Special Research Project

The experiment in which we observed the ability of β–galactosidase and alkaline phosphatase (AP) to catalyze their enzymatic activities on a native PAGE gel provides us with a convenient platform from which to ask an important question. Is the loss of AP activity the result of general enzyme denaturation or due to localized changes in enzyme structure induced by disulfide bond reduction?

Before considering AP further, a brief look at the legendary experiments performed by Christian Anfinsen on the denaturation and refolding of ribonuclease A in the 1950's (which led to a Nobel Prize) might provide us with some insights. A thorough explanation of Anfinsen's experiments is outlined in Lehninger Principles of Biochemistry (pps. 140 – 142), which you are advised to read. These landmark experiments provided strong evidence for the notion that the folded, or tertiary structure, of a protein is primarily determined by the amino acid sequence and the proclivity those amino acids have to form secondary structures, which are believed to act as a nucleation sites along the folding pathway (see pps. 142 – 143 in Lehninger) for collapse of the protein to its native tertiary structure. We must be careful, however, to realize that in Ainfensen's experiments an important procedure they carried out involved the inclusion of urea as a general protein denaturant, which in our experiment was not included.

The Special Research Project will be an attempt to answer the question posed above. In this project, each group will choose an experiment from the list below OR design their experiment in an attempt to provide some insight to the question we have asked. Each group will be required to decide which experiment they will perform by the last week of October, the experiments will be done on Tuesday, Nov. 17 and Thursday, Nov. 19, which gives ample time to become familiar with the experimental method and technique. The results of your experiments will be presented to class on Tuesday, November 24. It will be the responsibility of each group to become familiar with the experimental techniques for their project and design the experiment they will perform, with consultation with Dr. Hazzard, the TA's, and Dr. Chad Park (CD and fluorescence). Some experiments will require you to work in labs in BSW where that instrumentation is located for those two days.

The projects listed below are posed as questions the answers to which will come out of the investigations.

1. Can full enzyme activity be restored by removal of BME by dialysis? Is there a difference between simple dialysis using molecular oxygen as a sulfhydryl oxidizing agent or is greater activity restored in the presence of Cu^{2+}? The technique most useful for this would be enzyme activity assays using visible spectroscopy.

2. How is the structure of AP affected by disulfide bond reduction? This effect can be monitored by several techniques:
   a) changes in secondary structure determined by circular dichroism spectroscopy.
   b) changes in fluorescence of Trp residues.
   c) is there a general "unfolding" of the enzyme, or exposure of more hydrophobic regions of the enzyme based on the fluorescence of ANS.

3. A third set of experiments that are not related to the disulfide bond reduction is based upon another question. Is K_m the same as K_d for the dissociation of a substrate from an enzyme? Steady state kinetics provides a means to determine K_m for an enzyme and its substrate. However measuring K_d for that enzyme and substrate is difficult because of catalysis. In order to make this determination, it is necessary to render the enzyme catalytically inactive without interfering with substrate binding and dissociation. For Mg^{2+} containing enzymes it is possible to remove the metal using EDTA (as we will see). The Mg^{2+} can sometimes be replaced with Ca^{2+} without restoring enzymatic activity. Substrate binding does occur. Thus, by this method it might be possible to differentiate between K_m and K_d using a phosphorylated fluorescent substrate that undergoes an increase in fluorescence as a result of dephosphorylation by AP catalysis for determination of K_m and the change of anisotropy for that same substrate binding to the catalytically inactive enzyme for K_d determination.

4. A final set of experiments will involve doing mass spectrometry on the AP purified in class. We will determine which sample will assayed based on two criteria: (1) highest specific activity and (2) greatest purity based on SDS-PAGE gel analysis. For this experiment, we will take the fraction meeting the above criteria and carry out a tryptic digest of the sample, which will then be submitted to the Mass Spec facility in Old Chemistry for tandem mass spectrometry on the ESI-Ion Trap instrument.

In addition to the knowledge we gain about AP from these experiments, a significant purpose of this Project is instill in the course a more research based experience than one gets following well worked out experimental protocols, often called “cookbook” exercises. Frankly, we have no idea what the results of these experiments will be, but then that is the nature of science!