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pp. 81-89

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Rieske Iron-Sulfur Protein Isoforms in a Unicellular Cyanobacterium

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Abstract

Rieske iron-sulfur proteins (ISPs) are integrally involved in photosynthetic electron transport chains and thus closely linked to energy production and its regulation in cyanobacteria and plant chloroplasts. The cyanobacterium *Synechocystis* sp. PCC 6803 has three Rieske proteins, PetC1, PetC2, and PetC3, whose specific roles are not well understood. Here we describe two-dimensional (2D) gel electrophoresis and immunoblotting used to investigate the PetC1 Rieske protein in native *Synechocystis* and to confirm its absence in a mutant with an inactivated PetC1 gene. These studies revealed a "trail" of PetC1 Rieske protein spots migrating at the same molecular weight on 2D gels but at different isoelectric points. This "stuttering" phenomenon suggests covalent modifications that may reflect oxidative damage or modifications of the protein involved in biochemical signaling reactions.

Introduction

Energy is important for the survival of living organisms and the photosynthetic electron transport chain of cyanobacteria and plant and algal chloroplasts is a major source of energy for living organisms on earth. The electron transport system releases energy through a series of oxidation-reduction reactions that must be carefully controlled to prevent the formation of damaging oxygen radicals. These processes are not completely understood. *Synechocystis* sp. PCC 6803 and *Synechococcu*s sp. PCC 7002 are two unicellular cyanobacteria that obtain energy through photosynthesis. Cyanobacteria have also been called blue-green algae because of their physical appearance. The internal thylakoid membranes involved in photosynthesis use water as the electron donor and produce oxygen as an important byproduct of electron transport.

Rieske Iron-Sulfur proteins (ISPs) play an integral role in this electron transport mechanism. Thus further knowledge about their structure and function within photosynthetic cells can help to better understand globally important energy conversion processes as a whole. Rieske ISPs are among the thousands of proteins in cyanobacteria. These ISP proteins are critical components of the cytochrome *bf* electron-transfer complex. These protein complexes are located in the thylakoid membranes of plant chloroplasts and cyanobacteria. Proteins can be described in terms of primary, secondary, tertiary or quartenary structure. Typical Rieske proteins of cytochrome *bf* complexes have two soluble subdomains, one of which binds the iron sulfur cluster, and a membrane-spanning anchor. The 2Fe-2S cluster is a planar structure bound via its irons to two cysteines and two histidines in the protein (Carrell et al. 1997). These Rieske ISPs have a molecular weight of ~19,500 Daltons (19.5 kDa) and an isoelectric focusing point (pI) of ~5.6 (Schneider, 2004). Because they grow rapidly and can be easily manipulated genetically, cyanobacteria were used in the experiments reported here.

The genome of *Synechocystis* PCC 6803 contains three different *petC* genes, which encode different Rieske proteins. It is possible to delete *petC2* or *petC3*, and to a lesser extent *petC1*, without drastically changing functionality or phenotype. Removing both *petC2* and *petC1* results in irreparable damage and loss of function; however, removing either *petC1* and *petC3* or *petC2* and *petC3* still permits some functionality. Although all three *petC* genes are different, they do maintain uniformity to some degree. Thus *petC2* can partially replace *petC1*, but *petC3* is unable to replace any of the other two *petC* genes. For these reasons, it would be interesting to study all three genes, their deletions, and combinations of deletions to better understand the role of these genes and their protein products in cytochrome *bf* complexes and photosynthesis.

Identification, separation, and isolation of individual protein subunits can allow greater insight into individual intricacies of protein function and has been a traditional approach in biochemistry. Separation and identification can be accomplished through a number of techniques including one or two dimensional (1D or 2D) sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). After such separations, proteins can often be specifically visualized and detected through immuno- or "western-" blotting against specific antibodies. Recently, mass spectrometry techniques such as Matrix Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) and electrospray ionization (ESI) have become widely available and extremely valuable for identification and characterization of proteins and peptides.

In the present study, these techniques were used to investigate Rieske ISPs of the cyanobacteria *Synechococcus* PCC 7002 and *Synechocystis* 6803. We focused mainly on the PetC1 Rieske protein of *Synechocystis*. Our goal was to test the hypothesis that a mutant strain, ΔPetC1, with a defective *petC1* gene is truly capable of photosynthetic growth in the absence of the predominant, PetC1, Rieske protein of the cytochrome *bf* complex. Data presented here support this hypothesis and most interestingly, provide evidence for the existence of different forms of the PetC1 Rieske protein that may have specific regulatory functions or that may be the byproducts of oxidative damage resulting from electron transfer reactions.

Methods

Cyanobacterial Strains and Culture Conditions

Synechococcus sp. PCC 7002 and *Synechocystis* sp. PCC 6803 were grown as described in Nelson et al. (2005) or Schneider et al. (2004). *Synechococcus* is an abundant unicellular, marine cyanobacterium. *Synechocystis* 6803 is widely used as a model organism and was the first phototroph to have its entire genome sequence determined (referenced in Mulkidjanian et al. 2006). It was isolated from a fresh water lake and is spontaneously transformable, meaning it has the capability of incorporating foreign DNA into its own genome through homologous recombination. Additionally, *Synechocystis* is able to grow under a variety of conditions, including heterotrophically in the absence of light.

Polyacrylamide Gel Electrophoresis (PAGE)

PAGE procedures are widely used for separation of protein complexes, individual proteins, or peptide chains based on their overall conformation, surface charge, or molecular weight (Santoni et al., 2003; Molloy, 2000). SDS-PAGE

relies primarily on the properties of dithiothreitol (DTT), acrylamide, and sodium dodecylsulfate (SDS). Polyacrylamide is a cross-linked polymer of acrylamide that establishes the gel matrix. The higher the concentration, the longer the acrylamide polymers, and the more slowly proteins move through the gel. Although polyacrylamide is non-toxic, acrylamide is a neurotoxin. Thus gels must be handled carefully because of the possible presence of free acrylamide. The 1D gels were done under conditions similar to those reported by Santoni (2003).

PAGE under denaturing conditions most commonly uses sodium dodecylsulfate, or SDS and is referred to as SDS-PAGE. SDS is an anionic (negatively charged) detergent used to denature, or unfold, proteins prior to loading and as they move through the polyacrylamide gel matrix. SDS binds to protein in a ratio of about 1.4 g SDS per 1.0 g protein. Because of this direct proportionality, the mass to charge ratio is also uniform and proteins separate in the gel largely according to their size. Bromophenol Blue dye is added to the gel matrix or directly to the protein solution so a dye front moves down the gel. This allows one to see when the dye is about to exit the gel and provides time to stop the electrophoresis to prevent low molecular weight proteins from running out of the bottom of the gel. Dithiothreitol, or DTT, a reducing agent, is added to the gel loading solution. It helps denature proteins by breaking the disulfide bridges present in many proteins. This disrupts the tertiary or quaternary structures of proteins and can aid in breaking apart subunits.

One-dimensional (1D) SDS-PAGE

This procedure separates proteins only on the basis of their molecular weight but has the advantage of being relatively fast and easy. A critical parameter is the polyacrylamide concentration. Higher percentages result in better separation of high molecular weight proteins but sacrifice resolution of lower molecular weight proteins. Lower concentrations of acrylamide have the opposite effect. The PetC1 Rieske ISP, the protein of interest, has a low molecular weight of about 19,500 Da. Thus a higher percentage of acrylamide yields the best separation in this range. Generally 10–12% single concentration gels or 8–16% gradient gels were used. Discontinuous 1D gels were used, meaning they had a lower resolving gel layer (that actually separates, or resolved, the proteins) and an upper stacking gel layer that allowed proteins to pile up, or "stack," before they reached the resolving gel (Santoni et al., 2003). Before the stacking gel polymerized, a comb with a number of wells was inserted. Once polymerization occurred, the comb was removed and the denatured and negatively charged proteins were loaded into the wells. An electrical current (usually ~ 100 V and 20 mA per 10 cm long gel) was applied causing the SDS-bound proteins to move down the gel to the cathode, or positive end. A lane or well with a set of pre-stained protein standards was run alongside the protein samples to calibrate the relative molecular weights of the protein samples. Proteins appeared as bands on these 1D gels, with each band representing one or more proteins of a particular molecular weight.

Two-dimensional (2D) Isoelectric Focusing (IEF) SDS-PAGE

This procedure uses an immobilized pH gradient (IPG strip) to separate proteins based on their isoelectric points (pI, the pH at which a protein has no net charge) in one dimension, and by molecular mass or size in a second dimension

(Molloy, 2000). 2D-PAGE requires much more time, often several days, and is subsequently more difficult than 1D PAGE. However, it has the advantage of separating proteins not only by their size, but also by their isoelectric points. BioRad ReadyStrip (pH 4-7 or 3-10) IPG Strips were used in this study. Better resolution of PetC1 Rieske proteins (pI ~5.6) was obtained with the narrower range pH 4-7 strips. Most of the 2D protein mini gels (~12 x 10 cm) used in the current study were loaded with ~100 μg of protein to allow optimal resolution. A denatured protein solution was pipetted into a long narrow well and an IPG strip was placed gel side down on top of this solution with electrodes touching each end of the strip. A current was then applied to force proteins to migrate to their isoelectric points on the IPG strip.

SDS-PAGE gels for the second dimension did not use a stacking gel and multi-well comb, but rather one long well for an IPG strip and a small well for protein standards. Each IPG strip from the first dimension was equilibrated in SDS-PAGE sample buffer, then placed horizontally into the long well of an SDS-PAGE gel. The area around the strip was filled with agarose containing a small percentage of Bromophenol Blue to run as a dye front. An electrical current was applied, as in 1D gels, to force proteins to migrate through the gel. Gels were stained either with silver or Coomassie stains (Molloy, 2000; Santoni et al., 2003). Individual proteins appeared as discrete spots on these gels.

Immunoblotting and Mass Spectrometry

These procedures were used to identify which spots on the 2D gels corresponded to proteins of interest, in this case Rieske proteins. After electrophoresis, gels were either stained to visualize proteins, or the proteins were transferred ("blotted") to a membrane for antibody detection. This transfer employed an Owl Blotting Apparatus, which ran a constant current perpendicular to the gel plane and transferred the proteins onto a piece of Millipore Immobilon P membrane. Subsequently, a primary antibody (anti GST/Rieske B4#2, Holton et al., 1996) was used to bind specifically to Rieske proteins on the membrane. A second, enzyme-linked antibody bound specifically to the primary antibody. The secondary antibody was linked to an alkaline phosphatase enzyme. On blots incubated with BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt) and NBT (Nitro-Blue Tetrazolium Chloride), the BCIP substrate was converted to a visible, purple spot that marked the location(s) of PetC1 Rieske proteins.

Protein spots identified by immunoblotting as Rieske ISPs were excised from gels for possible further characterization by mass spectrometry. Material extracted from these spots was digested with trypsin and spotted onto a MALDI (matrix assisted laser desoption ionization) target plate for attempted MALDI-TOF (time-of-flight) mass spectrometry and protein identification by peptide mass fingerprinting (Pandey and Mann, 2000). Unfortunately, the data obtained were not of sufficient quality to yield significant protein identification scores in searches against the Mascot protein database.

Results and Discussion

Membrane Protein Solubilization

The SDS-PAGE and immunoblotting techniques described above were used to investigate the Rieske iron-sulfur proteins of *Synechocystis* PCC 6803. Most of the

current work focused on characterization of the predominant PetC1 Rieske protein from wild type *Synechocystis* and tests to confirm its absence from a mutant, ΔPetC1, with an inactivated *petC1* gene. Because Rieske ISPs are membrane proteins, they present special problems for gel electrophoresis, particularly for 2D SDS-PAGE where membrane proteins tend to precipitate at their isoelectric points. Such membrane proteins must be kept in solution with detergents but many detergents are incompatible with isoelectric focusing or are ineffective at extracting proteins from membranes (Molloy, 2000). Thus several detergents were tested for membrane protein extraction and compatibility with 2D SDS-PAGE. Figure 1 shows a 1D gel comparing two detergents, CHAPS and ASB14. In these experiments, the less expensive CHAPS proved to be as effective as ASB14 and was used in all subsequent work.

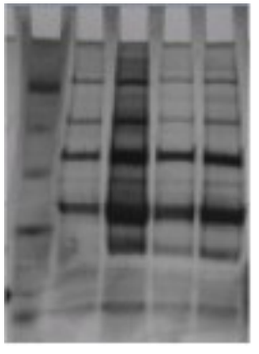

Figure 1

Silver stained 1D SDS-PAGE gel comparing solubilization of membrane proteins with the detergents CHAPS and ASB14. 10-20% acrylamide gradient gel. Wild type *Synechocystis* cells were harvested at either 3.12 OD_{750} units (lanes 2 and 3) or 0.53 OD_{750} units (lanes 4 and 5). From left to right:
1) Protein size standards, 2) membranes extracted with CHAPS, 3) ASB-14, 4) CHAPS, and 5) ASB-14.

Detection of the PetC1 Rieske Iron-sulfur Protein on 2D Gels

To test the hypothesis that the ΔPetC1 mutant of *Synechocystis* is indeed capable of growth in the absence of the predominant, PetC1, Rieske iron-sulfur protein of photosynthesis, 2D SDS-PAGE gels were run of membrane proteins extracted from *Synechocystis* wild type and the ΔPetC1 mutant. In addition, our larger goal was to develop conditions for effective separation of cyanobacterial membrane proteins. Figure 2 shows a representative 2D IEF-SDS-PAGE gel of *Synechocystis* membrane proteins. The overall separation in 2D gels is notably different that in 1D gels, because distinct spots appear on 2D gels, whereas 1D gels produce bands. Each spot represents a unique protein that has migrated to a specific pH (equal to its isoelectric point or pI) and to a specific molecular weight. If the pI and molecular weight of a protein of interest is known, for example, the PetC1 Rieske ISP, it can theoretically be identified on a 2D gel. However, this is often difficult because the resolution of 2D gels is not precise and even simple bacteria have thousands of proteins, many of which may migrate in proximity. Moreover, covalent protein modifications such as oxygen adducts from oxygen radical damage or phosphorylation involved in signaling alter protein surface charge and thus pI and migration on 2D gels.

Figure 2: Section of a 2D IEF-SDS-PAGE gel of wild type *Synechocystis* membrane proteins. Silver stained gel: horizontal dimension, pH 4-7 IPG strip (pH ~4.5 to 6 shown), vertical dimension, SDS-PAGE, 8-16% acrylamide (size range shown ~15,000 – 100,000 kDa).

To identify the *Synechocystis* PetC1 Rieske ISP on these gels, immunoblots were prepared and probed with a PetC1 Rieske antibody as described above. This approach was highly successful and spots corresponding to the PetC1 Rieske protein were readily detected from the native (wild type) *Synechocystis* cells (Figure 3). Note that the PetC1 protein spots migrated at a higher apparent size than the expected ~19.5 kDa. Rieske ISPs have long been known to migrate at higher apparent sizes under some SDS-PAGE conditions (Hurt and Hauska, 1981). In contrast, no signals were detected from 2D gel immunoblots of proteins from the *Synechocystis* ΔPetC1 mutant. In other words, the ΔpetC1 strain did not react against the PetC1 antibody. The ΔPetC1 mutant carries a deletion of the *petC1* gene and these data confirm the absence of the PetC1 Rieske protein, at levels below immunological detection, in this cyanobacterium.

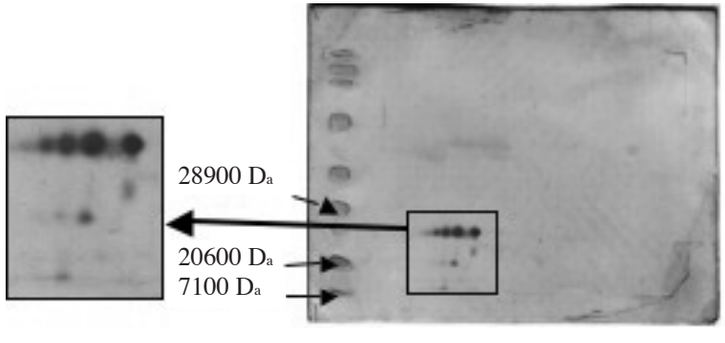

Figure 3: Immunoblot of a 2D IEF-SDS-PAGE gel of wild type *Synechocystis* membranes: Proteins were separated by 2D SDS-PAGE using pH 4-7 IPG strips and probed with an anti-PetC1 Rieske ISP antibody. The spots represent proteins that have reacted against the antibody.

Evidence for Isoforms of the PetC1 Rieske Protein in Cyanobacteria

The most unique and unexpected result from our experiments was the discovery of multiple PetC1 Rieske protein isoforms in the wild type *Synechocystis* 6803. These isoforms are apparent as a horizontal series of spots on 2D gel blots

against the PetC1 antibody as illustrated in Figure 3. This so-called "stuttering" phenomenon is observed in gels or blots as a horizontal series of spots (Ballasteros et al., 2001). On our gels, these very likely represent different forms of the same PetC1 Rieske protein because they all cross-reacted with the Rieske antibody and all migrated at the same molecular weight. They are "isoforms" because they migrated at different pH values in the horizontal, isoelectric focusing, direction. Because they have different isoelectric points (pIs) the surface charges of these protein isoforms must be different.

There are several possible, biologically significant, explanations for such shifts in pI. One is the misincorporation of acidic or basic amino acids into a protein causing a shift in surface charge and pI. Ballasteros et al. (2001) working with non-photosynthetic bacteria, suggested that stasis-induced carbonylation (covalent, double-bonded attachment of oxygen) targets specific proteins. During highly oxidizing conditions leading to oxygen radical formation from electron transport, carbon starved cells may become carbonylated due to the depletion of glucose. In the photosynthetic cyanobacteria, the cytochrome *bf* complex is a major site of oxygen radical production (Horn, 2005). These radicals may be formed during the electron transfer reaction involving the PetC1 Rieske iron-sulfur protein. Thus the Rieske ISP is a likely target for oxygen radical damage. Modifications such as carbonylation would result in a change of the Rieske ISP surface charge and pI, while largely maintaining its molecular weight. As a result, a stuttering pattern would appear on 2D gels.

An alternative explanation for the PetC1 Rieske isoforms is that thse proteins become modified as part of a signaling mechanism that senses and transmits information about the oxidation-reduction potential of the electron transport chain. Rieske proteins have been implicated in redox signaling (deVitry et al., 2004). Phosphorylation, for example, would alter protein surface charge and is widely used in cellular biochemical signaling pathways. Two forms of Rieske ISP have previously been detected from 2D gel analysis of spinach chloroplast proteins (Yu et al., 1994). The significance of such Rieske ISP modifications in that context or in cyanobacteria remains to be determined.

The Rieske ISP spots resulting from immunoblot analysis were excised from the original 2D gels and spotted onto MALDI target plates for mass spectrometry. Several problems were encountered with reliable data collection, mass calibration, and comparisons against the Mascot database, and useable data were not obtained. Since these initial experiments, mass spectrometry procedures have been optimized and these important techniques should now be more readily available for characterization of biologically significant protein modifications.

Conclusions

The present work has contributed to establishing 1D and 2D gel electrophoresis and immunoblot analysis of proteins from cyanobacteria which are model organisms for photosynthesis and globally important for oxygen production and carbon fixation. In this study, we have confirmed that the cyanobacterium *Synechocystis* sp. PCC 6803 is capable of photosynthesis and growth in the absence of the predominant, PetC1, Rieske iron-sulfur protein of the cytochrome *bf* complex. Apparently, the ΔPetC1 mutant lacking the PetC1 Rieske ISP can function, although less efficiently, with an alternative, PetC2, Rieske protein. Our most interesting finding

was the discovery of isoforms, evidenced as "stuttering" patterns on 2D gels, of the major PetC1 Rieske protein in wild type *Synechocystis*. These isoforms represent PetC1 proteins that have similar masses but different surface charges that may arise from oxidative damage or modifications such as phosphorylation with possible roles in biochemical signaling.

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