Protein–Polymer Hybrids

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Azurin–Poly(*N*-isopropylacrylamide) Conjugates by Site-Directed Mutagenesis and their Thermosensitive Behavior in Electron-Transfer Processes**

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Peptide and protein polymer conjugates have recently received increased attention.[1] The activity and structure of the biohybrid materials can be modulated as a function of the polymer attached. If "smart" stimuli-responsive polymers are bound, the corresponding bioconjugates respond to external stimuli such as light, pH, or temperature.[2] As "smart" polymer, the thermoresponsive poly(*N*-isopropylacrylamide) (PNIPAM)[3] has been used to prepare "intelligent" biohybrid materials.[4] Below 32 °C the polymer is soluble in water in the chain-extended hydrated state. Above the so-called lower critical solution temperature (LCST) polymer chains collapse and turn into their hydrophobic state. This phase switch can be used to induce biological responses of PNIPAM–biomolecule conjugates.[2,4,5]

Various approaches for conjugation of proteins with polymers have been followed:[1] 1) reaction of polymers bearing reactive end groups with proteins, 2) reaction of polymerization initiators with proteins and subsequent polymerization, and 3) cofactor reconstitution by using cofactor-terminated polymers.[6] If more than one reactive site in the protein is accessible to the activated polymer or initiator, nonspecific multiple modification is possible by applying approaches (1) or (2). This problem can be circumvented by using bioengineering to prepare modified proteins bearing defined reactive sites for selective attachment of the polymer or initiator.[1f,2,7]

Herein we present a conceptually different approach for preparation of protein–polymer conjugates with azurin, which is a well-characterizied blue copper protein involved in bacterial electron-transfer chains.[8] Recent progress in electrochemistry has enabled the efficient electrical communication between azurin and an electrode. Consequently, azurin is considered to be a potential component in biodevices.[9]

Canters and co-workers have shown that the imidazole of His117, which acts as a Cu ligand in the active site of the blue copper protein azurin of *Pseudomonas aeruginosa*, can be replaced by imidazole derivatives in His117Gly mutants.[10] The activity of imidazole-reconstituted mutants could be restored. Guided by these studies we decided to synthesize imidazole-terminated PNIPAM by nitroxide-mediated radical polymerization[11,12] for preparation of "smart" mutated azurin–PNIPAM conjugates by reconstitution of the His117Gly azurin mutant with imidazole-terminated PNIPAM. In this way PNIPAM will be introduced to the active site of azurin (Figure 1) and is therefore expected to be located between azurin and its redox partner protein, cytochrome c. The presence of PNIPAM should selectively alter the protein–protein interaction as a function of temperature.

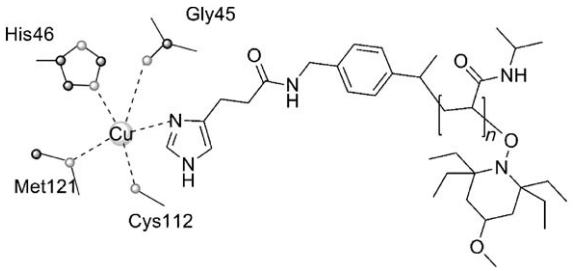

Figure 1. The active site of a reconstituted azurin–PNIPAM conjugate.

We show herein by kinetic measurements that PNIPAM bound to the active site of azurin can serve as a modulator of the electron transfer (ET) from cytochrome c to the azurin–PNIPAM conjugate as a function of temperature. The fact that short-chain PNIPAM cannot affect the ET process provides a clue of how PNIPAM disturbs the interaction between cytochrome c and azurin.

The synthesis of imidazole-terminated PNIPAM is described in Scheme 1. Radical bromination (Br_2, $h\nu$, CCl_4) of 4-ethylbenzonitrile gave **1**, which was converted to alkoxyamine **2** by using a known procedure.[5] Reduction of **2** with lithium aluminum hydride (LAH) and subsequent coupling with

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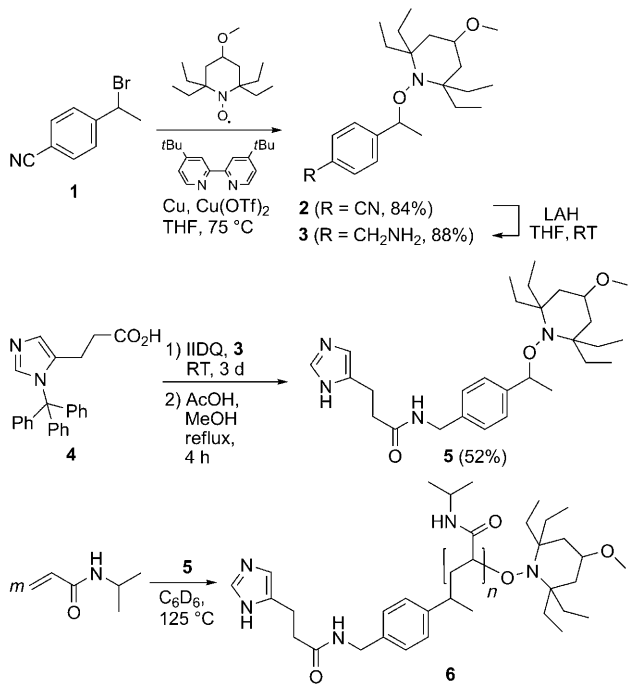

Scheme 1. Synthesis of imidazole–PNIPAM conjugates **6** with M_n = 3800–34 200 g mol^{-1}. IIDQ = isobutyl-1,2-dihydro-2-isobutoxy-1-quinoline carboxylate.

trityl-protected imidazole propionic acid **4**[13] gave radical initiator **5** after deprotection. Nitroxide-mediated polymerization was conducted in C_6D_6 with 0.5—2 mol % **5** at 125 °C in sealed tubes. Conversion was determined by ^{1}H NMR spectroscopy; the mean molecular weight (3800–34 200 g mol^{-1}) and polydispersity index (PDI = 1.14–1.39) were determined by mass spectrometry and gel permeation chromatography (GPC) (see the Supporting Information).[14,15] LCSTs of the imidazole–PNIPAM conjugates **6** were found to lie at around 32 °C as measured by ^{1}H NMR spectroscopy (imid–PNIPAM 3800 g mol^{-1}: 30.5 °C; imid–PNIPAM 9800 g mol^{-1}: 31.6 °C; imid–PNIPAM 34 200 g mol^{-1}: 32.6 °C; see the Supporting Information).[16]

The mutagenesis of His117 (base code CAC) into Gly (base code GGC) was carried out by polymerase chain reaction of azurin plasmid DNA by using primers with the necessary mutation. Azurin His117Gly apoprotein was expressed and purified in analogy to the protocol of Canters et al. for expression of azurin wild type (WT).[17] The purity of the apoprotein was checked by SDS-PAGE and UV/Vis spectroscopy (see the Supporting Information).

The reconstitution of azurin with **6** (3800 and 9800 g mol^{-1}) was carried out by a modified literature protocol:[18] $Cu(NO_3)_2 \cdot 3H_2O$ (1 equiv) was added to a solution of mutant apoprotein in 20 mM MES-NaOH (pH 6.0) leading to an increase of absorbance at 420 nm due to formation of type-2 copper azurin. A threefold excess of **6** and NaCl (0.2 M) were then added, and the solution was stirred at 4 °C until formation of the characteristic blue type-1 copper complex was complete. After desalting and precipitation of free **6** the resulting azurin–PNIPAM conjugates were analyzed. The reconstitution protocol had to be slightly modified for the longer-chain PNIPAM derivative (34 200 g mol^{-1}); a 20-fold excess of PNIPAM was used in that case. The reconstituted holoprotein precipitated when heated to 40 °C and could be separated readily from non-reconstituted apoprotein (about 43 % was reconstituted, see the Supporting Information). Since azurin–PNIPAM(34200) precipitated above the LCST, it was not suitable for ET measurements and was not further used in these studies.

UV/Vis spectra of azurin–PNIPAM (3800 g mol^{-1}) and azurin–PNIPAM (9800 g mol^{-1}) were recorded at 25 °C and 35 °C and compared with the spectra of azurin WT and the azurin mutant reconstituted with 4-methylimidazole (Figure 2). The ratio of absorbance at 280 nm to the absorbance at 620 nm is a measure for successful reconstitution. This ratio was nearly identical for the mutants and azurin WT which indicated complete reconstitution of the type-1 copper center. The azurin–PNIPAM hybrids showed no spectral change when heated above the LCST of the polymer. Therefore, expulsion of the imidazole–PNIPAM ligand (ligand exchange with water) of the mutant at higher temperature was ruled out.7

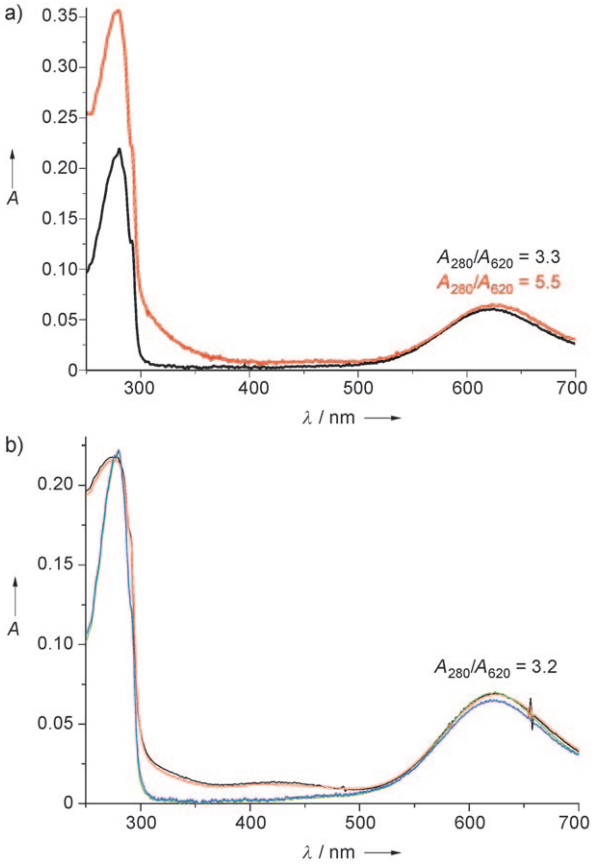

Figure 2. a) UV/Vis spectra of WT azurin (black line) and an azurin mutant reconstituted with 4-methylimidazole (red line). b) UV/Vis spectra of azurin reconstituted with PNIPAM(3800) recorded at 25 °C (black line) and at 35 °C (red line); UV/Vis spectra of azurin reconstituted with PNIPAM(9800) recorded at 25 °C (green line) and at 35 °C (blue line).

Reconstituted mutants were also analyzed by EPR spectroscopy. Azurin WT and the azurin–PNIPAM(9800) mutant showed very similar spectra (Figure 3). The coordination geometry at the Cu center of the mutant reconstituted with imidazole–PNIPAM was virtually identical to that of wild-type azurin as judged based on $A_{\parallel}(58\times10^{-4}\,cm^{-1})$, $g_{\parallel}$-(2.248), and $g_{\perp}$(2.055) values.[10a,18] Similar results were also obtained for azurin–PNIPAM(3800) (see the Supporting Information).

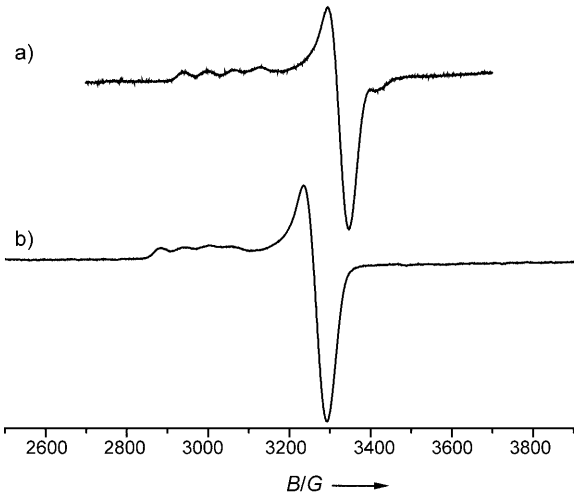

Figure 3. EPR spectra of the reconstituted azurin–PNIPAM(9800) mutant (a) and WT azurin (b) at 80 K.

Recorded CD spectra of WT azurin and azurin–PNIPAM-(9800) at 25 °C and 35 °C indicated that the mutant virtually retained the original protein conformation of WT azurin at 25 °C as well as at 35 °C (see the Supporting Information).

Finally, we studied ET processes between reduced cytochrome c and WT azurin and the reconstituted mutants at 25 and 35 °C. We used cytochrome c_{552} from *Thermus thermophilus* HB8 as the electron donor instead of the natural redox partner, cytochrome c from *Pseudomonas aeruginosa*, because cytochrome c_{552} shows high conformational stability under the experimental conditions. Moreover, the hydrophobic patch located at the surface around the heme in cytochrome c_{552} was expected to force hydrophobic interactions with azurin to allow electron transfer. Kinetic experiments were conducted in a UV cell under strictly anaerobic conditions to avoid atmospheric oxidation of reduced cytochrome c_{552} (see the Supporting Information). To this end, the oxidized form of a reconstituted azurin mutant was added to a solution of reduced cytochrome c_{552}, and the disappearance of the Soret band at 416 nm was recorded as a function of time at the given temperature (Figure 4).

For WT azurin the apparent rate constant, $k = 9.8\times10^{5}\,M^{-1}s^{-1}$, obtained at 25 °C is in agreement with literature values.[19] A similar k value was obtained at 35 °C ($1.0\times10^{6}\,M^{-1}s^{-1}$). For azurin–PNIPAM(3800) ET transfer was about an order of magnitude slower at 25 °C ($1.9\times10^{5}\,M^{-1}s^{-1}$) than with the wild-type azurin, showing that the solvated PNIPAM tail has a small but measurable effect on the activity of the protein. At 35 °C a slightly larger rate constant was obtained ($3.2\times10^{5}\,M^{-1}s^{-1}$). In this case the PNIPAM tail was most likely too small to induce any thermosensitive behavior.[20] Indeed, the ET rate constant could be altered as a function of temperature for azurin–PNIPAM(9800). At 25 °C we determined an apparent rate constant of $2.5\times10^{5}\,M^{-1}s^{-1}$ (similar to the value obtained for the mutant bearing the smaller tail). However, at 35 °C, the rate constant dropped to a value of $6.2\times10^{4}\,M^{-1}s^{-1}$.[21] A fourfold lower activity was obtained for the 9800 mutant at higher temperature.

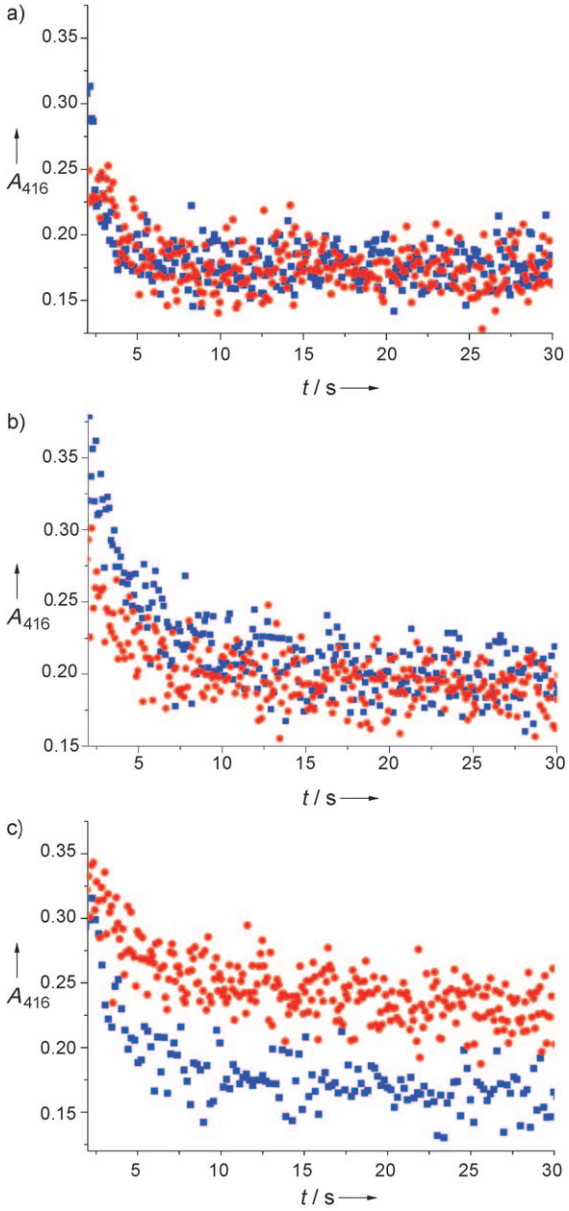

Figure 4. Electron-transfer processes from reduced cytochrome c_{552} to oxidized WT azurin (a), to the azurin–PNIPAM(3800) mutant (b), and to the azurin–PNIPAM(9800) mutant (c) at 25 °C (blue dots) and at 35 °C (red dots).

We believe that structural changes of the PNIPAM tail (for polymers with M_n around 10000 g mol^{-1}) on a temperature increase from 25 to 35 °C influences protein recognition of cytochrome c_{552} with azurin. Hydrophobic interactions at the active site of azurin, where the polymer is located in our mutant, are known to be important for the interaction of cytochrome c and azurin in the wild-type enzymes. The collapsed PNIPAM tail would partially block the interaction site on azurin, which significantly reduces the accessibility of cytochrome c_{552} to the site, while hydrated PNIPAM provides space for cytochrome c_{552} to access the site for ET although full contact between the proteins is restricted. The different results obtained with PNIPAM(3800) and PNIPAM(9800) presumably reflect to what extent the polymer tail covers the hydrophobic protein/protein recognition site.

In conclusion, we have shown that polymer–azurin hybrids can be prepared by His117Gly mutations and subsequent reconstitution with imidazole-terminated polymers. The polymer acts as a ligand for the copper ion in the active site. This is the first report on the preparation of a polymer–protein biohybrid by polymer–ligand reconstitution in a metalloprotein. Importantly, this approach allows the site-specific attachment of the polymer. Moreover, the polymer is located at the active site of the metalloprotein which is important for tuning enzyme activity. Structure and activity were restored upon protein reconstitution. For biohybrid materials bearing larger PNIPAM tails enzyme activity could be altered as a function of temperature. The ET rate constant decreased by a factor of 4 at higher temperature. For protein–polymer hybrids bearing smaller PNIPAM tails thermosensitivity was not observed.

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