APPLICATIONS OF CAPILLARY ELECTROPHORESIS TO REDOX PROTEINS AND NUCLEOTIDES IN PHOTOSYNTHETIC SYSTEM

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Abstract Several redox proteins and nucleotides involved in photosynthetic electron transfer were separated by capillary zone electrophoresis (CZE) with a noncoated fused silica capillary column. We found that reduced and oxidized forms of plastocyanin, cytochrome c, NAD and NADP could be separated by CZE. Two types of ferredoxin, commonly present among higher plants, were also clearly separated by this technique.

Key words: Capillary electrophoresis, redox proteins, membrane proteins, photosynthesis, nicotinamide nucleotides

The light reaction of oxygenic green plant photosynthesis is a combination of two sequential and coordinated photochemical reactions, photosystem 1 and photosystem 2. These two photochemical reactions activate the photosynthetic electron transport, which is a series of reductions and oxidations starting from the oxidation of water with the oxygen evolution as a by-product, ending with the reduction of NADP(nicotinamide adenine dinucleotide phosphate). The reduced NADP(NADPH₂) and ATP formed in the course of the electron transport are the hydrogen and energy sources for the dark reaction where a complex series of enzyme reactions known as Calvin cycle fixes carbon dioxide and produces carbohydrates such as starch and sugars.

The photosynthetic electron transport takes place mainly in proteins bound to membranes inside a chloroplast; many of the electron carriers and supporting proteins are hydrophobic proteins that form complexes. These subunit proteins have been analyzed almost exclusively by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); any other techniques have so far not been able to achieve as much resolution. Although the resolving power of SDS-PAGE is very high, SDS denatures and inactivates functional proteins. In case of highly hydrophobic membrane proteins, however, our effort to replace SDS-PAGE with CZE has not been successful so far. We found, on the other hand, some of the electron carrier proteins loosely bound to membranes, such as ferredoxin, plastocyanin and c-type cytochrome, can be separated by using CZE with mild detergents. Ferredoxin's two isozymes which could not be separated easily with conventional technique were clearly separated by CZE. We found it particularly useful that the reduced form could be separated from the oxidized form. Reduced and oxidized forms of non-protein electron carriers in photosynthesis and oxidative phosphorylation such as NADP and NAD were also separated by this technique.

EXPERIMENTAL

Apparatus CZE was carried out by using a Jasco Model CE-800 capillary electrophoresis system(1). The system composed of a high voltage power supply (Model 890-CE), an intelligent UV/VIS detector (Model 875/870-CE) using as an on-column detector and a data processing integrator (Model 807-IT). A fused silica capillary with protective polyimide coating outside (50 μ m i.d./370 μ m o.d.) was purchased from GL Sciences Inc., Tokyo. In most cases a column with the effective length of 50 cm was used. A part of the coating (5 mm) was peeled off at 20 cm from the end for the optical detection. The column was

washed before each measurement with the following media by using a sucking method with a hand-operated vacuum pump (MityVac, Nalgen): water for 1 min., 0.1N HCl for 1 min., water for 1 min., 0.2N NaOH for 2 min., water for 1 min., and finally a running buffer. An aliquot of a sample solution was injected into a capillary column by using a siphon method, dipping the end of the column in a sample tube and lifting it 10 cm above the detector level for a short period. By changing the lifting time, usually from 5 to 15 sec, the amount of a sample solution being injected could be varied. No effort was made to keep the column temperature constant besides the room temperature which was kept around 25 C. UV/VIS spectra of samples were measured by using either a Jasco Model Ubest-35 double-beam spectrophotometer or a Jasco Model Ubest-55 double monochromator spectrophotometer.

Reagents and samples Ferredoxins prepared from spinach and pokeweed (*Phytolacca americana*) leaves were kindly provided by Prof. K. Suzuki(2). Plastocyanin was prepared from spinach according to Katoh(3). Photosystem-1 reaction center preparations were prepared from spinach chloroplasts by using Triton X-100 as the solubilizing reagent according to (4). Horse heart cytochrome c, NAD, NADP, NADH₂ and NADPH₂ were purchased from Sigma Chemical Co. (St. Louis, MO., U.S.A). CAPS (3-cyclohexylaminopropanesulfonic acid) was a product of Dojindo Laboratories, Kumamoto, Japan. Zwittergents were products of Calbiochem, La Jolla, CA., U.S.A.

RESULTS AND DISCUSSIONS

Photosystem-1 reaction center preparations We attempted to resolve these complexes into consisting subunit proteins by CZE. Two types of reaction center complex preparation were tested: one consisting of more than six protein subunits and another, only two. With these preparations, no detergents including SDS have so far been able to give good resolutions. We feel that further effort to find a appropriate buffer system for highly hydrophobic membrane proteins will be needed.

Cytochrome c As a model for c-type cytochromes in photosynthetic systems, a mitochondrial cytochrome c was employed in an attempt to separate the reduced and the oxidized forms. A mitochondrial cytochrome (horse heart) was chosen because this much less hydrophobic protein is much easier to handle than cytochrome f, a hydrophobic membrane-bound c-type cytochrome in chloroplasts. An untreated cytochrome c preparation gave a single peak. Upon the addition of small amount of a reductant, dithiothreitol (DTT), another peak appeared. With increasing concentrations of DTT, the original peak was gradually replaced by this new peak, indicating the new peak was due to the reduced cytochrome c (data not shown). By checking the absorption spectrum of each sample, we confirmed that the original peak was indeed due to the oxidized cytochrome and the new one, the reduced. This was confirmed also by setting the detector wavelength at 550 nm, an absorbance maximum of the reduced cytochrome. The resolution as well as the reproducibility, however, has not been satisfactory enough and will have to be improved in the future.

Ferredoxins This electron carrier protein with iron-sulfur cluster as the prosthetic group is only loosely bound to chloroplast thylakoid membranes, and readily solubilized without detergent usually only by breaking chloroplasts. Ferredoxin has been known to exist in two different forms (isozymes) in a number of plants and algae(5). Their primary structures (amino acid sequences) are in many cases only slightly different each other; no significant difference in physiological activities has been found. In most cases, the difference of the two forms are so slight that the separation has been no easy task. The separation has so far been achieved either by derivatization of the proteins(5), by HPLC(6) or by non-denaturing gel electrophoresis(2). We found that CZE could readily separate these two forms. Fig. 1 shows that the migration time for the Form II ferredoxin from pokeweed is much shorter than that of Form I under these conditions. An electropherogram of the crude ferredoxin preparation (Fig 1-C) shows a clear separation of the two forms. As usual for CZE, much less amount of sample and much less time are needed in this analysis than any previously known methods. The running time can be made much shorter than the present 40 min. by using a shorter column, as the separation of the isozymes was quite good. This CZE system was found to be also quite effective for the analysis of spinach ferredoxin isozymes which could not be separated by a gel electrophoresis (data not shown). As the reduced form of ferredoxin is known to be quite unstable, no attempt was made to prepare reduced ferredoxin and run CZE with the reduced ferredoxin.

Fig.1 Electropherogram of ferredoxins from *Phytolacca americana* (pokeweed). Upper left, Ferredoxin II; upper right, Ferredoxin I; bottom, crude ferredoxin. The buffer system contains 20 mM sodium phosphate buffer, pH 7.0 and 200 mM Zwittergent 3-08. Sample concentration, approximately 0.1 mg/ml. Voltage, 15kV; average current, 10 uA. Detected at 200 nm.

Plastocyanin This copper-containing electron carrier protein is also only loosely bound to the membrane, and becomes water soluble like ferredoxin and cytochrome c once it is released from the membrane. Plastocyanin carries electrons to photosystem 1 from photosystem 2. The oxidized form has a distinct blue color characterized by an absorbance maximum at 597 nm, whereas the reduced form has no absorption in the visible region. We found that the oxidized and the reduced forms of plastocyanin had different migration times under certain experimental conditions. Fig 2 shows the separation of the reduced and the oxidized plastocyanins in an untreated preparation in which the majority is known to be in the reduced form. This was confirmed by running separately a preparation reduced by ascorbate and another oxidized by ferricyanide (data not shown). This was further confirmed by changing the detector wavelength to 597 nm, where only the oxidized form has absorbance.

Fig. 2 Electropherogram of plastocyanin from spinach. The buffer system contains 20 mM sodium phosphate buffer, pH 7.0. Sample concentration, 0.5 mg/ml. Voltage, 15 kV; average current, 25 uA. Detected at 200 nm.

Nucleotide coenzymes NADPH₂ is the final end product of the photosynthetic electron transport in green plant photosynthesis. In photosynthetic bacteria, the reduced form of nicotinamide adenine dinucleotide (NADH₂) is formed instead. To determine the ratio of the reduced and the oxidized forms of these nicotinamide coenzymes (electron carriers), cumbersome and time-consuming procedures have been used (7). Although a number of reverse phase HPLC systems have been reported for the determinations of NADP, NAD and other nucleotides, none of them was able to determine the reduced forms mainly due to highly acidic eluent systems that destroy unstable reduced forms. We developed a CZE buffer system with which these four components could be readily separated within 15 min (Fig 3). NAD migrated first followed by NADH₂, then NADP and finally NADPH₂. We found, in this alkaline buffer system, the reduced forms of NAD and NADP reasonably stable; no significant degradation was found to take place during this short running time.

Fig. 3 Electropherogram of nucleotide electron carriers. From left to right, NAD, NADH₂, NADP and NADPH₂. The buffer system contains 100 mM sodium CAPS, pH 10.5 and 100 mM sodium dodecyl sulfate (SDS). Sample concentrations, approximately 2 mg/ml each for each nucleotide. Voltage, 15 kV; average current, 29 μ A. Detected at 260 nm.

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REFERENCES

1. Y. Kurosu, K. Hibi, T. Sasaki and M. Saito, *J. High Resol. Chromatography*, **14**, 200-203 (1991).
2. S. Takaichi, K. Makino, A. Iwasaki and K. Suzuki, *J. Biochem.*, **83**, 1151 (1978).
3. S. Katoh, in *Methods in Enzymology*, Vol. XXIII, A. San Pietro ed., pp 408, Academic Press, N.Y. & London, 1971.
4. T. Hiyama, A. Katoh, T. Shimizu, K. Inoue and A. Kubo, in *Progress in Photosynthesis Res.*, J. Biggins ed., Vol. 2, pp 45, Martinus Nijhoff Publishers, Dordrecht, The Netherlands, 1987.
5. Y. Takahashi, T. Hase, K. Wada and H. Matsubara, *Plant Cell Physiol.*, **24**, 189-198 (1983).
6. M. Shin and N. Sakihama, in *Iron-Sulfur Protein Research*, H. Matsubara et al. eds., pp.91-96, Japan Sci. Soc. Press, Tokyo/Springer-Verlag, Berlin, 1986.
7. M. Klingenberg, in *Methods of Enzymatic Analysis*, H. U. Bergmeyer ed., Vol. 4, pp 2045, Academic Press, N.Y. & London, 1974.