



Crystal Structures of Human MD-2 and Its Complex with Antiendotoxic Lipid IVA

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Science **316**, 1632 (2007);

DOI: 10.1126/science.1139111

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The mechanistic basis for MPLA's failure to stimulate full MyD88 signaling, with associated proinflammatory effects, remains to be fully elucidated. However, we propose that an important component is the acquisition, relative to LPS, of an anti-inflammatory function that requires PI3K activity (Fig. 2D). This would be distinct from the loss of a proinflammatory activity, such as a simple failure to recruit Mal and/or MyD88 to TLR4. Discovering the precise mechanism of MPLA's ability to function as a low-toxicity adjuvant may permit improvements in the design of future TLR4-dependent vaccine adjuvants.

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28. This work was supported by the U.S. Public Health Service (grants AI51377 and AI059023), the Commonwealth of Kentucky Research Challenge Trust and Lung Cancer Research Funds, and the Barnstable Brown Foundation.

Supporting Online Material

www.sciencemag.org/cgi/content/full/316/5831/1628/DC1

Materials and Methods

Figs. S1 to S5

References

18 December 2006; accepted 10 May 2007

10.1126/science.1138963

Crystal Structures of Human MD-2 and Its Complex with Antiendotoxic Lipid IVa

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Endotoxic lipopolysaccharide (LPS) with potent immunostimulatory activity is recognized by the receptor complex of MD-2 and Toll-like receptor 4. Crystal structures of human MD-2 and its complex with the antiendotoxic tetra-acylated lipid A core of LPS have been determined at 2.0 and 2.2 angstrom resolutions, respectively. MD-2 shows a deep hydrophobic cavity sandwiched by two β sheets, in which four acyl chains of the ligand are fully confined. The phosphorylated glucosamine moieties are located at the entrance to the cavity. These structures suggest that MD-2 plays a principal role in endotoxin recognition and provide a basis for antiseptic drug development.

Innate immunity is the first line of defense against microbial infections (1). Defense responses are activated when microbial components are recognized by a variety of pathogen sensors, including the Toll family of receptors, Nod-like receptors, and double-stranded RNA sensors (2). Among microbial components, lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria is a potent stimulant of immune responses, and a small difference in LPS structure has a great influence on host responses against Gram-negative bacteria (3). Excessive responses to the endotoxic LPS frequently result in severe sepsis, a rapidly progressing inflammatory disease with up to 29% mortality, leading to more than 215,000 annual deaths in the United States alone (4). Toll-like receptor 4 (TLR4) and MD-2 form a complex, and both have been implicated

in LPS recognition because mice lacking either molecule are hyporesponsive to LPS (5, 6).

TLR4 is a type I transmembrane protein consisting of extracellular leucine-rich repeats and a cytoplasmic signaling domain similar to the type I interleukin-1 (IL-1) receptor (1). MD-2 is a 160-amino acid glycoprotein with a 16-amino acid secretion signal at the N terminus (7) and represents a class of MD-2-related lipid recognition (ML) proteins that also include mite allergen proteins (8). MD-2 forms a stable complex with TLR4 on the cell surface (9), and MD-2 alone as well as in the complex directly binds to LPS with nanomolar affinity (10). On the other hand, it has also been reported that TLR4 recognizes and binds to LPS (11, 12).

Lipid A, the primary immunostimulatory core of LPS, is diverse in several species (13), and these variations are discriminated by the TLR4-MD-2 complex as endotoxic or as antiendotoxic (14). Lipid A of the *Escherichia coli* type (Fig. 1) acts as a potent agonist in human macrophage cells and in mouse cells. However, its precursor lipid IVa (15), the tetra-acylated form of lipid A, acts as an antagonist in human cells but as an agonist in mouse cells (16). Despite identification of the complex as the LPS receptor, no information is available on the structures of liganded pathogen sensors. Here, we

report crystal structures of human MD-2 and its complex with lipid IVa. The structures suggest that MD-2 plays a principal role in LPS recognition.

We expressed human MD-2 in methylotropic yeast *Pichia pastoris*, as described (17). Polysaccharide moieties of MD-2 were trimmed off by endoglycosidase treatment, which leaves a single N-acetylglucosamine (NAG) at each glycosylation site. Monomeric MD-2 was crystallized into space group $P4_12_12$ with $a = 53.1$ Å, $c = 111.5$ Å, and one MD-2 molecule contained in the asymmetric unit. The structure of the native MD-2 crystal was determined at 2.0 Å resolution by multiple isomorphous replacement. A cocrystal with the lipid IVa complex was obtained from a mixture of MD-2 and lipid IVa and is nearly isomorphous to the native crystal. The structure of the complex was refined at 2.2 Å resolution. Details of the crystallographic analyses are given in (17).

Residues from Glu¹⁷ to Asn¹⁶⁰ are defined in the structure with two N-linked NAGs at Asn²⁶ and Asn¹¹⁴. MD-2 is folded into a single domain consisting of two β sheets in the immunoglobulin fold conserved among the ML proteins (8): One sheet consists of three antiparallel β strands, and the

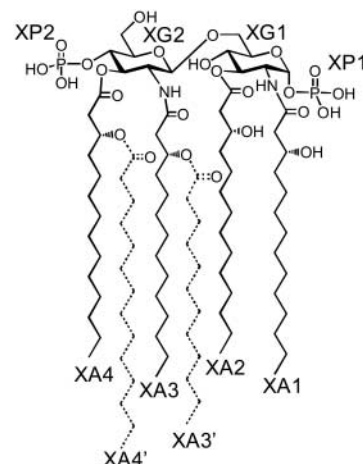


Fig. 1. Chemical structure of lipid IVa. Lipid A has additional XA3' and XA4' acyl chains.

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other consists of six antiparallel strands (Fig. 2). Between these sheets is a large and deep hydrophobic cavity. It has a volume of 1710 Å³ with approximate dimensions of 15 Å by 8 Å by 10 Å. The β6 and β7 strands line the entrance to the cavity. The cavity makes the span of the two sheets much wider than that in the previously predicted model of MD-2 (18). Three disulfide bridges are located between Cys²⁵ and Cys⁵¹, between Cys³⁷ and Cys¹⁴⁸, and between Cys⁹⁵ and Cys¹⁰⁵, in contrast to the predicted bridges between Cys²⁵ and Cys¹⁴⁸ and between Cys³⁷ and Cys⁵¹ (18). The sole free Cys¹³³ is located deep in the cavity and seems not to be involved in the oligomerization that has previously been reported (19).

In the native structure, unexpected electron densities were observed in the cavity; these were attributed to bound lipidic molecules that presumably copurified with MD-2 (fig. S1A). Three myristic acid molecules were built into the structure (fig. S2). In the structure of the lipid IVa complex, electron densities in the cavity (fig. S1B) were assigned to the different parts of lipid IVa (Fig. 1): two glucosamine, two phosphate, and four fatty acid chains. The glycosylation sites of both Asn²⁶ and Asn¹¹⁴ are distant from the cavity region, indicating that the glycosylation plays a role not in ligand binding, but (presumably) in the secretion and protection of MD-2.

Root mean square (RMS) positional deviations between the superposed native and complexed structures are 0.3 Å for the main-chain atoms and 0.7 Å for all the protein atoms. Thus, the MD-2 structure is not significantly altered upon lipid IVa binding. A major difference is in the side-chain conformation of Lys¹²² located at the entrance to the cavity: The side chain is shifted toward the second glucosamine moiety XG2 of lipid IVa, with RMS deviations of 0.3 Å for the main-chain atoms and 2.8 Å for the side-chain atoms. Overall *B* factors for the native and complexed structures are 39 Å² and 37 Å², respectively, and a slight decrease (3.3 Å²) in the averaged *B* factor is noticed for β7 upon complexation.

Details of the interactions between lipid IVa and MD-2 are shown in Fig. 3 and fig. S3. The phosphate and sugar groups are aligned in parallel with β7 in the order XP1, XG1, XG2, and XP2, with an XP1-XP2 distance of 12.5 Å, which explains how the peptide fragment from Phe¹¹⁹ to Lys¹³² can bind to LPS (20). Residues Phe¹¹⁹ to Gly¹²³ are important for the LPS recognition, and these residues, with the exception of Lys¹²², are conserved in all the species of MD-2 (21). Three hydrogen bonds to lipid IVa are noticed: Ser¹²⁰ N to XA1 O1' (distance of 2.87 Å), Lys¹²² N to XA3 O1' (3.07 Å), and Ser¹²⁰ O to XA3 O3' (2.66 Å). Water atoms mediating lipid IVa and MD-2 are located at the cavity entrance (Fig. 3). Among a total of 18 lysine and arginine residues of human MD-2, which is highly basic with an isoelectric point value of 8.7, only Lys¹²² and Arg⁹⁰ are located in the vicinities of the entrance, and their side chains cover XG2 and XP2. These interactions tether the hydrophilic moiety of lipid IVa to the cavity. Hydrophobic and electrostatic surface potentials in the vicinities of the entrance indicate that the

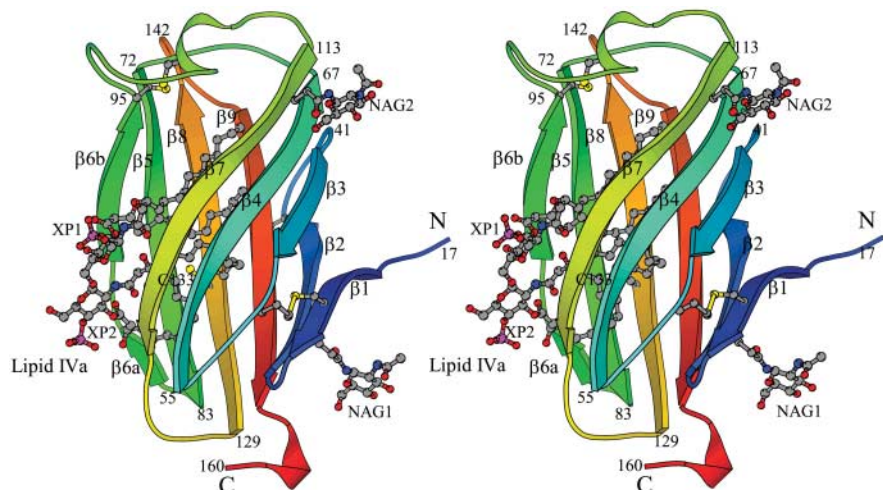


Fig. 2. Stereo ribbon model of human MD-2 in complex with lipid IVa. The N terminus is drawn in blue and the C terminus in red. The β strands are indicated with their labels, and some amino acid residue numbers are shown. Bound lipid IVa and NAGs as well as cysteine residues are drawn as ball-and-stick models. The two β sheets are inclined toward each other by about 45°.

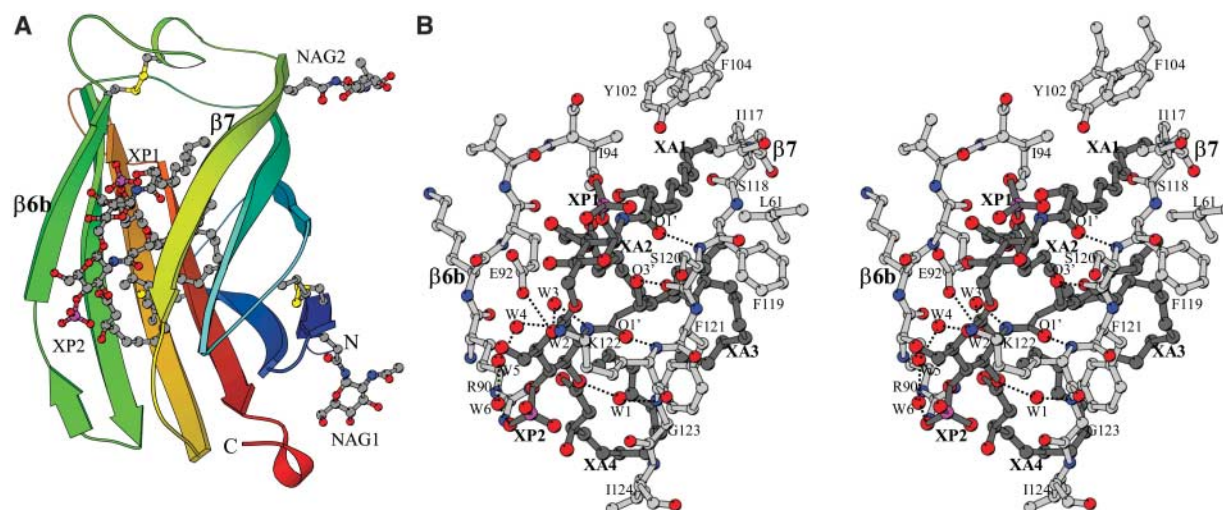


Fig. 3. Binding interface to lipid IVa. (A) Ribbon representation of the lipid IVa complex, as viewed from a 40° rotation with respect to Fig. 2. The representation is similarly drawn as in Fig. 2 so as to show that the entrance to the MD-2 cavity is lined by the β6b and β7 strands. (B) Stereo close-up view of the binding interface. Amino acid residues located in the vicinities of the entrance are drawn as ball-and-stick models with their

residue labels. The structure of the lipid IVa moiety is similarly drawn in darker gray, O atoms in red, N in blue, C in gray, and P in pink. Water O atoms involved in hydrogen bonds (broken lines) are also depicted: W1 between Gly¹²³ N and XG2 O3, as well as a group of W2, W3, W4, W5, and W6, in which W2 is hydrogen-bonded to Glu⁹² O^{e1}, W2 to W3, and W3 to XG2 N2.

entrance is positively charged and the inside of the cavity is highly hydrophobic (Fig. 4). None of the phosphate groups of lipid IVa, which are reported to be essential to the activation of immune responses (13), are involved in direct hydrogen bonds to MD-2 atoms. The lysine and arginine residues mainly contribute to the attraction of negatively charged lipid IVa.

Four fatty acid chains of lipid IVa are all deeply confined in the cavity. The XA1 chain is in an extended linear conformation and is stuck deeply into the cavity: Three of its four sides are surrounded by hydrophobic MD-2 side chains. The XA2 chain is also in the cavity and lined up with XA1. The XA3 and XA4 chains are curved, and the regions of XA3 C10'-11' and XA4 C11'-13' atoms are loosely packed in the cavity. The tip end of XA4 folds back toward the XG2 moiety, and that of XA3 hangs over XA4. The fatty acid chains in the cavity are packed next to each other through van der Waals contacts, as exemplified in the lipid molecules bound to the GM2 activator protein (22). The packed and confined fatty acid structures are distinct from the extended structures of the fatty acid chains of LPS associated with the membrane-embedded region of the FhuA ferri-chrome ion receptor (23). The cavity of MD-2 is divided into four sites on the basis of their interactions to the fatty acid chains: L1 through L4 respectively correspond to XA1 through XA4 (table S3). In the L1, L2, and L3 sites of the native structure, the fatty acid molecules assigned as myristic acids exist in nearly identical configurations to those of XA1, XA2, and XA3, respectively (figs. S2 and S3). The averaged *B* factor for the lipid IVa molecule is 46 Å²; comparable values are obtained for XA1 (33 Å²), XA2 (46 Å²), and XA3 (41 Å²), with a larger one for XA4 (53 Å²). These suggest that the L1, L2, and L3 sites have higher affinities to fatty acid chains. The surface area accommodating lipid IVa is very wide, 890 Å². This large value is comparable to that of ligands bound to the antibodies (24) and explains the nanomolar affinity of MD-2 toward LPS (10).

The MD-2 residues essential to the interaction with TLR4 are reported to be Arg⁹⁰, Lys⁹¹, Asp¹⁰⁰, Tyr¹⁰², Cys⁹⁵, and Cys¹⁰⁵ in the absence of the ligands (25). A synthetic peptide from Cys⁹⁵ to Cys¹⁰⁵, in the oxidized form, exhibits a decrease in LPS-induced activation (18) and is supposed to compete with MD-2 through the interaction with TLR4. These residues are located at the cavity entrance (Fig. 4).

The structure of CD14, which transfers LPS to MD-2, also has a hydrophobic cavity of dimensions nearly equal to those of MD-2 (26); hence, it is presumed to recognize acyl chains of LPS. The only differences between the structures of the antagonist lipid IVa and of the agonist lipid A are two additional acyl chains, XA3' and XA4' (Fig. 1). The MD-2 cavity likely could not accommodate more than four acyl chains. When the additional XA3' chain ester-linked to the XA3 O3' atom is directed toward the inside of the cavity, the hydrogen bonds of the XA3 O3' atom to both Ser¹²⁰ O and to XA1 O1' are disrupted, and hence the XA3 and XA4 portions are rearranged. This rearrangement would displace some portions of XA3 and XA4 toward the region near Val⁸², Leu⁸⁷, and Phe¹²⁶, which is reported to affect ligand-stimulated TLR4 clustering (27).

Binding sites other than L1 through L4 for the additional acyl chains or conformational changes enlarging the cavity are conceivable for lipid A. The additional lipid A acyl chains displaced from the hydrophobic cavity might be involved in activation upon MD-2 complexation with TLR4, and they may induce the reported oligomerization of TLR4 (28). This activation scheme is consistent with the increased MD-2 affinity to lipid A upon association with TLR4 (29). Recombinant human MD-2 in which Ser⁵⁷, Leu⁶¹, and Lys¹²² are replaced with the corresponding mouse residues (Thr⁵⁷, Val⁶¹, and Glu¹²²) is reported to be activated by lipid IVa and lipid A (30). The hydroxy O^γ atom of Ser⁵⁷ in the β4 strand is hydrogen-bonded to Glu⁵³ N, Leu⁶¹ is located deep in the cavity, and Lys¹²² is on the surface of the cavity entrance. The former two replacements

would bring subtle changes in the construction of the cavity, and the replacement with the glutamate side chain would change the electrostatic properties of the cavity entrance.

We hypothesize that the MD-2 structure unaltered by lipid IVa binding might be essential to antagonistic properties in human cells. The complexed structure that confines most of lipid IVa suggests that MD-2 plays a principal role in recognizing LPS. Moreover, it provides a basis for structure-based development of antiseptic drugs that might be effective in preventing endotoxin shock.

References and Notes

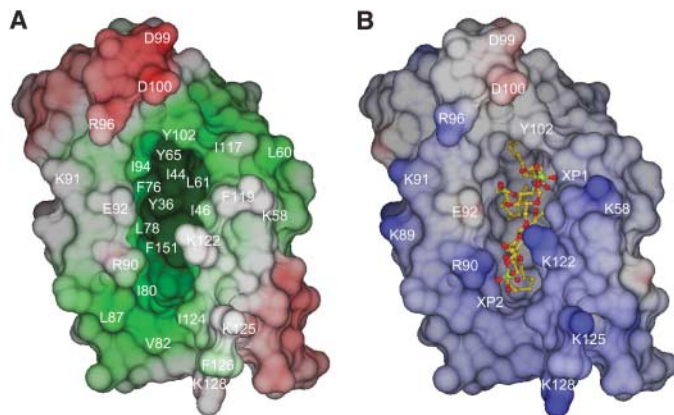
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31. Supported by Japanese Ministry of Education, Culture, Sports, Science and Technology grants-in-aid and a Protein 3000 grant (Y.S.), a JST-CREST grant (K.M.), and the JSPS scholarship and its grant (U.O.). We thank K. Hasegawa for technical assistance in the synchrotron experiment at SPring-8 BL38B1. Coordinates and structure amplitudes have been deposited in the Protein Data Bank with accession numbers 2E56 and 2E59 for the native and complexed structures, respectively.

Supporting Online Material

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20 December 2006; accepted 18 April 2007
10.1126/science.1139111

Fig. 4. Binding pocket and surface properties of MD-2. MD-2 is viewed from a 90° rotation with respect to Fig. 2, and residues of interest are indicated. (A) Protein surface showing hydrophobic and hydrophilic properties. The lipid IVa structure is removed from the complexed structure. Green and red represent hydrophobicity and hydrophilicity, respectively, and the extent is indicated by color darkness. (B) Electrostatic potential surface. Positive and negative potentials are shown in blue and red, respectively. Bound lipid IVa is drawn as a ball-and-stick representation: O in red, N in blue, C in yellow, and P in green.





Supporting Online Material for

Crystal Structures of Human MD-2 and Its Complex with Antiendotoxin Lipid IVa

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Published 15 June 2007, *Science* **316**, 1632 (2007)
DOI: 10.1126/science.1139111

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Materials and Methods

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Materials and methods

Protein expression and purification

The DNA sequence encoding human MD-2 (residues 17–160), placed downstream of a His₆ tag sequence with a factor Xa cleavage site, was inserted into the downstream of the *Saccharomyces cerevisiae* α -factor signal sequence in each of the *Pichia pastoris* expression vectors of pPIC9, pPICZ α , and pPIC6 α (Invitrogen, San Diego, CA). *P. pastoris* KM71 strains were transformed with these vectors sequentially into the strain genome, and transformants with higher expression levels were selected with Zeocin and Blasticidine (Invitrogen, San Diego, CA). MD-2 in the culture supernatant from 10-l-scale fermentation was purified with Ni-Chelating Sepharose fast flow column (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Polysaccharide moieties bound to MD-2 were trimmed off by the incubation for 3 hr at 20°C with 1/1000 (v/v) Endoglycosidase Hf (New England Biolabs., Ipswich, MA). Monomeric MD-2 was isolated with Sepharose CL-4B (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) size-exclusion chromatography, and was followed by tag cleavage with 1/1000 (v/v) Factor Xa (Novagen, Madison, WI) for 18 hr at 25°C. Further purification was performed with chromatography steps using Poros-CM (Applied Biosystems, Foster City, CA) cation-exchange and Superdex 75 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) size-exclusion columns. Purified monomeric MD-2 was concentrated to a 10 mg/ml solution of 10 mM Na-acetate pH 5.0 and 100 mM NaCl. The fraction of the lipid IVa complex was separated from an equimolar mixture of MD-2 and synthetic lipid IVa (S1) with the Poros-CM column, and concentrated to a 6 mg/ml solution of 10 mM Na-acetate pH 5.0 and 100 mM NaCl.

Crystallization

Crystallization attempts were carried out with the hanging-drop vapor-diffusion method at 4°C by mixing equal volumes of the protein and reservoir solutions. Native crystals were obtained using a reservoir solution of 20% PEG 8000, 120 mM Na-acetate, and 70 mM Na-cacodylate pH 6.3. Crystals of the lipid IVa complex were obtained using a reservoir solution of 15% PEG 3350 and 40 mM Na-citrate pH 5.4. The Crystals were transferred into a harvesting mother solution of 25% PEG 8000, 0.2 M Na-acetate, 0.1 M NaCl, 0.1 M Na-cacodylate pH 6.3, and 20% 2,3-butanediol. Four heavy atom derivatives were prepared as listed in Table S1.

Diffraction data collection and structure determination

Diffraction datasets were collected at 100 K on BL38B1 of SPring-8, Hyogo, Japan, with a Jupiter 210 CCD detector (Rigaku, Tokyo, Japan). All the crystals were cryoprotected with the mother solution. The datasets were processed with the HKL2000 package (S2). Phasing was performed with the SHARP program (S3) and improved with solvent flattening using DM in the CCP4 suite (S4). Data collection and phasing statistics are also summarized in Table S1. The mean figures of merit were 0.67 for acentric reflections and 0.61 for centric reflections. Structural models were built with COOT (S5) and refined with the maximum-likelihood refinement procedure implemented in REFMAC

(S6). Based on the electron densities (Fig. S1), the structures of three myristic-acid molecules were built into the native model (Fig. S2). The crystal structure of the lipid IVa complex was refined using the native structure as an initial model, and the structure of the lipid IVa molecule was also built in (Fig. S3). Three loop regions from Lys 39 to Gln 41, from Arg 96 to Asp 100, and from Gly 140 to Glu 143 are located on the surface and in lower electron-densities than the rest of the residues. Structural geometries were analyzed with program PROCHECK (S7). Neither the native nor lipid IVa complexed structures have residues in the disallowed regions of the Ramachandran plot. The cavity volume was calculated with CAST (S8), and the buried surface areas with ArealMol in the CCP4 suit (S4). Refinement statistics are summarized in Table S2. The fatty-acid binding sites of MD-2 are listed in Table S3.

Figs. 1, 2, S2, and S3 were prepared with MOLSCRIPT (S9), Figs. 3A and 3B with NOC (S10), and Fig. S1 with CueMol (S11).

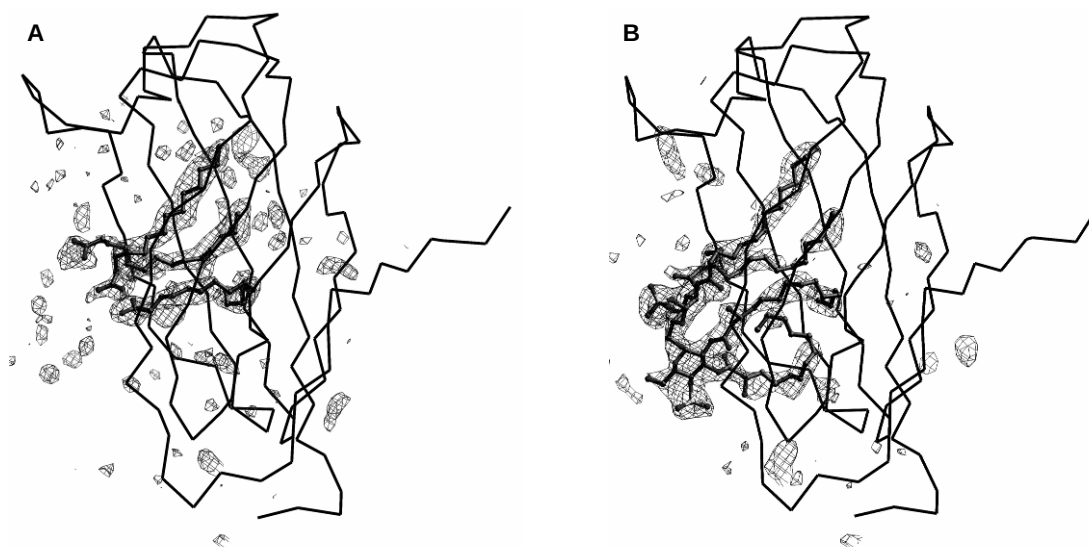


Fig. S1. Electron densities in the cavity of MD-2. (A) Native structure. (B) Lipid IVa complexed structure. MD-2 is shown as C α -traced lines. Structures of the bound myristic acids and lipid IVa are drawn as ball-and-stick models, and superimposed onto F_o-F_c difference-Fourier omit maps contoured at a 2.5σ level. Each view is from a direction nearly identical to that of Fig. 1.

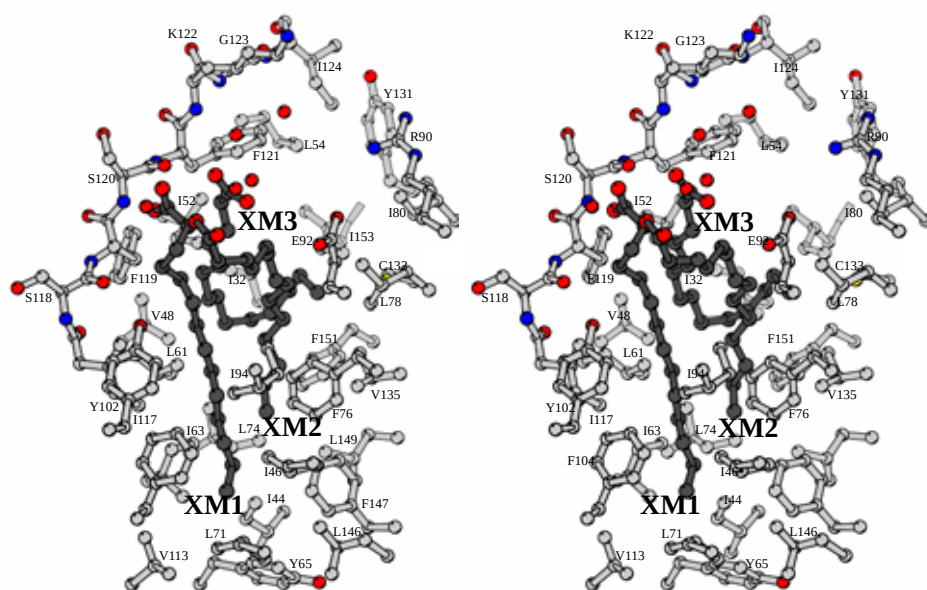


Fig. S2. Stereo view of the hydrophobic cavity of the native structure of MD-2. Side chains constructing the pocket wall as well as a part of the $\beta 7$ strand are drawn as ball-and-stick models with their residue labels. The structure of the myristic-acid molecules (XM1 through XM3) are similarly drawn with darker colors. O atoms are drawn in red, N in blue, C in gray, and Cys 133 S^{γ} in yellow. Water O atoms are also depicted. The view is from the direction opposite to that of Fig. 1.

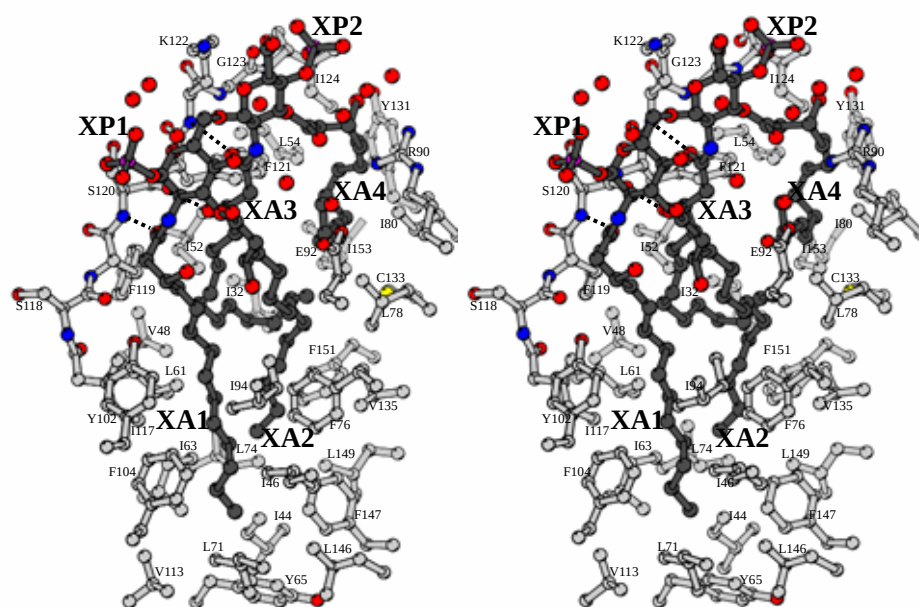


Fig. S3. Stereo view of the binding interface to lipid IVa. The view and drawing nearly correspond to Fig. S2. The structure of lipid IVa is drawn with darker colors and its P atoms in pink. Hydrogen bonds are drawn as broken lines.

Table S1. Data collection and phasing statistics^a.

	Native	KI	PCMBS ^b	HgCl ₂	K ₂ PtCl ₄
Data Collection					
Wavelength (Å)	1.0000	1.0000	1.0061	1.0061	1.0720
Resolution limit (Å)	2.0	2.5	2.4	2.6	3.0
Average $I / \sigma(I)$	11.3 (3.0)	7.5 (2.0)	8.8 (1.7)	6.4 (1.5)	6.2 (2.0)
$R_{\text{merge}}(I)$ ^c	0.105 (0.615)	0.079 (0.571)	0.100 (0.421)	0.097 (0.626)	0.100 (0.451)
Completeness (%)	99.5 (97.8)	98.0 (96.9)	93.6 (62.7)	98.9 (93.0)	97.4 (91.3)
Phasing					
R_{iso} ^d		0.171	0.285	0.246	0.225
Number of heavy-atom sites		9	6	6	3
Phasing power (acentric / centric)		1.8 / 1.3	1.7 / 1.3	1.5 / 1.3	0.5 / 0.6

^a The parenthesized values are for the highest resolution shell.

^b *p*-chloro-mercuribenzenesulfonate.

^c $R_{\text{merge}}(I) = \Sigma |I - \langle I \rangle| / \Sigma I$, where I is observed diffraction intensity.

^d $R_{\text{iso}} = \Sigma |F_{\text{PH}} - F_{\text{P}}| / \Sigma F_{\text{P}}$, where F_{PH} and F_{P} are structure amplitudes of the native and derivative datasets, respectively.

Table S2. Data collection and crystallographic refinement statistics^a.

	Native	Lipid IVa complex
Data collection		
Lattice parameters <i>a</i> (Å)	53.1	52.8
<i>b</i> (Å)	111.4	110.9
Resolution range (Å)	48.4–2.0	27.7–2.2
Number of observed reflections	140,303	85,660
Number of unique reflections	11,389	7,629
Average <i>I</i> / $\sigma(I)$	11.3 (3.0)	9.6 (2.6)
$R_{\text{merge}}(I)^b$	0.105 (0.615)	0.100 (0.423)
Completeness (%)	99.5 (97.8)	90.7 (57.9)
Refinement		
Number of reflections used	9641	6291
Completeness (%)	88.7 (64.7)	78.9 (32.0)
Number of non-hydrogen atoms		
Protein	1162	1162
Water	143	89
NAG	28	28
Ligand	48	95
<i>B</i> -factor (Å ²)		
Protein	37.5	36.2
Water	46.9	41.0
NAG	64.4	45.1
Ligand	50.5	45.9
R^c	19.4	20.6
R_{free}^d	24.9	26.3
Rms deviations		
Bond length (Å)	0.010	0.011
Bond angles (°)	1.54	1.50

^a The parenthesized values are for the highest resolution shell.

^b $R_{\text{merge}}(I) = \Sigma |I - \langle I \rangle| / \Sigma I$, where *I* is observed diffraction intensity.

^c $R = \Sigma |F_o - F_c| / \Sigma F_o$, where *F*_o and *F*_c are observed and calculated structure amplitudes, respectively.

^d R_{free} is an *R* value for a 5% subset of all the reflections, which was not used in the crystallographic refinement.

Table S3. Fatty-acid binding sites of MD-2^a.

L1 in contact with XA1		L2 in contact with XA2		L3 in contact with XA3		L4 in contact with XA4	
Leu 61	(23.0)	Ile 46	(7.3)	Val 48	(10.2)	Leu 54	(6.2)
Ile 63	(21.8)	Ile 63	(10.8)	Ile 52	(29.2)	Leu 78	(13.4)
Tyr 65	(4.9)	Phe 76	(33.8)	Phe119	(25.2)	Ile 80	(32.2)
Leu 71	(6.6)	Leu 78	(19.0)	Ser 120	(26.9)	Arg 90	(41.4)
Leu 74	(8.9)	Glu 92	(36.8)	Phe 121	(31.0)	Phe 121	(47.2)
Phe 76	(28.7)	Ile 94	(14.7)	Lys 122	(12.6)	Ile 124	(30.2)
Ile 94	(20.0)	Val135	(13.3)	Cys 133	(15.2)	Tyr 131	(12.8)
Tyr 102	(23.4)	Phe147	(7.4)	Phe 151	(25.8)	Cys 133	(19.0)
Phe 104	(15.0)	Phe 151	(19.1)	Ile 153	(12.7)	Ile 153	(16.9)
Ile 117	(18.9)						
Ser 118	(14.0)						
Phe 119	(19.3)						
Ser 120	(20.8)						
Phe 147	(10.1)						

^a The parenthesized values ($\text{\AA}^2$) are surface areas in contact with individual fatty-acid chains.

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