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The crystal structure of human protein α1M reveals a chromophore-binding site and two putative protein–protein interfaces

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ABSTRACT

Lipocalin α1-microglobulin (α1M) is a conserved glycoprotein present in plasma and in the interstitial fluids of all tissues. α1M is linked to a heterogeneous yellow–brown chromophore of unknown structure, and interacts with several target proteins, including α1-inhibitor-3, fibronectin, prothrombin and albumin. To date, there is little knowledge about the interaction sites between α1M and its partners. Here, we report the crystal structure of the human α1M. Due to the crystallization occurring in a low ionic strength solution, the unidentified chromophore with heavy electron density is observed at a hydrophobic inner tube of α1M. In addition, two conserved surface regions of α1M are proposed as putative protein–protein interface sites. Further study is needed to unravel the detailed information about the interaction between α1M and its partners.

1. Introduction

The lipocalins, widely found in animals, plants, and bacteria, are a superfamily of small, extracellular proteins that contains about 80 members [1–3]. The family shares a well-conserved, three-dimensional structure folding into an anti-parallel β-strand barrel. The active site of lipocalins recognizes lipophilic ligands, or substrates, that is located at the open mouth of the barrel [4]. This family performs a wide variety of biological functions, including retinol transport, cryptic coloration, olfaction, pheromone transport and the enzymatic synthesis of prostaglandins. Previous research revealed that some lipophilic ligands appear to diffuse across the membrane into cell, suggesting that lipocalins function as tumor protease inhibitors [5]. Additionally, some lipocalins, including Lipocalin 2, α1-microglobulin (α1M), alpha-1-acid glycoprotein and C8gamma, participate in the regulation of the immune response [6–9].

So far, α1M (also called protein HC), an evolutionarily well-conserved glycoprotein, has been found in many species of animals, including mammals, birds, fish and amphibians (Fig. 1) [3,10]. The conserved protein is mainly synthesized in the liver and then rapidly secreted and distributed to different organs and tissues, including liver, heart, eye, kidney, brain, lung, pancreas and skeletal muscle, suggesting that α1M plays an important role in physiological activities. Allhorn et al. reported that α1M could bind heme strongly and degrade it with the help of hemoglobin [8]. In addition, α1M combines with an unidentified chromophore and exhibits a yellow–brown color. The chromophore, presumably the degradation products of protoporphyrin, is bound covalently to cysteine and lysine residues, including Cys34, Lys92, Lys118 and Lys130, at the entrance of the heme-binding cavity [8,11]. α1M which also found in the extravascular compartments in a free form or as a component of protein complexes interacts with immunoglobulin A (IgA), α2-macroglobulin, fibronectin, albumin and prothrombin [12–16]. In human blood, about half of α1M binds to IgA via a covalent disulfide bond formed between the penultimate Cys residue of the IgA heavy chains and Cys34 of α1M [17]. Interestingly, the interaction of α1M with IgA does not inhibit its specific capacity to bind heme [18]. Up to now, many partners of α1M, such as chromophore, IgA, fibronectin, albumin and prothrombin, had been identified, whereas there is little knowledge about their interaction sites of α1M.

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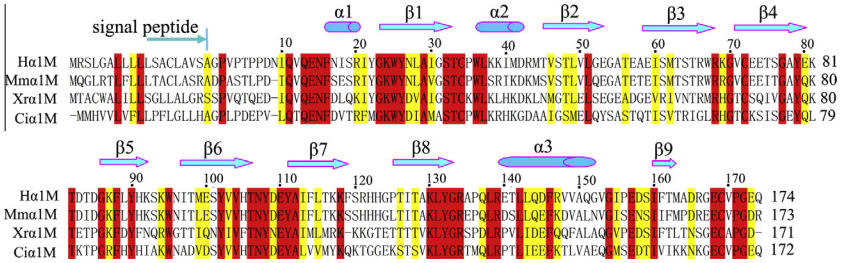

Fig. 1. α1M structure and structure-based sequence alignment. Sequence alignment of four α1M homologs of animal species, including Hα1M of *Homo sapiens* (accession number NM_001633), Mmα1M of *Mus musculus* accession number BAB23659), Xrα1M of *Xenopus ropicalis* (accession number AH64278), Ciα1M of *Ctenopharyngodon idella* (accession number ABW37743). Identical and similar residues are shown in *red* and *yellow*, respectively. Secondary structure elements of human α1M are shown above the sequences. (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

To further investigate the biochemical and physiological functions of α1M, we present here the crystal structure of the human α1M at 2.0 Å resolution. Due to the high resolution and crystallization in a low ionic strength solution, the unidentified chromophore with heavy electron density is observed at the hydrophobic inner tube. In addition, two conserved surface regions are proposed that serve as putative binding sites for target proteins.

2. Materials and methods

2.1. Protein expression and purification

Purification and crystallization of α1M were carried out as previously described [19]. α1M protein was expressed in *E coli* BL21 (DE3) induced by addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 293 K. The protein was purified by Ni–NTA affinity (Qiagen) and Hitrap Q (GE Healthcare) column successively at 277 K. Seleno-L-methionine substituted α1M protein (SeMet-α1M) was produced in *E coli* B834 (DE3) in inorganic media supplemented with seleno-L-methionine and purified with the above described method. The protein concentrations of α1M and SeMet-α1M were adjusted to approximately 10 mg ml^{-1} for crystallization trials, respectively.

2.2. Crystallization

Crystals of both the native and the SeMet-α1M proteins were grown using the hanging drop vapor diffusion method at room temperature. The initial crystallization conditions were screened by the sparse matrix sampling method using Crystal Screen, Index and PEG-Ion kits (Hampton Research). Ultimately, suitable crystals of native α1M for diffraction experiments were grown for approximately 15 days at 293 K with a reservoir solution containing 20% PEG 3350 (w/v), 1% 1,1,1,3,3,3-hexafluoro-z-propanol (v/v), 0.2 M ammonium citrate and 0.1 M Hepes pH 6.94 [19]. The SeMet-α1M crystals suitable for diffraction experiments were obtained using 20% PEG 3,350, 0.16 ammonium citrate, 0.04 M potassium sulfate and 0.08 M Hepes pH 6.80–7.0.

2.3. Data collection and structure determination

Crystals were transferred to a reservoir solution supplemented with 20% (v/v) glycerol and were immediately placed in a 100 K nitrogen gas stream. A multi-wavelength anomalous dispersion (MAD) data set was collected from a single SeMet-α1M crystal on beamline 3W1A of the Beijing Synchrotron Radiation Facility (BSRF). Data were collected at two wavelengths (λ_{peak} = 0.9792 Å and λ_{remote} = 1.000 Å) and were processed and scaled to 3.0 and 2.8 Å, respectively, using the program HKL2000 [20]. The native data set was collected on beamline BL17U1 of the Shanghai Synchrotron Radiation Facility (SSRF) and was processed and scaled to 2.0 Å using the program HKL2000. The final data-collection and processing statistics are given in Table 1.

The SeMet-α1M crystal used for the structure solution belonged to the $P4_3$. Using 3.0 Å MAD data and the program SOLVE, 12 selenium atoms in the asymmetric unit were located and the initial phases were calculated [21]. Initial phases were then improved by density modification and subsequently used for model building. Using the program RESOLVE [22], about 50% of the residues in the protein were automatically built. Further model building was performed manually using the program WinCoot, and the refinement was performed with Phenix [23,24]. The stereochemical quality of the final model of α1M was ascertained using MolProbity [25]. Structural factors and coordinates of α1M have been deposited into the Protein Data Bank with the accession code 4ES7. Structural refinement statistics are listed in Table 1. Ribbon cartoons and surface representations were generated using PyMol [26].

3. Results and discussion

3.1. Overall structure of α1M

The X-ray crystal structure of α1M was determined by MAD phasing and refined against the native data to a 2.0 Å resolution. The final model consisted of 172 amino acids, 54 water molecules and one PEG 300 molecule and exhibited R_{factor} and R_{free} values of 20.19% and 26.11%, respectively. The root mean square deviations (*rmsd*) from ideal values of bond lengths and bond angles were 0.007 Å and 1.234°, respectively. Ramachandran φ–ψ plots for the α1M structure indicated that 96.95% of the non-glycine and non-proline residues had main chain torsion angles in the most favored regions, and 3.05% of the residues were in the allowed region. Data collection and refinement statistics are summarized in Table 1.

The α1M monomer folds into a single-domain protein with the shape of approximately spherical molecules in dimensions of 36 × 41 × 43 Å. The overall structure and topology of α1M is shown in Fig. 2A. Secondary structural elements of α1M are composed of nine β-strands pulsing one short 3_{10} helix (α1, residues 18–21) and two long α-helices (α2, residues 35–43 and α3, residues 140–153) (Fig. 2A and B). The overall structure of human α1M reveals a classic lipocalin fold that differs slightly from those known lipocalin structures (Fig. 2B and C). The central barrel of the nine β-strands is flanked on one side by the α3 helix, and its mouth and bottom are occupied by α2 and α1, respectively. The strands form a nine-stranded antiparallel β-sheet that wraps into a cylinder surrounded by three helices. Seven β-sheets, from the second to the eighth, form anti-parallel β-strands. The α2 and α3 function as connectors between β2-β3 and β8-β9, respectively (Fig. 2A and

Table 1
X-ray data collection and refinement statistics for human α1M.

Space group	P43		
Cell dimensions (Å)	$a = b = 36.437$, $c = 112.667$		
Data set	Native	Peak	Remote
Wavelength (Å)	1.0000	0.9792	1.0000
Resolution (Å)[a]	35–2.0 (2.07–2.0)	50–3. 0 (2.07–3.0)	50–2.8 (2.85–2.80)
Total number of reflections used	284280	67334	88526
Number of unique reflections	9947	5687	7037
Completeness (%)[a]	99.9(98.9)	99.3(88.9)	99.4(87.0)
Average redundancy	7.1(7.3)	7.1(2.8)	7.1(3.0)
I/σ(outer shell)	16.7(8.5)	10.5	9.2
Rmerge (%)[a,b]	7.0(35.8)	9.3(27.5)	8.3(40.6)
Refinement			
Resolution	40–1.95		
R_{free}[c]	20.19%		
R_{factor}[c]	26.11%		
Bond length *rmsd* (Å)[d]	0.007		
Bond angle *rmsd* (°)[d]	1.234		

[a] Values in parentheses are for the highest-resolution shells.
[b] Rmerge = $\sum(I - < I >)/\sum I$, where I is the observed intensity and $\langle I \rangle$ is the statistically weighted average intensity of multiple symmetry related observations.
[c] R_{factor}: $R = \sum||F_{calc}| - |F_{obs}|/\sum|F_{obs}|$, where F_{calc} and F_{obs} are the calculated and observed structure factors, respectively. R_{free} is calculated similarly to R_{factor}, but from a test set containing 5% of data excluded from the renement calculation.
[d] *rmsd*, root-mean-square deviation from ideal geometry.

B). In addition, two conserved cysteines, Cys72 and Cys169, form a disulfide bridge to stabilize the amino terminal loop to tightly bound to the central barrel core (Fig. 2A).

3.2. Comparison of sequence and structure homologs

A Pfam annotation suggests that α1M belongs to a family of lipocalins, such as prostaglandin synthase, gelatinase, lactoglobulin, lipocalin2, lipocalin12 and lipocalin15, which recognizes specific hydrophobic molecules. A BLAST search reveals a large number of homologs of α1M. In addition to mammalian proteins, homologs of α1M are also found in birds, fish, and amphibians, suggesting that it is an animal-specific protein and performs essential biological functions.

Despite the low sequence identity, α1M exhibits structural similarity to some determined structures. The most similar proteins were found by the Dali server in the PDB, including prostaglandin H2 isomerase (PDB code 3o2y, *rmsd* = 1.8 over 159 Cα atoms), neutrophil gelatinase-associated lipocalin (PDB code 3s26, *rmsd* = 1.9 over 155 Cα atoms) and lipocalin 15 (PDB code 2xst, *rmsd* = 1.9 over 147 Cα atoms) [27]. Interestingly, these proteins adopt the typical β-barrel fold and perform chemically similar functions, such as recognizing ligands (or catalyzing substrates) which contain hydrophobic groups. In these proteins, the active sites conservatively settle at the mouth of the barrel, suggesting that α1M exhibits similar biochemical functions [28,29].

3.3. Mapping of the chromophore binding site

Previous research reported that α1M binds to an unidentified yellow–brown hydrophobic ligand; however, there is little knowledge about the binding site of this chromophore [8,11]. Berggård et al. revealed that the recombinant human α1M is also a yellow–brown color similar to that of the natural α1M (the protein purified from human blood or urine), implying that a high resolution crystal structure might provide key clues about the nature of the unidentified chromophore [11].

The electrostatic surface potential of human α1M displays prominent asymmetry. As shown in Fig. 2B, the largest pocket, located at the mouth of the barrel, can be divided into two distinct

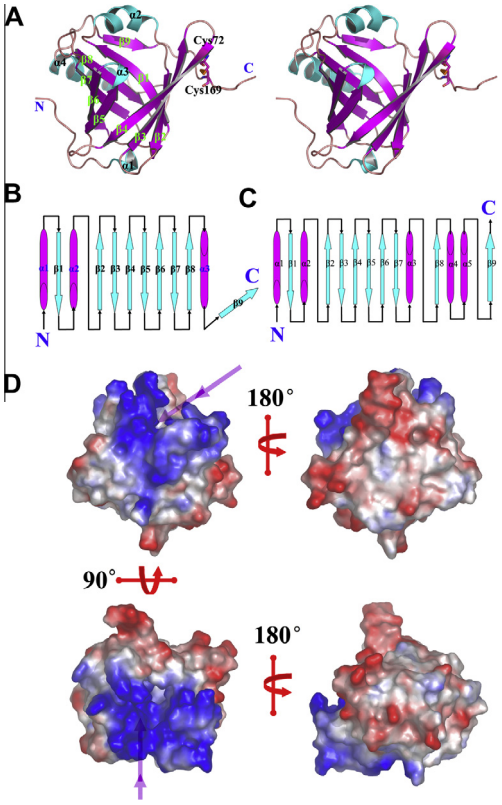

Fig. 2. Overall structure of human α1M. (A) A stereo view of human α1M. The secondary structure elements are numbered correspondingly. (B and C) The top structure of α1M and the most similar structure, 3O2Y, respectively. The "N" and "C" coloring in blue represent the amino and carboxyl terminus. (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

regions: the entrance and the inner tube. The entrance region exhibits a distinctly positive-charged feather. 11 polar residues, including Lys39, Arg43, Arg66 Arg67, Lys69, Lys92, Lys94, Lys118, Arg121, His123 and Lys130, contribute to the positive charge of the entrance region (Fig. 3A and B). At the inner tube region, 10 hydrophobic amino acids, including Phe16, Ile21, Leu49, Leu51, Met62, Phe88, Met99, Val103, Phe114 and Tyr132, form a markedly hydrophobic tube with dimensions of approximately 6×25 Å (Fig. 3B and C). Fortunately, unusually heavy electron density was observed in the tube region, indicating that a long-chain molecule colonizes at this region (Fig. 3C). Similar to the natural α1M, the recombinant protein also combines with a chromophore; nevertheless, the colored molecule has not been identified [8,11]. Consequently, we cannot unambiguously state which molecule is bound in the cavity; we thus tentatively state that the electron dense region is a PEG 300 molecule.

Previous research found that the chromophore of human α1M, with a molecular weight of 282 Da, contains a 12-carbon aliphatic chain substituted with a cyclohexene, a hydroxyl and an amine group [11]. Berggård also suggested that the hydroxyl and amine groups combine the same carbon atom of the aliphatic chain, which conflicts with some previous studies [11]. The recombinant α1M expressed from *E. coli* also combined with an unidentified chromophore that is similar to that of the natural α1M [30]. Interestingly, the groove on the mouth of the β barrel is a match to the unidentified chromophore, which confirms Berggård's deduction that the ligand is buried in the interior of the protein. The inner tube exhibits distinctly hydrophobic features, which coincides well with the hydrophobic 12-carbon aliphatic chain of the chromophore [11]. However, the hydrophobic tube with a diameter of

approximately 6 Å does not have enough space to simultaneously accommodate the hydroxyl and amine group, which combine the same carbon atom of the aliphatic chain (Fig. 3C). Accordingly, we put forward a new structural model that the 12-carbon aliphatic chain buries in the hydrophobic hole and the cyclohexene, combining with the two polar elements (hydroxyl and amine group) by covalent bonds, settles at the hydrophilic mouth of the β barrel. The double bond of the cyclohexene plus the lone pair electrons of oxygen or nitrogen from the hydroxyl and amine group forms a conjugate π bond that could absorb the characteristic wavelength and result in the yellow–brown. Interestingly, the entrance region of α1M contains a broad space and a charged surface that is suitable for accommodating the cyclohexene, hydroxyl and amine groups of the chromophore. Unfortunately, the electron density corresponding to the hydrophilic hexatomic ring group was not observed from the crystal structure (Fig. 3C).

Similar to known lipocalins, the substrate binding site of human α1M also localizes to the positive-charged groove on the mouth of the β barrel [28,29]. Interestingly, most of the 10 conserved residues including Cys34, Trp36, Leu37, Met62, Tyr79, Tyr90, Trp95, Met99, Lys118, Lys130 and Tyr132 settle at the secondary structural elements (α2, β3, β4, β5, β6, β7, β8), implying that the tube is extremely spatially steady and is a conserved structural feature of α1M homologs (Fig. 1). Berggård et al. suggested that Lys92, Lys118, and Lys130 of human α1M are partially buried at the entrance to the lipocalin pocket and bound to structurally heterogeneous chromophores by covalent bonds [11]. In this study, Lys 118 settles at the mouth of the barrel and Lys92 and Lys130 localize at the middle region of the hydrophobic tube. Additionally, both the recombinant and refolded α1M exhibit a yellow–brown color, further verifying that the chromophore might bind to α1M by a covalent bond [11]. Unfortunately, the weak electron density of the human crystal structure fails to provide additional evidence supports the hypothesis (Fig. 3A and 3B). Consequently, further study is needed to unravel the details concerning the structural model of the chromophore interaction with α1M.

3.4. The putative protein–protein interaction sites of α1M

α1M is known to also interact with several target proteins, including α1-inhibitor-3 (α2-macroglobulin), fibronectin, prothrombin and albumin [12–14]. As a lipocalin, the sequence, structure and biological functions of α1M are evolutionarily well-conserved [31,32]. Although only the α1M from human and rat have been partially characterized, their homologs in other animals may play similar roles in those organisms. Accordingly, it is deduced that the amino acids at the protein binding sites of α1M are conserved.

Four homologs of animals, with identity from 75% to 40%, were selected to delineate the conserved sites of α1M. As shown in Fig. 1, there are about 90 conserved residues that could be classified as exterior or interior. The interior conserved residues primarily maintain the stability of protein, whereas the exterior amino acids likely relate to protein functions, such as protein–protein interactions. The exterior conserved residues of α1M mainly distribute consecutively at the two adjacent regions (regions I and II), which act as the primary sites for protein binding (Fig. 4A and B). Other conserved exterior residues settle separately in space or near the positive charged cave that possibly correlates with the biological activity of the protein (Fig. 4B) [33].

As shown in Fig. 4B, both of the candidate sites for protein binding are on the opposite side of the heme binding site [33]. Region I is mainly formed by the residues located at β2, β3, β6, β7, β8 and the amino terminal loops. At these sites, there are 13 conserved residues, including 4 charged residues (Glu14, Asn15, Asn107 and Asp109) and 9 hydrophilic residues (Phe16, Val46, Leu51, Ile60, Val103, Tyr111, Leu115, Leu131 and Leu138), which make up the hydrophobic protein binding site. At region II, located adjacent to region I, there are 6 conserved residues at β1, α2 and the carboxyl terminal loop, which shape a distinct negatively charged protein–protein interaction site (Fig. 4B and 4C). Though more than half of the residues are located at the carboxyl terminal loop, a disulfide bond formed by Cys72 and Cys168 stabilizes the loop close to the barrel and provides a steady binding site (Fig. 2A).

In blood, about half of the α1M exits as a component of a protein complex via binding to IgA [8]. Calero et al. suggested that a disulfide bridge between α1M and IgA was formed by Cys34 of α1M and the penultimate Cys residue of IgA [8,17]. It is worth noting that the side chain of Cys34 orients to the positively charged inner surface of the chromophore binding groove, implying that there is a steric obstacle to the formation of a disulfide bond between Cys34 and IgA. In addition, due to the same interaction site, IgA binding to α1M produces steric hindrance that prevents α1M binding to heme [33]. Consequently, Larsson et al. revealed that the free α1M and α1M-IgA complex exhibit the similar capacity to recognize heme [18]. Collectively, the conflicting data suggest that Cys34 might not form a disulfide bond to stabilize the interaction between α1M and IgA. According to previous analysis, there are two adjacent, putative protein interaction sites locating at the back side of the heme binding sites. In region II, Cys72 and Cys168 form a disulfide bond to stabilize the negatively charged protein interface. Allhorn et al. revealed that there was a disulfide bridge that directly participated in the interaction between α1M and IgA, implying that the putative interface II of α1M might serve as the target site of IgA. When α1M binds to IgA, the disulfide bridge formed by Cys72 and Cys168 might break; then, the outboard residue, Cys168, along with one cysteine of IgA, will form a new disulfide bridge to stabilize the α1M-IgA complex. The detailed nature of the interactions between members of these protein complexes, such as α1M-IgA, α1M-α2 macroglobulin, α1M-fibro-

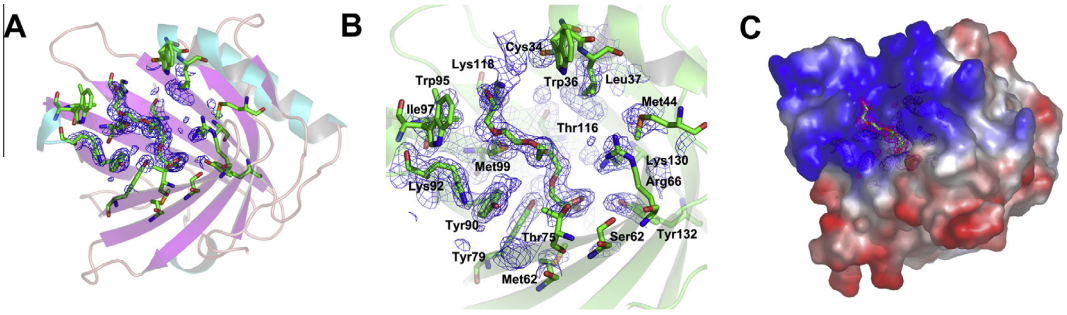

Fig. 3. The chromophore-binding site of human α1M. (A) The stereo view of the chromophore (magenta) binding site, with α1M residues (green) within a 4 Å distance that might be involved in binding chromophore. The 2Fo-Fc omit electron density map covering the chromophore (magenta) is shown contoured at 1.2 sigma (light blue mesh). (B) The electrostatic potential surface of α1M is shown with the same orientation as that of the Fig. 4A. *Saturating red* indicates $\Phi < -10$ kiloteslas/*e*, and *saturating blue* indicates $\Phi > 10$ kiloteslas/*e*, $T = 293$ K. (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

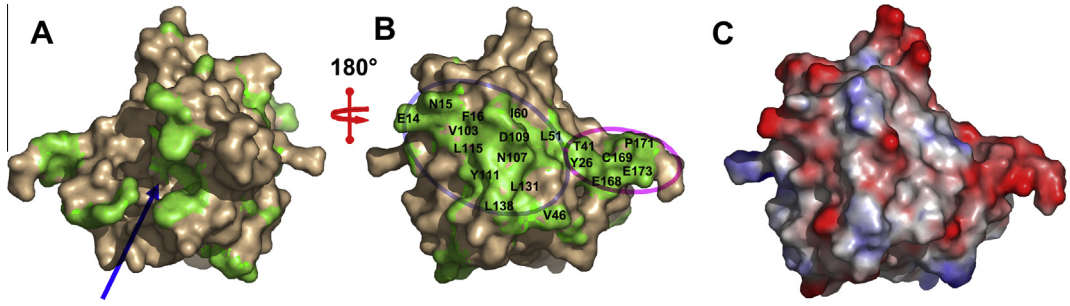

Fig. 4. Possible protein–protein interaction sites of human α1M. The conserved residues on the surface are indicated, and the presumed sites are circled for clearance. (A) The conserved exterior residues locate near the putative heme binding site. (B) The two conserved exterior residues at putative protein–protein interface. The light blue and light pink cycles represent the hydrophobic region I and hydrophilic region II, respectively. (C) The electrostatic potential surface of α1M is shown with the same direction as that of the Fig. 4B. *Saturating red* indicates $\Phi < -10$ kiloteslas/*e*, and *saturating blue* indicates $\Phi > 10$ kiloteslas/*e*, $T = 293$ K. (For interpretation of the reference to color in this figure legend, the reader is referred to the web version of this article.)

nectin, α1M-prothrombin and α1M-albumin, need further experiments to be clarified [12–14].

3.5. Protein data bank accession code

The atomic coordinates and structure factors for the structure of α1M have been deposited in the PDB with Accession Code 4ES7.

Acknowledgments

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