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Direct Observation of Intracellular Materials Using a Phase Contrast Transmission Electron Microscope (TEM).

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Intracellular materials can be visible with microscope once are labeled by specific markers. To observe biological specimens using the TEM, also it is requested to prepare samples with steps of fixation, dehydration, resin embedding, sectioning and staining and staining by specific markers. Their markers have many varieties include, heavy metal agents and immune or enzyme reactions. In most of these methods, chemical fixatives are necessary because of the difficulty for agents to penetrate into the cell. This procedure has, however, been introducing serious artifacts. The phase contrast TEM has been developed to solve this problem in our laboratory (1). It has been possible to observe biological objects without staining in the ice embedded state of whole cells (2). Phase contrast TEM suited with unstained and thick samples. We expect that the intracellular materials can directly observe intracellular materials with high resolution with phase contrast TEM without special treatment. As the first step, we have tried to observe inorganic heavy particles in or on biological specimens, which will be extended in future for the specific protein labeling together with the immunostaining method.(1)Danev and Nagayama (2002) J. Biol. Phys. 28: 627-635(2) Kaneko et al. (2005) J. Electron Microsc. 54: 79-84

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Light scattering can detect the thermal-dependent conformational changes of proteins.

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It is well known that the strucutre and function of protein is highly related each other. Proteins change their conformation or structure acording to temperature. We proposed here that temperature-dependent dynamic conformational changes of proteins can be measured by light scattering technology. Dynamic light scattering (DLS) can detect the difference of hydrodynamic diameter which directly reflects the structural information of the target. The advantage of light scattering technique is its high sensitivity, so that we can obtain information on the structural changes of proteins at very low volume and concentration. We will present here the comparison of DLS with differential scanning carolimetry (DSC) on the structural changes of lysozyme. Futhermore, DLS can be applied for the measurement of the affinity of the carbohydrate binding protein, Lectin, with monosaccharides.

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Nanosecond responses of proteins to ultra-high temperature pulses

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Observations of fast unfolding events in proteins are typically restricted to temperatures below 100 °C. We use a novel apparatus based on a thin gold film and pulsed laser to heat and cool enzymes within tens of nanoseconds to temperatures well in excess of the boiling point, without direct exposure to laser light. Thermal modelling and temperature measurements show temperature relaxation on a 40 nanosecond timescale. Temperature is measured with nanosecond resolution using a low intensity laser beam that monitors the reflectance of the gold film. The temperature spikes are too fast to allow water to boil but can affect protein function. Spikes of 174 °C for catalase and around 290 °C for horseradish peroxidase were required to produce irreversible loss of enzyme activity. Similar temperature spikes have no effect when restricted to 100 °C or below. The inactivation rate at high temperature is consistent with extrapolation of low temperature rates over twelve orders of magnitude using the Arrhenius relation. A similar system could be used to deliver thermal pulses with duration from nano- to milli- seconds to any macromolecule which can be localized near a metal surface.

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Construction of selenomethionyl protein expression system by *Pichia pastoris* for the protein crystallography.

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In protein crystallography, the protein whose methionine is replaced with selenomethionine (SeMet) is useful for the phase determination by the multiwavelength anomalous diffraction (MAD) and the trace of the polypeptide chain. Recently, the expression of selenomethionyl protein has been routinely done in *Escherichia coli*, however, in which many eukaryotic proteins cannot be expressed, if any, the proteins often lack their activities. On the other hand, yeasts have also been used for the recombinant protein expression, especially for the eukaryotic proteins. Until now, there are few reports on the SeMet incorporation by yeasts. Here, we attempt to construct an expression system of selenomethionyl proteins in methylotrophic yeast *Pichia pastoris*. As it is known that SeMet is highly toxic to cells, we first obtained SeMet-resistant *P. pastoris* mutants and expressed a human lysozyme, which has two methionine residues, in the mutants with SeMet containing media. Mass spectrometry of trypsin-digested recombinant human lysozyme and X-ray fluorescence analysis of crystal revealed that the methionine residues in the recombinant protein were partially replaced with SeMet. These results indicated that the mutants did not detoxify SeMet but can transfer the SeMet to protein.