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on the project

*"The Bioenergetics of Salt Tolerance"*Research Goals

The aim of this project was to try to understand the adaptive mechanisms that organisms develop in order to respond to a sudden transformation in their environment to a "salt shock." To study this problem we used a fresh water oxygenic photosynthetic cyanobacterium known as *Synechococcus* 6311. This organism suffers injury after this sudden exposure to high concentrations of sodium chloride equivalent to or even higher than that in sea water. Yet they are able to re-establish their photosynthetic activity which is partially injured and return to virtually normal growth rates. Identification of the temporal sequence of changes involved in adaptation to this stress was the rationale. Indeed this project employed a wide variety of biochemical and biophysical methods, including electron spin resonance techniques and nuclear magnetic resonance to study the bioenergetics and transport mechanisms, growth and energy changes in these organisms and how the structural components of the cells changed in response to adaptation to growth at high salinity.

The problem has relevance for higher plants because most of the arable farmland in the world is already under use and that which is not used is usually in saline environments. Hence, understanding basic mechanisms of salt tolerance is a fundamental biological problem with great applications for bioproductivity and agriculture.

The findings of our studies were as follows:

PROGRESS REPORT

Introduction

This review of our progress will cover work we have carried out over the last five years on the bioenergetics of stress responses in cyanobacteria.

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Synopsis

Understanding the mechanisms whereby organisms respond and adapt to stress is fundamental problem in biological systems. In particular, the response of photosynthetic organisms to salt stress has special relevance and importance because of the problem of salinity and plant productivity. In developed parts of the world, intense agricultural practices and irrigation cause salinity problems, whereas in the underdeveloped parts of the world, the land available for agriculture is often saline. Our laboratory has been using the fresh water cyanobacterium Synechococcus 6311 as a model system to study problems in this area. We expose the cells of this organism, grown in a low salt (control) growth medium containing about 15 mM sodium, to 0.50 M NaCl, an amount of salt slightly greater than that found in sea water. As expected, this type of change in culture conditions will lead to many cellular changes. The areas in which our studies have shown salt stress responses are as follows:

- *Bioenergetics (photosynthesis and dark respiration)
- *Permeability and ion transport
- *Osmoregulation
- *Gene Expression
- *Cell growth and organization

In our studies over the last few years, we have sought to identify the temporal sequence of the events that comprise the response to stress. Such information is needed to develop concepts concerning the mechanisms of inhibition of cell growth and how changes in gene expression permit the organisms to adapt to growth under drastically different ionic conditions, by accumulating the necessary osmoregulants, balancing the energetic demands of the cells for growth under harsh conditions, and eventually developing tolerance allowing growth under stress. It is also important to investigate deadaptation in order to separate the phenomenon of cell adaptation from the selection of particularly stress-resistant mutant cells in culture. A range of different techniques has been employed in an attempt to gain this type of information. Our experimental strategy has mainly emphasized the use of non-invasive methods and confining experimentation to the actual growth medium. Now, we are beginning to form a more comprehensive picture of the time dependence of the responses of the organism to salt stress. And, in preparing for this progress report, we succinctly summarize below the status of our knowledge of the specific responses of this freshwater cyanobacterium to growth in high salt.

Very early responses to salt shock - milliseconds to minutes. An immediate decrease followed by an increase in the internal aqueous volume was observed using ESR methods pioneered by Rolf Mehlhorn. Typically, the internal aqueous volume can be determined by the ESR signal of a small, membrane permeable nitroxide. The addition of a membrane impermeable paramagnetic broadening agent prior to measurement enables the visualization of only the extracellular signal of the nitroxide, so that the cell volume can be calculated accurately and quickly from the internal probe concentration as a fraction of the total probe concentration. Shrinkage is followed by cell swelling as sodium and presumably chloride enter.

Using NMR techniques where a special wide bore sample holder was equipped with 9 fiber optical light pipes and tubes for gas exchange to both illuminate or control gas exchange in the samples, we were able to study rapid changes in internal sodium and ATP levels. ^{23}Na -NMR experiments were made in the presence of a membrane impermeable dysprosium salt to chemically shift the sodium signal outside of the cells. Sodium entry is, accompanied by proton extrusion. This was first shown by Blumwald et al. () using 9-aminoacridine as a fluorescent pH sensitive probe. Photosynthetic O_2 evolution (polarographic and $^{18}\text{O}_2$ exchange studies) shows a transient but severe inhibition. Thus, it was not unexpected that we found, by ^{31}P -NMR, that ATP levels immediately fall after salt shock, and that the P_i/ATP ratio increases.

The inhibition of O_2 evolution is still a mystery although we know some details about the lesion in photosynthesis. One idea entertained by us was that uncoupling of the membrane-associated components could occur as a result of the high salt, causing a disassociation of phycobilisomes from thylakoid membranes. Indeed, we found (with Guy Dubertret) a decrease in fluorescence energy transfer from phycobiliproteins with increasing salinity. However, this is a transient event and does not account for the large and sustained inhibition of O_2 evolution. The transport system for nitrate uptake, has also been found (by I. Fry) to be inhibited. These early changes after salt shock indicate that the energetic system of the cell has been drastically affected.

In the subsequent 1-5 minutes after salt exposure, we begin to observe the early responses of the cells to salt adaptation. Photosynthesis is still decreased but dark respiration begins to increase. Then ATP levels begin to recover and the internal sodium concentration, which was at a very high level, begins to fall. Changes in membrane fatty acid composition begin. Also, at this time, a remarkable affect is observed by conventional thin section electron microscopy. The major granular accumulation products, such as glycogen and carboxysomes, which are conspicuous components in the electron micrographs of the cyanobacterium, disappear. Also, the density of the cytoplasm decreases and the DNA becomes more smooth in appearance. These astounding changes in cell structure observed one minute after salt exposure have not yet been explained. About one hour after salt exposure, glycogen in the cell begins to accumulate. We have shown this in by growing cells in ^{13}C bicarbonate and observing glycogen accumulation by ^{13}C -NMR, and also by chemical extraction of the glycogen. At this time period growth remains inhibited.

Afterward, in the time period between one to five hours, we believe that some of the key events occur which allow cells to recover and adapt to the salt shock. These include the beginning of the recovery of O_2 evolution, the continued increase of dark respiration activity, the accumulation of osmoregulatory substances, and the continuing decrease in the internal sodium concentration. The granular inclusions begin to reappear, particularly glycogen and carboxysomes, as seen by thin section electron microscopy. Freeze fracture electron microscopy reveal remarkable changes in the structure of the cytoplasmic membrane. Decreases in total number of particles and the preferential disappearance of small particles with a concomitant appearance of larger particles were found. Studies with flow cytometry showed that the changes in particle number were not accounted for by changes in the cytoplasmic membrane surface, which does not differ in salt as compared to control grown cells.

In the following period between 24 to 48 hours photosynthetic O_2 evolution returns to normal. The dark respiratory rate is much higher as shown by O_2 consumption, low temperature EPR Cu^{+2} signal and cytochrome c oxidation. Bioenergetic studies by W. Nitschmann showed that this dark respiration makes a greater contribution to the proton electrochemical potential than ATPase. In addition to cytochrome oxidase, components involved in NADPH oxidation to cytochrome c also show large increases in activity. A normal pH difference between the cytoplasm and the thylakoid compartment is reestablished during the adaptation stage. This was identified by novel ESR spin labeling methods in which Belkin, Mehlhorn and Nitschmann used 5-membered nitroxides which are weak acids and bases (reductants but are not readily reduced by one electron reactions with cell or electron transport. Their partitioning in the cell under various conditions of illumination and oxygen concentrations allowed us to follow changes in external pH, pH of the cytoplasmic and thylakoid compartments under these conditions. Intracellular sodium falls to its lowest level, 35 mM, which is seven times higher than control cells but, nevertheless, much lower than the amount initially intruded into the cells in the salt shock period. Osmoregulation seems to be completely accounted for by the accumulation of soluble sugars, potassium and sodium.

Marked changes in the cytoplasmic membrane are observed in the adaptation stage. These include changes in particle density and, especially, the accumulation of large particles which represent 60% of the total number of particles, a 3-fold increase over control cells. Also, other studies (Gunter A.

Peschek laboratory, Vienna) show marked changes in the polypeptide patterns. Studies by M. Huflejt in collaboration with the A. Tremolieres laboratory (Gif) have found specific increases in long chain fatty acids. G. Khomutov also showed with ESR methods measuring fluidity and order parameters of isolated cytoplasmic membranes that a decrease in membrane fluidity accompanies adaptation. The accumulation of glycogen which has occurred in massive amounts is strikingly seen by electron microscopy to be preferentially localized in the cell periphery in the space between the cytoplasmic and thylakoid membranes. At this stage, the growth rate has markedly improved.

Thus, remarkable changes occur in cells that become tolerant to growth at high salt. But the question whether these changes are reversible remains. Deadaptation studies made with cells that have been transferred back to a low salt medium, exhibit no change in O₂ evolution after 24 hours, but dark respiration rate returns to control levels. Also, the fatty acid composition begins to return to control values, so the changes in fatty acid composition occur much more slowly in deadaptation than in adaptation. Glycogen content also falls again to control levels.

Universal vs. specific stress responses - Although we have primarily focused the characterization of the responses of cyanobacteria to saline shock, we are also interested in the question of which of the responses to stress are universal and which are specific to the type of stress applied. In this regard, we have studied two other types of stress. In one series of experiments, the growth temperature at 40°C was suddenly changed to 20°C. This is followed by a decreased growth rate, a large increase in dark respiration, and a massive glycogen accumulation. Similarly, if the selenium to sulfur ratio is increased, growth is inhibited. Later, a large increase in respiration and a massive accumulation of glycogen occurs again. Changing the selenium to sulfur ratio back to normal causes the dark respiration and glycogen content to return to control levels, and the selenium to be eliminated from the cells. Studies in other laboratories (L. Sherman, and C. Wenner) suggest that, in addition to these kinds of responses to stress, another universal aspect of stress responses is nutrient deprivation.

Conclusions - Cyanobacteria such as Synechococcus 6311 consist of two distinct membrane systems, the cytoplasmic and the thylakoid membrane. The thylakoid contains the photosynthetic apparatus and, depending on the growth conditions and age of the culture, a portion of the respiratory electron transport components. The cytoplasmic membrane is the primary barrier or interface with the environment and the site of the various transport systems for nutrient assimilation, containing the majority of the respiratory electron transport activity. Although, historically, respiration has been considered to play a minor role in cyanobacterial bioenergetics, our studies show, however, that under "stress" situations a major portion of the trans (cytoplasmic) membrane pH gradient is redox potential driven by components of the respiratory electron transport pathway.

The importance of respiratory electron transport becomes apparent under stress when the cell requires energy for ion transport and biosynthesis. Under these conditions the cell increases up to 10 times the level of cytochrome-c-oxidase, as determined by the characteristic Cu²⁺ EPR signals. The oxidase is also a proton pump and the stress-increased activity is located presumably mainly in cytoplasmic membrane, where it contributes to the establishment of a new proton motive force equilibrium.

As a result of this DOE funded research program, a large number of publications in peer review journals and elsewhere appeared. A list is appended below. In addition, the Principal Investigator was co-organizer of a number of workshops on cyanobacteria that were sponsored by the European Molecular Biology Organization, four of them to be precise. In addition, together with a colleague on the Berkeley campus, the Principal Investigator co-edited a volume of Methods in Enzymology on "Cyanobacteria" Volume 148. This recent book is considered to be the "Bible" of the current methodology and state of the art in this research field.

CYANOBACTERIAL PUBLICATIONS:

Articles resulting from this grant

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Respectfully submitted,

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