structure as well as some protonation states of specific residues of the protein [1] and the far-IR domain (500–50 cm^{-1}) on the metal–ligand vibrations of the cofactors and the hydrogen bonding network [2–4]. When Infrared spectroscopy is coupled to electrochemistry, changes in secondary structure, protonation states of residues and metal ligand vibrations induced by the redox reaction can be monitored. The immobilization of proteins on rough metal surfaces leads to an enhancement of the intensity of the IR vibrational peaks (SEIRA effect) [5–8]. The magnitude of the enhancement depends on the topography of the metal surface as well as on the nature of the interaction between the protein and the surface. Covalent immobilization is usually more efficient than physisorption. We will describe the immobilization of complex I and QFR from *E. coli* on thin layers of gold sputtered on a silicon crystal and the reconstitution of the lipid bilayer. We have studied the effect of different types of lipids. All the immobilization procedure has been followed by SEIRAS. The redox behavior of these immobilized enzymes will also be reported.

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1P.30 Red complex I — Using cytochrome c_{550} from *Bacillus subtilis* as a fusion domain to study NADH:quinone oxidoreductase

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Complex I (NADH:quinone oxidoreductase) contains four very large membrane spanning protein subunits that hitherto have been difficult to express individually in any appreciable amounts in *Escherichia coli*. The polypeptides contain no prosthetic groups or visual redox pigments and are poorly antigenic. In this work we have constructed fusion proteins where the C-terminal end of complex I protein subunits NuoH, NuoL, NuoM and NuoN from *E. coli* were genetically fused to the cytochrome c domain of *Bacillus subtilis* cytochrome c_{550}. A naturally occurring transmembrane helix anchor was removed from the cytochrome c_{550} and was substituted by the membrane spanning polypeptide to be tagged. To facilitate purification of the expressed proteins, a C-terminal his-tag was added to the protruding cytochrome domain. The fusion proteins were expressed from plasmids in a wild type *E. coli* strain, together with a plasmid containing the operon encoding the *E. coli* cytochrome c maturation (*ccm*) proteins [1], enabling holo-cytochrome c synthesis under aerobic conditions. The rationale behind cytochrome tagging was to be able to monitor the proteins. The heme in cytochrome c is covalently bound to the polypeptide, renders the proteins visible by optical spectroscopy, and can be used to monitor and quantify the proteins, and to determine the orientation of the polypeptides when reconstituted in liposomes. Particularly the three large antiporter-like subunits NuoL, NuoM and NuoN, that previously had been particularly cumbersome to produce in *E. coli*, could be made in unprecedented amounts when expressed with a fused cytochrome c domain. Finally, a gene fragment encoding the NuoN-cytochrome c fusion protein was reintroduced into the *nuo* operon on the *E. coli* chromosome using the recombination plasmid pKOV [2] allowing the production and characterization of cytochrome-tagged whole complex I.

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1P.31 The evolution of respiratory chain complex I from an 11-subunit last common ancestor

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Respiratory chain complex I is a large complex enzyme that has evolved from the combination of smaller functional building blocks. The NuoE and F are part of a family of flavin-containing NADH dehydrogenases, NuoG resembles a Fe-only hydrogenase/formate dehydrogenase, NuoB and D are homologous to NiFe-hydrogenase [1] and NuoKLMN make up an antiporter module homologous to the Mrp antiporter complex [2]. Small membrane-bound hydrogenases consisting of homologues of NuoB, D, H, I and one antiporter-like subunit are also found in nature. The ancestor of complex I did however not resemble these present day small membrane-bound hydrogenases, instead the membrane bound hydrogenases and complex I have a common ancestor. We postulate that this last common ancestor was composed of 11 subunits, homologous to NuoA, B, C, D, H, I, J, K, L, M and N. It was noticed early on that chloroplasts and cyanobacteria contain such a complex I-like protein complex with 11 subunits [3]. We have conducted a detailed survey of the distribution of 11-subunit complex I in the tree of life, using the 656 whole genome sequences currently available in CMR. Notably, 11-subunit complex I are found both in the archeal and the eubacterial kingdoms, whereas the 14-subunit classical complex I is only found in some eubacterial phyla. A complex I-homologous enzyme, F_{420} dehydrogenase, that contains a 12th subunit, FpoF, is found in Euryarchaeota but not in Crenarcheyota that only contain 11-subunit complex I. A convergent evolutionary event has occurred in eubacteria, evident today in the ε-proteo-bacteria such as *Helicobacter* and *Campylobacter* where a NuoG-like 12th subunit has been added to the 11-subunit ancestor. The evolution of classical complex I in the eubacterial lineage has occurred in two steps, first NuoG is recruited to the 11-subunit complex I, followed by a second event, where the NuoE and F subunits are added, forming classical complex I.

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1P.32 Affinity of *Escherichia coli* complex I variants to NADH and NADPH

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The NADH:ubiquinone oxidoreductase (EC 1.6.5.3., complex I) couples the electron transfer from NADH to ubiquinone with a proton translocation across the membrane. It consists of two arms, the peripheral and the membrane arm. The structure of the bacterial complex I showed that the NADH binding site is made up by a novel type Rossman-fold providing binding sites for the FMN as well as for NADH. We solved the structure of the NADH binding site of the *Aquifex aeolicus* enzyme with bound substrates at 2 Å resolution. From the structure it is evident, that the Glu 183 (numbering according to *E. coli* complex I) provides one of the most prominent interactions with NADH by building a hydrogen bond to the hydroxyl group of C2'-ribose. The affinity of the *E. coli* complex I to NADH is 50 times higher than that to NADPH. The reason could be a sterical clash between the C2'-PO_3^{2-} of NADPH and the Glu183-side chain. This prediction is examined by testing the activity of protein variants with mutations at this position. The effect of the mutations on the NAD(P)H/ferricyanide, NAD(P)H/HAR and NAD(P)H:decylubiquinone oxidoreductase activity will be examined.

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1P.33 Characterization of the binding site of Δlac-acetogenin in bovine mitochondrial complex I by photoaffinity labeling

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In the course of wide structural modifications of natural acetogenins, we synthesized "Δlac-acetogenins" by deleting a γ-lactone ring that is a structural feature common to a large number of natural acetogenins. Characterization of the inhibition mechanism of Δlac-acetogenins revealed that they are a new type of complex I inhibitors; for instance, i) the level of superoxide production induced by Δlac-acetogenins is remarkably low and ii) the inhibitory potency for the reverse electron transfer is significantly weaker than that for the forward event [*1, 2*]. Considering the unusual inhibitory effects of Δlac-acetogenins, further exploration of their inhibition manner including identification of the binding site would provide valuable insights into the terminal electron transfer step of complex I. In order to clarify the binding site of Δlac-acetogenins, we here synthesized a photoreactive Δlac-acetogenin ([^{125}I]*dia*zirinylated Δlac-*a*cetogenin, [^{125}I]DAA), which has a small photolabile diazirine group attached to a pharmacophore (bis-THF ring moiety), and carried out photoaffinity labeling with bovine heart submitochondrial particles (SMP). Biochemical characterization revealed that DAA retains very potent inhibitory activity at nM level, whereas its inhibition mechanism differs slightly from that of the original Δlac-acetogenins. Analysis of the SMP photocross-linked by [^{125}I]DAA revealed that [^{125}I]DAA binds to the ND1 subunit in the membrane domain with a high specificity. The Lys-C and Asp-N digest of the cross-linked ND1 and careful analysis of the peptide fragments suggested that [^{125}I]DAA binds to two different sites in the ND1 subunit; one is located between Tyr127 and Phe198 (covering 4th and 5th transmembrane helices) and the other is located between Asp199 and Lys262 (covering 6th and 7th transmembrane helices). Consideration that a photolabile diazirine group is attached at hydrophilic moiety of amphiphilic [^{125}I]DAA, the cross-linked residues may be located in the loop region connecting the 5th and 6th transmembrane helices. In the presence of an excess amount of Δlac-acetogenin, the cross-linking solely of the region Asp199–Lys262 was completely suppressed. This result strongly suggests that Δlac-acetogenin binds this region with a high specificity.

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1P.34 EPR detection of protein-associated ubiquinone molecules in purified bovine heart NADH–ubiquinone oxidoreductase (complex I)

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It has been known that various energy transducing membrane systems utilize protein-associated quinone molecules. Based upon EPR spin-coupling studies, T. Ohnishi and her collaborators reported that bovine heart NADH–ubiquinone oxidoreductase (complex I) contains two different types of semiquinone. They called them fast relaxing semiquinone (SQ_{Nf}) and slow relaxing semiquinone (SQ_{Ns}) [1–4], and assigned each of them different roles. The former is for gated-proton transport, and the latter, for the converter between one-electron and two-electron transport. In recent years, complex I has been isolated and purified in many laboratories. A problem in the purification of complex I seems to be that quinone is easily lost during the purification. Recently, Yoshikawa's group reported that their highly purified, active bovine heart complex I preparations maintain one protein-associated ubiquinone per complex I molecule [5]. Using these preparations, we detected EPR signals of both semiflavin (SF) and semiquinone (SQ) radicals (S. T. Ohnishi et al. BBA. in press). We now report that we detected the signal from endogeneously-bound semiquinone, and succeeded in separating signals from two types of semiquinone. Possible implications are twofold. The first role: three types of quinone-inhibitors, Type-A (piericidin A), Type-B (rotenone) and Type-C (capsaicin and tridecylstigmatellin) are known [6–7]. This categorization of inhibitors may be explained by the binding specificity of these inhibitors to the Q_{Nf} and Q_{Ns} sites. For example, piericidin A binds to both Q_{Nf} and Q_{Ns}, rotenone binds preferentially to Q_{Nf} and TDS binds to Q_{Ns}. The second role is their involvement in electron–proton coupling. T. Ohnishi now hypothesizes that Q_{Ns} may also play an important role in indirect electron–proton coupling.

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