8-1 Molecular Mechanism of the Membrane Recruitment of GGA by ARF in Lysosomal Protein Transport

The human body is made up of some 60 trillion cells containing membrane-bounded organelles such as nuclei, mitochondria, the endoplasmic reticulum (ER) and the Golgi apparatus, which perform specific functions using proteins, DNAs, RNAs and other biological molecules. Protein sequences are coded in DNA but many eukaryotic proteins must be glycosylated (chemically linked to carbohydrates) after translation to become mature proteins. Proteins and N-GAT. (a) Schematic diagram of the GGA-GAT domain. The GAT domain is modeled from the structure of the ear domain of γ-adaptin (2), PDB: 1IU1) based on their similarity both in sequence and function.

was found in 2000 and later demonstrated to facilitate the vesicular transport of lysosomal hydrolases which degrade unwanted glycolipids in lysosomes. All GGAs have a common domain organization: VHS, GAT and GAE domains (Fig. 1(a)). The lysosomal hydrolases first need to be modified with oligosaccharides with mannose 6-phosphate moiety for transport to lysosomes. Mannose 6-phosphate receptors (M6PR) collect lysosomal hydrolases correctly labeled with the mannose 6-phosphate groups in the trans-Golgi network. Next, adaptor proteins recognize the cargo-loaded M6PRs [1] and package them with clathrin. Clathrin coated transport vesicles are then transported to early lysosomes which become mature lysosomes. The GGA-GAE domains modulate the packaging and membrane fusion processes through interactions with accessory proteins [2].

Figure 1
Domain organization of GGA and the proposed model of the interactions with its partners during vesicle formation. (a) Schematic representation of the domain organization of human GGA proteins and N-GAT. (b) Model of GGA proteins with its domains interacting with M6PR, ARF-GTP, clathrin N-terminal propeller and an accessory protein. The VHS domain recognizes sorting signals such as M6PR (PDB: 1JWG). The GAT domain interacts with a membrane-bound ARF (in this study). The subsequent hinge region interacts with clathrin (clathrin terminal domain complexed with clathrin-box peptide from β3-hinge of AP-3, PDB: 1C9I). The sequence S"LDDELMA interacts with the VHS domain (autoinhibition) when S" is phosphorylated. Finally, the C-terminal GGA1 GAE domain is modeled from the structure of the ear domain of γ-adaptin [2], PDB: 1IU1) based on their similarity both in sequence and function.
ARF-GTP bound to trans-Golgi network membranes recruits its effector, the GAT domain of GGA, thus making it easier for GGA to recognize the cargo-loaded receptors. Here we report the X-ray crystal structures of the human GGA1-GAT domain and the complex between ARF1 (ADP-ribosylation factor) and the N-terminal part of the GAT domain [3]. When unbound, the GAT domain forms an elongated bundle of three α-helices with a hydrophobic core (Fig. 2(a)). When combined with the preceding VHS domain, this domain structurally resembles CALM, an AP180 homolog involved in endocytosis, implying a canonical structural motif prevalent in vesicle transport. In the complex with ARF1-GTP, a helix-loop-helix of the N-terminal part of GAT (N-GAT) interacts with the switches 1 and 2 of ARF1 predominantly in a hydrophobic manner (Fig. 2(c)).

Incidentally, three other groups from the US National Institute of Health (NIH) [4], Cambridge University [5] and Oklahoma University [6], reported the structures of the GGA1-GAT domain separately within a period of 2 months. The structure reported by the NIH group is very similar to ours, but the other two studies report that the GAT domain folds into a 4-helix structure which resembles our combined model of the N-terminal GAT domain in complex with ARF1-GTP and the GAT domain alone. Along with our circular dichroism data which shows a partially folded state of the N-terminal part of the GAT domain, we believe that this part is in equilibrium between an α-helix and a disordered random coil, and that the interaction with ARF fixes it in the 4-helix structure for secure docking onto the TGN membrane. Our N-GAT/ARF structure differs from any of the ARF complexes reported so far, and indeed serves as the first reported structure of the complex between ARF and its effector. These data reveal a molecular mechanism underlying the membrane recruitment of adaptor proteins by ARF-GTP, which then facilitates the recruitment of cargo-bound receptors (Fig. 1) followed by clathrin-coated transport vesicle formation, budding, and membrane fusion.

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8-2 Structure of the Protein-Degradation Mediating Mammalian 20S Proteasome

The proteasome is the central mediator of nonlysosomal protein degradation in both the cytosol and the nucleus and is involved in the degradation of both misfolded proteins and short-lived regulatory proteins. The 26S proteasome consists of a central cylindrical 20S proteasome containing 28 subunits as a catalytic machine with a molecular mass of 700 kDa, and two large regulatory complexes termed PA700 (Proteasome Activator 700). The 26S proteasome, with a molecular mass of approximately 2 MDa, degrades target proteins flagged with poly-ubiquitin (the ubiquitin-proteasome system). This degradation requires ATP hydrolysis. The crystal structures of the 20S proteasome, from the archaea Thermoplasma acidophilum [1] and yeast Saccharomyces cerevisiae [2] were solved in 1995 and 1997. The proteasome structure in higher eukaryotic organisms, however, has not yet been determined. In higher eukaryotes, the proteasome acts as an antigen-processing enzyme, responsible for the generation of peptides presented by MHC class I molecules. Moreover, the mammalian 20S proteasome has three active subunits, β1, β2, and β5, which are replaced in the immunoproteasome by interferon-γ-inducible subunits β1i, β2i, and β5i.

To elucidate the sophisticated molecular mechanism underlying the mammalian proteasome and to study how it differs from the proteasome in lower organisms, we have analyzed the crystal structure of the 20S proteasome from bovine liver [3]. Data collection was performed at several synchrotron radiation facilities. Since the isomorphism between crystals was very poor, the partial datasets could not be combined. Only one crystal out of more than 1,000 was suitable for successful structure determination. The crystal belonged to the space group P2\(_{1}\)2\(_{1}\)2\(_{1}\), with cell dimensions of \(a = 315.7\) Å, \(b = 205.9\) Å, and \(c = 116.0\) Å. The \(R_{\text{merge}}\) was 9.5% with 96.3% completeness between 100 and 2.75 Å resolution. One 20S proteasome molecule was in an asymmetric unit. The structure was determined with the molecular replacement method using the structure of the yeast proteasome as a model. The overall shape of the bovine 20S proteasome was an elongated cylinder having large central cavities and narrow constrictions (Fig. 3). Its overall dimensions were approximately 150 Å in length and 115 Å in diameter, a size that has been conserved from the \(T\). acidophilum and yeast proteasome. The electron density map of the bovine proteasome clearly distinguished the constitutive subunits from the γ-interferon inducible ones. Furthermore, the bovine 20S proteasome was confirmed to have an identical arrangement of subunits as the yeast proteasome.

The structures of the β2, β1, β5, β6, and β7 subunits of the bovine enzyme were different from the yeast
Highlights

115 Å

150 Å

18-3 A Novel Interacting Mechanism between Mammalian Protein Phosphatase 1 and its Inhibitor Calyculin A

Protein phosphatase 1 and 2A (PP1 and PP2A) are two of the four major enzymes that dephosphorylate serine and threonine residues of proteins in the cytosol of eukaryotic cells [1]. The catalytic subunits of PP1 (PP1c) and PP2A are subjected to inhibition by various toxins, the so-called okadaic acid class of compounds, including okadaic acid, microcystin-LR (MCLR), and calyculin A. Among these inhibitors, calyculin A has a unique structure, consisting of a polyketide and a dipeptide with a phosphate group in the polyketide portion, and is known to adopt a pseudocyclic conformation by forming intramolecular bonds in the solid state as well as in solution. X-ray crystallography of the catalytic subunit of PP1 (α-isozyme; PP1α) complexed with MCLR, which was the first structure solved of the complex of PP1c and inhibitor, provided a detailed view of the interaction between the enzyme and the toxin [2]. In addition, a recent crystallographic study shows that okadaic acid is also accommodated into PP1γ (γ-isozyme of PP1) with essentially the same binding mode as MCLR [3]. Several binding models for calyculin A, as well as those for the other inhibitors, have been proposed on the basis of this crystal structure. Many of these models postulated that calyculin A is accommodated into the binding pocket for MCLR in such a way that the head component of calyculin A including the phosphate group forms hydrogen bonds or indirect coordinate bonds with the amino acid residues around the catalytic center, whereas the nonpolar polyketide tail is docked in the hydrophobic surface groove that extends from the catalytic center. However, calyculin A has a very flexible backbone structure, and these models are purely speculative.

The crystal structure of the complex between the PP1 catalytic subunit (PP1γ) and calyculin A has been

Figure 3
Co drawings of the bovine 20S proteasome. The enzyme consists of 28 subunits, [(α1–α7)(β1–β7)]. Fourteen of the different subunits are shown in different colors; α1, red; α2, yellow; α3, green; α4, sky blue; α5, blue; α6, pink; α7, gray; β1, orange; β2, dark sea green; β3, medium sea green; β4, dark sky blue; β5, purple; β6, magenta; and β7, light pink. (a) Top view, (b) Side view.

Figure 4
Overall structure of PP1γ-calyculin A complex.

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References
The lower eukaryote Physarum polycephalum changes morphology during its life cycle which includes the amoeba and plasmodia phases. Plasmodium is a giant creeping single cell, and is characterized by vigorous cytoplasmic streaming. Therefore, the wound healing process is indispensable for the survival of plasmodia. Plasmodia have a unique sealing system; when the cytoplasm is exposed to extracellular fluid, the calcium binding protein 40 (CBP40) seals damaged areas forming large aggregates Ca$^{2+}$-dependently [1]. CBP40 contains four EF-hand motifs and 218 residues in the N-terminal region. Part of the CBP40 is truncated N-terminal 32 residues by a proteinase in plasmodia (CBP40$\Delta$), which does not aggregate in the Ca$^{2+}$-bound form. In order to elucidate the mechanism of the Ca$^{2+}$-dependent oligomerization of CBP40, we have determined the crystal structures of CBP40$\Delta$ in both the metal-free and the Ca$^{2+}$-bound states at 3.0 Å resolution [2,3].

Both structures consist of three domains: coiled-coil, intervening and EF-hand (Fig. 6). The N-terminal coiled-coil domain is comprised of helices 1 and 2. In the Ca$^{2+}$-bound form, theomit map confirmed that four Ca$^{2+}$ are bound at the four EF-hands. On the other hand, the omit map of the metal-free form showed that there is negligible Ca$^{2+}$ remaining. The topology of the EF-hand domain is similar to that of the penta-EF-hand (PEF) protein family such as calpain. But, CBP40 does not have a fifth EF-hand. The structure of the metal-free form is almost identical with that of the Ca$^{2+}$-bound form, except for minor perturbations of the EF-hand loops. Since CBP40 has no linker loop between EF1/EF2 and EF3/EF4, these hands interact closely to form a stable and highly compact structure. This may be the reason why a large conformational change as calmodulin does not occur in CBP40. The large hydrophobic interface of the EF-hand region is about 10$^{2}$ M, and [Ca$^{2+}$] in the extracellular fluid is about 10$^{-3}$ M. Once the plasmodium is wounded, [Ca$^{2+}$] of the damaged area rises and CBP40 binds Ca$^{2+}$. Next, CBP40 aggregates and walls off the damaged area. Then, how is the self-assembly of CBP40 regulated by Ca$^{2+}$? We propose a hypothesis for the regulation mechanism. Helix 1 in the coiled-coil of CBP40$\Delta$ is 17 residues shorter than helix 2. According to secondary structure prediction, helix 1 is expected to become the same length as helix 2 in CBP40. Helical wheel analysis reveals that the additional region of helix 1 will make hydrophobic and electrostatic interactions with helix 2.

References

The interacting surface between the helices therefore will become much larger than that of CBP40Δ, resulting in a longer intertwined coiled-coil. We propose the mechanism of oligomerization from this structural feature; the extended coiled-coils of CBP40 make intermolecular helical bundles. In CBP40, the small structural change of the EF-hand domain upon Ca$^{2+}$-binding is transmitted to the coiled-coil, and induces the rearrangement of the coiled-coil helices, which enables coiled-coil to make intermolecular helical bundles. The model is shown in Fig. 7. Slight rearrangement of the relative position between coiled-coil helices is enough for making intermolecular four-helix bundles. There are some Gln and Lys residues on the hypothetical intermolecular surface. Recently, a transglutaminase was purified from plasmodia, and CBP40 was identified as a substrate. These Gln and Lys residues might be cross-linked by transglutaminase to form rigid clumps.

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References

8-5 A Novel Intramolecular Covalent Bond Found in Catalase-Peroxidase

Many microorganisms have catalase-peroxidases which exhibit both catalase and peroxidase activities to defend against oxygen toxicity by removing H$_2$O$_2$ from the cell.

\[
\begin{align*}
2\text{H}_2\text{O}_2 &\rightarrow 2\text{H}_2\text{O} + \text{O}_2 \quad \text{(catalase activity)} \\
\text{H}_2\text{O}_2 + 2\text{AH} &\rightarrow 2\text{H}_2\text{O} + 2\text{A} \quad \text{(peroxidase activity)} \\
&\text{(AH: substrate such as NADH)}
\end{align*}
\]

Catalase-peroxidases share an amino acid sequence similarity with monofunctional peroxidases, but not with typical monofunctional catalases such as bovine liver catalase and Escherichia coli hydroperoxidases II. Therefore, the catalase-peroxidase is classified as a member of the class I peroxidase superfamily, and its heme environment at the active site has been speculated to be similar to that of typical monofunctional peroxidases such as yeast cytochrome c peroxidase (CCP) and eukaryotic ascorbate peroxidase (APX) [1]. The present research has been undertaken to throw light on the structure of catalase-peroxidase which acquires catalase activity in addition to peroxidase activity.

The crystal structure of the catalase-peroxidase from halophilic archaea, Haloarcula marismortui, (HmCP) has been determined at 2.0 Å resolution [2]. An asymmetric unit of the HmCP crystal contains two identical subunits, each of which binds one heme b and ~700 solvent molecules (Fig. 8). Consistent with predictions from sequence analysis, each subunit is organized into two structurally similar domains. The topological arrangement of secondary structural elements in the two domains is identical to those of CCP and APX. There are three additional large loops in the N-terminal domain of HmCP. Due to these loops, the heme is buried inside the enzyme, and substrate access to the active site is through a narrow channel that prevents the access of a large substrate. The arrangement of a buried heme active site is similar to that in monofunctional catalases but is different from that in peroxidases.
In the vicinity of the active site of HmCP, continuous electron density is observed between O$_\varepsilon$1 of Tyr218 and C$_\eta$2 of Trp95, and between O$_\varepsilon$2 of Tyr218 and S$_\delta$ of Met244 in each subunit of the dimer (Fig. 9(a)). Regardless of whether the model was refined with or without steric restraint among the three side chains, the electron density map indicates the existence of covalent bonds between these side chains. These novel covalent bonds have not been reported previously. The guanidino group of Arg409 forms hydrogen bonds with O$_\eta$1 of Tyr218 and with the amide nitrogen atom of Met244. These hydrogen bonds stabilize the covalent bond between Tyr218 and Met244 by fixing the relative positions of these residues (Fig. 9(b)). Tyr218 anchors one of the large loops on the molecular surface by forming covalent bonds. The conservation of Trp95, Tyr218, Met244 and Arg409 suggests that the novel covalent bonds observed in HmCP may be common in other catalase-peroxidases. Furthermore, these covalent bonds may be important for the catalase activity of the catalase-peroxidase because only the catalase activity is lost upon mutation of Trp95 [3].

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References
The Diels-Alder reaction is a cycloaddition whose mechanism involves the overlap of the π-orbitals of the two unsaturated systems in which an alkene (dienophile) adds to a 1,3-diene to form a 6-membered ring. The reaction is synthetically very useful because it forms cyclic products with high regio- and stereoselectivity under mild conditions [1]. It has been applied to the synthesis of complex pharmaceutical and biologically active compounds. Catalytic methods with biomolecules such as RNA and protein antibody have also been developed. The reactions catalyzed by these biomolecules show remarkable enantio- and diastereoselectivity. Recently, natural Diels-Alderases such as solanapyrone synthase [2], lovastatin nonaketide synthase [3] and macrophomate synthase [4] (MPS) have been reported in the biosynthesis of secondary natural products. The function and catalytic mechanism of the natural Diels-Alderase are of great interest due to the diversity of molecular skeletons in natural Diels-Alder adducts [5]. However, the details of the catalysis of natural Diels-Alderases are still poorly understood.

The phytopathogenic fungus, *Macrocalloma commelinae*, isolated from spots on the leaves of *Commelina communis* has the ability to transform 2-pyrone derivatives into the corresponding benzoate analogues [6] (Fig. 10). This complex aromatic conversion is catalyzed by only one enzyme, macrophomate synthase (MPS), with oxalacetate as a substrate for the C3-unit precursor. MPS is a Mg\(^{2+}\)-dependent enzyme with 339 amino acid residues (Mw = 36244 Da) [7], the sequence of which showed no significant similarity with known proteins in a homology search. The catalytic mechanism of the whole pathway was investigated extensively, and it was shown that it proceeds through three separate steps including decarboxylation, two carbon-carbon bond formations, and decarboxylation with concomitant dehydration. In the absence of 2-pyrone, MPS simply acts as a decarboxylase with high catalytic efficiency (Fig. 10(a)). Furthermore, the involvement of a Diels-Alder reaction at the second step is proposed [4], based on the previously reported reaction type and the stereospecificity of the reaction. We present the first atomic resolution structure of a natural Diels-Alderase.

The molecule is hexameric with point group symmetry 32. The protomer core region consists of an 8 stranded \(\beta\)-barrel surrounded by 8+3 \(\alpha\)-helices with a \((\beta\alpha)\)\(_8\) barrel fold. The long \(\alpha\beta\)-helix (colored in magenta) belongs to the neighboring protomer related by the 2-fold axis and joins to the \(\beta\)-barrel core to form a complete \((\beta\alpha)\)\(_8\) barrel. (b) The functional unit of MPS with point group symmetry 32. (c) The residues in the active site pocket and proposed model for the very early transition state of the Diels-Alder reaction. 2-pyrone 2 (blue thick bond) is placed in parallel with pyruvate enolate (red thick bond) which is bound to Mg\(^{2+}\) (green ball). (d) The space-filling model of the active site with transition state of substrates pyruvate enolate (red) and 2-pyrone 2 (blue) and (e) reaction intermediate bicyclo[2,2,2]octane (yellow). The active site is delicately engraved for the entropy trap Diels-Alder reaction.
In the second step of the reaction, the cycloaddition of the enolate and the 2-pyrene takes place (Fig. 10). The steric complexion of the peptide backbone allows the 2-pyrene access only from one side of the enolate plane where the catalytic pocket is open. Figure 11(c,d) shows the proposed model for the very early transition state of the Diels-Alder reaction. In this binding model, two planes (2-pyrene and pyruvate enolate) are placed in parallel at $\pi$-orbital-overlapping distance. Several features are worth noting in this model. First, the 2-pyrene molecule is likely to be fixed in place through two hydrogen bonds between the carbonyl oxygen of 2-pyrene and Arg101, and the C5-acyl oxygen and Tyr169. Tyr169 is in turn placed in the proper orientation via stacking with Phe149. The C5-acyl oxygen and Tyr169. The stacking direction of 2-pyrene to pyruvate enolate is exactly the one expected from the product.

Both of R101S and Y169F mutants dramatically disturbed MPS activity while retaining the decarboxylase activity, suggesting the importance of these hydrogen bonds in the carbon-carbon bond-forming reaction. The experimental result that 2-pyrones lacking a C5-acyl group are not converted into normal aromatic products [8] gives further support for this binding structure. Generally speaking, the hydrogen-bonds between LUMO-energized substrate and some moieties in the reaction medium accelerate the Diels-Alder reaction. As shown in Fig. 11(d,e), the intermediate is substantially reoriented from the early transition state with respect to the enzyme because of the conformational constraints imposed upon the adduct. The rather large hydrophobic cavity of this enzyme enables this rotation (reorientation) to occur without any steric congestion. The enzyme also has substantial van der Waals contacts to this intermediate. The first natural Diels-Alderase is found to adopt several such ingenious strategies.

**References**


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**8-7 Crystal Structures of the Reaction Intermediate and its Homologue of an Extradiol-Cleaving Catecholic Dioxygenase**

Extradiol-cleaving catecholic dioxygenases (hereafter extradiol dioxygenases) are enzymes that play a key role in the degradation pathway of aromatic compounds. These enzymes catalyze the addition of two atomic oxygens to the catechol ring of the substrate, resulting in cleavage of the catechol ring. Extradiol dioxygenases typically contain a non-heme iron (Fe$^3$) in their active site, which is easily oxidized into Fe$^6$ under aerobic conditions. The oxidation of the Fe ion inactivates the enzymatic reaction. BphC is an extradiol dioxygenase derived from *Pseudomonas* sp. strain KKS102. Previously, we have determined the crystal structures of BphC and its substrate complex using inactive form enzymes [1,2]. These crystal structures revealed details of the extradiol dioxygenase’s active site, but high-resolution crystal structures with the active form enzyme are essential to precisely discuss the reaction mechanism of the enzyme. Here we report high-resolution crystal structures of BphC in substrate-free form, the BphC-substrate complex (the ES complex) and the BphC-substrate-NO complex (the ES-NO complex) [3,4].

The BphC used in the present study was reactivated just before crystallization [3]. The reactivated BphC was then crystallized under anaerobic conditions. The crystals of ES and ES-NO complexes were prepared using the soaking method under anaerobic conditions. Diffraction data of these crystals was collected at 100 K using the ADSC 2x2 CCD detector installed either at BL-6A or BL-18B. Crystal structures of BphC (substrate free form) and the ES-complex were determined at 1.45 Å resolution with R-factors of 16.1% and 16.3%, respectively. The crystal structure of the ES-NO complex was determined at 2.0 Å resolution (R-factor = 16.1%) [4] (Fig. 12).

The crystal structures revealed the followings. (A) The substrate (2,3-DHBP) directly coordinates to the Fe ion as a monoanionic form (Fig.12(b)). (B) Upon substrate binding, His194, which is indispensable for the catalytic reaction [5], makes a conformational change, forming a strong hydrogen bond with the hydroxyl group of the substrate (Fig.12(b)). (C) The NO molecule directly coordinates to the Fe ion (Fig.12(c)). The binding site of the NO molecule, which is highly likely to be the binding site of a dioxygen, is the vacant site of the octahedral coordination sphere of the ES complex.

On the basis of the present crystal structures, we propose a catalytic mechanism for BphC [4] (Fig. 13). In this mechanism, His194 seems to play three distinct roles. At the early stage of the catalytic reaction, His194 appears to act as a catalytic base, which likely deprotonates the...
hydroxyl group of the substrate (Fig. 13(b)). At the next stage, the protonated His194 seems to stabilize a negative charge on the O₂ molecule located in the hydrophobic O₂-binding cavity (Fig. 13(d)). Finally, the protonated His194 seems to function as a proton donor (Fig. 13(e)).

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References

Equilibrium and Kinetics of the Allosteric Transition of the Chaperonin GroEL Studied by Solution X-Ray Scattering

The GroEL from *Escherichia coli*, a tetradecameric protein complex of 14 identical 57-kDa subunits arranged in two heptameric rings stacked back-to-back with a central cavity, is one of the best characterized molecular chaperones (Fig. 15). The ATP-dependent control of the affinity of GroEL for its target protein and the resulting facilitation of protein folding are underpinned by the allosteric transitions of GroEL induced by ATP. However, these allosteric transitions have so far only been investigated by the ATPase assay of GroEL or by fluorescence spectroscopy of tryptophan mutants of GroEL, and there is no direct structural data for the real-time allosteric transitions in solution. Therefore, a number of mysteries remain unsolved, especially concerning the structural characteristics and the kinetics of the allosteric transitions. Another method, which can monitor directly the global structural changes of the protein molecule in real time, is certainly required if we are better to understand the allosteric mechanisms of GroEL.

Small-angle X-ray scattering (SAXS) is a powerful

Figure 14
GFP-based directed evolution and overall subunit structure of the Rv2002-M3 protein. (a) Fluorescence of the resuspended cells harboring genes encoding the wild type or mutant Rv2002 proteins in a GFP-fused form and expression test of the wild type or mutant Rv2002 proteins in a non-fused form. WT, wild type; M1-M5, soluble mutants. tot, total cell; ppt, precipitant fraction; sup, supernatant fraction. The arrow indicates the expressed Rv2002 proteins and the asterisk signifies the mutation at a conserved residue of the SDR family. (b) Ribbon diagram of the Rv2002-M3 monomer in complex with NADH and androstone.

indicate that Glu142 reverses the effect of Lys157 in influencing the pKa of Tyr153. This study raises the possibility that the Rv2002 gene product could be involved in steroid metabolism in *M. tuberculosis* as a unique member of the SDR family. T69K and I6T mutations could possibly increase the intrinsic solubility of the folded protein, since these substitutions occurring on the molecular surface enhance the polar characteristics of the molecule. The mutation V47M contributes to a tighter packing of the subunit hydrophobic core and consequently to the overall stability of the subunit. A major role of V47M mutation may be to lower the kinetic barrier in the folding pathway. All single mutants (I6T, V47M, T69K) were mostly insoluble, whereas two of the double mutants (I6T/V47M, V47M/T69K) were highly soluble.

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References
technique for studying directly any changes in the size and shape of a protein molecule and a protein complex in solution, and this technique should complement the structural information acquired via other spectroscopic (fluorescence, circular dichroism, etc.) techniques and via enzymatic assay. Recently, the use of synchrotron radiation has made it possible to combine a stopped-flow technique with SAXS measurements. Furthermore, combination of the SAXS technique with a two-dimensional (2D) charge-coupled device (CCD)-based X-ray detector has made it possible to improve the signal-to-noise (S/N) ratio of the stopped-flow SAXS data dramatically. This new technique should be useful for investigating the allosteric transitions of large protein complexes [1,2].

In the present study, we have investigated the ATP-induced allosteric transitions of GroEL with static and stopped-flow SAXS [3,4]. The results of the SAXS measurements show that the three allosteric states (the TT, the TR, and the RR states) are structurally different from each other (Fig. 16(a)); the two rings of GroEL assume two allosteric states, T (tense) and R (relaxed), depending on ATP concentration, and hence there are three allosteric states, TT, TR and RR, for the GroEL particle. The kinetics of the TT to TR transition has been observed for the first time directly using stopped-flow SAXS. The rate constant of the transition is 3.4 s⁻¹ (85 μM ATP and 4.8°C) (Fig. 16(b)), and hence this is shown to correspond to the second phase of the ATP-induced kinetics of tryptophan-inserted GroEL previously measured by stopped-flow fluorescence. We have also found by fluorescence spectroscopy that the first phase is a molecular process caused by non-cooperative binding of ATP to GroEL with a bimolecular rate constant of 5.8×10⁵ M⁻¹s⁻¹. The ATP-induced cooperative transition observed by fluorescence as well as SAXS measurements at low concentration of ATP (<400 μM) is well explained by a kinetic Monod-Wyman-Changeux (MWC) model that is a combination of the conventional transition state theory and the basic MWC model.

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References

8-10 Light-Induced Conformational Change of Photoactive Yellow Protein

Photoactive yellow protein (PYP), a photoreceptor protein found in the purple phototrophic bacterium, *Ectothiorhodospira halophila*, acts as a blue-light receptor for the negative phototaxis of the bacterium. The chromophore of PYP, a thioester-linked trans-p-coumaric acid, is isomerized to the cis-form upon photon absorption and PYP undergoes a photocycle. Among the photocycle intermediates, the last one (PYPₘ), whose absorption band is located in the near-UV region, is considered to be in a physiologically active form. The structure of PYPₘ has been analyzed by time-resolved crystallography [1]. The structural difference between PYP and PYPₘ in the crystal form is limited to the region around the chromophore. However, accumulated evidence strongly suggests that the structural change under physiological conditions is much larger than that in the crystal, probably because conformational change is restricted in the crystal. We have examined the light-induced conformational change of PYP in solution by small-angle X-ray scattering (SAXS) [2]. PYP was heterologously overexpressed by
Escherichia coli, and reconstituted by adding p-coumaric anhydride. Because of the short lifetime of PYP, its characterization is difficult. To stabilize PYP, the N-terminal 6, 15 or 23 amino acid residues of PYP were truncated by bovine pancreas chymotrypsin (T6, T15 and T23, respectively). Systematic characterization of the truncated PYPs provided information on the structural change in the N-terminal region. The SAXS measurements were carried out at BL-10C.

The square of the radius of gyration \( R_g^2 \) of PYPs in the dark was estimated by a Guinier plot (Fig. 17). Values of \( R_g^2 \) for T6, T15, and T23 under illumination (T6M, T15M, and T23M) were similarly estimated. The \( R_g^2 \) values for the dark states were slightly decreased by truncation. Upon illumination, the \( R_g \) values of truncated PYPs were markedly increased. The increase in \( R_g \) by illumination was 1.1 Å for T6 and 0.7 Å for T15 and T23. It should be noted that the difference in \( R_g \) between T6 and T15 was 0.3 Å, but that between T6 and T15M it was 0.7 Å. In contrast, the difference in \( R_g \) between T15 and T23 was 0.6 Å for both dark and light conditions. Namely, the decrease of \( R_g \) by removal of Gly7-Leu15 in the M state was larger than that in the dark state, but that of Ala16-Leu23 was constant. Therefore, Gly7-Leu15 in T6M is more responsible for \( R_g \) than it is in T6. This means that Gly7-Leu15 in the dark state is located more proximately to the center than in the M intermediate. In the dark state, Gly7-Leu15 forms a short \( \alpha \)-helix which lies parallel to the plane of the \( \beta \)-sheet (Fig. 18). The present experiment suggested that N-terminal loop is detached from the \( \beta \)-sheet upon formation of PYP.

The structural change for formation of PYPM is not merely a rearrangement of the surface charge distribution but involves a global conformational change, resulting in increases in the dimensions and changes in the shape. This may enable the interaction with other molecules such as membrane and proteins.

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References

8-11 Energy Transducing Conformation Changes of Myosin Motor Proteins

Elucidating the mechanism by which the actin-myosin motor system transduces the chemical energy of ATP hydrolysis to power the movement of animals and cells remains one of the major challenges in biological science. Skeletal muscle myosin (myosin II) consists of two globular heads linked by helical segments that supercoil to form a long helical rod. The rod segments assemble into the shaft of the myosin filaments, and the head portions project outward towards the actin filaments, forming the crossbridges that are responsible for the generation of a sliding force between the two filaments. Myosin heads play a key role in converting chemical energy in the form of ATP into mechanical energy driving the directional motion of myosin heads relative to the actin filaments. It has
The global conformational change of the myosin head (S1) during energy transduction. The motion of the light chain binding domain of S1 involves a tilt and a slew. The S1 molecule is shown with its catalytic domain facing the actin filament axis.

long been expected for the crossbridge mechanism of the acto-myosin motor system that global conformational changes of myosin heads interacting with actin occur in coupling with an ATP hydrolysis reaction. Prior to X-ray crystallographic studies, X-ray solution scattering of isolated myosin heads (subfragment 1, S1) first indicated that the long 12-helical regulatory domain containing two light chains deformed globally relative to its catalytic domain during the ATPase cycle [1]. The deformation involves both a tilt and a twist, resulting vectorially in a 5-nm spinning of the distal end of the regulatory domain (Fig. 19) [2,3].

Recently, X-ray scattering studies of S1s with various nucleotide analogs mimicking the ATP hydrolysis reaction. Prior to X-ray crystallographic studies, X-ray solution scattering of isolated myosin heads (subfragment 1, S1) first indicated that the long 12-helical regulatory domain containing two light chains deformed globally relative to its catalytic domain during the ATPase cycle [1]. The deformation involves both a tilt and a twist, resulting vectorially in a 5-nm spinning of the distal end of the regulatory domain (Fig. 19) [2,3].

As summarized in Fig. 20, a large conformational change of the regulatory domain of S1 occurs in a lever arm-like fashion both in the S1*ATP and S1*ADP states before and after the key intermediate state (S1**ADP.Pi) of its ATPase cycle. The direction of motion of the regulatory domain is opposite to that occurring in the S1**ADP.Pi state. When an actin filament is present, the contractile or sliding force is thought to be produced in the transition from an S1**ADP.Pi state to an S1*ADP state, that is, in the phosphate release step. The present results may indicate a working stroke of myosin heads amounting to ca. 10 nm in this transition. Recent X-ray crystallographic analysis of S1s from invertebrate muscle myosin with various nucleotide analogs has suggested the possible atomic mechanism of such a global movement of the regulatory domain. Support for such a lever arm hypothesis for the motor action of myosin heads is found in recent time-resolved X-ray diffraction and quick-freezing electron microscopic studies from contracting muscle fibers. On the other hand, we have stressed that the elastic dynamics of thin actin filaments is important for the acto-myosin motor activity under the action of myosin heads [8]. Coordinated conformational changes of actin and myosin are clearly required to perform efficient energy transduction in the acto-myosin motor system.

Y. Sugimoto, T. Arata and K. Wakabayashi (Osaka Univ.)

References

[7] Y. Sugimoto et al., to be published.

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Figure 19
The global conformational change of the myosin head (S1) during energy transduction. The motion of the light chain binding domain of S1 involves a tilt and a slew. The S1 molecule is shown with its catalytic domain facing the actin filament axis.

Figure 20
Global motion of the light chain binding domain of S1 with various nucleotide analogs mimicking the intermediate steps of the myosin ATPase reaction. The radius of gyration value (Rg) and the maximum chord length (D_max) of S1 with and without nucleotides were obtained respectively from Guinier plots and the p(r) functions of the X-ray solution scattering data. The top row of the table shows the representative intermediate states along the ATPase reaction of S1. The movement direction and magnitude of the distal end of the light chain binding domain are shown with an arrow and a value relative to the S1 with no nucleotide or the S1.ATP state in each transition of the states in the bottom row of the table. In the second row, AMPNP is adenylylimidodiphosphate, PPI is pyrophosphate and S1 ADP-PDM is an S1 trapping ADP by PDM (p-phenylenedimalimid) which crosslinks two reactive Cysteine residues (SH1 and SH2) of S1.