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De novo proteins designed to study aromatic side-chain redox chemistry

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Introduction

Proteins play key roles in essentially all biological processes. The foundation for this remarkable functional diversity is provided by the structural and chemical properties of twenty different amino acids. In recent years, a specific feature of amino-acid functionality has moved into focus as four residues – tyrosine, tryptophan, cysteine and glycine – have been shown to form catalytically active, one-electron oxidized radicals (Stubbe and van der Donk, 1998). The family of proteins in which side-chain redox chemistry forms a principal mechanistic theme catalyzes a number of fundamental reactions in biology. The ribonucleotide reductase enzymes, for example, utilize three, if not all four, of the known redox-active side chains in the conversion of ribonucleotides to deoxyribonucleotides in all living organisms (Stubbe and van der Donk, 1998). The aromatic side-chain redox cofactors have been implicated in DNA repair (Aubert et al., 2000), lignin degradation (Whittaker et al., 1999), and they serve as redox mediators in several heme peroxidases (Stubbe and van der Donk, 1998, Ivancich et al., 1999). In addition, studies performed in the Babcock laboratory have shown that redox-active tyrosines are integral to the catalytic cycles of photosystem II (PSII) and cytochrome *c* oxidase (CcO), the two key enzymes involved in the major water to dioxygen and dioxygen to water redox cycle in Nature. Thus, in the end of the 80's Babcock, Barry, Debus and coworkers showed that a redox-active tyrosine links the photochemistry at the PSII reaction center with the water-splitting chemistry at the $(Mn)_4$ cluster (Babcock et al., 1989). In 1995, Babcock *et al.* proposed that this tyrosine operates as a H-atom transfer cofactor in the catalytic cycle of PSII. The H-atom abstraction model for PSII has been discussed and developed in a series of articles (see Tommos and Babcock, 2000, Hoganson and Babcock, 2000 and references therein). More recently, the MSU group showed that the histidine cross-linked tyrosine located at the active site of CcO is oxidized in one of the key intermediate state of the catalytic cycle. In their mechanistic model for dioxygen reduction in respiration, the cross-linked tyrosine serves as a H-atom donor during the oxygen-oxygen bond-breaking step (Proshlyakov et al., 2000).

De novo designed radical proteins. In order to delineate the complex chemistry catalyzed by side-chain radical enzymes, detailed chemical knowledge of their radical cofactors is required. Here we describe a project aimed to characterize tyrosine and tryptophan redox chemistry by using *de novo* protein design. Two proteins, denoted α_3W and α_3Y, have been designed and synthesized. Their amino-acid sequences were

derived from α_3-1 (Johansson et al., 1998), which design was based on the three-helix bundle theme (DeGrado et al., 1999). α_3W and α_3Y comprise 65 residues each and are designed to fold into structures containing three interacting α-helices. The peptide sequences of α_3W and α_3Y are identical with the exception of residue 32, the position of the aromatic side chain. Structural characterization confirmed that α_3W and α_3Y form stable, highly helical structures with their single aromatic residue located within a well-packed protein core (Tommos et al., 1999).

Results and discussion

α_3W and α_3Y represent the "wildtype" proteins for the first family of *de novo* designed radical proteins. Recent developments of the three-helix bundles with respect to protein synthesis and purification, structural characterization, and functional mutations are summarized below. We end by discussing α_3Y as a PSII donor-side model system.

Protein synthesis and purification. Initially the three-helix protein samples were produced chemically by using solid-phase peptide synthesis (Tommos et al., 1999). At Stockholm University, our aim has been to develop an efficient system to produce large quantities of the three-helix bundles. Accordingly, the *de novo* proteins are synthesized biochemically by using bacterial protein expression. The synthesized α_3W gene was cloned into a pET11 vector (Novagen) and the resulting plasmid transformed into a BL21(DE3) protein expression cell line. The three-helix protein is expressed into the cytosol of the *E. coli* host strain following IPTG induction. α_3W and α_3Y are thermostable and > 80% of their secondary structures are still intact at 70° C (data not shown). This feature allows us to purify the three-helix bundles from the majority of contaminating cytosolic proteins by the following heat treatment. After cell lysis, the cytosol it placed at 70° C for one hour and then centrifuged to remove the heat-denatured proteins. The *de novo* designed protein is isolated from the resulting heatcut crude by a single size-exclusion chromatography step.

The amino-acid sequence of the expressed protein is identical to the sequence of the chemically synthesized protein (Tommos et al., 1999), with one minor modification. An alanine has been added to the N-terminus to facilitate the removal of the translation initiator methionine. Initially, α_3W was expressed with an unmodified peptide sequence. The molecular weight of the expressed protein showed that the methionine was not posttranslationally removed by the *E. coli* methionyl-aminopeptidase. α_3W is a small protein, 7.4 kDa, and the presence of the methionine severely decreases its solubility at acid pH. To address this problem, four mutants were constructed in which a small, polar residue (alanine, glycine, serine or threonine) was incorporated in the penultimate position (Hirel et al., 1989). Of these four constructs, the Ala-α_3W protein was expressed to the highest yield. Mass spectrometry data (not shown) on purified Ala-α_3W showed that the N-terminus methionine is efficiently processed from this protein. Expression systems of other three-helix proteins, including α_3Y, α_3F and the mutants described below, were derived from the Ala-α_3W/pET11 construct.

The α_3W gene was also cloned into a modified pET15 vector (Novagen), which codes for thioredoxin, a histidine-tag, and a thrombin cleavage site. The thioredoxin fusion-protein system has the advantage that it allows us to express mutants of the three-helix proteins in which the solubility, or other factors that may influence the expression yield, are less favorable. Together the two expression systems described

above provide a highly efficient, versatile vehicle to synthesize the *de novo* designed radical proteins.

Structural characterization of α_3W by NMR. A structural characterization of α_3W by NMR is in progress. A full data set on ^{15}N and ^{13}C labeled samples has been obtained and the data analysis is in its final stage (Qing-Hong Dai, Joshua Wand, pers. comm.).

Design of the α_3Y redox site. We define two major goals for the *de novo* radical proteins. First, the E_ms of the buried aromatic side chains should be sufficiently low to be oxidized by a biological oxidant, that is, < ~ 1.2 V. Second, the redox chemistry of the aromatic residues should be reversible. We note that the oxidation of tyrosine in solution is not a reversible process. The potential of Y_{32} in α_3Y is above the biological redox range (Tommos et al., 1999). In order to "activate" Y_{32}, the protein structure must be refined to lower the potential and make the redox process reversible. For tyrosine, three possible redox couples should be considered. $Y^{\bullet+}/Y$ has a potential of 1.38 V in water (Tommos et al., 1999), which is too oxidizing to be of biological relevance. The potential of $Y^{\bullet}/Y^{-}$ is lower, 0.68 V, however the design of a stable Y^{-} inside the protein core is considered as a more long-term goal. Thus, at present we focus experimentally on the oxidation of a protonated tyrosine to a deprotonated tyrosyl radical. The $Y^{\bullet}/Y$ redox couple is also the most relevant pair with respect to developing a PSII donor-side model system.

In order to lower the E_m of a buried tyrosine the phenolic proton should be transported from the protein redox site into the bulk upon oxidation (Tommos and Babcock, 2000). If the protonic charge remains inside the low dielectric protein medium, the solvation energy is expected to raise the potential. In addition, tight H-bonding between the tyrosine and a proton-accepting base is vital for an efficient and rapid oxidation event. This has been shown for tyrosine oxidation in PSII (Hays et al., 1999) and predicted theoretically (Blomberg et al., 1998; O'Malley, 1998; Cukier, 1999). We are in the process of making the following redox site designs to test these predictions. In a first approach, a cavity mutant of α_3Y is designed to allow water to enter the redox site. This design is intended to establish protonic contact between the tyrosine site and the bulk solvent. Our second approach directly mimics the PSII active site and introduces a histidine H-bonded to the tyrosine. Several histidine mutants of α_3Y have been made. Protein purification and characterization are in progress.

In addition to lower the potential, the tyrosine redox cycle should be made reversible. One strategy to hamper radical side reactions involves introducing covalently modified tyrosines in which the reactive sites of the aromatic ring are blocked. The blocking groups will be combined with a base function (Pessiki, 1994; Maki et al., 2001). A stable H-bond formed between the phenol oxygen and a covalently attached base should prevent the phenol oxygen from attacking the surrounding protein matrix. Moreover, the base can serve as a proton-transfer agent that shuttles the protonic charge out of the site, as intended with the designs described above. At present, we are investigating ionization potentials and H-bonding geometries for substituted tyrosines by quantum mechanical methods. The most promising compound identified by the calculations will be chemically synthesized and introduced into α_3Y. The protein core of α_3Y will be redesigned to fit the modified tyrosine bases on the information derived from the structural NMR studies.

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