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Transport of Ions Across the Inner Envelope Membrane of Chloroplasts
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When this grant began in 1992, very little was known about the mechanisms of the transport of inorganic ions across the inner membrane (IEM) of the chloroplast envelope. In view of the fact that changes in the concentrations of protons, Mg^{2+} and Ca^{2+} have been implicated in the regulation of photosynthesis, it was surprising that ion transport across the IEM had not received more attention.

My interest in transport across the IEM began with the study of the transport of glycolate and glycerate, the first and final products, respectively, of photorespiration. We found that we could load the fluorescent pH indicator, pyranine, into isolated IEM vesicles and that the transport of glycolate and glycerate, which takes place by proton symport could be followed by stopped-flow fluorescence. In DOE-funded research, pyranine fluorescence was used to study directly the transport of protons and indirectly the transport of bicarbonate, nitrite and sulfate.

Within a few weeks of his arrival, Dr. Richard Shingles showed, using pyranine-loaded IEM vesicles, that the membrane contains a proton-translocating ATPase. Although it had been known for some time that the IEM has ATPase activity, there was no evidence for a proton-translocating ATPase until Dr. Shingles' findings. A remarkable feature of the H^+-ATPase is that it is strongly inhibited by dithiothreitol (DTT). As little as 2 mM DTT nearly abolished ATP-dependent proton pumping in IEM. DTT is often added to isolation media and buffers. This fact may explain, at least in part why others did not detect ATP-dependent proton uptake in IEM vesicles. The H^+-ATPase of the IEM probably functions to regulate the pH of the stroma as well as to generate an electrochemical proton gradient across the membrane that could be the driving force for the uptake of a number of ions, including Ca^{2+}.

In the experiments on ATP-driven proton transport, the IEMs were loaded with pyranine by a freeze-thaw method. The sidedness of these vesicles had not been established. Using the latency of the plasma membrane H^+-ATPase as an indicator of sidedness, Dr. Shingles showed that vesicle prepared by the freeze-thaw procedure were predominately inside out. Vesicles were also prepared by extrusion of isolated plasma membranes through 100 nm polycarbonate membranes. These vesicles were predominately right side–out. Little ATP-dependent H^+ transport was seen in IEM prepared by extrusion. If, however, the extruded membranes were exposed to two freeze-thaw cycles, ATP-dependent H^+ transport was restored. Thus, it is very likely that the active site(s) of the H^+-ATPase of IEM is located on the stromal side of the membrane and that the enzyme pumps H^+ out of chloroplast. In view of the ease in which predominantly inside out and right side-out biological membranes may be prepared using

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freeze thaw or membrane extrusion, it is surprising that these techniques are not more commonly used.

Sulfate transport across the IEM was studied in intact chloroplasts by silicone oil centrifugation to separate radioactive sulfate in the medium from that inside chloroplasts. The phosphate translocator of the IEM catalyzes the export of glyceraldehyde 3-phosphate from the stroma to the cytosol coupled with the import of Pi. The translocator can also catalyze an exchange of 3-phosphoglycerate (PGA) for Pi, a process that results in proton transport. Dr. Shingles loaded IEM vesicles with pyranine and 5 mM Pi. Mixing of these vesicles with PGA caused a rapid acidification of the vesicles' internal space. The dependence of the rate of this acidification on PGA concentration was hyperbolic and 50% of the maximal rate was reach at 0.6 mM PGA. Vesicles loaded with 5mM sulfate and pyranine also showed internal acidification upon addition of PGA. Although the concentration of PGA that gave the half-maximal rate of acidification in sulfate-loaded vesicles was similar to that in Pi-loaded vesicles, the maximum rate in sulfate-loaded vesicles was about 45% that in Pi-loaded vesicles. These results confirm those with intact chloroplasts.

Several attempts were made to identify proteins in IEM that bind ATP. In addition to the H^+-ATPase, a putative Ca^{2+}-ATPase has been proposed to be associated with the IEM. The nucleotide analog, 5'-P-fluorobenzoyladenosine (FSBA), binds covalently to a number of proteins that bind ATP. IEMs were incubated with FSBA and the unreacted reagent removed by washing. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the presence of FSBA was checked by immunoblotting with antibodies against FSBA. Two proteins in the Mr range of 95-110, 000 bound FSBA. These proteins are good candidates for ion translocating ATPases. These early studies (1994-1995) formed the basis for an extensive proteomic analysis of the IEM that is currently under way. Immunoblotting and amino acid sequencing of IEM proteins separated by two-dimensional electrophoresis has allowed the identification of several proteins, including a novel isoform of legume ferritin.

Nitrate is taken up by the roots and transferred in the xylem to leaves. In the cytoplasm of leaf cells, nitrate is reduced to nitrite which in turn is reduced to ammonia in chloroplasts. How nitrite crosses the IEM was unclear. The addition of nitrite (final concentration, 2.5 mM) to IEM vesicles containing pyranine lead to very rapid acidification of the vesicle interior when the pH of interior volume of the vesicles was originally 8.0 and that of the nitrite solution mixed in a stopped-flow mixer with the vesicles was 7.0. The dependence of the initial rate of nitrite-induced acidification on pH closely paralleled that of the concentration of nitrous acid. As the pH was lowered from 8 to 5.5, with a constant intravesicular pH of 8.0, the rate of acidification rose dramatically. The pKa of nitrous acid is 3.3 at 25 C. Vesicles made of soybean lipids gave results quite similar to those obtained with IEM vesicles. These results make it likely that nitrate uptake can take place by diffusion of nitrous acid. Also, the rate of nitrous acid transport was faster than that of nitrite reduction in illuminated chloroplasts. These results do not exclude the possibility that the IEM contains a high affinity nitrite transporter.

Interestingly, erythrocyte ghosts are poorly permeable to nitrous acid, perhaps because of the high cholesterol content of the membrane. Nevertheless, nitrite must cross the red blood cell membrane since nitrite can oxidize hemoglobin to methemoglobin. The fluorescence of the compound, 6-methoxy-*N*-(3-sulfopropyl) quinolinium (SPQ), is quenched by chloride. Dr. Shingles, together with Michael Roh, an undergraduate, found that nitrite was even more effective than chloride as a quencher of SPQ fluorescence, but bicarbonate is ineffective. Red cell ghosts were loaded with SPQ and with either KCl or KNO_2 by extrusion. The loaded membranes were then mixed in the stopped-flow fluorometer with $KHCO_3$. Bicarbonate and chloride are substrates of the anion exchange transporter. Rapid increases in the fluorescence of the trapped SPQ upon addition of bicarbonate were seen in ghosts that also contained either chloride or nitrite. Treatment of the membranes with *N*-ethylmaleimide, an inhibitor of the exchanger, abolished the change in fluorescence. Thus, nitrite likely enters erythrocytes by heteroexchange with anions. Unfortunately, SPQ cannot be used to measure nitrite transport in IEM vesicles. The fluorescence of SPQ is quenched by the inner filter effect caused by carotenoids in the membrane.

Dr. James V. Moroney, Professor of Biology at Louisiana State University, joined the lab for the better part of a semester as a sabbatical visitor. Dr. Moroney worked on the adaptation of the alga, *Chlamydomonas reinhardtii* to growth at low concentrations of CO_2 and was interested in carbonic anhydrase activity and the possibility that the IEM from adapted cells might have a bicarbonate transporter. He and Dr. Shingles developed a sensitive assay of carbonic anhydrase activity based on the change in pH detected by pyranine. Stopped-flow mixing was used to initiate the reaction. Carbonic anhydrase and pyranine could be trapped in vesicles and the transport of carbon dioxide and the effects of carbonic anhydrase on both sides of the membrane on CO_2 flux determined. Acidification of pyranine trapped inside vesicles (IEM, red cell ghosts or soybean lipid) was most rapid when carbonic anhydrase was present on both sides of the membrane. These observations are consistent with hydration being a rate-limiting step in CO_2 flux across membranes.

The transport of Ca^{2+} and regulation of Ca^{2+} concentration in chloroplasts has received relatively little attention, despite the fact that changes in Ca^{2+} levels likely regulate enzymes of the reductive photosynthetic carbon cycle and NAD^+ kinase. Michael Roh, Michael Cleveland, a graduate student and Dr. Shingles used fura-2 fluorescence to monitor Ca^{2+} transport in vesicles prepared from soybean lipids or from IEMs. The soybean lipid vesicles showed no decrease in the fluorescence of fura-2 trapped inside the vesicles for 30 s after addition of Ca^{2+} by stopped-flow mixing. If, however, ionomycin, a Ca^{2+} ionophore were present, the addition of Ca^{2+} causes a rapid quenching of fura-2 fluorescence. Thus, as expected, the diffusion of Ca^{2+} across the lipid bilayer is slow. With IEM vesicles, a fluorescence decrease was seen when Ca^{2+} was added in the absence of ionomycin. When the pH on both sides of the IEM vesicles was maintained at 8.0, the initial rate of Ca^{2+} transport was stimulated more than two-fold by a K^+ diffusion potential of 16 mV. The potential-stimulated Ca^{2+} transport was strongly inhibited by ruthenium red, an inhibitor of the mitochondrial Ca^{2+} uniporter.

Thus, Ca^{2+} transport in chloroplast by a potential-sensitive uniporter is likely. Although there is no direct evidence for other mechanisms of Ca^{2+} transport in chloroplasts, a protein similar to a Ca^{2+}-translocating ATPase is present in the IEM.

Ferredoxin, iron-sulfur proteins and cytochromes, all components of the chloroplast electron transport chain, contain iron. Iron is also required for the biosynthesis of chlorophyll. Only recently, however, has iron transport by IEM been studied. When the probe, Phen Green SK, binds Fe^{2+} its fluorescence is dramatically quenched. Phen Green SK fluorescence is much less sensitive to Cu^{2+} or Zn^{2+}. Richard Shingles and Marisa North, an undergraduate, developed an assay for Fe^{2+} transport based on the trapping of the impermeant probe inside vesicles and stopped-flow fluorescence. Vesicles containing Phen Green SK were prepared from either soybean lipids or IEMs. When the vesicles were rapidly mixed with a solution that contained 5μM Fe^{2+}, little change in the fluorescence of the probe was seen in the soybean lipid vesicles, but substantial quenching was detected in the IEM vesicles. The quenching had a half-time of about 0.5 s. Calibration of the response allowed calculation of the amounts of Fe^{2+} transported. In IEM vesicles with 5 μM Fe^{2+}, the rate of transport was 3 nmol/min/mg protein, much lower than that of Ca^{2+} (9 μmol/min/mg protein).

Dr. Shingles and Marisa North then studied Fe^{2+} transport by IEM vesicles in more detail. The dependence of the rate of transport on Fe^{2+} concentration was hyperbolic, with half-maximal transport at 2 μM Fe^{2+}. A K^+-diffusion potential stimulated the initial rate of Fe^{2+} transport by more than 100%. When tested at the same concentration as the Fe^{2+} (5 μM), Cd^{2+}, Zn^{2+}, Cu^{2+} and Mn^{2+} significantly inhibited Fe^{2+} transport. The inhibition by Zn^{2+} was shown to be competitive with respect to Fe^{2+}. Fe^{2+} transport was not inhibited by diltiazem and, thus, the Fe^{2+} transporter and the Ca^{2+} transporter are like to be different proteins. The inhibition of Fe^{2+} transport by other cations could indicate that the transporter is a general divalent cation transporter, a possibility that is under active investigation.

In addition to the new information on transport by IEM, the DOE-funded research contributed methods that are useful to those who work on biological membranes and membrane transport in general. For example, procedures for the preparation of membrane vesicles capable of transport that are either mostly inside out or mostly right side out by simple methods were developed as part of the DOE-funded project. Also, the methods we have refined for the study of the transport of ions by IEM vesicles are applicable to other membranes, as we have shown in the case of nitrite transport by erythrocyte ghosts and for the flux of CO_2 across membranes. Novatek has express an interest in the carbonic anhydrase activity assay. Finally, Hopkins has very talented undergraduate students and several have worked on this project. Michael Roh, who was a coauthor of three research papers (and first author on one), will finish his Ph.D. at the University of Michigan this year and will complete his medical studies two years hence. He is headed for a career in academic medicine in which he will excel. Dr. Shingles deserves praise for the way in which he guides students who work with him and for the enthusiasm for experimental science he imparts.

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