Solution Structures of the DctD Receiver Domain

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Introduction

Two-component signal transduction is the predominant information-processing mechanism used by bacteria to control adaptive responses to environmental changes. It is also present in archaeons, single-celled eukaryotes, and plants. The kinds of behaviors controlled by two-component signal transduction range from the expression of single genes for nutrient acquisition to well-orchestrated developmental programs that totally redefine a cell's physiology and sometimes its anatomy. One group of two-component response regulators is formed by proteins known as σ54-dependent transcriptional activators, or bacterial enhancer binding proteins. Each of these contain an AAA+ ATPase domain that is used to couple ATP hydrolysis with conformational changes in the σ54-form of RNA polymerase. The conformational change permits the polymerase to isomerize into productive open complexes at promoters. We are studying one such protein, the dicarboxylate transport protein D (DctD) of *Sinorhizobium meliloti*. These bacteria reduce nitrogen to ammonia only when living in symbiosis with their legume host, alfalfa. DctD regulates the expression of a dicarboxylate transport protein encoded by dctA, which enables the bacteria to use the food that the host provides to fuel symbiotic nitrogen fixation [1].

DctD, like about 50% of the 600 known σ54-dependent activators, has three functional domains. An N-terminal "receiver" domain regulates the functional state of a central AAA+ ATPase output domain, and these are guided to the right promoter region by a C-terminal DNA-binding domain. Analytical ultracentrifugation studies indicated that the DctD receiver domain exists in dimeric but distinct states in both its apo (inactive) and phosphorylated (active) forms. Crystallography revealed a novel dimeric structure for the inactive receiver domain [2, 3]. Substitution E121K destabilized the dimer about 20-fold, and partially activated the ATPase domain. Beryllium fluoride mimicked phosphorylation of the receiver domain, also partially activating the ATPase domain [4]. Together, beryllium fluoride and the substitution E121K sufficiently stabilized the active form of the receiver domain to obtain a crystal structure [5]. Unfortunately, it was unclear which of the four possible dimers present in the crystal lattice represented the solution state [5, 6]. Small-angle x-ray scattering (SAXS) and wide-angle x-ray scattering (WAXS) experiments were explored during a workshop held in the spring of 2002. The purpose was to learn if such data would distinguish between the possible dimers of the DctD receiver domain in its active state and to determine which form exists in solution. Although the studies were only begun during this workshop, they were found to be feasible and were completed and expanded upon in 2003. This report describes the initial studies that were conducted during the workshop in 2002 and relates them to subsequent studies that grew from the workshop experience.

Methods and Materials

Residues 2-144 of the DctD protein (called DctDNL since it contains the N-terminal receiver domain and adjacent linker), with or without substitution E121K, were expressed as a 6His-tagged recombinant protein from vector Pet21a in *E. coli* strain BL21/DE3/pLysS (Novagen). The proteins were purified as reported [3, 4], then simultaneously concentrated to 8 mg/mL and dialyzed against 10 mM HEPES (pH 7.0). Protein samples were mixed with various combinations of magnesium chloride (4 M stock solution), carbamyl phosphate (1.3 M stock solution), or sodium fluoride (2.5 M stock suspension) and beryllium chloride (1 M stock solution). Mixed samples were centrifuged for 5 min at 10,000 rpm and then filtered with 0.1-μm Anatop filters (Whatman) before data collection. A third protein, a replication factor for the poliovirus, was provided by C. Cameron of Pennsylvania State. It was present in a 20 mM Tris-HCl buffer supplemented with 2 M sodium chloride. SAXS experiments were conducted on the Bio-CAT undulator beamline 18-ID at the APS. Aliquots of DctDNL protein (100 μM dimer concentration) were incubated in the presence of $MgCl_2$ (100 mM) and carbamyl phosphate (100 mM) at room temperature. At various times, the buffer and sample were exposed to focused x-rays (12,000 ±20 eV; ~1 × 1012 photons/s) for 12.2 s, at a specimen-to-detector distance of 2.78 m. Two-dimensional scattering patterns were obtained by using a 5 × 9-cm charge-coupled device (CCD) detector [7]. For exposure, 100 μL of sample was contained in a 1.5-mm-diameter borosilicate glass capillary. To minimize radiation damage, the sample was oscillated at 4 μL/s by using a programmable syringe pump (Hamilton model 541C). Scattering over the q range from 0.004 to

0.13 $Å^{-1}$ was calculated from radial integrations of the 2-D scattering patterns by using the routines in the FIT2D data analysis program [8]. Integrated scattering profiles from sample plus buffer and from buffer alone were scaled by using incident flux values integrated over the exposure time. Radius of gyration (R_G) was calculated conventionally as the square root of -3 times the slope from plots of ln(I) versus q^2, where I is the difference in intensity in scattering patterns from sample plus buffer minus buffer alone. Density distribution functions were obtained by using GNOM [9], and they were processed to yield envelope shapes by using DAMMIN [10].

Results

Analytical ultracentrifugation studies had been conducted for both the wild-type and E121K-substituted DctDNL proteins prior to attempting the SAXS experiments [3]. Those data indicated a stable dimeric state with no signs of larger species for loading concentrations as high as 1 mg/mL. It was thus unexpected when the scattering profile for the wild-type DctDNL protein gave R_G values typical of a significantly larger species, tentatively identified as a tetramer. Data were obtained for mixtures of apo and phosphorylated protein by sampling phosphorylation reactions over a 45-min interval, during which phosphorylation typically proceeds to 95% completion. The R_G values calculated for these data indicated that phosphorylation stabilized a shift from putative tetramer species to a dimer species. The protein bearing the E121K substitution, which had been seen to destabilize the solution dimer by about 20-fold, did not scatter photons consistent with a tetramer. Instead, those data were consistent with a dimer state. Phosphorylation was seen to shift the R_G values to a slightly larger size, still typical of a dimer and also similar to the size for a phosphorylated wild-type protein. Incubation of both wild-type and E121K-substituted proteins with beryllium fluoride gave consistent results, indicative of a dimeric state. The poliovirus protein behaved as single species with an R_G that is expected of a monomer of its molecular mass. The data yielded a solution envelope that fit a monomer from the crystal structure and suggested that the ubiquitin tag that had been added to aid purification was still present. Since there were adequate DctDNL samples and adequate time available for some exploration of WAXS data, the camera was moved closer to the sample stage, and attempts were made to obtain data for the wild-type protein that had been phosphorylated for up to 1 h; also, the temperature was decreased to 5ºC to collect data under conditions that had been found to change circular dichroism spectra. Because these were unplanned experiments, useful data were not obtained, but SAXS and WAXS data were collected and combined.

Discussion

The possibility that SAXS data could discriminate between alternate dimer forms of the DctD two-component receiver domain was explored, and the participant, a molecular biologist, learned about the SAXS/WAXS methodology. A second problem was also investigated for a colleague, who wanted to know if the poliovirus replication protein 3C was monomeric or dimeric. Technical problems were experienced when the method was applied to distinguish between dimeric solution-states of the DctD receiver domain. The poliovirus protein 3C was found to be monomeric under the employed buffer conditions. These data were further processed by using the GNOM and DAMMIN programs [9, 10] and shown to provide a solution envelop that fit a monomer of the known dimeric crystal structure very nicely. This experience, these data, and the solution envelope for the poliovirus protein were incorporated into a course that the applicant teaches to graduate students at Pennsylvania State.

The last six residues of the DctDNL linker contribute to tetramerization in the crystal lattice of unphosphorylated protein. These residues of the linker region were removed, and small-angle neutron scattering (SANS) data similar to the SAXS data described above showed that the tetramers disappeared (September 2002; Center for Neutron Research of the National Institute of Standards and Technology in Bethesda, MD; Nixon and Krueger unpublished). Data analysis indicated that higher-resolution SAXS/WAXS data would likely distinguish between the four dimer conformations, thus identifying the one relevant to solution. SAXS/WAXS data were obtained (April 2003; together with studies of the receiver and ATPase domains of the NtrC1 protein of *Aquifex aeolicus*; Nixon, Callahan, and Kondrashkina unpublished). These data do identify the relevant dimer in the crystal lattice of the DctD receiver domain. They also confirm that activation of the NtrC1 protein occurs by a similar mechanism, with alternate dimers of the receiver domain either dictating an off-state dimer of the AAA+ ATPase or permitting it to self-assemble into an active heptamer [12]. Preliminary data also show structural responses in the assembled ATPase when different nucleotides or nucleotide analogs are bound. This provides a sensitive assay to optimize nucleotide binding for future biochemical or crystallographic studies. Processing these data with GNOM and GASBOR [11] programs strongly suggests that the approach provides an alternative to cryogenic electron microscopy for obtaining low-resolution solution structures of AAA+ ATPases (e.g., see Refs. 13 and 14).

These low-resolution structures promise to reveal conformational changes in the σ54-dependent ATPases that are coupled with conformational change in the RNA polymerase, which permits isomerization into productive open complexes.

Acknowledgments

Use of the APS was supported by the DOE Office of Science, Office of Basic Energy Sciences, under Contract No. W-31-109-ENG-38, Bio-CAT, and National Science Foundation Grant No. MCB-9727745 to Nixon.

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