Substrate specificity and the effect of calcium on *Trypanosoma brucei* metacaspase 2

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Keywords
calcium binding; kinetic parameters; metacaspase; structural modification; substrate specificity

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(Received 30 November 2012, revised 19 February 2013, accepted 11 March 2013)
doi:10.1111/febs.12248

Introduction

Metacaspases are cysteine peptidases that are distantly related to the mammalian caspases but found only in plants, fungi and protozoa [1]. Together with the caspases and paracaspases [1], they are endopeptidases which have been grouped into structural family C14 in clan CD of the MEROPS peptidase database [2]. In *Trypanosoma brucei*, five metacaspase genes (*TbMCA1–5*) have been reported with three, *TbMCA2*, *TbMCA3* and *TbMCA5*, predicted to code for active peptidases based on the conservation of an intact cysteine-histidine catalytic dyad [3,4]. Interestingly, in *TbMCA1* and *TbMCA4* the catalytic cysteine is replaced by a serine, indicating that the coded proteins would not exhibit cysteine peptidase activity [3,5]. Whilst plant metacaspases have been shown to be involved in cell death pathways, trypanosomes appear to lack regulated cell death [6] and in these organisms metacaspases have evolved alternative functions [5,7].

Abbreviations
Abz, *ortho-*aminobenzoic acid; EDDnp, *N*-2,4-dinitrophenyl-ethylenediamine; FRET, fluorescence resonance energy transfer; 
*TbMCA2*, *Trypanosoma brucei* metacaspase 2.
Despite being related to the caspases, metacaspases have been shown to be both structurally and functionally distinct. Metacaspases are known to cleave their substrates after Arg and Lys residues in the P4 position [8], as demonstrated for metacaspases from Trypanosoma brucei [9], Trypanosoma cruzi [10], Leishmania major [11], plants [12–14] and yeast [13], while the caspases cleave their substrates after Asp [15]. Other functional differences between the two families of enzymes are that, unlike the caspases, metacaspases do not necessarily require processing or dimerization for activity and they are activated by calcium [9,12–14,16].

Results

Effects of temperature, pH and Ca$^{2+}$ concentration on the activity of TbMCA2

The activity of TbMCA2 was initially assayed using the reference substrate Abz-KARSSAQ-EDDnp over a range of temperatures (20–37 °C), pH values (pH 5 to pH 11) and CaCl$_2$ concentrations (0–5 mM).

Aliquots of TbMCA2 were incubated in the presence of Ca$^{2+}$ at four different temperatures (20, 25, 30 and 37 °C) for 15 min and the residual activity was measured at 2.5 min and then at 5 min intervals showing that the remaining activity of TbMCA2 decreases most rapidly at higher temperatures (Fig. 1). At higher temperatures TbMCA2 undergoes much more extensive autoprocessing, as observed by SDS/PAGE analysis (data not shown), suggesting that while minimal autoprocessing is not detrimental [9], unlimited autoprocessing will ultimately inactivate TbMCA2. It was also noted that TbMCA2 is stable at 37 °C for at least 60 min when incubated in the presence of a saturating substrate concentration (data not shown). However, in order to reduce TbMCA2 activity loss during the reactions all assays were conducted at 25 °C.

The relationship between pH and $k_{cat}/K_M$, for the hydrolysis of Abz-KARSSAQ-EDDnp by TbMCA2, indicates an optimum pH for TbMCA2 activity of 7.7 and the participation of two ionizing groups with $pK_a = 6.70 \pm 0.02$ and $pK_a = 8.70 \pm 0.02$, which can be directly attributed to the histidine ($pK_a$ 6.7)–cysteine ($pK_a$ 8.7) catalytic dyad (Fig. 2). This bell-shaped pH profile and the calculated $pK_a$ values are similar to those

![Fig. 1. The thermal stability of TbMCA2. The activity of TbMCA2 was measured over a 15-min period using time intervals of between 2.5 and 5 min at 37 °C (○), 30 °C (■), 25 °C (□) and 20 °C (●).](image-url)
observed for caspases [21,22] indicating that TbMCA2 shares a similar pH dependence and signifies the existence of one active form of the enzyme with the increase in activity most probably a result of the de-protonation of the catalytic cysteine [22]. Such interpretation is supported by previous site directed mutagenesis studies [9] and more recently by the analysis of the crystallographic structure of TbMCA2 where the position of the Cys213Ala and His158 catalytic dyad was shown to be conserved with the caspases [17].

To investigate the effect of Ca$^{2+}$ on the activity of TbMCA2, the kinetic parameters for the hydrolysis of Abz-KARSSAQ-EDDnp were calculated using CaCl$_2$ concentrations in the range 0–5 mM. Both $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ were found to increase sharply up to $\sim$ 500 $\mu$m of CaCl$_2$ but above this concentration both $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ started to decrease (Fig. 3A,B). $K_M$ increases with CaCl$_2$ concentration up to 1 mM and reaches a plateau above this CaCl$_2$ concentration (Fig. 3C). The fact that high CaCl$_2$ concentrations affect TbMCA2 efficiency and turnover but not substrate affinity suggests that, at high concentrations, CaCl$_2$ does not alter the substrate binding site.

**TbMCA2 substrate specificity**

The substrate specificity of TbMCA2 was studied using six individual series of FRET peptides derived from Abz-KARSSA-Q-EDDnp (Table 1). This peptide was chosen from a series of fluorogenic peptidase substrates that we had available in our laboratory and was modified in order to provide a simple and appropriate reference peptide for our work with TbMCA2. The peptides were synthesized by substituting a single amino acid residue in the Lys-Ala-Arg-Ser-Ser-Ala (KARSSA) portion of the substrate by all of the natural amino acids (with exception of Cys and Trp) at positions P3 to P$^{\prime}_3$, with the Arg-Ser (R↓S) bond being the cleavage site unless otherwise indicated. Where peptide synthesis was successful, the peptides were assayed and the resulting kinetic constants are shown in Table 1. In addition, the products of hydrolysis of the cleaved peptides were analysed by HPLC coupled to an ion spray mass spectrometer in order to verify the cleavage site(s) in each substrate.

**P$_1$ residue: hydrolysis of the Abz-KAXSSQA-EDDnp series**

The substrates with basic residues ($X = \text{Arg or Lys}$) in P$_1$ were the only peptides to be hydrolysed by TbMCA2 in this series and the cleavage was exclusively at peptide bond Arg-Ser and Lys-Ser, with a clear preference for the peptide containing Arg at the P$_1$ position. All other peptides tested were resistant to cleavage. These results confirm the previously described substrate specificity of TbMCA2 [9] and of other metacaspases [8,13,23]. Peptides in the series that
Table 1. Kinetic parameters for the hydrolysis of FRET peptides derived from Abz-KARSSAQ-EDDnp by TbMCA2. An asterisk indicates the peptides that present substrate inhibition. The substrate inhibition parameters were calculated using Eqn (1). \( K_s \), \( K_{cat} \) and \( k_{cat}/K_s \) are given here but the substrate inhibition constants \( K_{sI} \) are in the text. A dash represents peptides that were not assayed.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>( P_1 (K) )</th>
<th>( P_2 (A) )</th>
<th>( P_1 (R) )</th>
<th>( P_1 (S) )</th>
<th>( P_2 (S) )</th>
<th>( P_3 (A) )</th>
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<td>R ( K_s (\mu M) )</td>
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<td>0.19 ± 0.01</td>
<td>0.29 ± 0.03</td>
<td>Cleavages R1\textsubscript{R} and R1\textsubscript{S}</td>
<td>Cleavages R1\textsubscript{S} and R1\textsubscript{A}</td>
<td>Cleavages R1\textsubscript{S} and R1\textsubscript{Q}</td>
</tr>
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<td>( k_{cat} (s^{-1}) )</td>
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<td>0.400 ± 0.005</td>
<td>0.600 ± 0.009</td>
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<td>2105</td>
<td>2068</td>
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<td>0.29 ± 0.02</td>
<td>0.49 ± 0.02</td>
<td>Cleavages R1\textsubscript{K} and K\textsubscript{J} ( S )</td>
<td>Cleavages R1\textsubscript{S} and K\textsubscript{J} ( A )</td>
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<td>( K_c = 7.5 \ \mu M )</td>
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<td>( 612 )</td>
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<td>( K_c = 7.8 \ \mu M )</td>
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<td>( K_c = 9.4 \ \mu M )</td>
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<td>( k_{cat}/K_s (mM^{-1} \cdot s^{-1}) )</td>
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<td>( K_c = 7.5 \ \mu M )</td>
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<td>( k_{cat}/K_s (mM^{-1} \cdot s^{-1}) )</td>
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<td>( K_c = 7.5 \ \mu M )</td>
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<td>( k_{cat}/K_s (mM^{-1} \cdot s^{-1}) )</td>
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<td>( K_c = 7.5 \ \mu M )</td>
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</table>
were found to be resistant to hydrolysis by TbMCA2 (where $\text{X} \neq \text{Arg}$ or Lys) were all assayed as inhibitors and found to be competitive inhibitors, for which the $K_i$ values are shown in Table 1. It should be noted that lower $K_i$ values were obtained with Abz-KADSS-

\[ \text{S} \text{AQ-EDDnp} \] or Abz-KAESSSAQ-EDDnp, indicating that the negatively charged side chain of Asp or Glu interacted with TbMCA2 in an unusual inhibitory manner, such as through allostery.

**P$_2$ residue: hydrolysis of the Abz-KXRSSQAQ-EDDnp series**

Peptides containing basic amino acids Arg or Lys showed the highest specificity constants in this series ($k_{\text{cat}}/K_M = 6200 \text{ mm}^{-1}\text{s}^{-1}$ and 2100 mm$^{-1}\text{s}^{-1}$, respectively), although the substrate containing an Ala substitution was also efficiently hydrolysed by TbMCA2 with $k_{\text{cat}}/K_M = 2100 \text{ mm}^{-1}\text{s}^{-1}$. Peptides containing aromatic residues (Phe and Tyr) were also efficiently hydrolysed with $k_{\text{cat}}/K_M = 1400 \text{ mm}^{-1}\text{s}^{-1}$ and 900 mm$^{-1}\text{s}^{-1}$, respectively, but these FRET peptides exhibited substrate inhibition at concentrations higher than 10 $\mu\text{M}$ and the observed $K_{IA}$ constants determined for the unproductive binding were 14.7 $\mu\text{M}$ (Tyr) and 27.1 $\mu\text{M}$ (Phe). The peptide that contained Asp at the P$_2$ position had the lowest $k_{\text{cat}}/K_M$ value (8 mm$^{-1}\text{s}^{-1}$) and highest $K_M$ (12 $\mu\text{M}$) and the peptide Abz-KERSSAQ-EDDnp was resistant to hydrolysis; thus a negatively charged group at the P$_2$ position of the substrates is very unfavourable for TbMCA2 activity.

**P$_3$ residue: hydrolysis of the Abz-XARSSAQ-EDDnp series**

Of the peptides tested in this series, the substrates containing basic residues Arg or Lys at the P$_3$ position were the most susceptible to hydrolysis by TbMCA2. Together with the results for the P$_2$ series, this suggests a preference of TbMCA2 non-prime sites for positively charged basic residues. In accordance with this observation, the peptides containing Glu and Asp were resistant to hydrolysis but inhibited the enzyme with a $K_i$ of around 4 $\mu\text{M}$. All the other peptides tested in this series were hydrolysed but with low $k_{\text{cat}}/K_M$.

**P'$_1$ residue: hydrolysis of the Abz-KARXSAQ-EDDnp series**

All the peptides in this series susceptible to hydrolysis were cleaved at the Arg-X peptide bond except for the substrates where $\text{X} = \text{Arg}$ or Lys; these substrates were also hydrolysed by TbMCA2 at the Arg-Arg (or Arg-Lys) (60%) and Arg-Ser (or Lys-Ser) (40%) positions. The kinetic parameters for peptides hydrolysed at more than one position were not calculated. The reference peptide with Ser at the P'$_1$ position was the most susceptible substrate to hydrolysis by TbMCA2 ($k_{\text{cat}}/K_M = 2100 \text{ mm}^{-1}\text{s}^{-1}$), while peptides containing aliphatic residues were poorly hydrolysed or even resistant to hydrolysis (Pro and Ile). In addition, TbMCA2 did not cleave peptides containing the aromatic residues Phe and Tyr at this position, and those with negatively charged side chains (Asp and Glu) were poorly hydrolysed. These results suggest that the $S_1'$ binding pocket has a preference for residues with small side chains.

**P'$_2$ residue: hydrolysis of the Abz-KARXSAQ-EDDnp series**

Of the substrates tested in this series, the one that was most susceptible to hydrolysis by TbMCA2 was the peptide containing Ile at the P'$_2$ position. The peptide containing Glu at this position was also well hydrolysed by TbMCA2, with $k_{\text{cat}}/K_M = 2400 \text{ mm}^{-1}\text{s}^{-1}$; however, this contrasts sharply with the low hydrolysis observed when an Asp is present in P'$_2$ ($k_{\text{cat}}/K_M = 153 \text{ mm}^{-1}\text{s}^{-1}$). The substrate containing Pro in P'$_2$ presented substrate inhibition and the estimated inhibition constant for this unproductive enzyme-substrate interaction is $K_{IA} = 5.9 \mu\text{M}$. In addition, peptides with Ser, Gly or Val at this position were hydrolysed with relatively high $k_{\text{cat}}/K_M$ values, whereas peptides with the aromatic residues Tyr and Phe were resistant to hydrolysis.

**P'$_3$ residue: hydrolysis of the Abz-KARXSSAQ-EDDnp series**

In this series, the peptide containing Phe at the P'$_3$ position showed the highest $k_{\text{cat}}/K_M$ (9375 mm$^{-1}\text{s}^{-1}$) amongst all of the assayed peptides, mainly due to the high value of $k_{\text{cat}}$ (6.0 s$^{-1}$), but this substrate also showed substrate inhibition kinetics with $K_{IA} = 3.7 \mu\text{M}$. TbMCA2 also efficiently hydrolysed the peptide containing Tyr at the P'$_3$ position; however, this happened without substrate inhibition. Peptides with Ala, Ile, His and Met were also efficiently hydrolysed by TbMCA2 ($k_{\text{cat}}/K_M \geq 900$) with the peptide containing Ile also displaying substrate inhibition ($K_{IA} = 0.5 \mu\text{M}$).

**Influence of CaCl$_2$ on TbMCA2**

The effects of Ca$^{2+}$ on the activity of TbMCA2 were further investigated using the fluorogenic peptides.
Z-RR-AMC and Z-FR-AMC, which are both hydrolysed at Arg↓AMC. Different profiles of activation were observed for these two substrates: the hydrolysis of Z-RR-AMC produced a $k_{cat}/K_M$ that significantly increased up to a concentration of 250 µM CaCl$_2$ and then decreased at higher concentrations, whereas the $k_{cat}/K_M$ values of Z-FR-AMC increased until around 6 mM CaCl$_2$ and declined after this (to the measured 20 mM) (Fig. 4A). As these two peptides differ only at the P$_2$ position, the influence of this residue on the activation of TbMCA2 by Ca$^{2+}$ was further explored.

Initially, the FRET peptides Abz-KRR↓SSAQ-EDDnp, Abz-KFR↓SSAQ-EDDnp and Abz-KAR↓SSAQ-EDDnp were assayed over a range of Ca$^{2+}$ concentrations (0–5 mM) and the activation profiles for the hydrolysis of these longer peptides were found to be similar to those observed with Z-RR-AMC and Z-FR-AMC (Fig. 4B). This confirmed that the P$_2$ residue had an effect on the activation of TbMCA2 by Ca$^{2+}$.

To confirm these results the effect of Ca$^{2+}$ concentration on the kinetic behaviour of TbMCA2 was tested with all substrates presented in Table 1. Kinetic assays were carried out with TbMCA2 using the series of peptides at low (50 µM) and high (1 mM) Ca$^{2+}$ concentrations but only substrates with residues that varied in P$_2$ (P$_2$-varying substrates, Abz-KXRSSAQ-EDDnp) presented a differential Ca$^{2+}$ effect as represented in Fig. 5 and Table 2. Analysing the results from these assays revealed that the P$_2$-varying substrates could be split into three distinct groups:
substrates that show no inhibition regardless of the Ca\(^{2+}\) concentration (Ala, Thr and Gly); substrates that show inhibition at low (50 \(\mu\)M) but not high (1 mM) Ca\(^{2+}\) concentrations (Leu, Ile and Gln); and substrates that inhibit TbMCA2 regardless of the Ca\(^{2+}\) concentration (Tyr and Phe). These differential Ca\(^{2+}\) effects are exemplified using a representative substrate from each group (Ala, Leu and Tyr) by plotting substrate hydrolysis velocity (\(V\)) versus substrate concentration [S] at both low (50 \(\mu\)M) and high (1 mM) Ca\(^{2+}\) concentrations and by calculating the substrate inhibition constants \(K_{iA}\) over a range of Ca\(^{2+}\) concentrations (Fig. 5 and Table 2, respectively).

Kinetic assays using substrates represented by Ala at the P2 position produced hyperbolic plots at both low (50 \(\mu\)M) and high (1 mM) Ca\(^{2+}\) concentrations (Fig. 5A, B) and showed no substrate inhibition (Table 2). However, for peptides represented by Leu in P2, the kinetics for hydrolysis presented substrate inhibition at low Ca\(^{2+}\) concentration (50 \(\mu\)M) which was completely abolished in assays performed in the presence of a high concentration of Ca\(^{2+}\) (1 mM) (Fig. 5C, D). This effect was shown to be gradual, with the \(K_{iA}\) values rising with increasing Ca\(^{2+}\) concentration until around 0.5 mM after which substrate inhibition was abolished (Table 2). Additionally, kinetic assays performed with peptides represented by Tyr in P2 showed that excess substrate inhibition kinetics were observed independent of the Ca\(^{2+}\) concentration, as shown in Fig. 5E,F and Table 2.

**Examining Ca\(^{2+}\) binding in TbMCA2**

It has been shown previously that the addition of Ca\(^{2+}\) to TbMCA2 activates the enzyme and leads to autoprocessing events [9]. To better understand how Ca\(^{2+}\) might activate TbMCA2, we analysed TbMCA2 using far-UV circular dichroism spectra and intrinsic fluorescence assays (Fig. 6). Prior to analysis, a sample of TbMCA2 was treated with 1 mM CaCl\(_2\) and

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**Table 2.** Substrate inhibition constants (\(K_{iA}\)) determined at various calcium concentrations with the three representative substrates containing changes at the P2 position. ND, not determined.

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<th>(K_{iA}) ((\mu)M)</th>
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</tr>
<tr>
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**Fig. 6.** The effect of CaCl\(_2\) on the structure of TbMCA2. (A) Circular dichroism spectra of TbMCA2 in the absence (■) or in the presence (□) of 1 mM CaCl\(_2\) or in the presence of 1 mM CaCl\(_2\) plus 10 mM EGTA (○). Ellipticity is reported as mean residue molar ellipticity \(\Theta\) (deg\(\cdot\)cm\(^2\)\cdot\)dmol\(^{-1}\)). (B) Fluorescence emission spectra of TbMCA2 (\(\lambda_{ex}=280\) nm) in the absence (■) or in the presence (□) of 1 mM CaCl\(_2\) or in the presence of 1 mM CaCl\(_2\) plus 10 mM EGTA (○). (C) Changes in the maximum fluorescence intensity at an emission wavelength of 330 nm upon increasing the CaCl\(_2\) concentration (log scale).
peptidase activation was confirmed by visualizing the autoprocessing products on SDS/PAGE (data not shown). The far-UV circular dichroism spectra of TbMCA2 in the absence and presence of 1 mM CaCl$_2$ are shown in Fig. 6A and clearly show the secondary structure modification in the presence of Ca$^{2+}$. This effect was completely reversed by subsequent addition of 10 mM EGTA, indicating that TbMCA2 undergoes a reversible structural change on binding Ca$^{2+}$.

Intrinsic fluorescence emission spectra of TbMCA2 were measured in the absence of Ca$^{2+}$ or in the presence of 10 mM EGTA, 0.1 mM CaCl$_2$ or 1 mM CaCl$_2$ (Fig. 6B). A shift in the intrinsic fluorescence of TbMCA2 was observed in the presence of Ca$^{2+}$, which was more pronounced at higher Ca$^{2+}$ concentrations (Fig. 6B). When the intrinsic fluorescence was examined more closely and the shift in fluorescence was plotted against a range of Ca$^{2+}$ concentrations (0–10 mM), the curve revealed two points of inflexion, consistent with the existence of two Ca$^{2+}$ binding sites in TbMCA2 (Fig. 6C). Dissociation constants ($K_D$) for these two sites were calculated using the Adair equation giving $K_{D1} = 3.0 \pm 0.3 \mu M$ and $K_{D2} = 900 \pm 100 \mu M$. Data fitting could only be obtained using $n = 2$ (two binding sites), confirming the visual observation of two points of inflexion.

**Docking of VRPR into the active site of TbMCA2**

Docking studies of VRPR into the active site of TbMCA2, lacking five N-terminal residues, resulted in the ligand binding in several conformations all of which had similar predicted binding affinities. However, a 3D alignment of TbMCA2 with the structures from other clan CD [2] members revealed that the position of the catalytic dyad is conserved between all structures in the family [17]. In addition, where these structures have been determined with inhibitors bound in their active sites (caspase-7, PDB ID 1F1J [24]; paracaspase, PDB ID 3UOA [20]; gingipain-R, PDB ID 1CVR [25] and MARTX toxin, PDB ID 3GCD [26]), the orientation of the ligand and the position of the P$_1$ residue are all consistent (data not shown). Consequently, from the docking results, a conformation of VRPR in the active site of TbMCA2 was chosen that bound to the enzyme in a similar manner to other clan CD members.

VRPR docked into the crystal structure of TbMCA2 revealed that the P$_1$ Arg of VRPR binds in the predicted, acidic S$_1$ binding pocket of TbMCA2 with its carbonyl oxygen situated between the catalytic dyad (Fig. 7). It does this by forming hydrogen bonds, via its guanidinium group, to the carboxylic acid groups of Asp95 and Asp211 (Fig. 7A). Cys92 also sits within hydrogen bonding distance of the functional group on the P$_1$ Arg (Fig. 7A) and the prime side of the bound ligand sits near a narrow channel that includes residues H214 and T160. The electrostatic surface potential of TbMCA2 was calculated with APBS [42] contoured at $\pm 3 kT/e$ and shows the positively (blue) and negatively (red) charged surface areas. Structural figures were prepared using PYMOL [43].
Discussion

Using a series of peptide substrates spanning the residues P_3–P_3 of a TbMCA2 substrate, we have unequivocally demonstrated that TbMCA2 has a strict preference for basic charged amino acids (Arg or Lys) at the P_2 position, with a preference for Arg. Similar specificity occurs in metacaspases from other organisms [8,13,23]. The 3D crystal structure of TbMCA2 revealed that the basis for this strict specificity results from its S_1 site being a negatively charged pocket lined by the functional groups of residues Cys92, Ser156, Asp95 and Asp211 [17]. To demonstrate how a substrate with Arg at the P_1 position might bind in the S_1 pocket of TbMCA2 we docked the peptide VRPR into the active site of TbMCA2 (Fig. 7). This revealed that the ligand bound to the active site through its P_1 Arg forming hydrogen bonds to Asp95 and Asp211 (Fig. 7B), which were previously shown to be essential for TbMCA2 activity [17].

In addition to the clear specificity of TbMCA2 for Arg or Lys in P_1, it has also been shown that TbMCA2 shows a preference for peptides containing basic amino acids at the non-prime side (P_2 and P_3). This was also shown for the P_2 position for substrates of *Arabidopsis thaliana* metacaspase 9 (AtMCA9) where VRPR-AMC was identified as an optimized substrate [27], and the k_{cat}/K_M values for TbMCA2 (Table 1) suggest that, from this series of peptides, Abz-KKRSSA-EDDnp is one of the most effective substrates for the enzyme. Furthermore, peptides containing negatively charged residues Asp and Glu at the P_2 and P_3 positions were resistant to hydrolysis by TbMCA2. This result appears to be different in protein substrates, where autoprocessing in TbMCA2 was found to occur at sites where Asp residues occupy the P_3 positions [9]. This is presumably due to the differences found in cleaving a large protein substrate as opposed a small peptide, the character of which will be dominated by the small number of residues present. In addition, results from the docking studies of VRPR into the active site of TbMCA2 suggest that the regions most likely to form the S_2 and S_3 subsites are reasonably hydrophobic but interspersed with several polar residues (Ser, Thr and Gln). This suggests that, even in the absence of highly acidic or basic functional residues lining the S_2 and S_3 pockets of TbMCA2, the enzyme specializes in, and has a preference for, basic substrates and that negatively charged residues, at least on a small peptide, will interfere with its mechanism.

Peptides in our study containing non-basic residues at P_1 were found to be competitive inhibitors of TbMCA2. As the S_1 binding pocket has been shown to be negatively charged (Fig. 7A and [17]) and is almost certainly structured to be highly specific in accepting basic P_1 residues, it is most likely that these peptides are involved in binding to an allosteric subsite which subsequently prevents substrate binding to the active site.

Substrate inhibition kinetics were also observed for several other peptides in the series, perhaps most notably for substrates with aromatic residues (Phe and Tyr) at positions P_1 and P_2. These peptides were resistant to cleavage by TbMCA2 and their ability to function as competitive inhibitors suggests that aromatic residues in these positions would also result in the substrate being involved in an alternative mode of allosteric binding. In addition, kinetic assays for the P_1 series suggested that the S_1 binding pocket may favour residues with small side chains. This is also confirmed by the maximum efficiency of TBMC2A, against this series of FRET peptides, being achieved with the substrate k_{cat}/K_M Abz-KARSIA-EDDnp. This can be further explained by examining the position of the prime side of the docked VRPR in the structure of TbMCA2 (Fig. 7A) where a residue at this position would sit near to the small groove in the protein that would occlude larger aromatic side chains. In addition, FRET peptides containing basic amino acids at the prime side of the P_1 Arg resulted in a second cleavage site by TbMCA2, which meant that the resulting kinetic parameters could not be calculated. However, these additional cleavage sites were not observed in assayed peptides that contained the P_1 Arg along with basic residues at positions P_2 or P_3. This shows that it is important for TbMCA2 to form interactions in its extended active site in order to execute substrate hydrolysis.

TbMCA2 is dependent on Ca^{2+} for activation [9] and the kinetic behaviour of TbMCA2 tested using the entire series of FRET peptides revealed that the concentration of Ca^{2+} had a differential effect on the activity of TbMCA2 only when the series of peptides that differed in P_2 were used. In this series it was found that both the type of residue at P_2 and the concentration of Ca^{2+} used could affect the activity of TbMCA2 (Fig. 4, 5 and Table 2). These results suggest that the substrate inhibition exhibited by these peptides occurs in the vicinity of and/or via a Ca^{2+} binding site in TbMCA2 and that general allosteric inhibition may occur by preventing Ca^{2+} binding to the correct site(s) in the enzyme. In addition, the change in the far-UV circular dichroism spectra of TbMCA2 in the presence of 1 mM CaCl_2 demonstrated that Ca^{2+} binding to the enzyme causes...
a reversible structural modification. These data were backed up by intrinsic fluorescence assays, performed in the presence of various Ca\(^{2+}\) concentrations, which also indicated a structural shift on Ca\(^{2+}\) binding and the presence of at least two Ca\(^{2+}\) binding sites in TbMCA2. One of the binding sites was shown to have a high affinity for Ca\(^{2+}\) (\(K_{D1} = 3.0 \pm 0.3\) μM) while the other had a much lower affinity (\(K_{D2} = 900 \pm 100\) μM).

Structural studies on (an inactive mutant of) TbMCA2 identified a single Ca\(^{2+}\) binding site on the surface of the protein (residues Asp173, Asp189, Asp190 and Asp220), which was shown to be essential for both the activity of the enzyme and inhibitor binding [17] and is assumed to be the high affinity Ca\(^{2+}\) binding site identified in this study. This site is found at a location distinct from the active site and substrate binding pocket of TbMCA2 and the available structural data suggest that Ca\(^{2+}\) binding this site would not have a direct effect on the conformation of TbMCA2. Despite a thorough search for Ca\(^{2+}\) binding sites in TbMCA2 crystals, none was identified. However, TbMCA2 crystals that were soaked in various concentrations of CaCl\(_2\) showed a large conformational change in a loop that is positioned near to the predicted substrate binding pocket (denoted the 280-loop) [17]. In the absence of bound substrate the 280-loop was highly disordered in the crystal structures and it is assumed that the low affinity Ca\(^{2+}\) binding site and the conformational changes observed, using far-UV circular dichroism and intrinsic fluorescence emission spectra, can be attributed to movement in this region.

In this study we have shown that peptides that are resistant to cleavage can act as inhibitors of TbMCA2. TbMCA2 has a highly specific P\(_i\) substrate binding pocket and consequently the mechanism of inhibition must be allosteric. In addition, certain substrates (e.g. FRET substrates with Leu in P\(_2\)) were found to inhibit TbMCA2 at low concentrations of Ca\(^{2+}\) but not at high, which suggests that the allosteric inhibition of TbMCA2 is most likely to occur around the high affinity Ca\(^{2+}\) binding site. In these cases, the substrate is assumed to be binding to both the active and the allosteric site at low Ca\(^{2+}\) concentrations and as the concentration of Ca\(^{2+}\) increases the substrate is displaced from the high affinity Ca\(^{2+}\) binding site allowing the enzyme to retain full activity.

In another family of well-characterized calcium-dependent proteases, the calpains, a two-stage activation mechanism is governed by two different types of Ca\(^{2+}\) binding [28]. In view of our data, it seems possible that a similar bi-modal Ca\(^{2+}\)-dependent mechanism could operate in TbMCA2. In the parasite, TbMCA2 is found to reside mainly in the Rab11-positive endosomes [7] where the basal level of Ca\(^{2+}\) is reported to be ~100 nM [29]. This is 30 times lower than the calculated \(K_{D1}\) (3.0 ± 0.3 μM) leading to the supposition that under regular conditions TbMCA2 is inactive. This phenomenon was also observed in the calpains where the levels of Ca\(^{2+}\) required to activate the enzymes were much higher in vitro than those measured intracellular, leading to the hypothesis that certain cellular associations or events could lower the Ca\(^{2+}\) activation threshold [30]. Consequently, we assume that in vivo TbMCA2 will be activated in response to certain cellular stimuli that will either raise the Ca\(^{2+}\) levels or lower the Ca\(^{2+}\) activation threshold in order to occupy both Ca\(^{2+}\) binding sites, and thus intracellular Ca\(^{2+}\) levels will be used to control the physiological function of MCA2 [9]. The Ca\(^{2+}\) dependence of TbMCA2 (and presumably the other metacaspases) is key to unravelling the role of these proteases.

Overall, this work has investigated the substrate specificity of TbMCA2 from S\(_3\) to S\(_3\) using a new series of FRET substrates and the docking studies of VRPR into the active site of the enzyme. As no in vitro substrate for trypanosome MCA2 has been identified to date, these studies provide insights into the properties of potential TbMCA2 substrates. They also demonstrate the importance of the calcium ion for TbMCA2 activity, revealing a reversible structural modification on Ca\(^{2+}\) binding and the presence of a high and low affinity binding site. Furthermore, this work revealed that FRET peptides that were resistant to hydrolysis by TbMCA2 acted as competitive inhibitors and that substrates that differed in the P\(_2\) position could produce differential Ca\(^{2+}\) effects on the activity of TbMCA2, suggesting that the enzyme is susceptible to allosteric inhibition via the high affinity Ca\(^{2+}\) binding site under certain conditions.

**Experimental procedures**

**Expression of recombinant TbMCA2**

The expression plasmid for recombinant TbMCA2 with an N-terminal His-tag has been described previously [9]. This plasmid was transformed into chemically competent Escherichia coli BL21 (DE3) pLysS cells (Novagen, Darmstadt, Germany) and initially grown in 10 mL Luria broth medium supplemented with kanamycin (50 μg·mL\(^{-1}\)), chloramphenicol (50 μg·mL\(^{-1}\)) and glucose (5 mM) (supplemented LB, sLB) and incubated at 30 °C overnight (~ 16 h) with shaking at 150 r.p.m. The overnight cultures were...
transferred to 1 L of sLB medium at 30 °C and grown until the culture density reached an A600 of 0.4. The temperature was reduced to 20 °C until the cell density reached an A600 of 0.6-0.7 and the bacterial cells were then harvested by centrifugation (4000 g for 10 min at 4 °C). Finally the cells were resuspended in 1 L of fresh sLB medium (without glucose). At this point isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM and the cells were incubated at 20 °C for 14-16 h before being harvested by centrifugation at 4000 g for 10 min at 4 °C. The pellet was subsequently stored at −70 °C until required.

### Purification of recombinant TbMCA2

The cell pellet was resuspended in 20 mL of 50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole and 1 mg mL⁻¹ lysozyme and incubated on ice for 30 min before the addition of 5 μg mL⁻¹ RNase, 5 μg mL⁻¹ DNase and 0.02% Triton X-100. The solution was further incubated for 30 min on ice before centrifugation at 26 000 g for 20 min at 4 °C and the supernatant was recovered.

The supernatant from the cell lysis was loaded onto a 1 mL HisTrap Ni²⁺-Sepharose column (GE Healthcare, Amersham, UK) previously equilibrated in 20 mM sodium phosphate pH 8.0, 500 mM NaCl (buffering) using a flow rate of 0.5 mL min⁻¹. The column was then washed with 5 mL of binding buffer and recombinant TbMCA2 was purified and eluted using a step gradient consisting of 5 mL of 50, 100, 150 and 500 mM imidazole in the same buffer. Recombinant TbMCA2 was found to elute between 100 and 150 mM imidazole (as confirmed by SDS/PAGE analysis) and the fractions containing the protein were pooled. In order to desalt and further purify the sample, the pooled protein was loaded directly onto a Sephadex G-75 size-exclusion column, packed with 100 mL of resin (GE Healthcare) and equilibrated in 50 mM Tris pH 7.4, 150 mM NaCl (NaCl/Tris buffer). Elution fractions were analysed using SDS/PAGE and the fractions containing recombinant TbMCA2 were collected. The fractions containing pure, recombinant TbMCA2 were concentrated (~10-fold) using an Amicon filtration unit (Millipore Corp., Billerica, MA, USA) equipped with a 10 kDa exclusion membrane, and the recovered protein was stored in NaCl/Tris buffer at −70 °C. The working aliquots were diluted in NaCl/Tris buffer containing 30% glycerol and stored at −20 °C. Repeated freeze–thaw cycles can decrease TbMCA2 activity and were consequently avoided.

### Peptide synthesis

FRET peptides of the type Abz-peptidyl-Gln-EDDnp, where Abz is the fluorescent group ortho-aminobenzoxyc acid and EDDnp is the C-terminal quencher moiety N-(2,4-dinitrophenyl)-ethylenediamine, were synthesized by solid-phase synthesis (as described in [31]) using a bench-top automated solid-phase peptide synthesizer (PSSM-8, Shimadzu, Tokyo, Japan). The molecular mass and purity of the peptides were checked by analytical HPLC (Shimadzu 10AVP HPLC system) and by MALDI-TOF MS using a microflex LT system (Bruker Daltonics, Bremen, Germany). Stock solutions of the peptide substrates were prepared in distilled water and the concentration was measured spectrophotometrically at 365 nm using the molar extinction coefficient of the Dnp group of 17 300 m⁻¹ cm⁻¹.

### Kinetic assays

The activity of TbMCA2 was monitored by measuring the release of fluorescent groups, Abz or AMC (7-aminobenzoic acid), from the hydrolysis of the FRET substrates Abz-peptidyl-Gln-EDDnp and benzoxycarbonyl(Z)-peptidyl-AMC respectively by the enzyme. Measurements were made using a Shimadzu (Tokyo, Japan) RF-5301PC spectrophotometer with the excitation and emission wavelengths set at 320 and 420 nm, respectively, for the hydrolysis of the FRET substrates and 380 and 460 nm, respectively, for the hydrolysis of the Z-peptidyl-AMC substrates.

A 1 cm path-length cuvette containing 1 mL of the substrate solution was placed in a thermostatically controlled cell compartment for 5 min before the enzyme solution was added, and TbMCA2 was pre-incubated with 2.5 mM dithiothreitol. The kinetic measurements of peptide hydrolysis were performed at 25 °C in 50 mM Tris/HCl pH 7.4, 100 mM NaCl, 1 mM CaCl₂, unless otherwise stated. The progress of the reaction was continuously monitored by the fluorescence of the released products. The rate of increase of fluorescence was converted into moles of hydrolysed substrate per second, based on the fluorescence curves of standard peptide solutions before and after total enzymatic hydrolysis.

The enzyme concentration for the initial rate determination was chosen at a level intended to hydrolyse <5% of the substrate present. TbMCA2 concentrations varied from 1 nm to 100 nm and substrate concentrations were adjusted in order to obtain a V × [S] plot with minimum substrate concentration ranging from 0.1 km to 10 km. The inner filter effect was corrected for using an empirical equation which has been described previously [32]. Kinetic parameters (kcat, km and kcat/km) were calculated according to Wilkinson [33] and by the use of Eadie–Hofstee plots.

The kinetic parameters for the peptides that presented substrate inhibition kinetics were calculated using

$$V = V_{\text{max}}[S]/\{K_M + [S]/(K_{IA})\}$$

where V is velocity, Vmax is the maximum velocity, [S] is the substrate concentration, km is the Michaelis constant and KIA is the substrate inhibition constant.
All of the data obtained were fitted to nonlinear least-squares equations, using Grafit5 (Erithacus Software [34]).

**Determination of inhibition parameters**

The substrate Abz-KARSSAQ-EDDnp was used as a reference substrate in the cleavage reactions with TbMCA2, and any derivatives of this that were found to be resistant to hydrolysis by TbMCA2 were assayed as competitive inhibitors. For these peptides the inhibition parameters were determined in a continuous assay using Z-FR-AMC as the substrate. The equation used to calculate $K_i$ values was $K_i = k_{\text{app}}/(1 + [S]/K_M)$, where $[S]$ is the molar concentration of the substrate, $K_M$ is the Michaelis constant and $k_{\text{app}}$ is the apparent inhibition constant. $k_{\text{app}}$ was calculated using the equation $V_0/V_i = 1 + [I]/k_{\text{app}}$, where $V_0$ is the velocity of hydrolysis without the inhibitor, $V_i$ is the velocity of hydrolysis in the presence of the inhibitor and $[I]$ is the molar concentration of the inhibitor. A plot of $(V_0/V_i) - 1$ versus $[I]$ yields a slope of $1/k_{\text{app}}$ [35].

**The pH dependence of the kinetic parameters**

The $k_{\text{cat}}/K_M$ parameters were determined in the pH range 5–10 using Abz-KARSSAQ-EDDnp as the substrate in a four-component buffer system of constant ionic strength (25 mM glycine, 25 mM acetic acid, 25 mM MES and 75 mM Tris). The kinetic data were analysed by nonlinear regression using Grafit5 software [34] and

$$k = k_{\text{limit}}[1/(1 + 10^{pK_1-pH} + 10^{pK_2-pH})]$$  (2)

Equation (2) fits the data when the pH–activity profile depends upon two ionizing groups in a bell-shaped curve and the activities at low and high pH are zero. $k_{\text{limit}}$ corresponds to the pH-independent maximum rate constant, $K_1$ and $K_2$ are the dissociation constants of a catalytically competent base and acid, respectively, and $k = k_{\text{cat}}/K_M$ [35].

**The effect of calcium on the intrinsic fluorescence of TbMCA2**

Structural changes in TbMCA2 were studied by monitoring the intrinsic fluorescence in 50 mM Tris/HCl pH 7.4, 100 mM NaCl with the addition of varying concentrations of CaCl$_2$ (50 µM to 2 mM) and a final protein concentration of 2 µM. The emission spectra were collected in the range 300–400 nm at 25 °C using a fixed excitation wavelength of 280 nm (2.5 nm slit width for both excitation and emission). These data were analysed using the Adair equation that describes the binding of ligands to multiple sites on a receptor using Grafit5 software [34].

**Circular dichroism**

Circular dichroism spectra were recorded on a Jasco J-810 spectropolarimeter (Jasco, Easton, MD, USA) with a Peltier system for controlling cell temperature. The absorbance spectra of TbMCA2 (10 µM) were collected in the far-UV range (190–260 nm) using a 1 mm path-length cell. The system was routinely calibrated with an aqueous solution of twice crystallized d$_{10}$-camphorsulfonic acid. Ellipticity was recorded as the mean residue molar ellipticity $\theta$ (deg cm$^2$ dmol$^{-1}$). The spectrometer conditions typically included a sensitivity of 100 mdeg, a resolution of 0.5 nm, a response time of 4 s and a scan rate of 20 nm min$^{-1}$, and each curve is an average of four sequential scans at 25 °C. The control baseline was obtained with all buffer components prepared without the protein.

**Protein concentration**

For circular dichroism, intrinsic fluorescence and kinetic assays the protein concentration was determined spectrophotometrically based on the absorbance at 280 nm and on the calculated molar extinction coefficient of 29 910 M$^{-1}$ cm$^{-1}$ (as calculated by ProtParam [36]).

**Determination of cleavage sites in the substrates**

The scissile bonds of hydrolysed peptide substrates were identified by isolation of the fragments using analytical HPLC followed by determination of their molecular mass by liquid chromatography mass spectrometry using an LCMS-2010 EV mass spectrometer (Shimadzu) equipped with an electrospray ionization probe. For the substrates hydrolysed at two sites the percentage of each cleavage product was calculated from the individual integrated areas taken from the chromatograms.

**Protein docking studies**

A protein model of TbMCA2 was prepared by taking a structure from the Protein Data Bank (PDB [37]; PDB ID 4AF8 [17]) and removing all heteroatoms, including water molecules, and N-terminal residues 31–35 inclusive. Hydrogen atoms were added to this model and a 3D grid was designed with the predicted $S_1$ binding site [17] at the centre, in order to facilitate receptor docking, using AutodockTools4 [38]. The peptide inhibitor benzoxy carbonyl-Val-Arg-Pro-Arg-fluoromethyl ketone (Z-VRPR-FMK) has been shown to be an inhibitor of TbMCA2 [17] and was consequently chosen as the ligand for docking studies. The 3D structure of VRPR was taken from the crystal structure of a mammalian paracaspase [20] and AutodockTools4 was used to assign flexible torsions, Gasteiger charges and nonpolar hydrogen atoms. Docking of the ligand into the prepared structure of TbMCA2 was then carried out using Autodock...
VINA [39]. All 3D structural alignments were carried out using SSM SUPERPOSE [40] and COOT [41].

**Acknowledgements**

This work was supported by FAPESP grant 2008/57336-2 to MFMM and by CNPq grant 470044/2010-1 to MFMM. VO was supported by FAPESP grant 2011/51718-3. JCM, KM and CXM were supported by the Wellcome Trust (091790) and the Medical Research Council (0700127). The Wellcome Trust Centre for Molecular Parasitology is supported by core funding from the Wellcome Trust (085349).

**References**


