Photoinduced electron transfer in singly labeled thiouredopyrenetrisulfonate azurin derivatives

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Abstract A novel method for the initiation of intramolecular electron transfer reactions in azurin is reported. The method is based on laser photoexcitation of covalently attached thiouredopyrenetrisulfonate (TUPS), the reaction that generates the low potential triplet state of the dye with high quantum efficiency. TUPS derivatives of azurin, singly labeled at specific lysine residues, were prepared and purified to homogeneity by ion exchange HPLC. Transient absorption spectroscopy was used to directly monitor the rates of the electron transfer reaction from the photoexcited triplet state of TUPS to Cu(II) and the back reaction from Cu(I) to the oxidized dye. For all singly labeled derivatives, the rate constants of copper ion reduction were one or two orders of magnitude larger than for its reoxidation, consistent with the larger thermodynamic driving force for the former process. Using 3-D coordinates of the crystal structure of *Pseudomonas aeruginosa* azurin and molecular structure calculation of the TUPS modified proteins, electron transfer pathways were calculated. Analysis of the results revealed a good correlation between separation distance from donor to Cu ligating atom (His-N or Cys-S) and the observed rate constants of Cu(II) reduction.

Key words: Azurin; Electron transfer; Time-resolved spectroscopy; Transient kinetics; Pathway calculation

1. Introduction

The mechanism of intramolecular electron transfer in proteins has been a subject of extensive study in recent years and a variety of experimental approaches have been employed to investigate electron transfer between redox sites in proteins [1–6]. The progress in the field has been significantly enhanced by the use of redox proteins to which additional redox centers were attached, e.g. ruthenium complexes coordinated to surface histidine residues [4–7]. Utilization of ruthenium photochemistry provided experimentalists with a useful tool for monitoring time-resolved processes of electron transfer within different proteins. The focus of those investigations has been on the electron transfer reactions within small soluble proteins, such as myoglobin [8,9], azurin [10], plastocyanin [11], and cytochrome *c* [5,6], as well as on electron transfer reactions between metalloproteins in polyprotein complexes [12–17]. Up until recently chemical modification with ruthenium complexes was practically the only approach allowing investigators to examine the dependence of the electron transfer rate in non-photosynthetic proteins on the distance separating two, natively occurring or chemically introduced, redox centers.

We have recently introduced a new method to study electron transfer reactions in non-photosynthetic proteins in solutions with submicrosecond time resolution. The initiation of the reaction was achieved by photoexcitation of a thiouredopyrenetrisulfonate (TUPS) molecule covalently bound to cytochrome *c* [19]. Single photon excitation of TUPS yields the low potential triplet state of the dye with high quantum efficiency [18]. The high yield, long life-time and low redox potential of the triplet state make TUPS an efficient initiator of electron transfer processes. The TUPS moiety has been covalently attached to eight different lysine residues of cytochrome *c*, which were separated and identified. The rate constants for the electron transfer reaction from the photoexcited triplet state of TUPS to the oxidized heme group and the back reaction from the ferrous heme to the oxidized dye were determined by transient absorption spectroscopy. The dependence of the electron transfer rate constants on the distance separating the dye and the heme iron was in line with a model, where a protein can be treated as homogeneous barrier to electron transfer tunneling.

In the present study the TUPS was employed for initiating the intramolecular electron transfer reactions in azurin. Azurin is a small (~14.6 kDa) protein constructed of β-sheets which contains a single blue type I Cu(II) ion. The high resolution structure of azurin isolated from *Pseudomonas aeruginosa* has been determined by X-ray crystallography in both the oxidized Cu(II) and reduced Cu(I) state [20]. The redox active copper ion is coordinated to side chains of Cys-112, His-117, His-46, and Met-121. It is characterized by strong absorption around 620 nm with an extinction coefficient $\varepsilon \sim 6000\ M^{-1}\ cm^{-1}$ in the oxidized state and a relatively high redox potential of ~305 mV. The above mentioned properties of the protein make it an excellent model for electron transfer studies. Earlier electron transfer studies have been conducted on the native or single site mutated protein, where the electron transfer from Cys3-Cys26 disulfide to the Cu(II) was generated by pulse radiolysis [23–25] and on ruthenium modified azurins [21,22].

In our present study the dye was covalently linked to the protein through chemical modification of its lysines with isothiocyanopyrenetrisulfonate, a reaction that yields the thiouredo adduct. Singly labeled azurin TUPS derivatives were

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Abbreviations: IPTS, 1-isothiocyanatopyrene-3,6,8-trisulfonate; TUPS, thiouredopyrene-3,6,8-trisulfonate; TUPS*, excited triplet state of thiouredopyrene-3,6,8-trisulfonate; TFA, trifluoroacetic acid

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separated from one another and purified by ion exchange HPLC. Transient absorption spectroscopy was employed to directly measure the time course of the photoinduced electron transfer from the excited triplet state of TUPS to the Cu(II) ion and for the back reaction from the Cu(I) to the oxidized dye. Analysis of the obtained electron transfer rate constants to Cu(II) in azurin revealed a good correlation between them and the length of the pathway, as well as the copper ligand atoms (His-N or Cys-S) to which the pathway leads.

2. Materials and methods

2.1. Materials

1-Isothiocyanatopyrene-3,6,8-trisulfonate (IPTS) was purchased from Lambda Fluorescence (Graz, Austria).

2.2. Preparation of azurin

P. aeruginosa azurin was expressed in *Escherichia coli* strain K12 TG1, and purified from the sonified bacterial cells essentially as described [26]. The absorbance ratio 625/280 nm, which is taken as a criterion of purity for the azurins, was 0.59 for the protein obtained following the last purification step.

2.3. Preparation of TUPS-azurin derivatives

TUPS-azurin adducts were prepared by reaction of azurin with IPTS as follows: chromatographically purified azurin (1 mM) was mixed with freshly prepared ITPS (1 mM) and incubated for 15 min at 25°C in 8–10 ml of 50 mM borate buffer (pH 11.0), 0.5 M KCl. The labeled protein mixture was separated from non-bound dye and salt on a 50×10 mm G-25 Sephadex 'medium' column, equilibrated with ammonium acetate (pH 3.9). A mixture of labeled derivatives eluted in the void volume and was subjected to further HPLC purification.

2.4. Purification of single TUPS-azurin derivatives

The mono-TUPS-azurin derivatives were resolved by ion exchange HPLC. For initial separation 50 mg of protein was incubated with 1 mM ferricyanide for 1 min and then loaded onto a 250×4.6 mm Phenomenex W-Porex 5DEAE HPLC anion exchange column equilibrated with 10 mM ammonium acetate (pH 3.9). Azurin and its derivatives were eluted at room temperature at a flow rate of 0.8 ml/min by a linear gradient of ammonium acetate (pH 3.9) from 0.2 to 0.8 M over a period of 1 h. The gradient was generated by a dual pump system (Model 626), and the elution of azurin fractions was monitored at 628 nm ($\varepsilon_{628} \sim 5700\ M^{-1}\ cm^{-1}$) [27] on a UV/visible detector (Model PDA 996), both from Waters (Waters Chromatography Division of Millipore, Milford, MA, USA). Fractions corresponding to individual peaks were collected, and the label content in the fractions was determined by absorption spectroscopy. The TUPS content was quantitated on the basis of an extinction coefficient of $\varepsilon_{372} = 30\,000\ M^{-1}\ cm^{-1}$ [18] and the azurin content on the basis of the above mentioned extinction coefficient. The main peaks, eluted from the column in 350–600 mM ammonium acetate, containing 1/1 labeled azurin were passed through the 50×10 mm G-25 Sephadex 'medium' column, equilibrated with 5 mM HEPES (pH 7.5). The secondary purification of fractions was carried out on the same column, using a linear ammonium acetate gradient from 0.2 to 0.8 M. The purified derivatives were collected and passed through the 50×10 mm G-25 Sephadex 'medium' column in 5 mM HEPES (pH 7.0) to separate them from ammonium acetate.

2.5. Identification of the lysine residues modified by TUPS

The different singly modified proteins (100 μg/ml) were reduced with ascorbate (1 mM) and incubated with 100 μg/ml trypsin in 50 mM ammonium bicarbonate (pH 8.3) for 17 h at 37°C. The tryptic digests were separated at room temperature on a C18 reversed-phase HPLC column, 250×4.6 mm (Vydac, Hesperia, CA, USA). The peaks were eluted at a flow rate of 0.6 ml/min by a linear gradient of acetonitrile from 4 to 50% (0.1% TFA). The elution was followed, monitoring the absorbance of the peptide bond and the dye at 214 and 372 nm respectively, by a UV/visible detector (Waters Model PDA 996). The peak containing TUPS label was collected and analyzed by mass spectroscopy in order to confirm the peptide purity. The fractions containing more than one peptide were rechromatographed on the small anion exchange HPLC column (Phenomenex W Porex 5 DEAE, 30×4.6 mm) equilibrated by 10 mM Tris-HCl buffer, pH 8.0. The labeled peptide was monitored at 372 nm and collected after elution by linear KCl gradient from 0.1 to 0.3 M for 30 min. The purified labeled peptides, originating from all TUPS-azurin derivatives were sequenced on a PROCISE-HT protein sequencer at the Protein Center of the Israel Institute of Technology. The TUPS-modified lysine, obtained by Edman degradation, elutes earlier than any of the unlabeled amino acids when separated by HPLC. This property enabled the identification of the position of the label in the azurin sequence.

2.6. Transient absorption kinetics

Samples (1.5 ml), containing 5 mM HEPES (pH 7.0) and the TUPS-azurin derivative (30 μM), were placed in a four-face 1 cm quartz cuvette. The oxygen dissolved in the solution was removed by continuous bubbling of Ar at 15 ml/min through the cuvette for 15 min before and throughout the experiment. Excitation of the dyes was done by the third harmonic frequency of a Nd:Yag laser (355 nm, 2 ns full-width at half-maximum, 3 mJ/pulse), which was focused on a surface area of 0.3 cm^2 the side of cuvette. The redox state of the azurin's copper was monitored at 633 nm. As the TUPS triplet and the oxidized radical form of the dye slightly absorb the 633 nm light, a correction was made to account for changes in the redox state of azurin. The transient associated with the dye was monitored at 487 nm where azurin does not absorb. The absorbance of the TUPS triplet and the dye's radical at 487 nm is 7.8 times higher than that at 633 nm (not shown). The correction was made by subtracting the 7.8 times reduced 487 nm transient from that at 633 nm. The probing beam, either the 633 nm band of a helium-neon laser or the 487 nm band of an Ar laser, crossed the pulse-irradiated face of the cuvette perpendicular to the excitation beam. The probing beam was directed to a monochromator photomultiplier assembly, and transients were stored and averaged by a Tektronix TDS 520A digital oscilloscope as previously described [18]. The response time of the detection system was 20 ns. The analyzed transients are the average of 20 pulses collected at a frequency of 0.02 Hz. Reduction of azurin was quantitated by the absorbance coefficient difference of $\varepsilon_{633-487} = 5500\ M^{-1}\ cm^{-1}$.

2.7. Molecular modeling

Coordinates for *P. aeruginosa* azurin at 1.9 Å resolution were taken from the Brookhaven Protein Data Base (5 azu) [20] and used as the starting structure for the molecular modeling of the TUPS-derivatives (Lys-70, Lys-92, Lys-101 and Lys-122). The general structure of single-residue mutated azurins [20,28,29] and of azurin modified at His-83 with Ru(2,2′-bipyridine)$_2$(imidazole) [30] has been shown to be close to that of the native protein. Furthermore, mutations or chemical modifications outside the copper coordination sphere have so far been found to have no gross effect on the overall structure of the metal site [20,28–30]. Therefore only changes at the TUPS-modified residue and its immediate surroundings ($\sim$0.5 nm) were considered in the molecular modeling calculations while the Cu(II) ion and its ligating atoms were always maintained as they are in the native protein. The molecular modeling (energy minimization and molecular dynamics) was performed with the programs Discover and InsightII (Biosym Tech., San Diego, CA, USA), applying the consistent valence force field, excluding cross-term energies and Morse potentials [31]. The steepest descent and the conjugated gradients minimization algorithms were used until a maximum derivative of less than 40 J mol^{-1} nm^{-1} was achieved. Molecular dynamics calculations were performed in order to overcome local energy minima. The Leapfrog integration algorithm was used with typically 2000 iterations at 1000 K and 1.0 fs time steps. Several high energy conformations were analyzed. The minimization was started with all atoms fixed except those of the modified residue after which the neighboring side chains were allowed to shift position. This procedure was repeated until the interaction energy of all residues (side chain and backbone) within 5 Å was minimized.

2.8. Electron transfer pathways

Electron transfer pathways were calculated for native and modified azurins with the program Pathways 2.1 [31–34]. The program uses a combination of covalent bonds (C), hydrogen bonds (H) and through-space jumps (S) to link the donor and the acceptor in an LRET

process. Each kind of link (C, H, S) is assigned a certain decay parameter, and the overall electron transfer probability is calculated as the product of decay factors. The optimum paths are those with the *largest* EC value.

3. Results

3.1. Preparation of TUPS-azurin derivatives

Isothiocyanates are widely used in protein modification studies as a specific modifier of ε-amine of lysine residues. Incubation of the protein with stoichiometric concentrations of IPTS yields a mixture of singly and multi-labeled TUPS-azurin derivatives. The individual singly labeled derivatives were eluted from the column by a linear gradient of KCl from 0.2 to 0.6 M (Fig. 1). The TUPS content was determined on the basis of the extinction coefficients given in Section 2. Spectral analysis proved that nine main peaks, eluted from the column, contained singly labeled azurin. Derivatives carrying several TUPS residues had a strong negative charge, and were therefore eluted from the column at much higher (1.5 M) salt concentrations. Most of the singly labeled azurin fractions collected from the primary separation were contaminated with neighboring ones. The second chromatography of the fractions yielded six fractions, which were at least 95% pure single TUPS-azurin derivatives. Three fractions, however, remained contaminated even after several repetitive purification steps.

3.2. Identification of the modified lysines

The modified lysine residue in the six purified derivatives was identified by sequencing of the labeled peptides obtained by tryptic digestion of the derivative. The peptides absorbing at 372 nm (absorbance maximum of TUPS [18]) were rechromatographed and sequenced on a protein sequencer. Prior to the sequencing procedure, the sample was analyzed by mass spectroscopy in order to confirm its purity. The characteristic HPLC elution time of TUPS-modified lysine, obtained by Edman degradation, is different from that of the unlabeled amino acids. This enables recognition of the modified lysine residue in the sequencer records. The same TUPS identification strategy was applied for the six TUPS-azurin derivatives, and the results are summarized in Table 1.

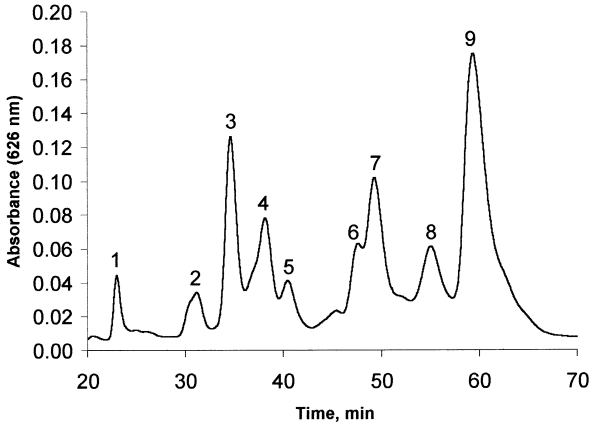

Fig. 1. HPLC separation of TUPS-azurin derivatives. The crude reaction mixture of TUPS-azurin derivatives (10 mg) was chromatographed on a 250×4.6 mm HPLC W-Porex 5DEAE anion exchange column at pH 3.9 with a linear gradient from 0.2 to 0.8 M ammonium acetate at a flow rate of 0.8 ml/min. Elution of TUPS-azurin derivatives was followed by the absorbance at 628 nm. The gradient was generated by a dual pump system (Model 626), and the elution of the fractions was monitored on a UV/visible detector (Model PDA 996) both from Waters (Milford, MA, USA).

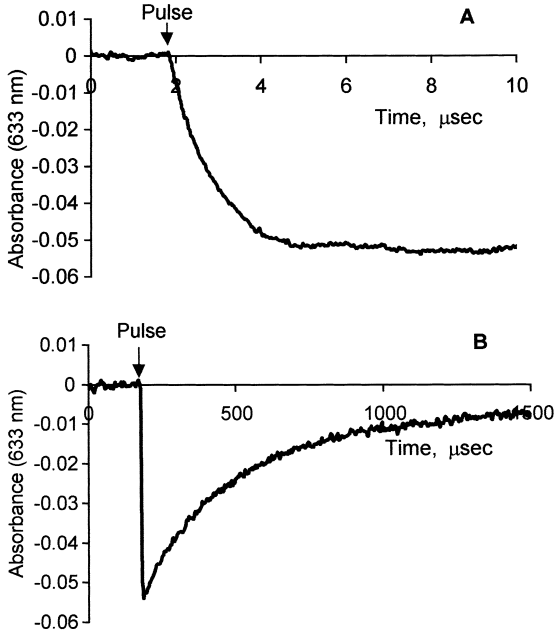

Fig. 2. Transient kinetics of the TUPS(Lys-122)-azurin derivative. The TUPS(Lys-122)-azurin derivative eluted as peak 9 from the HPLC separation (see Fig. 1). The TUPS(Lys-122)-azurin (30 μM) solution containing 5 mM HEPES (pH 7.0) was bubbled with Ar for 15 min and irradiated by a 3 mJ pulse of a Nd:Yag third harmonic frequency. The absorbance transients were monitored at 633 nm by He-Ne laser. A: The transient represents the Cu(II) reduction by TUPS excited state. B: The transient accounts for reoxidation of Cu(I). The transients are the average of 20 pulses collected at a frequency of 0.02 Hz.

3.3. Photoinduced electron transfer kinetics

We have earlier shown [18] that photoexcitation of TUPS generates the low potential triplet state of the dye (TUPS*) with high (∼30%) quantum efficiency. When covalently linked to an azurin molecule, TUPS* was now found to initiate the sequence of intramolecular redox reactions. Fig. 2 depicts the transient adsorbance changes observed in a solution of the Lys-122 azurin derivative. Photoexcitation of TUPS-azurin derivative resulted in the electron transfer from TUPS* to the copper(II) ion. The reduction time course of Cu(II) induced by the laser pulse is illustrated in Fig. 2A as an absorbance decrease at 633 nm. The time course of the transient could be fitted to a single-exponential decay having the rate constant $k_{obs} = (1.1 \pm 0.1) \times 10^6\ s^{-1}$. This rate constant was independent of the concentration of the TUPS-azurin derivative from 10 to 100 μM, which indicates that it is an intramolecular electron transfer process. This branch of the photochemical process generates two redox-active species: Cu(I) and the oxidized form of TUPS. Recombination, namely, intramolecular reoxidation of the copper(I) ion by the oxidized dye, brings the system to its initial prepulsed state. Because of the rather low excitation energy, this redox cycle initiated by photoexcitation of TUPS can be repeated several hundred times without altering the observed rates of the intramolecular electron transfer reactions. The transient

Table 1
Electron transfer rate constants of TUPS-azurin derivatives

Modified residue	Rate constant[a], 10^3 s^{-1}		Distance[b], Å	Sigma pathlength F_R [c]
	Cu(II) reduction	Cu(I) reoxidation		
Lys-92	250	1.6	15	22 (N_δ)
Lys-101	190	1.5	23	32 ($S\gamma$)
Lys-122	940	3.0	12	11 (S_δ)
Lys-70	1500	2.0	19	19 ($S\gamma$)
Lys-24[d]	–	–	32	
Ala-1[d]	–	–	23	

[a]Measured as in Fig. 2.
[b]Estimated between a nitrogen atom of the α-amino group of the corresponding lysine or of the α-amino group of Ala-1 and the copper ion using the 3-D model of azurin [20]. Error limits estimated to be ±10%.
[c]F_R pathlength calculated as the number of covalent bonds connecting N_δ of the TUPS-modified lysine to the donor atom of the copper ligand. Hydrogen bonds are counted as being equal to two covalent bonds.
[d]The rate constant of the Cu(II) reduction is slower than 10^3 s^{-1}; the value cannot be estimated with reasonable accuracy.

absorption change accounting for reoxidation (Fig. 2B) of the copper ion could also be fitted by a single-exponential absorption increase having a rate constant $k_{obs} = (2.5 \pm 0.1) \times 10^3$ s^{-1}. The rate constants for copper(II) ion reduction and reoxidation were determined in a similar fashion for the five other singly labeled derivatives and the results are summarized in Table 1. The rate constants of the electron transfer in both directions were found to depend on the position of the label on the protein molecule. The amplitudes of the transients, however, were found to be the same for all derivatives tested. The efficiency of the perturbation is high and about 30% of the azurin molecules undergo intramolecular reduction in a single pulse.

4. Discussion

The question of electron transfer rate dependence on the separating protein medium has been addressed by a large body of theoretical studies [1–6,35]. In the pathway model the linkage between the electron donor (D) and acceptor (A) is analyzed in terms of covalent bonds, hydrogen bonds and van der Waals contacts, each given a certain electronic

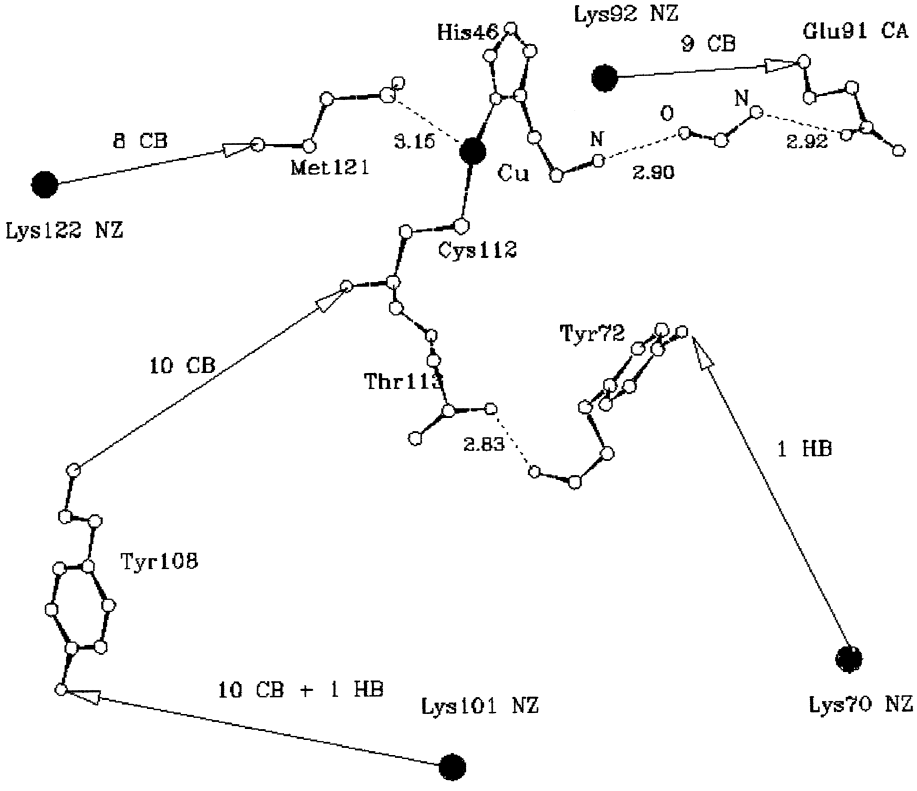

Fig. 3. Calculated electron tunneling pathways from the TUPS moiety attached to Lys-70, Lys-92, Lys-101 and Lys-122, respectively, to the copper center. For clarity parts of the protein structure have been replaced with arrows indicating the number of covalent bonds (CB) and hydrogen bonds (HB) in the routes that are not shown. The pathways from Lys-92, Lys-101 and Lys-122 start at the N_ε atom, while in the case of Lys-70, molecular modeling predicts a hydrogen bond between one of the sulfonate groups on the TUPS moiety and Tyr-72. Some interatomic distances (in Å) are also shown.

coupling decay value. Based on 3-D coordinates obtained from the high resolution crystallography structure of *P. aeruginosa* azurin and energy minimization calculations on the TUPS-modified protein, electron transfer pathways from donor D to acceptor A giving rise to the highest electronic coupling were calculated. The rate constants of the intramolecular reduction of Cu(II) by the triplet state of the dye (TUPS*) are given in Table 1.

Evaluation of the points of electron uptake or release from the redox centers is a rather complicated problem. In the TUPS label, the electron donating and accepting orbitals may be delocalized or associated with certain atoms. However the small molecular size of the TUPS (diameter ~ 7 Å) compared to the size of ruthenium complexes commonly used in electron transfer studies makes differences in the estimated electron transfer distances for the forward and the backward reactions relatively insignificant.

We have performed the above pathway calculations in order to examine the correlation between the rate constants and separation distances for electron transfer in the modified azurin, where the driving force (~ 0.8 eV) and reorganization energies may be assumed invariant in the four azurin modifications under study. The calculated pathways are shown in Fig. 3 and their lengths (σ distance) are given in the last column of Table 1. As Fig. 3 shows, the electron transfer pathways connecting Lys-101 and Lys-70 derivatized with TUPS* to Cu(II) go via the Sγ of Cys-112, while the pathway from Lys-92 leads to the copper center via N_δ of His-46. Finally, the pathway going from Lys-122 ends at the copper ligand Met-121. It has earlier been demonstrated both experimentally [36] and theoretically [37] that the high degree of anisotropic covalency in the copper coordination sphere would enhance the electron density between the metal ion and the thiolate ligand and therefore give rise to a more efficient electronic coupling than between the Cu(II) ion and its histidine ligands. Thus, the pathway which yields $F_R = 19$ Sγ of Cys-112 is expected to be more efficient than the one with $F_R = 12$ of N_δ of His-46. The S_δ of the Met-121 bond to copper is unusually long, 2.82 Å, and the Cu-3d orbitals exhibit minimal mixing with any of the Met orbitals which would make the Lys-122 to Cu(II) pathway highly unfavorable in spite of the short route. So, in spite of the shorter $F_R = 11$ pathway, the rate of intramolecular electron transfer is significantly slower compared with that from Lys-70 to Cu(II) (see Table 1).

The Cu(II) reduction in azurin-TUPS derivatives proceeds at a much slower rate than Cu(I) reoxidation because of the markedly driving forces. The free energy values for the forward and reverse electron transfer reactions are estimated to be 1.19 and 0.59 eV, respectively. These estimations are based on $E^o = -0.9$ eV for TUPS*/$TUPS_{ox}$ [18], $E^o = 0.88$ eV for TUPS/$TUPS_{ox}$ [38], and $E^o = 0.29$ eV for azurin. The ratio of the rates for Cu(II) reduction and Cu(I) reoxidation was found to be different for the TUPS-azurins tested (see Table 1). One possible reason for that may be a difference in the red/ox potential of the dye induced by a local protein environment.

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