SHORT COMMUNICATIONS

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Subcloning, crystallization and preliminary X-ray analysis of the signal receiver domain of ETR1, an ethylene receptor from *Arabidopsis thaliana*

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Abstract

The signal receiver domain of ETR1, an ethylene receptor from *Arabidopsis thaliana*, has been subcloned and expressed in *E. coli* and purified by affinity chromatography. Crystals of both native and a selenomethionine-substituted form of the receiver domain have been obtained. Native crystals grew in 1.6 *M* Li_2SO_4 and 0.1 *M* HEPES pH 7.5 and once flash-frozen diffract to 2.1 Å resolution. They belong to space group $P4_12_12$ with unit-cell dimensions $a = b = 48.4$, $c = 112.3$ Å.

1. Introduction

Ethylene, a gaseous phytohormone, controls a variety of plant developmental processes ranging from seed germination to cell elongation, fruit ripening, and fruit and petal senescence (Abeles *et al.*, 1992). In addition, environmental stresses such as chilling, flooding, wounding and pathogen invasion induce ethylene production as part of the adaptation and defense of the plant. While the ethylene biosynthetic pathway is well understood (Yang & Hoffmann, 1984), the mechanisms of ethylene action are currently being elucidated. Since 1967, when it was postulated that the ethylene receptor is a metalloprotein with a preference for an unsaturated bond at the terminal carbon of the ligand (Burg & Burg, 1967), efforts to understand ethylene sensing and signal transduction have made little headway (Sisler, 1987; Harpham *et al.*, 1996). However, recent molecular genetic analysis of ethylene-insensitive *Arabidopsis thaliana* mutants has revealed at least two functional ethylene receptors, ETR1 and ERS (Chang *et al.*, 1993; Hua *et al.*, 1995). Two genes from tomato (*Lycopersicon esculentum*), *NR* and *eTAE1* (Wilkinson *et al.*, 1995; Theologis, 1996), are similar to *ERS* ansd *ETR1* of *A. thaliana*, respectively. All four ethylene receptors contain domains similar to the diverse family of two-component signaling systems (for review, see Swanson *et al.*, 1994) responsible for adaptation to environmental and endogenous stimuli in eubacteria, halobacteria, yeast, slime molds and plants.

ETR1 from *A. thaliana* forms a disulfide bridge dimer and each monomer consists of at least three distinct domains: an N-terminal ethylene-binding transmembrane domain, a histidine kinase domain, and a signal receiver domain (Schaller & Bleecker, 1995; Schaller *et al.*, 1995). ERS is similar but lacks the receiver domain. The histidine kinase and receiver domains of ETR1 are reminiscent of the bacterial two-component systems responsible for chemotaxis, sporulation, and nitrogen and osmolarity regulation. The prototypical two-component system consists of a sensor and a response regulator that participate in a His–Asp relay of a phosphate group (Swanson *et al.*, 1994). The sensor contains often an extracellular sensing domain and a cytoplasmic histidine kinase domain. The histidine kinase is a conserved 250-amino-acid domain that responds to an external signal by autophosphorylating a His residue using ATP. The response regulator contains a 120-amino-acid receiver domain (Volz, 1993), which coordinates an Mg^{2+} and catalyzes the transfer of the phosphate from the His residue of the histidine kinase to an Asp residue on the receiver domain, and in turn regulates the activity of the cognate output domain, often a DNA-binding domain. Usually, the two components of bacterial two-component systems reside on two separate proteins. However, ETR1, like several eukaryotic two-component systems, is a hybrid kinase where both domains are contained in a single polypeptide. Recent progress into the mechanisms of two component-systems shows a four-step, His–Asp–His–Asp phosphorelay responsible for signal transduction as exemplified by the pathways of virulence control in *Bordetella pertussis* (Uhl & Miller, 1996), complementary chromatic adaptation in *Fremyella diplosiphon* (Kehoe & Grossman, 1997), and osmolarity regulation in *Saccharomyces cerevisiae* (Appleby *et al.*, 1996; Posas *et al.*, 1996). Interestingly, the two-component pathway in *S. cerevisiae* is linked to another regulatory system, a MAP kinase pathway. Downstream regulators in the ethylene signaling pathway are presently being cloned and ordered (Roman *et al.*, 1995; Kieber, 1997; Chao *et al.*, 1997), and have shown that CTR1 is a Raf-like Ser/Thr protein kinase active downstream of ETR1 (Kieber *et al.*, 1993). Thus, yeast and plants have combined two-component systems with MAP kinase pathways.

Three-dimensional structures of domains of several two-component pathways are known. The ligand-binding domain of the aspartate receptor, Tar (Milburn *et al.*, 1991; Bowie *et al.*, 1995; Yeh *et al.*, 1996); the receiver domains, CheY (Stock *et al.*, 1989), CheB (West *et al.*, 1995), NtrC (Volkman *et al.*, 1995), NarL (Baikalov *et al.*, 1996), and SpoOF (Feher *et al.*, 1997); the phosphotransfer domain of the histidine kinase CheA (Zhou *et al.*, 1995); the CheY binding domain of CheA (McEvoy *et al.*, 1996); the C-terminal HPt domain of ArcB (Kato *et al.*, 1997); as well as the DNA-binding domain of OmpR (Martinez-Hackert & Stock, 1997). However, to date no structural information exists on eukaryotic two-component systems. We report herein the subcloning, purification, crystallization and preliminary X-ray diffraction analysis of the signal receiver domain of ETR1 from *A. thaliana*.

2. Materials and methods

The portion of the *ETR1* cDNA that encodes the receiver domain, corresponding to residues 605–738 of ETR1 (Chang *et*

al., 1993), was amplified in a polymerase chain reaction (PCR). The 5′ PCR primer introduced both *Bam*HI and *Nde*I restriction sites with an in-frame ATG start codon at the 5′ end of the receiver domain. The 3′ PCR primer introduced a TAA stop codon, and a *Bam*HI restriction enzyme site at the 3′ end. The amplified DNA was cloned into the *Bam*HI site of pBluescript SK+ (Stratagene, La Jolla, California) and sequenced. Once verified, the fragment corresponding to the receiver domain was subcloned into the *Nde*I and *Bam*HI restriction sites of pET16b (Novagen, Madison, Wisconsin) which introduces an N-terminal in-frame His-tag followed by a factor X_a recognition site. Recombinant *E. coli* was grown at 310 K to an $OD_{600} = 0.6$ and then induced with 1 m*M* IPTG for 4 h. The cells were harvested, resuspended in 1X Binding Buffer (1XBB), and sonicated (as described in the pET System Manual, Novagen, 1992). The soluble extract was incubated with 3 ml of Ni^{2+}-charged chelating Sepharose (Pharmacia, Piscataway, New Jersey) for 1 h. The protein–resin mixture was poured into a disposable column and washed extensively with 100 m*M* imidazole in 1XBB. The protein was then eluted with 400 m*M* imidazole in 1XBB. The sample was desalted using a PD-10 column (Pharmacia) and incubated with 1%(*w*/*w*) factor X_a (Haematologic Technologies, Essex Junction, Vermont). After 48 h, the cleaved protein was loaded directly on a 300 ml Sephadex G-75 (Sigma, St Louis, Missouri) gel-filtration column and peak fractions representing the receiver domain were simultaneously buffer-exchanged and concentrated to 20–22 mg ml^{-1} in 25 m*M* HEPES pH 7.5, 1 m*M* DTT. After cleavage by factor X_a, the molecular weight determined by electrospray mass spectroscopy was 15 272 Da, which is in exact agreement to the calculated molecular weight. This leaves an N-terminus of HMSNF- with the Ser residue representing the first amino acid from the receiver domain of ETR1.

CheY, the receiver of the bacterial chemotaxis pathway, crystallizes using ammonium sulfate as the precipitant. Therefore, the purified receiver domain of ETR1 was subjected to a Grid Screen A/S (Hampton Research, Laguna Hills, California). After 3 d, a shower of crystals appeared in the pH range 5–7 and 1.6 *M* $(NH_4)_2SO_4$. Optimizing the protein concentration to 22 mg ml^{-1} and switching to the sitting-drop format with a final drop size of 10 µl decreased nucleation events and yielded large well behaved crystals. The final crystallization conditions were 1.6 *M* Li_2SO_4, 0.1 *M* HEPES pH 7.5, 0.03% NaN_3. Lithium sulfate maintained better crystal morphology than did ammonium sulfate over intervals up to four weeks prior to data collection. In this format, crystals were obtained with dimensions 0.4 × 0.4 × 0.8 mm. After testing PEG, glycerol, and 1-MPD, a saturated solution of meso-erythritol (Fluka, Buchs, Switzerland) was used as a cryoprotectant (Rogers, 1994). A flash-freezing technique was utilized.

X-ray diffraction experiments were performed on a Rigaku R-axis IIC imaging plate using graphite-monochromated Cu $K\alpha$ radiation from a Rigaku RU-300 rotating-anode generator. The generator was operated at 50 kV and 100 mA. The flash-frozen crystals diffracted beyond 2.1 Å. A native data set was collected and the space group was determined to be $P4_12_12$. The unit-cell parameters of the crystals as determined by the program *SCALEPACK* (Otwinowski, 1993) were $a = b = 48.4$, $c = 112.3$ Å. There was a single molecule of the receiver domain of ETR1 in the assymetric unit and the solvent content of the crystal was 42%. The data were 98.8% complete in the last resolution shell, 2.64–2.55 Å with an R_{merge} $[R_{merge} = \sum |I(h) - \langle I(h)\rangle| / \sum I(h)$, with $I(h)$, observed intensity and $\langle I(h)\rangle$, mean intensity of reflection h over measurement of $I(h)]$ of 5.3% for all reflections between 30 and 2.55 Å.

A selenomethionine(SeMet)-substituted form of the protein has been produced wherein the seven methionines of the signal receiver domain of ETR1 have been replaced with SeMet. This will allow phase determination using the MAD technique (Hendrickson, 1991). The protein expression plasmid described above was transformed into the methionine auxotrophic *E. coli* strain B834 (DE3) (Novagen) and grown in minimal medium in the presence of SeMet. The SeMet protein was purified as described for the native protein. The SeMet incorporation efficiency was 100% as determined by mass spectroscopy. The observed molecular weight was 15 613 Da while the calculated molecular weight was 15 611 Da. The SeMet protein was concentrated to 19 mg ml^{-1} and crystrallized using microseeding techniques in the same conditions as described for the native protein. The protein derivative crystals diffract to 2.5 Å at our home source.

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References

Abeles, F. B., Morgan, P. W. & Saltveit, M. E. (1992). Editors. *Ethylene in Plant Biology*. New York: Academic Press.

Appleby, J. L. Parkinson, J. S. & Bourret, R. B. (1996). *Cell*, **86**, 845–848.

Baikalov, I., Schroder, I., Kaczor-Grzeskowiak, M., Grzeskowiak, K., Gunsalus, R. P. & Dickerson, R. E. (1996). *Biochemistry*, **35**, 11053–11061.

Bowie, J. U., Pakula, A. A. & Simon, M. I. (1995). *Acta Cryst.* D**51**, 145–154.

Burg, S. P. & Burg, E. A. (1967). *Plant Physiol.* **42**, 144–152.

Chang, C., Kwok, S. F., Bleecker, A. B. & Meyerowitz, E. M. (1993). *Science*, **262**, 539–544.

Chao, Q. M., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W. & Ecker, J. R. (1997). *Cell*, **89**, 1133–1144.

Feher, V. A., Zapf, J. W., Hoch, J. A., Whiteley, J. M., McIntosh, L. P., Rance, M., Skelton, N. J., Dahlquist, F. W. & Cavanah, J. (1997). *Biochemistry*, **36**, 10015–10025.

Harpham, N. V. J., Berry, A. W., Holland, M. G., Moshkov, I. E., Smith, A. R. & Hall, M. A. (1996). *Plant Growth Reg.* **18**, 71–77.

Hendrickson, W. A. (1991). *Science*, **254**, 51–58.

Hua, J., Chang, C., Sun, Q. & Meyerowitz, E. M. (1995). *Science*, **269**, 1712–1714.

Kato, M., Mizuno, T., Shimizu, T. & Hakoshima, T. (1997). *Cell*, **88**, 717–723.

Kehoe, D. M. & Grossman, A. R. (1997). *J. Bacteriol.* **179**, 3914–3921.

Kieber, J. J. (1997). *J. Exp. Bot.* **48**, 211–218.

Kieber, J. J., Rothenberg, M., Roman, G., Feldman, K. A. & Ecker, J. R. (1993). *Cell*, **72**, 427–441.

McEvoy, M. M., Muhandiram, D. R., Kay, L. E. & Dahlquist, F. W. (1996). *Biochemistry*, **35**, 5633–5640.

Martinez-Hackert, E. & Stock, A. M. (1997). *Structure*, **5**, 109–124.

Milburn, M., Privé, G., Milligan, D., Scott, W., Yeh, J., Jancarik, J., Koshland, D. E. Jr & Kim, S.-H. (1991). *Science*, **254**, 1342–1347.

Otwinowski, Z. (1993). In *Data Collection and Processing: Proceedings of the CCP4 Study Weekend*, edited by L. Sawyer, N. Issacs & S. Bailey. Warrington: Daresbury Laboratory.
Posas, F., Wurglermurphy, S. M., Maeda, T., Witten, E. A., Thai, T. C. & Saito, H. (1996). *Cell*, **86**, 865–875.
Rogers, D. W. (1994). *Structure*, **2**, 1135–1140.
Roman, G., Lubarsky, B., Kieber, J., Rothenberg, M. & Ecker, J. R. (1995). *Genetics*, **139**, 1393–1409.
Schaller, G. E. & Bleecker, A. B. (1995). *Science*, **270**, 1809–1811.
Schaller, G. E., Ladd, A. N., Lanahan, M., Spanbauer, J. M. & Bleecker, A. B. (1995). *J. Biol. Chem.* **270**, 12526–12530.
Sisler, E. C. (1987). *Plant Hormone Receptors*, edited by D. Klambt, pp. 297–301. Heidelberg: Springer-Verlag.
Stock, A.M., Mottonen, J. M., Stock, J. B. & Schutt, C. E. (1989). *Nature (London)*, **337**, 745–749.
Swanson, R. V., Alex, L. A. & Simon, M. I. (1994). *Trends Biochem. Sci.* **19**, 485–490.
Theologis, A. (1996). *Curr. Biol.* **6**, 144–145.
Uhl, M. A. & Miller, J. F. (1996). *J. Biol. Chem.* **271**, 33176–33180.
Volkman, B. F., Nohaile, M. J., Amy, N. K., Kustu, S. & Wemmer, D. E. (1995). *Biochemistry*, **34**, 1413–1424.
Volz, K. (1993). *Biochemistry*, **32**, 11741–11753.
West, A. H., Martinezhackert, E. & Stock, A. M. (1995). *J. Mol. Biol.* **250**, 276–290.
Wilkinson, J. Q., Lanahan, M. B., Yen, H. C., Giovannoni, J. J. & Klee, J. H. (1995). *Science*, **270**, 1807–1809.
Yang, S. F. & Hoffman, N. E. (1984). *Annu. Rev. Plant Physiol.* **7**, 155–189.
Yeh, J. I., Biemann, H.-P., Prive, G. G., Pandit, J., Koshland, D. E. Jr & Kim, S.-H. (1996). *J. Mol. Biol.* **262**, 186–201.
Zhou, H., Lowry, D. F., Swanson, R. V., Simon, M. I. & Dahlquist, F. W. (1995). *Biochemistry*, **34**, 13858–13870.