fixed concentration (12.1 M) of AdoMet with sandwich protein as variable substrate (Fig. 6). The V_{max} observed with the sandwich protein (95 nmole/min/ml) was only 22% of the value observed with calmodulin (436 nmole/min/ml) in the presence of calcium. However, the apparent km for sandwich protein (579 nM) was only 1.7-fold higher than apparent Km for calmodulin (334 nM) in the presence of calcium.

Addition of TMP to the reaction inhibited the observed methylation rate substantially (Fig. 7), raising the possibility that the low level of methylation observed may have been due to a small percentage of sandwich protein that has an unfolded DHFR domain but still has an intact calmodulin domain that allows methyltransferase recognition. However, a modest decrease in the rate of calmodulin methylation is also observed with TMP, and a direct effect of this reagent on the methyltransferase or on calmodulin cannot yet be ruled out. Regardless, the results strongly suggest that the insertion of calmodulin into the DHFR protein

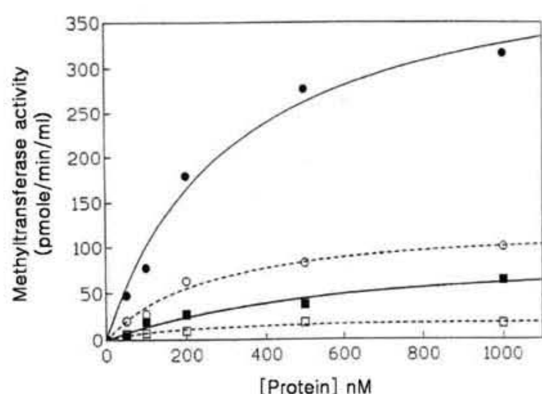

Fig. 6. Kinetics of the methylation of calmodulin-DHFR sandwich protein. Methylation activities were measured with a saturated concentration (12.1 μM) of AdoMet. Calomodulin in the presence of 0.1 mM $CaCl_2$ (●); calmodulin in the presence of 1 mM EGTA (○); calmodulin-DHFR sandwich protein in the presence of 0.1 mM $CaCl_2$ (■); calmodulin-DHFR sandwich protein in the presence of 1 mM EGTA (□).

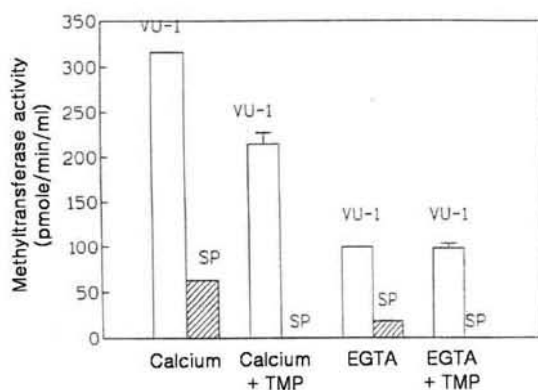

Fig. 7. Calmodulin methyltransferase activities in the presence or absence of trimethoprim (TMP). Methyltransferase activities were measured with a saturated concentration (12.1 μM) of AdoMet with VU-1 calmodulin (1 μM, VU-1, open bars) or calmodulin-DHFR sandwich protein (1 μM, SP, cross hatched bars) in the presence or absence of 3.4 μM of TMP, and in the presence of either 0.1 mM $CaCl_2$ or 1 mM EGTA.

significantly perturbed the methyltransferase recognition site.

Discussion

A calmodulin-DHFR sandwich fusion protein was generated by insertion of calmodulin into the β-bulge region of DHFR to observe the effects of structural constraints placed on the calmodulin molecule enforced by DHFR folding. The use of the DHFR protein for this study is ideal because it is structurally well defined, folding can be selected by TMP resistance, and because several inserts have been successfully integrated into the β-bulge.

The calcium binding properties of the sandwich protein were virtually indistinguishable from wild type calmodulin. This is not surprising since the two lobes of calmodulin do not interact and have similar structural and calcium binding properties regardless of whether they are part of the same molecule or are separate fragments [6, 7, 13, 16]. Therefore, if there is no structural perturbation within the lobes of calmodulin, the calcium binding characteristics of the calmodulin domain of the sandwich protein should be normal.

Despite normal calcium binding characteristics, the sandwich protein showed modest defects in its ability to activate NAD kinase, and substantial defects in it ability to recognized by the methyltransferase. Upon binding target peptide, the long central helix (residues 65-93) of calmodulin is disrupted into two helices connected by a long flexible loop (residues 74-82), thereby enabling the two domains to clamp the bound peptide [11, 14]. Further, the helix can unfold to a various extent to accommodate different target structures [15]. The lower activator properties of the sandwich protein might result from a constraint of two domains of calmodulin by the DHFR structure. However, this constraint does not drastically affect activation of the NAD kinase tested in this study. Previous work with various calmodulin-dependent enzymes shows that they are extremely variant in their sensitivities to mutations in calmodulin structure. For example, while some enzymes (e.g. PDE) are very tolerant of alterations in calmodulin structure, others are extremely sensitive to the same mutations [3]. In this regard it will be interesting to investigate the interaction of the sandwich protein with a variety of calmodulin-binding peptides and calmodulin-dependent enzymes.

The sandwich protein was poorly methylated by the methyltransferase, and this effect seemed to be enhanced in the presence of TMP. Since the recognition site of the methyltransferase resides solely in the C-terminal lobe of calmodulin, this result is probably not due to the alterations in the central helix or constraints

of the two lobes relative to one another. Perhaps steric hindrance of the C-terminal domain within the DHFR-sandwich protein structure prevents the methyltransferase from binding. Also we cannot exclude the possibility of a distortion of the methyltransferase recognition sequence or even local interactions between calmodulin and the DHFR structure.

The results of this study have provided important leads in the types of interaction and general structural elements that are necessary for methyltransferase binding. Further, it is clear that the recognition site is not a simple linear peptide sequence, and that it requires structures that are unique to the C-terminal lobe. With these findings as a starting point, finer detailed mutagenesis studies can now be designed to start to elucidate further the specific residues within the C-terminal lobe that are essential for methyltransferase specificity. In addition, the availability of a purified methyltransferase enzyme should facilitate molecular cloning experiments so that the structural and functional properties of the methyltransferase can be further pursued.

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