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Characterization of the Active Site of Ascorbyl Free Radical Reductase Purified from *Pleurotus ostreatus*

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Ascorbyl free radical reductase was purified from the white rot fungus, *Pleurotus ostreatus*. The enzyme contained FMN as a prosthetic group, which was reduced by NADH and reoxidized by ascorbyl free radical. Reduction of ascorbyl free radical by the enzyme was observed by EPR spectroscopy. Addition of NADH to the enzyme caused the formation of flavin semiquinone of a red anionic form with line width of 15 G. EPR signal of flavin semiquinone disappeared at room temperature and showed power saturation behaviour at liquid nitrogen. The catalytic mechanism of this enzyme was ping-pong type. The second-order rate constants of the enzyme towards NADH and ascorbyl free radical were estimated to be 2.9×10^7 and 7.2×10^8 $M^{-1}s^{-1}$ from Dalziel equation, respectively. The enzyme had 1 mol thiol group/mol of subunit in the active site and pK_a value of thiol group was determined to be 6.9 by the chemical modification study with thiol reagents. Thiol reagent-treated enzyme was not reduced by NADH, indicating that thiol group is involved in the electron transfer from NADH to FMN. On the basis of the changes of NADH fluorescence intensities, lysine and arginine were thought to participate in the interaction with NADH and ascorbyl free radical. The hypothesis on the electron flow in the active site of the enzyme was proposed.