Thermodynamics of apocalmodulin and nitric oxide synthase II peptide interaction

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Abstract The Ca^{2+}-free form of calmodulin (CaM), apocalmodulin (ApoCaM), regulates a variety of target proteins including nitric oxide synthase II (NOS-II). The CaM-binding site of NOS-II can bind ApoCaM with high affinity. Substitution of hydrophobic amino acids by charged amino acids at crucial positions 3, 9 and 13 within the CaM-binding motif did not abolish the ApoCaM interaction that occurred with significant affinity, though the affinity of the interaction was decreased remarkably. Isothermal titration calorimetry revealed that interaction of ApoCaM and synthetic NOS-II peptides was driven entropically.

Keywords: Apocalmodulin; Calmodulin-binding motif; Nitric oxide synthase

1. Introduction

Cellular Ca^{2+}-signals are monitored by Ca^{2+}-sensor proteins that transduce the signal to a corresponding target protein. The ubiquitous sensor calmodulin (CaM) controls a variety of target proteins and thereby regulates many cellular processes [1–3]. However, CaM not only operates as a modulator of cell signaling in its Ca^{2+}-bound form: the Ca^{2+}-free form, denoted apocalmodulin (ApoCaM), but is also able to regulate the function of specific target proteins [3]. A prominent example of an ApoCaM target is the inducible nitric oxide synthase (NOS-II), which binds CaM with high affinity and is active in the absence of Ca^{2+} [4].

All NOS isoforms harbor a CaM-binding site that fits well into the sequence motif of a classical Ca^{2+}-CaM-binding site, i.e., they consist of approximately 20 amino acids that form an amphipathic α-helix. Hydrophobic amino acid residues are mainly found at the positions 1-8-14 or 1-5-10. It is noteworthy that the Ca^{2+}-independent interaction of CaM with NOS-II occurs at a CaM-binding site that does not correspond to the IQ motif (IQxxxRGxxxR), a typical ApoCaM binding motif found in many CaM regulated proteins [2,3].

In a recent peptide binding study, we identified critical amino acid substitutions in the 1-8-14 motif of NOS-I that convert the classical Ca^{2+}-CaM-binding site into a site for ApoCaM-binding [5]. Substitutions were inspired by a comparison of NOS-I and NOS-II CaM-binding sites. For example, substitution of a negative charge to a positive charge is sufficient to create a target site for ApoCaM, further introduction of hydrophobic amino acids increases the apparent affinity for ApoCaM by almost one order of magnitude. Based on these observations, we reasoned that corresponding substitutions in the (Apo)CaM-binding site of NOS-II could either decrease the affinity for ApoCaM or even disable any ApoCaM binding. Therefore, we designed peptides with the corresponding amino acid exchanges and studied their ApoCaM binding characteristics.

2. Materials and methods

2.1. Peptide synthesis

Peptides were synthesized, purified and characterized as described [6]. Purity was $\geqslant 95\%$ by HPLC analysis. Concentration of peptides was determined by measuring the absorption of the peptide bond at 205 nm using the formula $c\ (\mathrm{mg\ ml^{-1}}) = A_{205}d/\varepsilon$, where d is 1 cm and ε is 31 [7].

2.2. Isothermal titration calorimetry (ITC)

Principles of experimental design and theory of data analysis of ITC has been described [8,9]. The experimental steps were performed as described for NOS-I peptides [5]. Briefly, the VP-ITC MicroCalorimeter from MicroCal Incorporated (kindly provided by Prof. M. Bott, IBT, Forschungszentrum Jülich) consists of a sample cell, a reference cell and a spinning syringe. One binding partner was placed in the sample cell and the other (reactant) in the spinning syringe. The calorimeter determines the temperature difference between the sample cell and the reference cell, when the reactant is injected into the sample cell. The binding enthalpy and the equilibrium constant can be derived from a titration experiment, where the concentration of one binding partner is kept constant in the sample cell and the concentration of the reactant is increased by repetitive injections. Peptides and CaM were dissolved in water and diluted in buffer to a final concentration of 200 μM and 8 μM, respectively. Titration steps were 0.5 or 1 nmol per injection. The buffer contained 10 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, 10 mM EGTA and 0.005% (v/v) Tween 20 (EGTA-buffer). All measurements were done at 25 °C. Data analysis was done with Microcal Origin Version 5.0 as described [5,8,9].

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Abbreviations: CaM, calmodulin; ApoCaM, Ca^{2+}-free calmodulin; NOS-II, nitric oxide synthase type II; dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; ITC, isothermal titration calorimetry; HPLC, high performance liquid chromatography

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2.3. Protein determination

CaM stock solutions were prepared from a lyophilized powder purchased from Sigma. Protein concentration was determined by a Coomassie Blue dye binding assay [10] using a CaM standard curve. The concentration of CaM standards were adjusted by using the molar extinction coefficient of CaM at 277 nm of $\varepsilon = 3006\ M^{-1}\ cm^{-1}$. Alternatively, the BCA Protein Assay Reagent Kit of Pierce was used as described previously [5].

2.4. Fluorescence measurements

CaM was derivatized with dansyl-chloride (dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl) according to a published procedure [11] with some modifications as described [5]. Fluorescence experiments were performed similarly as described [5,11] using a Shimadzu RS-1501 fluorescence spectrometer. Excitation wavelength was at 335 nm, the emission spectrum was recorded from 420 to 650 nm. Dansyl-CaM (1 μM) was first measured in buffer without calcium in the absence of peptides. Afterwards, measurements were done in the presence of varying peptide concentrations. Titration with peptides was performed in 0.5 or 1 μmol L^{-1} steps until the signal reached saturation. Concentration of free Ca^{2+} in EGTA containing buffer solution after addition of peptides was checked via determination of fluorescence lifetime with the Ca^{2+}-indicator calcium green 1 [12]. The free Ca^{2+} in EGTA-buffered solutions was <50 nM and did not change after addition of peptides.

3. Results

3.1. Binding of mutant peptides to ApoCaM monitored by changes in fluorescence emission

The CaM-binding site of mouse macrophage NOS-II (aa 503–528) consists mainly of hydrophobic amino acids. When displayed in a helical wheel plot (Fig. 1A), it is immediately apparent that the motif does not represent a typical amphipathic helix as it is found in the CaM-binding site of NOS-I or other classical Ca^{2+}-CaM-binding sites [2,3]. We showed previously that amino acid positions K733, E736, K739 and K743 in NOS-I from rat determine binding ability and affinity to ApoCaM [5]. Corresponding positions in the NOS-II CaM-binding site are V511, K514, F517 and M521 (Fig. 2). Substitutions of these amino acids by the amino acids present in NOS-I increased the amphipathic character of the binding site (Fig. 1B). We used synthetic peptides of the indicated sequences (Fig. 2) to investigate whether the introduced mutations alter ApoCaM binding properties.

First, we tested whether peptides had lost or retained the ability to interact with ApoCaM. Dansyl-CaM was employed in fluorescence measurements to study interaction of ApoCaM with target peptides. Dansyl-CaM in a Ca^{2+}-free buffer has a maximum of fluorescence emission at 530 nm (Fig. 3A and B). Addition of equimolar amounts of WT NOS-II peptide shifted the maximum emission wavelength to 506 nm and increased the fluorescence intensity by 1.6-fold (Fig. 3A) as reported previously [5]. Addition of mutant peptides caused a similar shift of the maximum emission wavelength to 500 nm (all peptides) and similar increase of the fluorescence intensity like 1.8-fold for NOS-II M521K, 1.9-fold for NOS-II V511K, 1.6-fold for NOS-II F517K, 1.7-fold for NOS-II 3K and 1.8-fold for NOS-II E3K (Fig. 3A and B). These experiments showed that all peptides carrying the substitutions bound ApoCaM.

3.2. Isothermal titration calorimetry

Second, we analyzed the thermodynamics of these target peptide interactions by isothermal titration calorimetry. A representative titration in Ca^{2+}-free buffer is shown in Fig. 4. CaM was present in the sample cell in Ca^{2+}-free buffer, 0.5 or

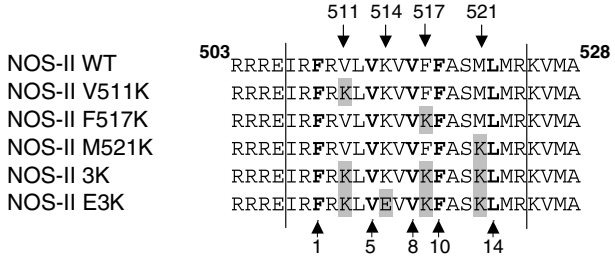

Fig. 2. Amino acid sequences of the peptides used in this study. At critical positions (511, 514, 517, 521; gray boxes), amino acids are changed to the corresponding residues present in NOS-I. Positions 1-5-8-10-14 of classical CaM-binding motifs are indicated (bold letters and small arrows). Vertical lines mark the boundaries of the corresponding helical wheel diagram in Fig. 1.

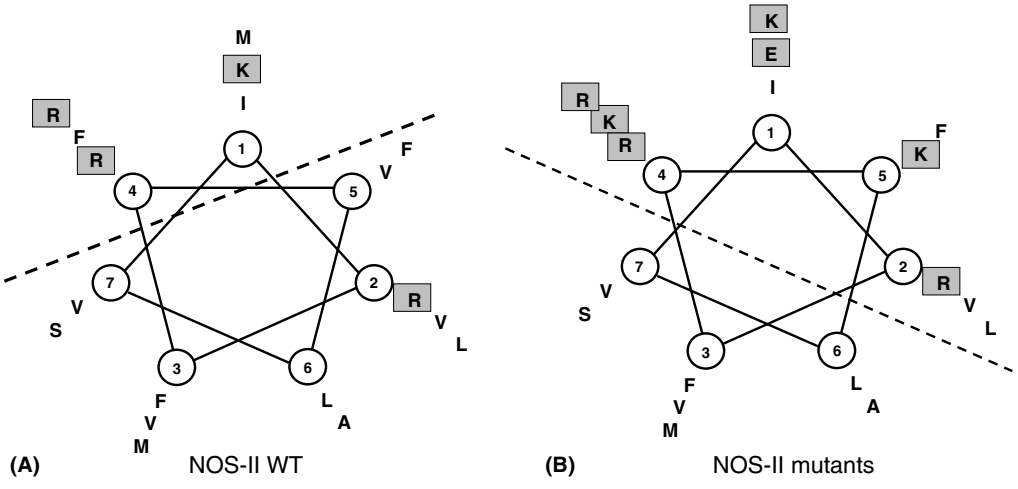

Fig. 1. α-Helical wheel diagram of the CaM-binding site of mouse macrophage NOS-II (amino acids I507-R524). Charged amino acids are highlighted by gray boxes. (A) WT NOS-II. (B) Mutant NOS-II E3K. Amino acid substitutions (see text) convert the hydrophobic WT peptide into the more amphipathic mutant peptide. Amphipathic character is highlighted by the dashed line.

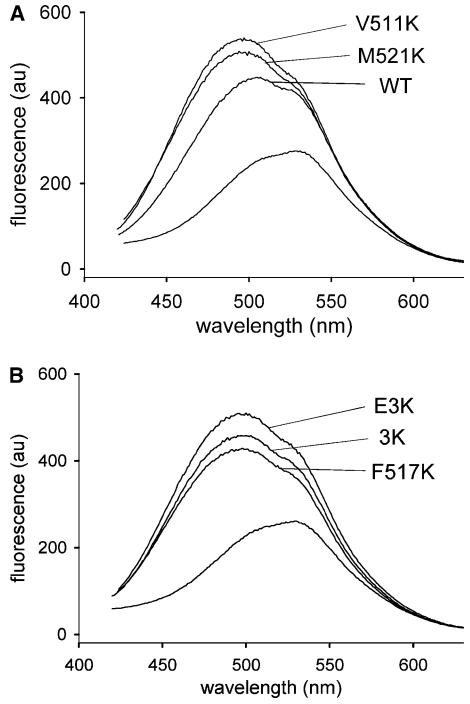

Fig. 3. Fluorescence emission spectra of dansyl-ApoCaM in the absence and presence of NOS-II peptides. Recording of 1 μM dansyl-ApoCaM alone (lower traces in A and B) and in the presence of equimolar amounts of either WT NOS-II, NOS-II M521K or NOS-II V511K (A) or equimolar amounts of either NOS-II F517K, NOS-II 3K or NOS-II E3K (B).

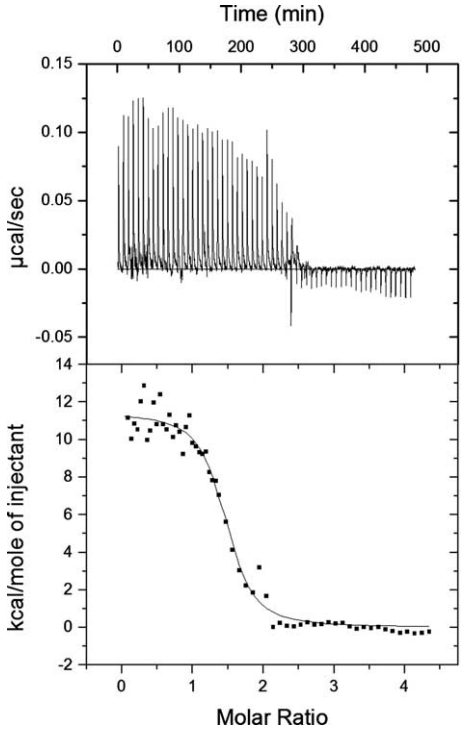

Fig. 4. ITC experiment with peptide NOS-II F517K and ApoCaM. Upper part: repetitive injections of NOS-II F517K into a solution of 8 μM ApoCaM caused endothermic heat pulses. Lower part: changes in enthalpy (kcal mol^{-1} of injectant) of the corresponding injections shown above as a function of the molar peptide to ApoCaM ratio. Data were fitted by the following equation (Q is the heat absorbed or evolved; V_0 is the volume of reaction cell; X_t is the total concentration of ligand; K is the binding constant K_A; M_t is the total concentration of macromolecules in cell; n is the number of binding sites)

$$Q = \frac{nM_t\Delta HV_0}{2}\left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t}\right)^2 - \frac{4X_t}{nM_t}}\right]$$

A detailed description of data analysis has been given [8,9]. The titration series above resulted in the following parameter: $K_A = 5.041 \times 10^6$ M^{-1}, $K_D = 198$ nM, $\Delta H = 11.42$ kJ mol^{-1}, $\Delta S = 68.96$ J mol^{-1} K^{-1}.

1 nmol of NOS-II F517K dissolved in buffer was repeatedly injected. Binding of CaM to this peptide caused endothermic heat pulses that decreased to a stable baseline when saturation was achieved (Fig. 4, upper part). When all peptides were analyzed in the same manner, we registered that amino acid substitutions caused a decrease in affinity in the following order:

NOS-II M521K > NOS-II F517K > NOS-II V511K > NOS-II E3K > NOS-II 3K (for K_D values see Table 1). Change in K_D from NOS-II M521K to NOS-II 3K was 25-fold. Interaction of ApoCaM with WT NOS-II peptides was of similar affinity as the interaction of NOS-II M521K with ApoCaM ($K_D \approx 40$ nM as reported in [13,14] for WT NOS-II and $K_D = 66.8$ nM for NOS-II M521K). We also tried to measure binding of ApoCaM to WT NOS-II peptides (mouse macrophage and human hepatic types) by ITC. However, due to the tendency of these peptides to aggregate soon after solubilization, we were unable to record reliable ITC titration series.

All binding events employing mutant peptides were endothermic with positive ΔH values (between 12 and 33 kJ mol^{-1}) and positive entropy (between 178 and 232 J mol^{-1} K^{-1}) resulting in a negative value of the Gibbs free energy (for a complete summary of data see Table 1). Thus, positive values of ΔH are compensated by large positive values of ΔS. Conversion of the hydrophobic NOS-II CaM-binding site into a site that displays a more amphipathic character (Fig. 1) did not abolish the principal ability to bind ApoCaM, but it changed significantly the apparent affinity of interaction.

Table 1
Thermodynamics of ApoCaM–peptide interaction

Peptide	K_A (M^{-1})	K_D (nM)	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG (kJ mol^{-1})	n
NOS-II V511K	$1.3 \times 10^6 \pm 2.0 \times 10^5$	804 ± 124	25.9 ± 0.65	203.5 ± 3.4	−34.8	2
NOS-II F517K	$4.4 \times 10^6 \pm 6.4 \times 10^5$	232 ± 33.7	31.2 ± 1.6	231.6 ± 56.9	−37.9	2
NOS-II M521K	$1.9 \times 10^7 \pm 9.0 \times 10^6$	66.8 ± 31.2	11.9 ± 1.3	178.3 ± 8.6	−41.3	2
NOS-II 3K	$6.0 \times 10^5 \pm 1.1 \times 10^5$	1660 ± 295	33.3 ± 1.0	222.5 ± 62.5	−33.0	3
NOS-II E3K	$8.3 \times 10^5 \pm 7.0 \times 10^4$	1199 ± 101	27.0 ± 9.6	203.9 ± 31.4	−33.7	2

4. Discussion

The different hydrophobic character of CaM-binding sites of NOS isoforms have been recognized for several years, but it is an unresolved issue as to how these differences determine the target recognition by CaM. Structural elucidation of CaM/target peptide complexes has unravelled an unexpected variety of three-dimensional conformations (see [1] for recent review) making it difficult to assign a general model of CaM/target interaction. The unique versatility of CaM as Ca^{2+}-sensor and signaling protein is also evident from observations that ApoCaM can bind to and control the activity of many proteins. Using the CaM-binding site of NOS-II as model for ApoCaM binding, we here show that the successive exchange of hydrophobic amino acids to charged amino acids (K and E) led to a decrease of apparent affinity without loss of ApoCaM binding ability. The binding event was entropically driven meaning that the driving force for binding was the translocation of solvent exposed hydrophobic side chains to a less water-exposed environment. A similar conclusion was derived for the interaction of NOS-I mutant peptides with ApoCaM, where the positive enthalpic term was also compensated by a larger entropic term [5]. Furthermore, successive exchange of charged amino acids to hydrophobic ones in a NOS-I peptide increased the affinity to ApoCaM, whereas the replacement of hydrophobic amino acids by charged amino acids decreased the affinity.

All NOS isoforms contain the classical '1-8-14' CaM-binding sequence motif harboring the key hydrophobic residues for CaM interaction. Aoyagi et al. [15] proposed in a recent study that hydrophobic residues V517 and L523 (human NOS-II) might represent additional important hydrophobic anchors for interaction with ApoCaM. The corresponding amino acids in our study are V511 and F517 (mouse NOS-II). Indeed, conversion of these hydrophobic into charged residues decreased the affinity of the peptides for ApoCaM significantly, but it did not disable binding to ApoCaM. The simultaneous introduction of three charged amino acids (3K) further weakened the binding to ApoCaM, but it did not prevent it. Interestingly, the K514E conversion of a positive to a negative charge at position 6 did not change much the affinity and the thermodynamic data when NOS-II E3K is compared with NOS-II 3K (Table 1). When we investigated a NOS-II mutant peptide with a K>E conversion at position 6 but no other mutations (NOS-II K514E), we observed no binding to ApoCaM at all (data not shown). Thus, a positive charge at this position appears to be important for interaction with ApoCaM consistent with our previous study on peptide NOS-I E736K [5]. However, a lack of a positive charge at position 6 can be compensated by a positive charge at position 13 (for example, in peptide NOS-II E3K), which might be explained by the spatial proximity of these two positions according to the helical wheel presentation (Fig. 1B). Thus, position 6 seems to be important for binding of a peptide to ApoCaM per se, but it is less important for the affinity of this interaction.

The site M521 (position 13) also appeared to be less crucial, since the mutant peptide NOS-II M521K retained the high affinity of WT NOS-II for ApoCaM. This is consistent with the fact that position 13 varies among all NOS isoforms [15] and therefore does not require a hydrophobic amino acid.

Our results are complementary to our previous observations made with NOS-I peptides [5] and demonstrate the importance of hydrophobic residues at positions 3, 9 and 13 for ApoCaM affinity and of position 6 for ApoCaM recognition. Furthermore, interaction of targets with ApoCaM can be fine-tuned by modifications at these positions leading to distinct affinities. Finally, the interaction of all studied peptides (NOS-I and NOS-II) with ApoCaM is entropically driven.

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