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Expression of recombinant SnRK1 in *E. coli*. Characterization of adenine nucleotide binding to the SnRK1.1/AKINβγ-β3 complex

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ABSTRACT

The SnRK1 complexes in plants belong to the family of AMPK/SNF1 kinases, which have been associated with the control of energy balance, in addition to being involved in the regulation of other aspects of plant growth and development. Analysis of complex formation indicates that increased activity is achieved when the catalytic subunit is phosphorylated and bound to regulatory subunits. SnRK1.1 subunit activity is higher than that of SnRK1.2, which also exhibits reduced activation due to the regulatory subunits. The catalytic phosphomimetic subunits (T175/176D) do not exhibit high activity levels, which indicate that the amino acid change does not produce the same effect as phosphorylation. Based on the mammalian AMPK X-ray structure, the plant SnRK1.1/AKINβγ-β3 was modeled by homology modeling and Molecular Dynamics simulations (MD). The model predicted an intimate and extensive contact between a hydrophobic region of AKINβγ and the β3 subunit. While the AKINβγ prediction retains the 4 CBS domain organization of the mammalian enzyme, significant differences are found in the putative nucleotide binding pockets. Docking and MD studies identified two sites between CBS 3 and 4 which may bind adenine nucleotides, but only one appears to be functional, as judging from the predicted binding energies. The recombinant AKINβγ-βs complexes were found to bind adenine nucleotides with dissociation constant (Kd) in the range of the AMP low affinity site in AMPK. The saturation binding data was consistent with a one-site model, in agreement with the in silico calculations. As has been suggested previously, the effect of AMP was found to slow down dephosphorylation but did not influence activity.

1. Introduction

SnRK1 is a Ser/Thr protein kinase that belongs to the AMPK/SNF1/SnRK1 family of protein kinases. As the mammalian and yeast counterparts, SnRK1 is involved in signaling energy stress at the cellular and organismal level to maintain homeostasis. In response to external unfavorable conditions, changes in gene expression are coordinated and regulated by SnRK1 [1,2]. In addition, different proteins are phosphorylated and regulated by SnRK1, including metabolic enzymes and biotic and abiotic stress interactors [3–5]. SnRK1 is a heterotrimeric complex formed by highly conserved α-catalytic and β and γ regulatory subunits [6]. In *Arabidopsis thaliana*, two catalytic subunits, AKIN10/SnRK1.1/AtSnRK1α1 and AKIN11/SnRK1.2/AtSnRK1α2, are mainly associated with SnRK1 complex activity. In both subunits, the catalytic domain is present in the N-terminal lobe and includes highly conserved key residues required for kinase activity. The lysine K48/K49 in SnRK1.1/1.2, respectively, is involved in the phosphotransfer reaction, and a threonine (T175/176) present in the activation loop is important for kinase activation [7]. Mutation of K48/K49 in the two Arabidopsis catalytic subunits abolishes their activity completely, and the same effect has been observed using the SnRK1·1 T175A mutant [8]. Interestingly, it has been widely accepted that replacement of the phosphorylated threonine with a negatively charged amino acid (T175D) results in a constitutively phosphomimetic mutant [8]. Recent experiments have shown that T175D mutants exhibit only weak complementation of yeast *snf1*, and when tested in plants, they are unable to recover a double AtSnRK1/2 mutant [9]. These data suggest that even if this mutant can phosphorylate a synthetic peptide *in vitro*, the

Abbreviations: SnRK1, Snf1 related kinase 1; AMPK, AMP activated kinase; SNF1, Sucrose non fermenting 1; CBS, cystathionine β-synthase; SnAK2, SnRK1-activating kinase 2

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activity is not sufficient to sustain a response *in planta*. The regulatory domain present in the Arabidopsis α subunits does not share high conservation at the amino acid level with human AMPK and yeast SNF1. It contains an ubiquitin-associated domain (UBA), which in AMPK and SNF1 corresponds to an autoinhibitory domain (AID). While the function of the UBA is not known in SnRK1, the AID is central in the regulation of mammalian AMPK [10]. The C-terminal segment of the SnRK1 α subunits is involved in the interaction with the regulatory subunits, but in the AMPK the AID and the C-terminal domain are connected by an α-linker, which is not conserved in the plant kinase [11]. Three β subunits are present in Arabidopsis. Two of them, AKINβ1 and AKINβ2, have a variable *N*-terminus containing an *N*-myristoylated site (N-MYR) and a carbohydrate-binding module (CBM). The C-terminal domain is important for the interaction with the α and γ subunits. The third subunit, AKINβ3, lacks the N-terminal MYR site and CBM, but it is able to form a heterotrimeric complex [12,13]. Post-translational modifications of the SnRK1 β subunits have been poorly studied; however, it is recognized that an N-terminal myristoylation (N-MYR) motif is present in both AKINβ1 and AKINβ2 subunits and it is modified *in vivo*. Mutant seedlings that are deficient in myristoylation show a marked increase in SnRK1 activity, indicating that N-MYR is a negative regulator of SnRK1 [14]. In addition, phosphorylation of the AKINβ1 subunit by adi3, a protein kinase linked to cell death suppression in tomato cells, resulting in complex inactivation [15]. In AMPKβ1 subunit, multiple phosphorylation sites have been identified, and the mutation of some of them (S24, S25, S182) shifts the location of AKINβ1 to the nucleus [16]. The γ regulatory subunit contains four-cystathionine beta synthase (CBS) motifs arranged in two tandem pairs called Bateman domains. In AMPK, these disc-like structures generate four potential nucleotide-binding sites. However, crystallographic studies of truncated and full-length heterotrimers have revealed the existence of three nucleotide-binding sites; one of them does not seem to be exchangeable, whereas the other two can accommodate AMP, ADP or ATP [17]. The Arabidopsis SnRK1γ subunit does not behave functionally as a γ-type subunit since is unable to complement yeast *snf4* mutants and has not been identified as part of the SnRK1 complex [6]. Instead, an AKINβγ containing an N-terminal CBM domain [18] and a pair of Bateman domains at the C-terminus seem to be part of the heterotrimeric complex [6,19]. No allosteric activation by AMP has been identified in SnRK1, but it has been demonstrated that AMP protects against dephosphorylation [20]. A recent study using trimeric complexes confirmed that AMP or ADP does not activate the kinase. Additionally, a comparison between the Bateman domains of AKINβγ and AMPKγ1 indicated that only one of the putative adenylates binding sites is conserved [6]; however, no experimental data evaluating binding are available. In this work, we expressed the SnRK1 subunits as recombinant proteins in *E. coli*. The recombinant SnRK1.1 and SnRK1.2 catalytic subunits were highly active only after phosphorylation with SnAK2. These phosphorylated proteins were able to bind to a preformed AKINβγ/β dimer with a further increased in activity. Despite the observation that both catalytic subunits had increased activity as a consequence of complex formation, SnRK1.1 demonstrated differential regulation. SnRK1.1/AKINβγ-β2 and SnRK1.1/AKINβγ-β3 are the most active complexes, with specific activities close to 40 nmolPi/min/mg, which is the highest value reported to date for a plant SnRK1 complex. Because the complex SnRK1.1/AKINβγ-β3 is unique to plants, a three-dimensional model of its structure was developed, based on the experimental structure of the complete mammalian complex and modeled it using the crystal structure of the full-length mammalian complex. From the model, extensive hydrophobic contacts were found to dominate an AKINβγ-β3 interfacial contact. In addition, using the dimeric protein AKINβγ-β3, we observed that the binding of AMP, ADP and ATP occurred with different affinities. In agreement, docking and Molecular Dynamics simulations (MD) identified one potential binding site, mainly at the boundaries between CBS4 and CBS3. Using a soluble heterotrimeric complex, the binding of AMP or ADP but not ATP, was shown to protect the catalytic subunit against dephosphorylation by a plant PP2CA phosphatase.

2. Material and methods

2.1. Cloning SnRK1 subunits for expression in E. coli

The SnRK1 catalytic subunits SnRK1.1 (At3G01090) and SnRK1.2 (At3G29160) were cloned into the pRSFDuet-1 vector (Novagen), whereas AKINβg (AT1G09020) was cloned into the pCDFDuet-1 vector (Novagen). Beta-type subunits were cloned into vectors that integrate a His-tag at the C-terminal end, AKINβ2 (AT4G16360) and AKINβ3 (AT2G28060) in the pET101 vector (Invitrogen) and AKINβ1 (AT5G1170) in the pET28b vector (Novagen). The SnRK1 activated kinase, SnAK2 (AT3G45240), was cloned into the pGEX4T-2 vector (GE healthcare). The catalytic domain (KD) from SnRK1.1 and SnRK1.2 (1023 and 1026 bp, respectively) was cloned into the PGEX4T-2 vector (Amersham). The sequences of the primers used for amplification of all recombinant proteins and for directed mutagenesis are described in Supplementary Table SI.

2.2. Expression and purification of the recombinant AtSnRK1 complex from E. coli

The catalytic and regulatory subunits were expressed independently in strain BL21 (DE3) *E. coli*. Co-transformation of SnRK1.1/1.2 and SnAK2 was performed, and colonies carrying both plasmids were selected on Lennox LB plates (Sigma) containing 50 μg/ml ampicillin and 25 μg/ml kanamycin. Plasmids carrying AKINβγ with AKINβ1, AKINβ2 or AKINβ3 were co-transformed and selected in 25 μg/ml spectinomycin and 25 μg/ml kanamycin and in 25 μg/ml spectinomycin and 50 μg/ml ampicillin, respectively. Single colonies were used to inoculate 50 ml of LB Lennox broth with the appropriate antibiotics, and the culture was incubated at 37 °C overnight at 200 rpm. One liter of LB medium was used to inoculate the overnight culture to achieve an OD600 = 0.700–0.800. Induction was performed by adding isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM for 4 h at 25 °C at 200 rpm. The cells were pelleted in an Avanti JXN-26 Centrifuge Beckman Coulter™ at 3000 × *g*, for 10 min at 4 °C. The bacterial pellet expressing the catalytic subunits containing SnAK2 was resuspended in lysis buffer (50 mM tricine, 50 mM NaCl, 50 mM NaF, 1 mM EDTA 1 mM EGTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF, 0.02% Brij 35, 5% glycerol and 1 mg/ml lysozyme). The bacterial pellet expressing the regulatory subunits was resuspended in LEW buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8 and 1 mg/ml lysozyme). The cells were incubated on ice for 30 min and sonicated using a Vibra Cell Sonicator with amplitude of 21% for 1 min at 10-s intervals. The soluble fraction was obtained after centrifugation at 10,000 × *g* in a Thermo Scientific Heraeus R16 centrifuge for 30 min at 4 °C. The catalytic subunits were purified by FPLC using the HiPrep16/60 Sephacryl S-300 molecular exclusion column. Fractions with SnRK1 activity were pooled and loaded into a Source 15Q 4.6/100PE anion exchange column. Fractions with activity were analyzed by SDS-PAGE, and the presence of SnRK1.1/1.2 was corroborated by Western blotting. The regulatory subunits were purified using 1 ml of Protino Ni-TED resin (Macherey-Nagel), and unbound proteins were washed with ten column volumes of LEW buffer. Elution was performed using LEW buffer with increasing imidazole concentrations. Proteins were dialyzed, concentrated and stored in 30% glycerol at −80 °C until use. To analyze different heterotrimeric complexes, a 2:1 ratio of regulatory versus catalytic subunits was incubated in a reaction containing 40 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 200 μM ATP, 4 mM DTT, 0.5 μM okadaic acid, 1 × protease inhibitor cocktail (Sigma) and 1 μg of SnAK2 where indicated, at 30 °C for 30 min. Kinase activity was then measured. Parallel experiments were performed in which the same amount of bacterial protein extracts expressing the catalytic subunits was mixed