Intramolecular Electron Transfer in a Novel Cytochrome P450cam System with a Site-Specific Branched Structure

Hidehiko Hirakawa, Department of Chemistry, The University of Kansas, 1251 Wescoe Hall Drive, Lawrence, KS 66045, **Noriho Kamiya**, Applied Chemistry & Center for Future Chemistry, Kyushu University, 744 Motooka, Fukuoka, 819-0395, Japan, **Tsutomu Tanaka**, Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan and Teruyuki Nagamune, Department of Bioengineering, School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

Cytochrome P450s are heme containing monooxygenases involved in various physiological processes from archaea to mammals. They catalyze diverse types of reactions containing hydroxylations, epoxidations, dealkylations and heteroatom oxidations, which make P450s attractive industrial catalysts. P450s require electrons from NAD(P)H to convert molecular oxygen into reactive species. However, excess amounts of electron transfer partner proteins are necessary to obtain sufficient reaction rates and coupling efficiencies and these properties are bottlenecks for practical applications of P450s.

The P450 from *Pseudomonas putida* (P450cam) that catalyzes stereoselective hydroxylation of *d*-camphor is a well-studied cytochrome P450. P450cam requires two electron transfer partner proteins, putidaredoxin (Pdx) and putidaredoxin reductase (Pdr), for the catalysis. Pdr accepts two electrons from NADH, and Pdx transports one electron at a time from Pdr to P450cam. Although one P450cam only requires one molecule each of Pdx and Pdr stoichiometrically, excess amounts, i.e., noncatalytic amounts of Pdx and Pdr are required because the reduced Pdx acts as a substrate for P450cam.

A fusion protein composed of these three component proteins could achieve an extremely high local concentration, and also bring a high catalytic activity owing to the increase in the effective concentration. Furthermore, this could bring a high coupling efficiency because catalytic amounts of Pdx and Pdr minimize the reoxidation of

these proteins by molecular oxygen as well. In general, fusion proteins can be prepared by (i) a genetic method, in which the component proteins are genetically linked and expressed as a single polypeptide chain, and (ii) a chemical method, in which the component proteins are cross-linked by chemical reagents after individual expression of each protein. To date, the former method has not yet provided sufficiently active fusion proteins due to the structural constraint, while the latter method is hard to control the cross-linking sites in general.

To construct a new type of P450 fusion protein we utilized an enzymatic method involving a transglutaminase (TGase). TGases catalyze the formation of an ε-(γ-glutamyl)lysine bond between the side chains of a glutamine residue and a lysine residue. The TGase from *Streptomyces mobaraensis*, which is widely used in food industry, has been reported to recognize apo-myoglobin at the sequence around the F-helix of horse heart myoglobin (HEAELKPLAQSHATKHKIPIK) and catalyze site-specific cross-linking of tethered proteins with tag peptides derived from the F-helix under protein-friendly conditions. Such TGase-mediated site-specific protein cross-linking enables the formation of a branched fusion protein with spatially equal geometry of the three component proteins, which should result in less structural constraint than a tandem linear fusion protein, as well as an intramolecular electron transfer.

Here, we show a site-specific branched fusion protein of P450 with its electrons transfer proteins constructed using enzymatic cross-linking with transglutaminase. A branched fusion protein of P450 system, which was composed of one molecule each of P450cam, Pdx and Pdr, showed higher catalytic activity (306 min^{-1}) and coupling efficiency (99 %) than the reconstitution system due to the intramolecular electron transfer. Many substrates for P450s are highly hydrophobic and P450-catalyzed reactions are preferable to be conducted in noaqueous media. The regenaration of NADH is also required due to its cost. We finally examined the hydrixylation of *d*-camphor by the fusion protein using NADH-regeneration driven by a hyper thermostable alcohol dehydrogenase in a reversed micella system. The hydroxylation of *d*-camphor and the regenetion of NADH were obserbed even in a reversed micellar system, while those were not obserbed using the reconstitution system.

This unique site-specific branched structure simply increased local concentration of proteins without serious loss of freedoms and activities of each protein. Therefore, enzymatic posttranslational protein manipulation can be a powerful alternative to

conventional strategies for the creation of multi-component enzyme systems with novel proteinaceous architecture.