S1f1-3

Development of a Time-Resolved Infrared Spectrophotometer Applicable to Enzyme Solutions

O Satoru Yamaguchi, Michihiko Aki, Yoko Kusumi, Kyoko Shinzawa-Itoh, Shinya Yoshikawa, Takashi Ogura
The Graduate School of Life Science, University of Hyogo

Infrared spectroscopy of enzymes is essential to elucidate their reaction mechanisms, since the vibrational spectra sensitively reflect the reactivity of amino acid residues. Time-resolved IR (TRIR) spectroscopy gives dynamical structure of the reaction intermediates. However, TRIR spectrophotometer using a flow cell applicable to any kind of enzyme has not been developed, due mainly to the high background of water and protein itself. In the present study, we have developed a novel TRIR spectrophotometer, using a multi-channel MCT detector and a CaF2 flow cell. As a test of the system, we have successfully recorded TRIR spectra of the recombination dynamics of CO of carbonmonoxy cytochrome c oxidase (CCO) at Δt after photolysis between 0 and 40 ms through observation of the vCO mode. In addition, we have succeeded in observing the carboxylate signal of Asp51 of CCO, which is a key residue in proton pumping. Thus, a methodology of TRIR spectroscopy for amino acid residue in proton pumping protein is now established.

S1f1-4

Cell-free synthesis of functional Paracoccus denitrificans cytochrome c oxidase

O Yukie Katayama1,2, Takashi Ogura1, Shimokata Kunitoshi1,2, Sae Matsuo Makoto1, Tomitake Tsukihara1, Shiya Yoshikawa1, Hideo Shimada1
1 Dept. of Biochem., Sch. of Med., Keio Univ., 2IBIC, 2Dept. of Life Sci., Univ. of Hyogo, 3Inst. for Protein Res., Osaka Univ.

Cytochrome c oxidase, the terminal oxidase of the cellular respiration, catalyzes the reduction of dioxygen to water coupled to proton pumping across the membrane, generating proton motive force that drives ATP synthesis. For elucidation of the proton-pumping mechanism, changes in protonation state and polarity of each amino acid driving the process must be followed by time-resolved infrared spectroscopy. For assignment of the amino acid residue, it is prerequisite to site-specifically label the residues with cell-free protein synthesis. Subunits I, II and III of the Paracoccus denitrificans enzyme were synthesized in the E. coli cell-free system supplemented with E. coli membrane fractions, heme A and Cu2SO4, the latter two for the redox center formation. Blue native-PAGE and following SDS-PAGE of dodecylmaltoside solubilized 15S-labeled proteins revealed a protein complex with a normal subunit stoichiometry. MonoQ column chromatographic analyses of the synthesized proteins demonstrated that the fraction at the elution volume of the authentic enzyme contained the three subunits and exhibited ferrocyanochrome c oxidation activity comparable to that of the native enzyme and a normal absorption spectrum, indicating successful synthesis of the three-subunit complex structurally and enzymatically almost indistinguishable from the native one.

S1f1-5

Infrared Spectroscopic Analyses of calcium-binding protein structure

O Masayuki Nara

Troponin C (TnC) is the Ca2+-binding regulatory protein of the troponin complex in striated muscle tissue. Vertebrate fast skeletal muscle TnCs bind four Ca2+, while Akazara scallop (Chlamys ninponensis akazara) striated adductor muscle TnC binds only one Ca2+ at Site IV, because of the other EF-hand motifs are short of critical residues for the coordination of Ca2+. In the present study, Fourier-transform infrared spectroscopy (FT-IR) was applied to examine coordination structures of Akazara scallop TnC and site-directed mutants (E142Q, E142D) with an inactivated site IV in D2O solution. The region of the COO' antisymmetric stretch provides information about the coordination modes of a COO' group to a metal ion. The wild type showed a band at 1543 cm⁻¹ in the Ca2+-bound state, indicating that the side-chain COO' group of Glu142 (the position 12 of Site IV) serves as the ligand for Ca2+ in the bidentate coordination mode. On the other hand, the E142Q and E142D mutants showed no band around 1543 cm⁻¹ in the Ca2+-loaded state, suggesting that the shortage of a methylene group is critical for the Ca2+ coordination structure of TnC. The metal-ligand interaction at Site IV is discussed in comparison with synthetic peptide analogues of Site IV of Akazara scallop TnC and those of Site III of rabbit skeletal muscle TnC.

S1f1-6

New Developments of Resonance Raman Spectroscopy in Applications to Photolabile Protein Species

O Takashi Ogura1, Wataru Hashizume1, Kenichiro Ikenura1, Masahiro Mukai2, Hideo Shimada1, Kyoko Shinzawa-Itoh1, Shiya Yoshikawa1

1. Fiber Optic Device: In the application of resonance Raman spectroscopy to photolabile species, we have to minimize the photon flux. For that purpose, a spinning cell or a flow cell system is widely used. However, such systems are not applicable to protein crystals. We have adopted a special fused optical fiber with which we could obtain resonance Raman spectra of the fully reduced and CO-bound cytochrome c oxidase in the crystalline state.

2. Red Excitation: We have succeeded in observing the V500 mode of compound II of peroxidase from horseradish and Arthromyces ramosus, the P intermediate of cytochrome c oxidase from bovine heart and Paracoccus denitrificans, and ferryl myoglobin upon 590 nm excitation. We have measured an excitation profile of the mode between 400 and 650 nm for horseradish enzyme. It has two peaks. One is near 400 nm as reported and another near 580 nm. We found that the intensity of the V500 mode relative to those of porphyrin modes are significantly higher upon red excitation. Moreover, red light causes less damage on the sample than blue light does. In conclusion, we demonstrate that red-excited resonance Raman spectroscopy is a general and powerful tool to study the structure of highly oxidized heme enzymes.