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Intramolecular Electron Transfer in a Covalently Linked Mutated Azurin Dimer

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Received: June 16, 2003

We have employed a novel recombinant azurin mutant where the cysteine residues 3 and 26 forming the internal disulfide bridge were replaced by alanines and a cysteine replaced the native alanine 42, thus leading to an azurin dimer linked via the disulfide formed between these residues and at a site closer to the copper center. An intramolecular electron transfer has been induced between the disulfide radical ion produced by pulse radiolytic reducing radicals and the Cu(II) ion. Analysis of this intramolecular long-range electron transfer (LRET) rates and their temperature dependence resolves a clear difference in the nature of this process as compared to that occurring in earlier-examined azurins inasmuch as the induced LRET proceeds along a shorter and structurally distinct part of the protein. Still, we demonstrate here that the results do fit the accepted model of how the heterogeneous matrix in a folded polypeptide mediates long-range electronic coupling.

Introduction

Azurins (Az) are blue single-copper proteins, present in several bacteria where they function as mobile electron transport mediators.[1] Three-dimensional structures of several azurins, both wild type (WT)[2,3] and mutated,[4−10] have been determined at high resolution. The copper ion is bound at one end of the β-sandwich-shaped molecule, separated from the solvent by a patch of hydrophobic residues. At the opposite end of the molecule at a distance of 2.6 nm from the copper ion, a conserved disulfide bridge is present which can be reduced pulse radiolytically by, for example, CO_2^- radicals to yield the $RSSR^-$ radical anion.[11] The electron is then intramolecularly transferred to the copper(II) ion. This reaction has therefore been employed for investigating the role of protein structure in intramolecular electron transfer (ET) using a large number of wild type and single site mutated azurins.[11−19] Extensive ET studies using azurin were carried out by the H. B. Gray group, mainly by employing surface mutation and conjugation of external redox centers.[20−22] Hence, azurin became a major research system for studying the dependence of long-range electron transfer (LRET) rates on the chemical nature and structure of a predominantly β-sheet protein matrix separating the copper ion and its redox partner.

Mutants where a different internal disulfide bridge can be produced, such as the Asn42Cys mutant of *Pseudomonas aeruginosa* azurin, enable novel applications of this protein for LRET studies. The latter mutant has been constructed, expressed, and under oxidizing conditions found to form a dimer where two azurin monomers are covalently linked via the Cys42−Cys42 disulfide bridge.[23] The three-dimensional (3-D) structure of this dimer has been determined, and the short intermolecular disulfide link was found to cause a strong steric constraint, forcing apart the hydrophobic surface patches covering the blue copper centers which are assumed to be involved in the electron self-exchange.[24] Indeed, the intramolecular Cu(I) to Cu(II) electron exchange in this dimer was found to be considerably slower (less than 10 s^{-1}) than that observed for electron exchange between the free monomers. This difference is assigned to the large through-bond distance of 2.4 nm separating the two copper ions in the dimer.[25] We have now employed this type of engineered azurin dimer with a Cys42−Cys42 disulfide bridge to investigate ET between the pulse radiolytically produced disulfide radical ion and the copper(II) ion over a considerably shorter distance than in the monomer (1.28 nm for Cys42 to Cu in the dimer as compared with 2.59 nm for Cys3/Cys26 to Cu in our previous studies). To eliminate possible interference from reduction of the native Cys3/Cys26 disulfide bond, a triply mutated azurin was constructed and expressed where the latter two cysteines were substituted by alanines, Cys3Ala/Cys26Ala/Asn42Cys. Earlier 3-D studies of an azurin mutant where this disulfide bridge has been eliminated (Cys3Ala/Cys26Ala) established that the overall protein structure is not affected except for a difference in the immediate proximity of the mutated residues.[10]

Results of the present experiments where intramolecular ET was induced in the triply mutated azurin dimer show that the observed rate constant is in good agreement with the tunneling pathway model developed by Beratan and Onuchic.[25] The activation parameters of this process have been determined and

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10.1021/jp0357018 CCC: $25.00
Published on Web 08/12/2003

TABLE 1: Rate Constants and Acivation Parameters for Internal ET in Different Copper-Containing Proteins and Enzymes

protein	ET process	k_{298} s^{-1}	$\Delta H^{\ddagger}$ kJ mol^{-1}	$\Delta S^{\ddagger}$ J K^{-1} mol^{-1}	ET dist. nm	ref
P. aeruginosa azurin	$RSSR^- \rightarrow Cu^{2+}$	44 ± 7	47.5 ± 4.0	-56.5 ± 7.0	2.56	12
C3/26A−N42C dimer	$RSSR^- \rightarrow Cu^{2+}$	7200 ± 100	17.7 ± 2.0	-112 ± 6	1.28	this work
CuNiR	T1Cu(I) → T2Cu(II)	185 ± 12	22.7 ± 3.4	-126 ± 11	1.27	31
Ascorbate oxidase	T1Cu(I) → T2/T3Cu(II)	201 ± 8	9.1 ± 1.1	-170 ± 9	1.22	32
Cyt. *c* oxidase	Cu_A(I) → heme-c(III)	$13\,000 \pm 1200$	11.4 ± 0.9	-121 ± 11	1.96	33

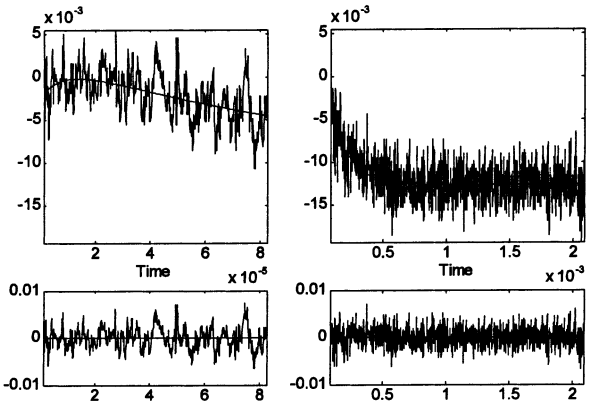

Figure 1. Time-resolved absorption changes reflecting the internal LRET between $RSSR^-$ of the C3/26A−N42C azurin dimer and Cu(II). The protein concentration was 20 μM in an N_2O-saturated solution containing 100 mM formate and 10 mM phosphate; pH 7.0. Temperature 26 °C. Pulse width 1.5 μs. Optical path length 3 cm. Wavelength 625 nm.

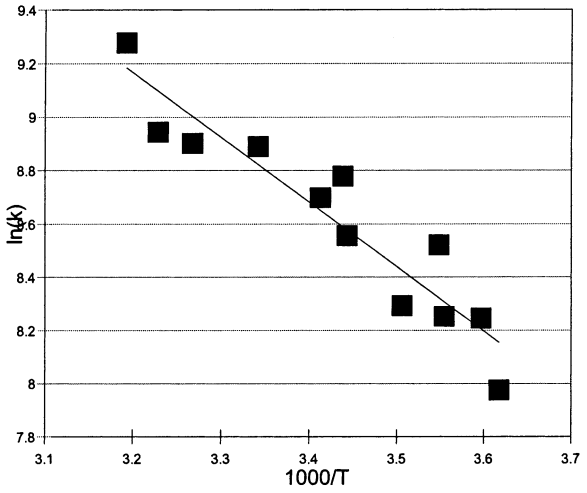

Figure 2. Temperature dependence of the intramolecular ET rate constants.

are discussed in relation to those obtained earlier for LRET in other copper proteins and enzymes.

Materials and Methods

Proteins. Cloning and expression of the *P. aeruginosa* azurin gene was performed as reported previously.[23] The site-directed mutagenesis, protein isolation, purification, and characterization of the N42C azurin mutant have also been described earlier.[23]

Kinetic Measurements. Pulse radiolysis experiments were carried out using the Varian V-7715 linear accelerator of the Hebrew University in Jerusalem. All technical details have been described in earlier publications.[12−18] Aqueous solutions, 0.1 M in sodium formate (pH 4.0), were deaerated and saturated with N_2O in glass syringes. The concentrated protein stock solution was then added, and the pH was adjusted to pH 7.0 with NaOH. N_2O bubbling was continued for another 5 min, and the solution was then transferred into the pulse radiolysis cuvette under anaerobic conditions. All optical measurements were carried out at 410 nm ($RSSR^-$ absorption, with $\epsilon_{410} = 10\,000$ M^{-1} cm^{-1}) and 625 nm (Cu(II) absorption, with $\epsilon_{625} = 5000$ M^{-1} cm^{-1}) under purified argon at a pressure slightly in excess of 1 atm. Each kinetic run was repeated at least four times. The time-dependent absorption changes were fitted to a sum of exponentials using a nonlinear least squares program written in MATLAB. The temperature of the reaction solutions was controlled by a thermostating system and continuously monitored by a thermocouple attached to the cuvette. All chemicals were of analytical grade and used without further purification. Milli-Q water was used throughout the studies.

Results and Discussion

All reactions were studied at pH 7.0 and over a dimer concentration range from 5 to 54 μM and monitored at both 410 and 625 nm (cf. Figure 1). When azurin dimer solutions are exposed to pulse radiolytically produced CO_2^- radical anions, the intermolecular disulfide bridge becomes reduced, forming the $RSSR^-$ radical in an essentially diffusion-controlled reaction ($k_1 \approx 10^9$ M^{-1} s^{-1}). In contrast to monomeric azurins, no competing bimolecular reduction of the blue copper(II) center by CO_2^- is observed. Disulfide reduction is followed by a concentration independent, intramolecular $RSSR^-$ to Cu(II) electron transfer:

$$RSSR^- - Az[Cu(II)] \xrightarrow{k_2} RSSR - Az[Cu(I)]$$

The internal ET rate constant, k_2, was calculated to be 7200 ± 100 s^{-1} at 25 °C. Each azurin solution was subject to only a few pulses, leading to less than 10% reduction; thus, the probability of reducing more than one of the Cu(II) ions in a dimer is negligible. The temperature dependence of the internal ET rate has been studied in a range from 3.2 to 40.0 °C, from which the activation parameters were derived (cf. Figure 2 and Table 1).

The semiclassical Marcus theory for nonadiabatic intramolecular ET reactions predicts that rates are governed by the standard free energy of reaction ($\Delta G°$), the nuclear reorganization energy (λ), the distance separating electron donor (D) and acceptor (A), and the electronic coupling (H_{DA}) between D and A at the transition state:[26]

$$k = \frac{2\pi}{\hbar} \frac{H_{DA}^2}{(4\pi\lambda RT)^{1/2}} e^{-(\Delta G^0 + \lambda)^2/4\lambda RT} \quad (1)$$

The electronic coupling energy, H_{DA}, is expected to decay exponentially with the distance separating D and A as:

$$H_{DA} = H_{DA}^0 e^{-\frac{\beta}{2}(r - r_0)} \quad (2)$$

When the distance between A and D is 1.0 nm or larger, only a very limited electronic coupling will exist. Still, intramo-

lecular ET reactions have been observed to proceed over distances of 2.0 nm or more.[27]

The polypeptide matrix separating the redox centers in proteins is comprised of an array of covalent and hydrogen bonds as well as van der Waals contacts. Beratan and Onuchic have developed a general model for the electronic coupling in which the structural complexity of the protein medium separating donor and acceptor is decomposed into elements linked by such interactions.[25] In this tunneling pathway model, the optimum coupling is identified by a searching algorithm analyzing the 3-D protein structure. We have earlier demonstrated that the rate of ET between the Cys3/Cys26, $RSSR^-$ radical, and Cu(II) in *P. aeruginosa* azurin fits quite well with the tunneling pathway model.[12−18] It is therefore of interest to extend the analysis using this model to the triple mutant studied here.

The polypeptide chain in the azurin dimer links S_γ of Cys42 with N_δ of His46, one of the copper ligands. It consists of 17 covalent bonds, resulting in an effective tunneling path length of 2.4 nm. Driving force optimized rate constants for ET in a β-sheet protein can be described by an average coupling decay constant of 7.3 nm^{-1}.[28] Thus, we may calculate an activationless, $k_{MAX} = 10^5\ s^{-1}$ (i.e., when the driving force, $-\Delta G°$, equals the reorganization energy, λ). Alternatively, using the structureless protein ET model developed by Dutton et al.,[29] in which an average distance decay of 14 nm^{-1} was found, we calculate an expected $k_{MAX} = 1.4 \times 10^5\ s^{-1}$. The concordance between the two models is interesting and probably implies the operation, in this particular case, of a direct S_γ to Cu(II) ET pathway. We have previously determined the reorganization energy and driving force for intramolecular ET between the Cys3/Cys26 $RSSR^-$ and the copper center in WT *P. aeruginosa* azurin and found $\lambda_{TOT} = 1.0$ eV and $-\Delta G° = 0.71$ eV.[16] Assuming these values are also applicable to the present mutant dimer, a rate constant of $4 \times 10^4\ s^{-1}$ at 298 K is calculated. This is 5-fold larger than the experimentally observed rate, $k_{298} = 7200\ s^{-1}$. Though not an unreasonably large discrepancy, the following rationale may be considered for the divergence.

In native *P. aeruginosa* azurin, the ET pathway includes the S_γ thiolate ligand of Cys112, while tunneling from the new C42/42 disulfide bridge to the copper center proceeds via the N_δ of His46. It has already been pointed out earlier that there is a high degree of anisotropic covalency in the blue Cu(II) center:[30] The wave function of the Cu center into which the electron is transferred has a high amplitude at the cysteine sulfur and a much smaller one at the histidine ligands; therefore, the ET rate is increased if the "pathway" runs through the thiolate ligand instead of through one of the histidine imidazoles. Hence, an ET pathway leading to His46, as in the current case, would drastically diminish the electronic coupling in the dimer compared with that in native azurin and cause a lower rate than calculated.

Finally, an interesting observation emerges from a comparison of the activation parameters calculated here for the azurin dimer with those obtained earlier for azurins as well as for related blue copper-containing enzymes (cf. Table 1). While in all azurins studied so far the $RSSR^-$ to copper(II) rates of LRET are controlled by a relatively large activation enthalpy, in other copper proteins including the presently studied dimer, the activation enthalpies are relatively small and large negative activation entropies are rate determining, although in these proteins the connecting ET pathways are considerably shorter. One rationale for this difference could be that ET in the monomeric azurins involves major solvent reorganizations at the intramolecular Cys3/Cys26 disulfide bridge which is more solvent exposed than is the intermolecular Cys42/Cys42 disulfide of the dimer. This notion is corroborated by the good linear correlation between activation enthalpies and entropies presented in Table 1, which suggests an overriding influence of solvent effects.

Acknowledgment. We wish to thank Gertrud Warmerdam for her excellent technical assistance in preparation of the triple mutant. O.F. wishes to thank the Danish Natural Science Research Foundation. I.P. acknowledges a grant from the German-Israeli Foundation and the continuous support by the E.&B. Shoor Foundation.

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