Insights into the anthrax lethal factor–substrate interaction and selectivity using docking and molecular dynamics simulations

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Received 23 March 2009; Accepted 14 May 2009

DOI: 10.1002/pro.169

Published online 7 July 2009 proteinscience.org

Abstract: The anthrax toxin of the bacterium *Bacillus anthracis* consists of three distinct proteins, one of which is the anthrax lethal factor (LF). LF is a gluzincin Zn-dependent, highly specific metalloprotease with a molecular mass of ~90 kDa that cleaves most isoforms of the family of mitogen-activated protein kinase kinases (MEKs/MKKs) close to their amino termini, resulting in the inhibition of one or more signaling pathways. Previous studies on the crystal structures of uncomplexed LF and LF complexed with the substrate MEK2 or a MKK-based synthetic peptide provided structure-activity correlations and the basis for the rational design of efficient inhibitors. However, in the crystallographic structures, the substrate peptide was not properly oriented in the active site because of the absence of the catalytic zinc atom. In the current study, docking and molecular dynamics calculations were employed to examine the LF-MEK/MKK interaction along the catalytic channel up to a distance of 20 Å from the zinc atom. This residue-specific view of the enzyme-substrate interaction provides valuable information about: (i) the substrate selectivity of LF and its inactivation of MEKs/MKKs (an issue highly important not only to anthrax infection but also to the pathogenesis of cancer), and (ii) the discovery of new, previously unexploited, hot-spots of the LF catalytic channel that are important in the enzyme/substrate binding and interaction.

Keywords: anthrax lethal factor; docking; molecular dynamics; MEK; MKK; enzyme-substrate interactions; protein plasticity

Introduction

Anthrax is an infectious disease of animals and humans caused by the gram-positive bacterium *Bacil-*

lus anthracis. *B. anthracis* spores can survive in the ground for long periods. Disease develops when *B. anthracis* endospores enter the host through abrasions in the skin or by inhalation or ingestion. While cutaneous anthrax is rarely lethal, inhalation of the spores is often fatal.¹ The only current therapeutic intervention for anthrax is antibiotics, which must be given early after infection when the host experiences flu-like symptoms. The major virulence factors are the poly-D-glutamic acid capsule and the anthrax toxin (ATx).^{2–4} The latter is a three-protein component exotoxin comprising 83 kDa protective antigen (PA), the 90 kDa anthrax lethal factor (LF), and the 89 kDa calmodulin-activated edema factor adenylate cyclase (EF). None of

Abbreviations: ATx, anthrax toxin; BSA, buried surface area; LF, lethal factor; MD, molecular dynamics; MEKs/MKKs, mitogen-activated protein kinase kinases; NAD, nicotinamide adenine dinucleotide; RMSD, root mean square deviation; RMSF, root mean square fluctuation.

Additional Supporting Information may be found in the online version of this article.

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Figure 1. (A) Ribbon representation of LF showing the N-terminal domain that binds to PA (domain I, white), the central domain, which is probably involved in substrate recruitment (domain II, orange), and domain III (cyan), which covers the active-site cleft of the catalytic domain (domain IV, blue). (B) and (C) Different angle views of the LF active site showing the Zn ion as orange sphere and the coordinated residues His686, His690, and Glu735 as yellow stick models. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the proteins are known to be toxic alone. The combination of PA with either LF or EF results in lethal toxin (LF-PA) and edema toxin (EF-PA), which cause different pathogenic responses in animals and cells. To exert its toxic effect, LF must enter the cytosol, and this is facilitated by PA. These three components act in concert to kill host macrophages, although recent reports indicate that the toxin has physiologically relevant effects on a broad range of cell types.⁵

Cytosolic LF acts as a highly specific Zn^{2+} -dependent endoprotease, which cleaves members of the mitogen-activated protein kinase kinases (MEKs/MKKs) at their N-terminus, disrupting their ability to interact with and phosphorylate their substrates. The overall effect is the alteration of signaling pathways and ultimately apoptosis,^{6,7} macrophages being lysed via a mechanism that is not entirely understood.⁸

In the crystal structure of LF and of its complex with MEK2,⁹ the molecule is organized in four α -helix-rich domains [Fig. 1(A)]. The active site is a broad, deep 40 Å long groove created by the vestigial NAD-binding pocket of domain II and the interface between domains II, III, and IV. The groove has an overall negative charge, as it contains clusters of glutamic and aspartic acid residues.⁹ Domain IV (residues 552–776) is the heart of the proteolytic activity of LF that contains two zinc-binding motif (H686–E687–F688–G689–

H690 and E735–F736–F737–A738–E739) separated by a spacer of 44 residues¹⁰ and bound to a single Zn ion. The X-ray structure of the MEK2 complex shows that a part (16 residues) of MEK2 is accommodated in the groove with the N-terminal near domain II and the C-terminal between domains III and IV [Fig. 1(A)]. The Zn-free complex of LF and MEK2 is one of the few examples of an uncleaved substrate bound to a protease.⁹ In the LF–substrate complex crystallized in the absence of Zn cofactor, the substrate is inserted in the substrate channel, but its scissile bond is distant from the catalytic zinc [Fig. 2(A)], suggesting the structure of a “precleavage” complex.⁹

LF's substrates are polypeptides of the MKK family comprising MEKs [MAPK/ERK (extracellular-signal-regulated kinase) kinase] 1 and 2 (MEK1, MEK2) and MKKs [MAPK (mitogen-activated protein kinase) kinases] 3, 4, 5, 6, and 7.^{11,12} MEK1 and MEK2 specifically phosphorylate and activate ERK1 and ERK2, respectively. MKK3 and MKK6 are specific for MAP kinase p38, whereas MKK4 and MKK7 phosphoactivate the stress-activated protein kinase JNK (c-Jun N-terminal kinase), although MKK4 can also phosphorylate p38.¹³ LF cleaves all mammalian MEKs and MKKs except MKK5, and shuts down the ERK, JNK, and p38 pathways. Table I shows all known LF cleavage site sequences in mammalian MEKs/MKKs. Cleavage

Figure 2. (A) Stereo representation of LF-MEK2 complex from PDB ID [1JKY](#) showing the catalytic residues' carbon atoms in cyan and the substrate's carbons in orange. (B) Stereo representation of the initial energy-minimized simulated complex of LF-MEK2. Zinc is shown as grey sphere and the catalytic zinc-bound water as red sphere. (C) Stereo representation of a TLN-phosphonamidate peptide inhibitor complex from PDB ID [5TMN](#) showing the active site residues as cyan sticks and the inhibitor in orange. All panels are aligned according to the active-site residues of TLN. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

occurs within a stretch of residues that approximately fits the consensus sequence $++++XhX\downarrow h$ (basic and hydrophobic residues are indicated, respectively, by + and h , X indicates any amino acid, and the cleavage site is indicated by $\downarrow$, Table I).¹² MKK4 and MKK7 are the only LF substrates that are cleaved twice.^{11,12,14–18} MKKs are cleaved by LF at specific sites outside their catalytic domains (Supporting Information Fig. S1).¹²

In addition to its involvement in the pathogenesis of anthrax, activation of the MAPK signaling pathways is central to the tumorigenesis, metastasis, and angiogenesis of many human malignancies.¹⁹ Inhibition of MKKs is, therefore, an attractive anticancer strategy,

as it may block undesirable signal transduction pathways that may arise from an oncogenic aberration. Elucidation of MKK inactivating mechanisms is, therefore, of general interest and not restricted to the anthrax disease.

It remains unclear which residues of LF are critical for efficient catalysis, and the way that certain MEK/MKK residues contribute to LF–substrate interaction. In the present study, docking and molecular dynamic (MD) simulations were used to analyze interactions between the LF active site and MKKs. Although the orientation of the MEK/MKK substrates, adopted in this study, is suggested by the crystal

Table I. *LF Cleavage Sites in Mammalian MKKs (Underlined)*

Substrate	P8	P7	P6	P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'	P6'	P7'	P8'
MEK1 ₍₈₋₉₎	M	P	K	K	K	P	T	<u>P</u>	<u>I</u>	Q	L	N	P	A	P	D
MEK2 ₍₁₀₋₁₁₎	A	R	R	K	P	V	L	<u>P</u>	<u>A</u>	L	T	I	N	P	T	I
MKK3b ₍₂₆₋₂₇₎	S	K	R	K	K	D	L	<u>R</u>	<u>I</u>	S	C	M	S	K	P	P
MKK6b ₍₁₄₋₁₅₎	K	K	R	N	P	G	L	<u>K</u>	<u>I</u>	P	K	E	A	F	E	Q
MKK4 ₍₄₅₋₄₆₎ ^a	Q	G	K	R	K	A	L	<u>K</u>	<u>L</u>	N	F	A	N	P	P	F
MKK4 ₍₅₈₋₅₉₎ ^a	P	P	F	K	S	T	A	<u>R</u>	<u>F</u>	T	L	N	P	N	P	T
MKK7β ₍₄₄₋₄₅₎ ^a	P	R	P	R	P	T	L	<u>Q</u>	<u>L</u>	P	L	A	N	D	G	G
MKK7β ₍₇₆₋₇₇₎ ^a	A	R	P	R	H	M	L	<u>G</u>	<u>L</u>	P	S	T	L	F	T	P
Consensus		+	+	+	+	X	h	<u>X</u>	<u>h</u>							

Conserved residues are shown in bold and, on the bottom line (consensus), are marked + for basic, *h* for hydrophobic, or *X* for any amino acid.

^a MKK4₍₄₅₋₄₆₎ and MKK4₍₅₈₋₅₉₎ are referred to in the text as, respectively, MKK4₍₁₎ and MKK4₍₂₎, while MKK7β₍₄₄₋₄₅₎ and MKK7β₍₇₆₋₇₇₎ are referred to as MKK7β₍₁₎ and MKK7β₍₂₎.

structure of LF with the MEK2, our modeling in not based on these crystallographic coordinates but examined de novo the exact positioning of the MKK substrate(s) in the LF catalytic channel in an attempt to elucidate the factors that govern LF–substrate binding and specificity.

Results

The currently available X-ray structures of LF show the binding modes of small-molecules or short-peptidic inhibitors.^{20–24} The binding subsites S₁ and S₁' of LF are well characterized, and the conformational changes at the active site of the protease are documented.^{20–23} However, the additional sites away from the active site of LF may be required for efficient proteolysis.²⁵ Because of the reorganization of the enzyme upon substrate binding, prediction of the interactions that dominate the complex is a challenging task. A fragment- and knowledge-based docking method followed by MD calculations, which was used previously to study the ACE–GnRH complexes,²⁶ was also employed in the present study. In this approach, each peptide fragment can explore all possible conformations allowing entry into the catalytic cavity and adopt the one that provides the most important interactions. The positioning of the peptides is governed by the forces and types of interaction, which differ in nature and strength. The net result was that each of the eight docked peptide substrates exhibited different contacts in the catalytic cavity characterized by the nature of its residues P₆–P₆' and their interactions with the S₆–S₆' subsites. Explicit solvent treatment facilitated the observation of water-mediated interactions between LF and its substrates. In this way, it was possible to monitor local motions of LF in the presence of the substrate on the nanosecond timescale and investigate the structural characteristics that govern their interaction.

Accommodation and interactions of the substrate peptide in LF catalytic groove

A number of LF sites that contributed significantly to the stabilization of the LF-MEK/MKK complex are

reported in Table II. As observed in all MD simulations, residues P₆–P₃ of MEKs/MKKs interacted with residues in helix 3α2 of LF via electrostatic and hydrophobic contacts. This helix is surrounded by two important areas of the enzyme, namely residues Lys673–Val675 and the loop L2 (Val653–Lys656) [Fig. 1(A)]. In addition to Tyr728, Val675, and Leu677, which lie in coils of LF, residues in helices 3α2 (Asp328, Phe329, Ser331), 4α4 (Gly683, His686, Glu687, His690), and 4α7 (Thr731, Asn732, Glu735, Glu739) were predicted to contribute appreciably to the specificity of LF for the NH₂-terminal cleavage of MEKs and MKKs. Interestingly, residues in the loop L2 and β-strand 4β3 displayed strong hydrogen bonds and favorable hydrophobic interactions with the substrates [Fig. 1(A)]. MD simulations suggested that the same regions of LF (loop L2 and β-strand 4β3) could further stabilize the complexes by also establishing major contacts with the P₃'–P₆' moiety. Finally, hydrophobic residues in the P₃'–P₆' fragment were well positioned for contacts with Val653, Leu658, Val660, Pro661, and Leu707 in LF.

Zinc is coordinated to the side chains of His686, His690, and Glu735 and to one water molecule [first coordination shell, Fig. 1(B,C)], adopting tetrahedral coordination geometry, as in the gluzincin prototype, thermolysin (TLN).²⁷ The second shell of residues surrounding Zn includes a glutamate (Glu687) that is proposed to act as a general base to activate the Zn-bound water molecule during catalysis and a tyrosine residue (Tyr728) [Fig. 2(A,B)].

The MD simulation data showed that the Zn-bound water molecule is predicted to form hydrogen bonds with the carbonyl oxygen of Ile(P₁') (MEK1, MKK3b), Leu(P₁') (MKK4₍₁₎), and Leu(P₂') (MKK6b) as well as with the hydroxyl group of Thr(P₂') (MKK4₍₂₎). This implies that the carbonyl oxygen of the neighboring substrate residues may contribute to the enhancement of the nucleophilicity of the Zn-bound water via a hydrogen-bonding interaction. Our MD data suggest that this hydrogen bond is an additional contact to the already existing polarization of the carbonyl oxygen between Zn and Glu687 carboxylate, which is

Table II. LF Residues That Exhibit Major Electrostatic Interactions with MEKs and MKKs

	S6	S5	S4	S3	S2	S1	S1'	S2'	S3'	S4'	S5'
MEK1	Lys Glu334 Gln384	Lys —	Lys —	Pro Ser655 Phe329 Val653 Val675	Thr Val675 Tyr728 His686 Tyr728 Glu739	Pro Lys656 Leu677 His686 Glu687	Ile Gly657 Val653 Leu658	Gln His690	Leu Tyr659 Gln642 Gln646 Leu658 Val660	Asn His645 <u>Gln646</u>	Pro Leu707
MEK2	Arg Glu333 Ile383 Gln384	Lys Glu333	Pro Thr332 His654	Val His654 Lys656 Ser331 Val653	Leu Asp328 Lys656 Val675 Leu677	Pro Phe329 Val653 Tyr728	Ala —	Leu Val653 Leu658 Val660 Pro661	Thr Glu651	Ile Leu707	Asn Pro661
MKK3b	Arg Glu333	Lys Lys673	Lys Asp328 <u>Lys673</u>	Asp Asp328 <u>Lys335</u> Val675	Leu Lys656 Asp328 Val653	Arg Val675 Tyr728 <i>Glu739</i> <u>Glu687</u> Leu677 His686 Tyr728	Ile Ser331 Gly657 Tyr659 Val653 Leu658	Ser Asn732 <u>Glu735</u>	Cys Tyr659 Gln646 Leu658 Val660	Met <u>Glu642</u> <u>Gln646</u> <u>Glu651</u>	Ser Asp694
MKK6b	Arg Gln390 Gln393 Asp394 Asn445	Asn Asp328 His654	Pro Lys335 <u>Leu330</u>	Gly Lys673	Leu Asp328 Glu687 Val675 Leu677 Tyr728	Lys Asp328 Glu651 <u>Val653</u> Ser655 Leu658	Ile Tyr728 Thr731	Pro Leu658 Pro661 Tyr659 His690	Lys <u>Leu707</u>	Glu <u>Lys377</u> <u>Glu651</u>	Ala His645 Glu646
MKK4 ₍₁₎	Lys Glu333	Arg Lys673	Lys Thr332	Ala Leu330 Lys335 Lys656 Ser672	Leu Lys656 Val675 Leu677	Lys Gly683 Glu687 Asp328 Lys656 Leu677 Tyr728	Leu Asp328 <u>Ser655</u> Phe329 Thr731	Asn Tyr659 His690	Phe Tyr659 Pro661 Leu707 Ala734 Asp694 Tyr659	Ala Lys377	Asn Glu651
MKK4 ₍₂₎	Phe —	Lys Thr332	Ser Phe329 Ser331 Ser672 <u>Ser655</u>	Thr Asp328 <u>Lys673</u> Ser672 Lys673	Ala Lys656 Gly657 Val675 Tyr728	Arg Val675 Asp328 Tyr728 <i>Glu739</i> Leu677 His686 Tyr728	Phe Ser655 Gly657 Asp328 Phe329 Val653	Thr Tyr659 Glu735 Tyr659 Pro661	Leu Glu651 Val653	Asn <u>Glu651</u>	Pro Pro661 Glu662

(Continued)

Table II. (Continued)

	S6	S5	S4	S3	S2	S1	S1'	S2'	S3'	S4'	S5'
MKK7β ₍₁₎	Pro —	Arg <u>Glu333</u> <u>Gln384</u>	Pro Lys673 Val675	Thr <u>Lys656</u> <u>Lys673</u> <u>His654</u>	Leu <u>Asp328</u> Val675 <u>Leu677</u> Tyr728	Gln <u>Glu651</u> <u>Val653</u> <u>Lys377</u> Ser655 Leu658	Leu Tyr728 Thr731 Asn732	Pro Tyr659 His690 Pro661	Leu Gln646 Glu651 Gln652 Leu658	Ala —	Asn <u>Ser705</u>
MKK7β ₍₂₎	Pro —	Arg <u>Thr332</u> <u>Glu333</u>	His <u>Lys673</u> <u>Glu334</u> His654	Met <u>Lys656</u> <u>Leu330</u> Asp328 Tyr728 Val675	Leu <u>Val675</u> Leu677 Lys656 His686	Gly <u>Ser655</u> <u>Gly657</u>	Leu Val653	Pro Tyr659 His690 Pro661	Ser <u>Gln642</u> <u>Tyr659</u>	Thr <u>Glu651</u>	Leu Ser705 Leu707

LF residues involved in salt bridge interactions are shown in *italic*, those making hydrophobic contacts in **bold italic**, those involved in van der Waals interactions in **bold**, and those making water-mediated contacts are underlined.

proposed to promote the attack of water on the carbon of the scissile bond.²⁸ This interaction was not observed in the other MKK-LF complexes examined because of the initial docking orientation from the docking simulations, which was different for each peptide: the negatively charged active site groove of LF limited the possible orientations of the substrate in the catalytic groove through electrostatic interactions with the peptide.

No specific interaction was observed between the substrate nitrogen of the scissile bond and any LF residue because the N—H bond lies in the side of the groove exposed to the solvent. Such interactions occur in other Zn proteases, such as TLN, neprilysin,²⁹ and ACE.³⁰ In ACE and TLN, one Ala stabilizes the scissile bond nitrogen of the substrate by hydrogen bonding to its carbonyl oxygen. However, water-mediated hydrogen bonding interactions between the scissile bond's N—H and neighboring residues or a possible reorganization of the scissile bond upon activation may compensate for the interaction that is not observed in our simulations.

Interactions between LF and substrates in the S₆–S₄ subsites

The P₆, P₅, and P₄ residues of MEKs/MKKs are mostly positively charged and make strong electrostatic interactions with the negative residues of the S₆, S₅, and S₄ subsites of LF. Such electrostatic interactions were observed during the entire course of the MD simulations involving particularly the acidic residues Glu333, Glu334, Gln384, and Asp328 of LF. The proteolytic site of LF is acidic in nature, and nicely complements the basic residues at multiple positions of the N-termini of the six MEKs/MKKs recognized by LF.

In particular, our MD data revealed two LF subsites in which the positive side chains of MKKs could be accommodated and form strong electrostatic interactions. Thus, when the P₆ MKK residue was arginine or lysine (MEK1, MEK2, MKK3, and MKK4₍₂₎), its side chain formed favorable electrostatic interactions with the LF subsite comprising Glu333, Glu334, and Gln384 in helix 3α2. It is interesting that, during the MD simulations, the distance between the side chains of Glu333 and Arg(P₅) of MKK7 and Lys(P₅) of MEK1 and MEK2 decreased because of the electrostatic contacts, indicating the importance of Glu333 for the stabilization of the substrates [Fig. S6(A)]. Major ionic interactions were also seen at the S₆ subsite comprising Gln390, Gln393, and Asp394 in helix 2α3. These residues are predicted to participate in the binding of some substrates by forming salt bridges with Lys(P₆) (MEK1) or Arg(P₆) (MKK6). Although the P₆ and P₅ residues were well positioned to interact with the S₆ and S₅ residues of LF, this was not the case for Lys(P₄) (MEK1, MKK3, MKK4₍₁₎), suggesting that Lys(P₄) does not play a major role in the binding of the substrates and therefore in enzyme–substrate

recognition. In addition, MD data analysis revealed that the residues at position P_4 in the other substrates mainly formed water-mediated interactions or van der Waals contacts in the S_4 subsite.

Interactions between LF and substrates in the S_3 and S_2 subsites

In general, residues at positions P_3 and P_2 formed antiparallel β -sheet-like interactions with the $4\beta 3$ and $4\beta 4$ strands of LF. As discussed earlier, position P_3 was occupied by different residues. Hydrophobic residues at P_3 were predicted to establish major hydrophobic contacts with Phe329, Val653, and Val675 in LF domain IV, while other residues were predicted to form hydrogen bonds or electrostatic interactions with Asp328, Lys335, Lys656, Ser672, and Lys673 [Fig. S6(B)]. Interestingly, Lys656 was predominantly hydrogen bonded with most MKKs. Similar interactions involving Lys656 and inhibitors containing thioamide hydroxypyrothiones were recently reported in a molecular modeling study.³¹ It is shown that the sulfur atom of the thioamide group acts as a hydrogen bond acceptor, interacting with the backbone amide N—H group of residue Lys656. Substrates that contained a hydrophobic residue at position P_2 (except MEK1) also exhibited major hydrophobic interactions with the catalytic channel of LF, especially because of the accommodation of P_2 in a cage of hydrophobic residues comprising Val675 and Leu677.

Interactions between LF and substrates in the S_1 subsite

The S_1 pocket is highly hydrophobic at its neck and acidic at its base, allowing the fitting of the long side chains of Arg or Lys in P_1 of some MKKs.⁹ The S_1 subsite included Tyr728, the hydroxyl group of which was within hydrogen bonding range of the carbonyl oxygen of the scissile bond [Fig. S6(C)]. A key residue of the S_1 pocket was Glu739 (in helix $4\alpha 7$), located four positions after the Zn-bound Glu735. This residue formed: (i) a salt bridge interaction with Arg742 and (ii) a hydrogen bond with the side chain of His686 that stabilized the geometry of Zn coordination. A negative residue (aspartic acid) may be present in the same position in the proposed second binding motif of TLN and ACE,³² providing important interactions with the ligands. The charged groups of Glu739 in LF and Arg(P_1) in MKK3b and MKK4₍₂₎ formed a salt bridge at the beginning of the MD simulations [Fig. S7(A)]. During the MD simulations, the guanidinium group of Arg(P_1) extended slightly to strengthen the salt bridge by forming favorable hydrogen bonds with Glu739, as shown in Figure S7(B). On the basis of these observations, it seems that Glu739 is a major determinant in the stabilization of substrates bearing an Arg at P_1 . This type of contacts has been observed only when Arg occupies P_1 .

When Lys is found at P_1 , according to the MD simulations, its primary amine forms a salt bridge with the carboxylic group of Glu687 [Fig. S6(C)] but not with that of Glu739. Moreover, the Lys(P_1) amine group may also form a H-bond with the carboxylic group of Asp328. Regarding Lys(P_1) of MKK6, the side chain amine of which is located inside the groove, initially a salt bridge was formed with Asp328 at the beginning of the simulation course [Fig. S6(C)]. After 2 ns of MD, MKK6 bound conformation was stabilized by water-mediated interactions and hydrogen bonds with LF residues: (i) Glu651 and Val653 established water-mediated electrostatic interactions with the Lys(P_1) amine group and (ii) Asp328 provided the side-chain carboxyl group for H-bond formation with the amide group of Lys(P_1). Thus, the high density of negatively charged LF residues spread over the catalytic channel (e.g., Glu687 and Glu739) mediates the accommodation of positively charged substrate residues and facilitates hydrolytic cleavage in P_1 .

However, in the absence of a basic P_1 residue, a number of important, mainly hydrophobic, interactions were observed between LF and the P_1 residues of MEK1 and MEK2. Pro(P_1) of MEK1 formed van der Waals contacts with the side chains of Lys656, His686, and Glu687 and hydrophobic contacts with Leu677. Pro(P_1) of MEK2, with its side chain exposed in the groove, formed van der Waals contacts with Tyr728 and hydrophobic contacts with Phe329 and Val653.

The two cleavage sites of MKK7 β (MKK7 $\beta_{(1)}$ and MKK7 $\beta_{(2)}$) contain the consensus sequence Leu-X↓Leu-Pro, where X is Gln or Gly, and the cleavage site is indicated by ↓ (Table I). In this case, LF residues Glu651 and Val653 formed a H-bonding network with the MKK7 $\beta_{(1)}$ Gln(P_1) side chain, which, throughout the major part of the MD simulation, was oriented toward the LF active site groove. Leu658 also contributed to the stabilization of the LF-peptide complex by forming van der Waals interactions with Gln(P_1). For the P_1 residue of MKK7 $\beta_{(2)}$, our simulation revealed that the amide group of Gly(P_1) formed a hydrogen bond with the hydroxyl group of Ser655.

Interactions between LF and substrates in S_1' - S_6'

Ile(P_1') of MEK1 displayed hydrophobic contacts with Val653 and Leu658, whereas MEK2 Ala(P_1') did not exhibit specific interactions, possibly because of its small aliphatic side chain. A Leu residue at position P_1' in both MKK7 cleavage sites exhibited a number of potential hydrophobic contacts with Val653, Lys656, Val675, Leu677, and Tyr728 [Fig. S6(D)]. Pro(P_2') of both MKK7 $\beta_{(1)}$ and MKK7 $\beta_{(2)}$ was further stabilized by hydrophobic and van der Waals contacts with LF residues Tyr659, Pro661, and His690. In general, the hydrophobic residues of the MEK/MKK P_1' - P_6' moiety were predicted to interact with the large loop L2 and

the strand 4 β 3 (residues Gln646, Glu651, Gln652, Val653, Leu658, Tyr659, Val660, Pro661) as well as with Leu707, which further stabilized the conformation of the enzyme.

Discussion

A series of docking calculations in combination with MD simulations of the free form of LF in relation with eight MEK/MKK peptide substrates posed in the catalytic groove of LF for NH₂-terminal endoproteolytic cleavage provided novel insights into the interactions of LF with potential substrates.

Analysis of the per-residue atomic fluctuations (RMSF) for the free form of LF in comparison with all eight complexes revealed a substantial number of LF domains with high flexibility. One of these domains includes residues 349–365 (helix 3 α 3, domain III) exhibited high RMSF, suggesting that substrate binding to the loop influences the mobility of these residues allowing the accommodation of MEKs/MKKs. Another highly flexible enzyme region was that composed of residues 303–320 (helix 3 α 1 in domain III). The data indicated that this region was affected by the highly flexible 349–365 region, since these helices were close to one other and underwent structural changes when substrates were bound to LF. In contrast, the regions comprising the catalytic channel of LF displayed low RMSF, presumably because of the enhanced compactness resulting from substrate accommodation. As a result, domain IV in the complex exhibited lower conformational plasticity than domain III. In contrast, in the absence of substrates, domain III and IV exhibited higher RMSF values indicated higher flexibility. The RMSF values were higher for the residues in domains III and IV, as these residues are more flexible in the absence of the substrates.

Residues from domain III that are distant from the Zn-binding (up to 20 Å) domain were involved in favorable interactions with the substrates, not illustrated in any available X-ray LF model. More specifically, this study suggests that a cluster of negatively charged residues in LF domain III, including Glu333, Glu334, and Asp328, is implicated in major electrostatic interactions with all the substrates. This remarkably high-electron density in the LF substrate channel comprises the primary anchor site for MEK/MKK substrates bearing one or more positively charged Arg or Lys residues at the P₈ to P₄ sites. The crystal structures of LF-inhibitor complexes did not attribute any importance to these particular residues in terms of substrate binding and specificity. Importantly, our MD simulations revealed that occupation of P₁ by Arg, but not by any other residue, allowed the formation of a salt bridge with Glu739, suggesting a major role of the Arg residue in the selectivity of LF for MKK3 and MKK4₍₂₎. Moreover, a Leu residue at P₂, as in MKK3, may sterically hinder the interaction of the amide group of Arg(P₁) with the carbonyl group of Asp328 of

Figure 3. Organization of the LF active site residues (A) at the X-ray crystal structure of LF in the absence of inhibitors (PDB ID: 1J7N), and (B) at a simulated LF-substrate complex. The catalytic Zn ion is represented as a green sphere, water molecules as red spheres and the active-site residues are shown as cyan sticks; the backbone of a substrate is shown in yellow. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

LF. This was not the case for MKK4₍₂₎, as the corresponding P₂ residue was Ala, the small side chain of which does not interfere with the H-bond formation between the amide of Arg(P₁) and the carbonyl group of Asp328 of LF. These data are in good agreement with the computational alanine-scanning mutagenesis results for MEKs/MKKs, which showed that positions P₆, P₅, P₄, and P₁ are important for efficient proteolysis by LF and do not allow any substitution with other residues.

Mutagenesis experiments by Tonello *et al.*³³ suggest that Tyr728 is essential for the metalloproteolytic activity of LF. Together, our structural data presented in this study suggested an orientation of the substrate such that interaction of Tyr728 with the carbonyl group of the scissile bond was probable, as the Tyr728 phenolic group was close to the scissile bond. As shown in Figure 3(A), when LF is crystallized in its native form,⁹ Tyr728 is hydrogen bonded to a coordinated water molecule (W1), while Glu687 interacts with another solvent molecule (W2). In our models, the carbonyl group of the substrate's scissile bond replaced W1, and then a water molecule (W) was

Figure 4. LF cleavage specificity of MEKs1–2, MKKs3–4, and MKKs6–7 and a search for a similar amino acid sequence in the 120-residue N-terminal part of MKK5. Scissors indicates the cleavage site, while basic residues are indicated in blue. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

coordinated in such a position that facilitates nucleophilic attack on the C=O carbon of the scissile bond. This water molecule (W) was observed to be hydrogen bonded to the carboxylate group of Glu687 throughout our simulations. In this orientation, the hydroxyl group of Tyr728 was within hydrogen bonding distance of the carbonyl group of the substrate's scissile bond. The orientation of the active site residues shown in Figures 2(B) and 3(B) was similar in all the eight models of LF-MEK/MKK complexes studied and is in accordance with a proposed mechanism for TLN [Fig. 2(C)].^{34,35} The corresponding TLN tyrosine (Tyr157) is in the vicinity of the carbonyl group of the scissile bond and is proposed to stabilize the tetrahedral intermediate via hydrogen bonding interactions.³⁴ Thus, the present study suggests that Tyr728 is essential for the catalytic activity of LF because it stabilizes the carbonyl group of the scissile bond. Furthermore, in addition to the polarization of the Zn²⁺-bound water between the metal ion and the carboxylate group of Glu687, the nucleophilicity of the water may be enhanced by its hydrogen bonding with the carbonyl oxygen of P₂, P₁', and P₂'.

Taking into account the hydrophobic character of MEKs/MKKs at P₂ (6 Leu, 1 Ala, and 1 Thr), P₁' (3 Leu, 3 Ile, 1 Ala, and 1 Phe; see Table I), and, in some cases, P₁ (Pro in MEK1/2 and Gln/Gly in MKK7β), our data show that the hydrophobic residues of LF at positions S₂ to S₂' (Phe329, Val675, Leu677, Val653, Val658, and Tyr728) are critical for enzyme–substrate stabilization. Their interactions with the substrate's hydrophobic groups were stable for 70–80% of the MD simulation time. A similar observation can be made in the available X-ray structures, in which the bulky, hydrophobic groups of many LF inhibitors are accommodated at these subsites.^{9,20}

Comparison of the LF cleavage sites in MEK1–2, MKK3–4, and MKK6–7 revealed certain similarities at

the level of the primary structure (consensus amino acid sequences, Fig. 4). The cleavage site for all substrates is in the first 40–50 N-terminal residues, even for MKK4 and MKK7β, which contain a secondary LF cleavage site that is, respectively, 13 or 32 residues C-terminal of the first. In all cases, the cleavage site is preceded by a series of basic residues followed by an aliphatic residue. On this basis, the 120 residue N-terminal part of MKK5 that is not cleaved by LF was analyzed and a four-residue fragment that could match the P₂–P₂' consensus of the MEK/MKKs sequences was identified. This sequence was Ile21–Lys22–Ile23–Pro24, the last three residues corresponding to P₁, P₁', and P₂' (Fig. 4). With the exception of Ala in MKK4₍₂₎ and Thr in MEK1, the residue at position P₂ is Leu. The hydrophobic Ile21 of MKK5 could therefore be accommodated as an alternative hydrophobic residue at the S₂ subsite. However, the basic residues that occupy the P₈ to P₄ sites of the other kinases and make ionic interactions with Asp328, Glu333, Glu334, and Glu336 in the substrate channel of LF are missing. The only basic residue near this sequence is Arg20. If the Ile21–Lys22–Ile23–Pro24 sequence of MKK5 is accommodated in the P₂ to P₂' sites, Arg20 will occupy P₃. However, the presence of a basic residue at P₃ is unprecedented among all known LF MEKs/MKKs substrate (Table I). A single basic residue in the “right” position may permit binding and cleavage. This is the case for MKK4₍₂₎, that contains only a single basic residue (Lys), at P₅. Considering that MKK4₍₂₎ can be cleaved by LF, while MKK5 cannot these observations suggest that, in addition to the total number of basic residues, their exact position in the MKK peptide sequence may determine the binding affinity of the substrate and its cleavage by LF. MD simulations were performed on LF-MKK5 complex suggesting that in the absence of basic residues, it was found that residues P₆, P₅, and P₄ did not exhibit any interactions

with LF. These findings are pointing out the importance of basic residues at these P sites. Taking into account that, in all the other MD simulations of LF with MKKs, important electrostatic interactions with Glu333, Glu334, and Asp328 were observed, the absence of these contacts between MKK5 and LF might play a major role in the inability of LF to carry out endoproteolytic cleavage of MKK5.

Methods

Preparation of structures

From the nine available LF crystal structures with PDB codes 1J7N and 1JKY,⁹ 1PWP, 1PWQ, 1PWU, 1PWV, and 1PWW,^{20,21} 1YQY,²² and 1ZXV,²³ the coordinates for LF were taken from 1YQY. This structure has the highest resolution, (2.30 Å), the smallest number of missing atoms, and no active-site mutation or disordered regions. The final LF-substrate complexes consisted of LF residues 265–778 (domains II, III, and IV). Domain I (residues 1–263) was excluded from the calculations because it is far from the catalytic site and not involved in proteolysis.²²

The LF model was prepared after removing the hydroxamate inhibitor and all water molecules from the PDB file. Hydrogen and other missing atoms were added using the LEaP module of AMBER 9.^{36,37} The system was initially relaxed with 500 steps of energy minimization using the steepest descent method and positional restraints with a harmonic force constant of 50 kcal mol⁻¹ Å⁻² on all heavy atoms except those not determined in the X-ray structure. The generalized Born implicit solvation model GB^{HCT} was employed³⁸ with a 16 Å cutoff for the nonbonded interactions.

To examine enzyme–substrate interactions up to 20 Å from the catalytic Zn, the eight MEKs/MKKs listed in Table I were used. Because of the large size of MEKs/MKKs, we considered only a 12-peptide segment for each substrate, centered in the LF catalytic site (six upstream and six downstream of the cleavage site). The initial coordinates of the substrates were generated using the LEaP module of AMBER, and then were subjected to 2000 steps of unrestrained energy minimization with conjugate gradients in implicit solvent.

Docking of substrates

An efficient approach for obtaining the docked conformation of a peptide–substrate in the active site of a metallopeptidase has been recently described for the modeling of angiotensin-converting enzyme (ACE)–gonadotropin-releasing hormone (GnRH) complexes.²⁶ This fragment- and knowledge-based docking approach was used in this study with slight adaptation so as to obtain the conformations of the LF substrates in three tetrapeptidic fragments. The established nomenclature for enzyme and substrate subsites is used

throughout this study.²⁶ Briefly, the conformation of each substrate fragment P₂–P₂' (Table I) was initially chosen so that the carbonyl oxygen of the scissile bond was close to the zinc ion. The top-ranked solution, with the proper position was then included in the subsequent docking calculations for the P₆–P₃ and P₃'–P₆' fragments (Table I). In this way, the selection of the bound conformation was guided by a short distance between P₃ and P₂ and between P₂' and P₃'. The docking results obtained for each MEK/MKK peptide fragment in complex with LF are summarized in Table S1. The appropriate top-ranked conformations of the three tetrapeptide fragments were then linked, and a covalent bond between the P₁ carbonyl oxygen and Zn was created. A water molecule coordinated to Zn, which is necessary for catalysis, was added and the system was subjected to restrained energy minimization as described previously.²⁶

AutoDock 3.05³⁹ was used for the docking calculations and AutoDockTools 1.4⁴⁰ was used for visual inspection of the docking results. Three overlapping grid maps, one for each of the three tetrapeptide fragments of the substrate, were calculated using AutoGrid 3.0 with 81 × 81 × 81 grid-points of 0.25 Å spacing. The central grid box for the docking of P₂–P₂' moieties was centered on the metal ion of the protease. After calculating the appropriate docked conformation of each P₂–P₂' moiety, their structures were included in the calculation of the two flanking grid boxes for the S₆–S₃ and S₃'–S₆' subsites. Their centers were adjusted so that both the P₂ and P₂' residues were inside the corresponding grid box. Typically, 100 – 200 docking rounds were performed using the Lamarckian Genetic Algorithm and the parameters described in Ref. 26.

Molecular dynamics simulations

All calculations were performed using either the SANDER or PMEMD programs of AMBER 9 with the force field of Cornell *et al.*⁴¹ for the protein atoms. Force field parameters for the Zn-binding domain were taken from Ref. 26. The protein–substrate complexes were immersed in isometric truncated octahedron TIP3P-water boxes and the proper number of counter ions was added using LEaP. Periodic boundary conditions were imposed using the particle mesh Ewald method with an 8 Å limit for the direct space sum. Numerical integration was performed with a 2-fs time step, and all bonds involving hydrogen atoms were constrained with SHAKE.⁴² Temperature and pressure controls were imposed using a Berendsen-type algorithm⁴³ with 1 ps coupling constants. The same procedure²⁶ was used for the equilibration of the systems. Production runs were carried out in the isothermal-isobaric ensemble ($P = 1$ atm, $T = 300$ K) for a total simulation time of 3 ns. Processing of the MD trajectories was performed using the PTRAJ module of AMBER 9, while VMD 1.8⁴⁴ was used for their

examination. Solvent-accessible and buried surface areas (BSAs) were calculated with a 1.4 Å probe radius using the program MSMS 2.5⁴⁵ (Figs. S4 and S5).

MD trajectories were analyzed to identify important intermolecular interactions by extracting their geometric features (distances and angles) as a function of simulation time. The hydrogen bonding interactions were monitored using a 3.4 Å distance cutoff and 120 degrees as the angle cutoff. The hydrophobic interactions were included for a pair of carbon atoms separated by a distance less than 4.0 Å. Only interactions present for more than half of the simulation time were considered. Water-mediated hydrogen bonds were considered if observed in at least 25% of the trajectory frames.

The root mean-square deviation (RMSD) of the C α atoms of LF and all atoms of MEK/MKKs from their initial position are shown in Figure S2, while the RMSF values that correspond to the per-residue protein flexibility and their comparison with crystallographic data are provided in Figure S3.

Conclusions

Overall, the present computational study explored the plasticity of eight LF-substrate complexes by means of MD calculations, so as to predict the intermolecular interactions that might contribute significantly to their binding. This approach identified a wide spectrum of interactions, not only proximal to the Zn-binding site, but also in the large catalytic channel of LF, and indicate those structural factors that might define the docking geometry of the substrate, and therefore determining thus the enzyme's specificity. These interactions reveal novel "hot-spots" of LF that offer new possibilities for the design and screening of novel bioactive compounds and extend our knowledge of enzyme-substrate binding.

The interactions between LF and substrate residues at positions P₆–P₂' found to be crucial for substrate binding and selectivity and may also explain the lack of proteolysis of MKK5 by LF. In summary, LF interacts strongly with the substrates bearing one or more basic residues at positions P₈–P₄ and a hydrophobic residue at position P₂; position P₃ may be occupied by any residue. These requirements explain why MKK5 is not cleaved by LF, while other similar kinases are. Thus, this work provides important information for key residues that are distant from the Zn-binding domain (Table II), as for example; S₆–S₃ the residues Asp328, Glu333/334, Asp394 (implicated to electrostatic interactions), S₃–S₂ the residues Phe329, Val653/675, Leu677 (hydrophobic interactions), S₃'–S₅' the residues Glu642/646/651 (water-mediated and electrostatic interactions). These data can be further exploited in a structure-activity relationship studies by driving single point and/or multiple mutations. In addition, our MD simulations revealed that Tyr728 is essential for the catalytic activity of LF because it sta-

bilizes the carbonyl group of the scissile bond. Finally, the present study suggests that occupation of P₁ by Arg allowed the formation of a salt bridge with Glu739, suggesting a major role of the Arg residue in the selectivity of LF for MKK3 and MKK4₍₂₎.

Acknowledgment

We thank Tom Barkas for English editing.

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