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Structural Insights into the Regulation of ACC2 by Citrate

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Acetyl-CoA carboxylases (ACCs) play critical roles in fatty acid synthesis and oxidation by the catalytic activity of the carboxylation of acetyl-CoA to malonyl-CoA. It is known that ACCs are inactivated through reversible phosphorylation by AMP-activated protein kinase (AMPK) and allosterically activated by citrate. Here, we determined the crystal structures of biotin carboxylase (BC) domain of human ACC2 phosphorylated by AMPK in the presence of citrate in order to elucidate the activation mechanism by citrate. This structure shows that phosphorylated Ser222 is released from the dimer interface, and thereby facilitating the dimerization or oligomerization of the BC domain allosterically. This structural explanation is coincident with the experimental result that the phosphorylated Ser222 was dephosphorylated more easily by protein phosphatase 2A (PP2A) as the citrate concentration increases.

Key Words : Acetyl-CoA carboxylase, Biotin carboxylase, Phosphorylation, Citrate

Introduction

Acetyl-CoA carboxylases (ACCs) are large multicomponent enzymes that catalyse the carboxylation of acetyl-CoA to malonyl-CoA. In prokaryotes, ACCs are multi-subunit enzymes consisting of biotin carboxylase (BC), biotin carboxyl carrier protein (BCCP), and carboxyl transferase (CT) as separate proteins, whereas eurokaryotic ACCs are multi-domain enzymes containing the three domains of each catalytic activity within a single polypeptide chain in the order of BC, BCCP, and CT domain.[1] The BC domain catalyses the ATP-dependent carboxylation of the biotin moiety incorporated at the BCCP domain and the CT domain transfers the carboxyl group from the biotin moiety to acetyl-CoA to produce malonyl-CoA.[2]

In mammals, there are two isoenzymes of ACC. ACC1 is present in lipogenic tissues such as liver and adipose, whereas ACC2 is present in oxidative tissues such as liver, heart and skeletal muscle.[3,4] ACC1 is involved in the biosynthesis of fatty acids and malonyl-CoA produced by ACC1 is used as a building block to extend the chain length of fatty acids by fatty acid synthase (FAS).[5] In contrast, malonyl-CoA produced by ACC2 acts as an inhibitor of carnitine palmitoyl transferase (CPT I), which is an enzyme importing fatty acids into the mitochondria for β-oxidation to acetyl-CoA.[6]

In mammals, ACC activities of both isoforms are regulated by reversible phosphorylation and allosteric activation by citrate. The crystal structures of yeast and human BC domain in complex with soraphen A, a natural product with the potent inhibitory activity against eukaryotic ACCs, showed that the inhibitor binds to the BC dimer interface and inhibits the BC activity allosterically by disrupting the polymerization of this domain.[7,8] The crystal structure of the phosphorylated BC domain revealed that the phosphorylated Ser222 binds to the dimer interface of BC domain and disrupts the polymerization as soraphen A, confirming that the polymerization of ACCs is the integral part of its regulation.[9] The active form of mammalian ACCs is a large, linear polymer, with a molecular weight of about 8 million Da.[10] This polymer is made up of 10-20 protomers, which are dimers of the ACC enzymes. Citrate is known to promotes the polymerization of ACC, and thereby leads to enzyme activation.[11]

To decipher the molecular mechanism of the ACCs activation by citrate, we have determined the crystal structure of the phosphorylated ACC2 BC domain in the presence of citrate. In this structure, the phosphorylated Ser222 was released from the dimer interface and disordered as Ser222 in the structure of the unphosphorylated BC domain, implying that citrate promotes ACC polymerization by this structural change.

Experimental Section

Reagents. Active AMPK and PP2A were purchased from Merck Milipore (Billerica, MA). The phosphorylated peptide MRPSMpSGLHLVKRG, derived from the BC domain with phosphorylated Ser222, was synthetized in Peptron, Inc (Daejon, Korea).

Protein Expression and Purification. The gene encoding the BC domain (amino acids 217-775) of human Acetyl-CoA Carboxylase 2 was amplified by PCR and then subcloned between the *NdeI* and *XhoI* site of a modified pET21b expression vector encoding additional residues MRGSGS at the N-terminus for improving the expression level and LEHHHHHH at the C-terminus for purification purposes. The recombinant protein was expressed in BL21 *Escherichia coli* cells at 18 °C and first purified on a nickel agarose (Pharmacia) and then further fractionated by gel-filtration

chromatography (Superdex-200, Pharmacia) and anion ion exchange (Mono Q, Pharmacia). In order to phosphorylate the human BC domain, the protein purified as an unphosphorylated form was treated with 1.4 unit AMPK, 20 mM $MgCl_2$, 5 mM ATP at 4 °C and further purified with nickel agarose and anion ion exchange.

Crystallization and Structure Determination. The proteins were concentrated to 12 mg/mL in the solution containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 5% Glycerol, 2 mM DTT, and 20 mM citrate. Crystallization was achieved at 22 °C by vapor diffusion using hanging-drop method and a protein to reservoir solution ratio of 1:1, with the reservoir solution containing 1.5-1.8 M ammonium sulfate and 0.1 M MES (pH 6.0-6.8). Crystals were transferred to the cryoprotectant solution containing the reservoir solution plus 25% (v/v) glycerol for a few seconds, then looped from the drop and flash-frozen in liquid nitrogen. The X-ray diffraction data for the crystals of the phosphorylated form in the presence of citrate were collected at the 7A beam line of PLS-II. Using the CNS package, the structure of the phosphorylated form with citrate was solved after one cycle of a rigid body refinement using the structure of the unphosphorylated form (PDB code 2HJW) as a probe. Subsequent rounds of model adjustment, simulated annealing, and thermal parameter refinement were performed and during the final stage of refinement, water molecules were inserted in the protein models. Table 1 summarizes the statistics for data collection and structure refinement. The coordinates and structure factors have been deposited in PDB (http://www.rcsb.org/pdb) under the accession code of 4HQ6.

Native Gel Assays. The phosphorylated form of the human BC domain was concentrated to 12 mg/mL and incubated with 1-12 mM of citrate at 4 °C for 1 h prior to electrophoresis. For electrophoresis in a 12% polyacrylamide gel (pH 8.8), 1 μL of the protein solution, and 4 μL of the loading buffer (0.125 M Tris [pH 6.8], 20% [v/v] glycerol, and 0.04% [w/v] bromophenol blue) were loaded for each lane. The electrophoresis was performed in a Tris-glycine running buffer under 70 V at 4 °C for 12 h.

PP2A Enzyme Assays. The phosphatase activity of PP2A was measured colorimetrically in 96-well polystyrene plates by detecting the released inorganic phosphates. The phosphorylated BC domain and the synthetized peptide with phosphorylated Ser222 (100 μL and 100 μM) were treated with 1 unit PP2A with 0-32 mM of citrate at 20 °C for 20 min prior to detecting phosphate ions. The incubations were terminated by addition of 100 μL 1 M HCl. Aliquots (0.1 mL) of the incubated solution were mixed with 0.1 ml malachite green reagent. The malachite green reagent was prepared just prior to use and consisted of 10 mL water, 2 mL 1.3 mM malachite green in 3.6 M H_2SO_4, 0.5 mL 7.5% ammonium molybdate and 40 μL 11% Tween 20. The plate was allowed to stand for 2 h at 20 °C and the absorbance at 650 nm was determined.

Table 1. Statistics on Data Collection and Structure Refinement

Data Collection	
Wavelength (Å)	1.1271
Space Group	$P3_221$
Unit cell (a, b, c) (Å)	75.60, 75.60, 188.78
Resolution (Å)	2.70
Observations	85927
Unique reflections	16550
Completeness (%)	92.2 (73.0)
Average I/σ(I)	28.3 (4.3)
Rsym[a] (%)	9.7 (41.8)
Structure Refinement	
Resolution (Å)	20.0-2.70
Reflections (|F| > 0σ)	15395
Rcryst[b] (%)	22.1 (37.1)
Rfree[c] (%)	27.3 (41.9)
R.m.s.d.[d]	
Bonds (Å)	0.008
Angles (°)	1.3
Average *B*-factor (Å^2)	55.85

Values in parentheses are for the outer resolution shell. [a]$R_{sym} = \Sigma_h\Sigma_i|I_{h,i} - <I_{h,i}>|/\Sigma_h\Sigma_i\Sigma_{h,i}$ for the intensity (I) of *i* observations of reflection h. [b]$R_{cryst} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. [c]R_{free} = R-factor calculated using 5% of the reflections data chosen randomly and omitted from the start of refinement. [d]Root-mean-square deviations from ideal geometry.

Results and Discussion

Structure of the Phosphorylated BC Domain in the Presence of Citrate. It is known that citrate promotes the polymerization of ACC, and thereby leads to enzyme activation. From several experimental data, it has been suggested that the polymerization of ACC is thought to occur more easily as a result of a conformational change in the ACC protomer caused by citrate.[11] However, there is no structural information about the putative conformational change of ACC by citrate. To reveal the activation mechanism of citrate at a molecular level, we have determined the crystal structure of the phosphorylated BC domain of human ACC2 in the presence of citrate. As previously reported, the loop containing the phosphorylation site was disordered maybe due to its intrinsic high flexibility in the unphosphorylated BC domain.[8] However, in the structure of the phosphorylated BC domain by AMPK, the phosphorylated Ser222 was bound to the putative dimer interface of the BC domain.[9] In the structure of the phosphorylated BC domain in the presence of citrate, the electron density corresponding the phosphorylated Ser222 have disappeared, and therefore resulting in the same disorder of the N-terminus (residues 217-238) as in the case of the unphosphorylated structure (Fig. 1). Moreover, the citrate binding site could not be identified since the extra electron density corresponding to citrate was not shown anywhere in the structure. As a result of the release of the phosphorylated Ser222 from the putative dimer interface by citrate, the dimerization of the BC domain would not be interrupted. Our structural

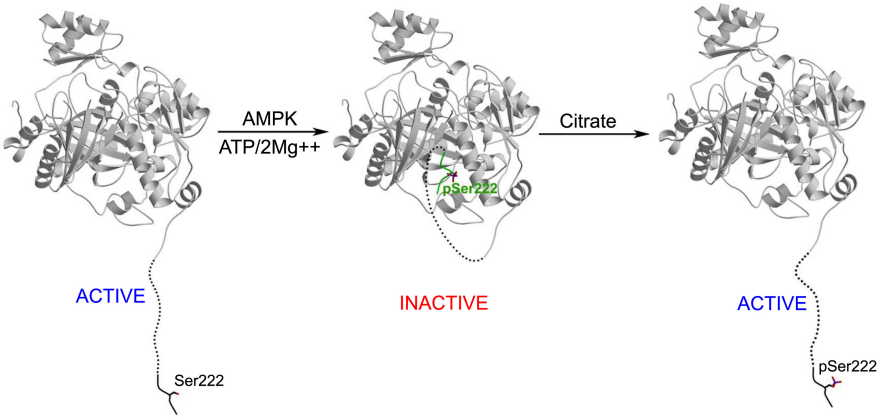

Figure 1. Structural change of the phosphorylated BC domain by citrate. The disordered N-terminal region is expressed as a dotted line and colored black, whereas the bound N-terminal residues including the phosphorylated Ser222 are colored green. The unphosphorylated and phosphorylated Ser222 are shown in a stick model and labeled Ser222 and pSer222, respectively.

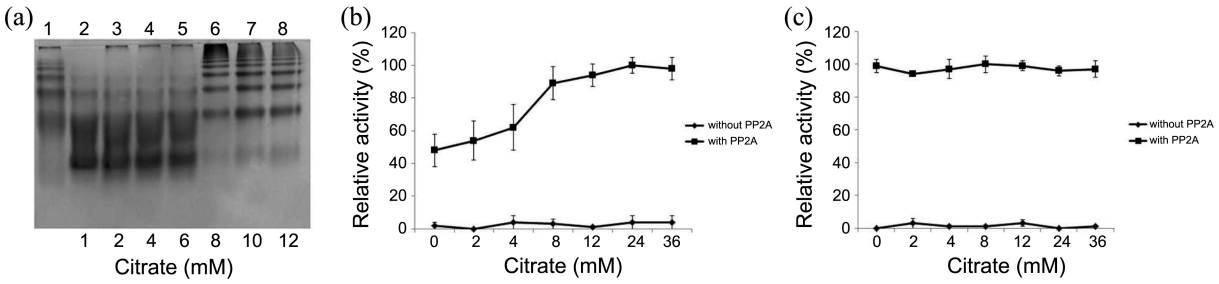

Figure 2. The effect of citrate on the native gel electrophoresis and PP2A activity (a) The native gel showing the effect of citrate in the electrophoretic mobility of the phosphorylated BC domain. Lane 1, unphosphorylated BC domain. Lanes 2-8, phosphorylated BC domain in the presence of citrate (1-12 mM). (b) The dephosphorylation activity of the phosphorylated BC domain in the presence of citrate (0-36 mM) with or without PP2A. (c) The dephosphorylation activity of the phosphorylated 14 mer peptide in the presence of citrate (0-36 mM) with or without PP2A. Assays were performed in triplicate, and error bars denote the standard deviations.

information suggests that citrate is likely to promote the polymerization of ACC by facilitating the dimerization or oligomerization of the BC domain allosterically.

Citrate Raises the Oligomeric State of the Phosphorylated BC Domain of ACC2. To obtain an experimental evidence for the effect of citrate on the oligomeric state of the BC domain phosphorylated by AMPK, we also examined the mobility of the phosphorylated BC domain in a native gel electrophoresis with increasing citrate concentration (Fig. 2(a)). When the concentration of citrate surpassed 8 mM, the collapsed bands by phosphorylation were drastically raised with the polymeric state similar to that of the unphosphorylated BC domain. This result is similar to the previous result of the native gel electrophoresis with the mutants of the key residues for binding of the phosphorylated Ser222, implying citrate expels the phosphorylated Ser222 from the putative dimer interface of BC domain and thereby recovering the oligomeric state of the phosphorylated form to the similar level of the unphosphorylated form.[9]

Citrate Facilitates the Dephosphorylation of the Phosphorylated Ser222 by PP2A. It is known that the phosphoryl groups on the inactivated ACCs are dephosphorylated by protein phosphatase 2A (PP2A) and this activation of ACCs by dephosphorylation requires citrate.[12] To understand the effect of citrate in dephosphorylating the phosphorylated BC domain *in vitro*, we have carried out the enzymatic assay by PP2A with increasing citrate concentration. In the absence of PP2A, the phosphoryl group was not hydrolyzed at all, regardless of the citrate concentration, indicating that citrate itself does not cause the spontaneous dephosphorylation of the BC domain and the disordered loop containing the phosphorylated Ser222 is still in a phosphorylated form (Fig. 2(b)). When the phosphorylated BC domain was treated with PP2A, the increase of the citrate concentration caused a moderate increase of the activity of PP2A toward the phosphorylated BC domain. However, when the 14-mer peptide containing the phosphorylated Ser222 was treated with PP2A, the change of the citrate concentration could not affect the activity of PP2A (Fig. 2(c)). These enzymatic assay results are coincident with the structural studies, indicating that citrate induces the release of the phosphorylated Ser222 from its binding site and exposes it to solvent to be recognized by PP2A more easily.

Proposed Mechanism of the ACC Activation by Citrate. From the structural information, native gel assay, and PP2A enzymatic assay, we could suggest a scenario of the ACC

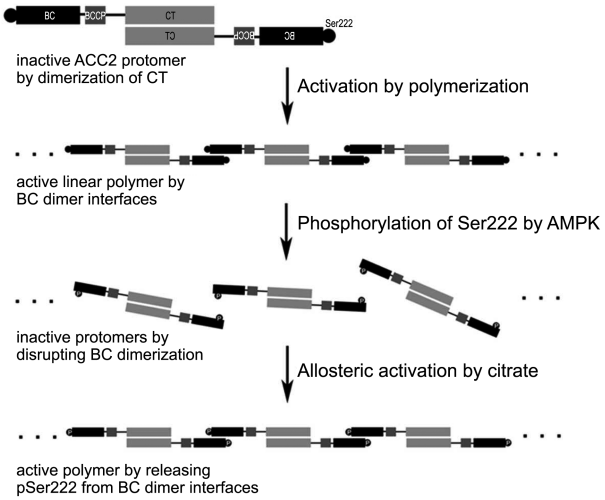

Figure 3. The proposed mechanism of ACC2 polymerization and the role of citrate. Unphosphorylated Ser222 is depicted as a black circle attached to BC domain and phosphorylated Ser222 is labeled P inside the circle. In the presence of citrate, the phosphorylated Ser222 is released from the dimer interface of the BC domain and the protomers can make interactions at the dimer interfaces of the BC domain, and thereby the linear polymer can be generated again with the recovery of the enzymatic activity of ACC2.

activation by citrate (Fig. 3). It is known that the activity of ACC is absolutely dependent on the state of polymerization and the active form of mammalian ACCs is a large, linear polymer made up of 10-20 protomers, which are dimers of the ACC enzymes.[10] As previously proposed, the strong interaction in the dimer interface of CT domain may serve as a driving force for the generation of ACC protomer, and the weak interaction of the dimer interface of BC domain would be suitable for the reversible polymerization.[9] The unphosphorylated ACC would form an active linear polymer as Ser222 does not bind to the dimer interface of BC domain. The phosphorylation by AMPK would disrupt the active linear polymeric form of ACC through inhibiting the BC dimer interactions by the binding of the phosphorylated Ser222. Finally, citrate restores the dimer interactions between BC domains and promotes polymerization by detaching the phosphorylated Ser222 from the dimer interface of the BC domains.

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