Molecular Crystals and Liquid Crystals
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl18

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Version of record first published: 04 Oct 2006.

To cite this article: Ole Farver & Israel Pecht (1991): Long Range Electron Transfer in Blue Copper Proteins, Molecular Crystals and Liquid Crystals, 194:1, 215-224

To link to this article: http://dx.doi.org/10.1080/00268949108041167

Mol. Cryst. Liq. Cryst., 1991, vol. 194, pp. 215–224
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Printed in the United States of America

LONG RANGE ELECTRON TRANSFER IN BLUE COPPER PROTEINS

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ABSTRACT

Long range electron transfer (LRET) within proteins is investigated. Resolution of the detailed pathways and the effect of separating medium, distance and energetics on the rates are the focal questions. Two types of systems are studied: In azurins, it is the intramolecular LRET from an $RS\text{-}SR^-$ radical, produced pulse radiolytically, to the Cu(II) center. Differences in amino acid sequences amongst naturally occurring azurins make it possible to examine the parameters controling the reaction rates along this non physiological pathway.

In the second system, the intramolecular LRET from the type 1 Cu(I) site to the type 3 binuclear center is studied in the multi sited blue copper oxidase, laccase. This enables a structure-reactivity correlation for the LRET along a pathway optimized by evolution.

Keywords: Azurin, laccase, pulse radiolysis, disulfide radical, copper oxidase

INTRODUCTION

Long range electron transfer (LRET) within proteins has been observed and investigated in numerous laboratories[1-9]. This led to the recognition that LRET plays is important in diverse biochemical processes. The roles that driving force, distance, structure and nature of the medium separating the electron donor and acceptor have in determining the electron transfer (e.t.) rates, are subjects of intense research activity.

In order to dissect and determine the relative roles of the above parameters, LRET is beeing studied in several model systems including synthetic polypeptides and metallo proteins of known three dimensional structure[1-9]. The efficiency and selectivity of e.t. were assumed to depend on the existence of pathways coupling electron donor and acceptor by specific combinations of covalent, hydrogen, and non-covalent contacts[10]. The roles played by particular interactions are then resolved experimentally by studying systems with given structural differences.

Blue copper proteins function as e.t. agents in nature[11,12], and here we report studies of LRET within two types of these proteins: electron mediators and multi-sited oxidases.

Azurins are blue single copper proteins that mediate electrons in the respiratory chain of several bacteria. More than a dozen different azurins have so far been sequenced[13] and found to be highly homologous. Having also the three dimensional structures determined for three different azurins, makes this family an ideal subject for examining the influence of structure and driving force on the rate of e.t. All azurins

sequenced to date[13] contain an intra-chain disulfide bridge connecting residues Cys3-Cys26. In the three dimensional fold, this disulfide is at one end of the β-barrel structured protein, some 20 A from the copper center[14] (Fig. 1). This disulfide can be reduced by strong reductants forming the $RS\text{-}SR^-$ radical anion, which by a slow intramolecular process

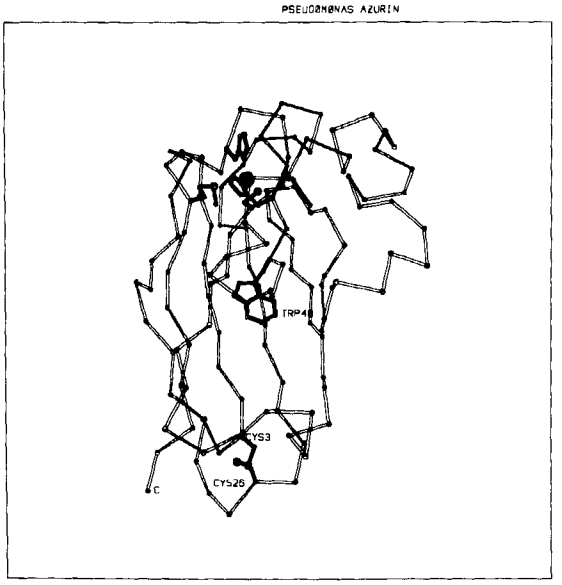

FIGURE 1. Three dimensional structure of Pseudomonas aeruginosa azurin[14]. Shown are the copper ion (large shaded circle) and (in bold lines) its four ligands, as well as Trp48 and the Cys3-Cys26 bridge. The data are taken from the Brookhaven Protein Data Bank, file 1AZU.

transfers the electron to the Cu(II) site[7]. Azurins with well defined structural differences provide therefore a most interesting system for investigation of the factors controlling this LRET.

The blue copper oxidases carry three distinct copper binding sites and catalyse the four-electron reduction of dioxygen yielding two molecules of water[11,12]. Single electrons are taken up from specific organic substrates by one specific copper site, called Type 1 (T1) copper, which is also responsible for the intense blue color. The three remaining copper ions are in close proximity, two forming a copper pair (T3) with strong electronic interaction making the Cu(II) state EPR undetectable, but with a strong near UV absorption. The Type 2 (T2) copper site is EPR detectable, but its absorption is too weak compared with T1 and T3 Cu(II) to be detectable.

The crystal structure of the multi copper enzyme ascorbate oxidase (A.O.) has recently been determined[15]. From the sequence homology around the copper centers it can be safely assumed that the metal sites in lac-

case are constructed in a similar way[12,15]. This makes a study of the intramolecular e.t. from T1 to T3 both interesting and significant.

EXPERIMENTAL

Preparations.
Azurins were isolated and purified from Pseudomonas aeruginosa, Pseudomonas fluorescens (biotype B), Alcaligenes spp. (Iwasaki), and Alcaligenes faecalis according to the method of Ambler and Wynn[16]. Laccase was isolated and purified from the acetone extract of the Japanese Rhus vernicifera lacquer tree according to the procedure of Reinhammar[17]. Prior to the pulse radiolysis experiments, all proteins were dialysed against the medium used in the kinetic experiments. The pH of the reactant solutions was adjusted by titrating the unbuffered solutions with formic acid or sodium hydroxide. Triply distilled water was used throughout.

Kinetic studies.
The pulse radiolysis experiments were carried out on the Varian V-7715 linear accelerator at the Hebrew University in Jerusalem. The experimental details are given in an earlier publication[7].

The copper redox state in azurin was monitored at 625 nm (ε_{625} = 5,700 M^{-1} cm^{-1} for Ps. and 4,100 M^{-1} cm^{-1} for Alc. azurin)[11]. The formation and decay of RS-SR^- were followed at 410 nm (ε_{410} = 10,000 M^{-1} cm^{-1})[7]. For laccase the absorption maximum for the T1 copper(II) is 614 nm (ε_{614} = 5,700 M^{-1} cm^{-1}) and for the Type 3 Cu(II) pair, the maximum is at 330 nm (ε_{330} = 2,400 M^{-1} cm^{-1}) [11].

In aqueous solutions irradiated with high energy electrons the following primary products are being formed

$$H2O \longrightarrow e^-_{aq}\ (2.65),\ OH\ (2.75),\ H\ (0.65),\ H_2\ (0.45),\ H_2O_2\ (0.32)$$

The numbers in parantheses represent number of molecules formed pr 100 eV of absorbed energy (G-values).

In N_2O saturated solutions e^-_{aq} is converted into OH radicals by the reaction:

$$e^-_{aq} + N_2O + H^+ \longrightarrow N_2 + OH$$

OH and H are converted into the milder reducing formate radical, CO_2^- (E' = -1.05 V) by reaction with formate ions:

$$OH/H + HCOO^- \longrightarrow CO_2^- + H_2O/H_2$$

CO_2^- is thus the sole radical produced in N_2O saturated formate solutions. Using pentaerythritol instead of formate produces an even milder reducing neutral radical:

$$(CH_2OH)_4C + OH \longrightarrow (CH_2OH)_3C\text{-}CHOH + H_2O$$

All reactions were performed under pseudo first order conditions using a large excess of protein over radical concentration.

RESULTS AND DISCUSSION

Azurin.

Cu(II)-azurin was found to be reduced by CO_2^- radical ions in two phases as monitored via the 625 nm band (Fig. 2). The fast phase was found to be first order in protein and in CO_2^- concentrations.

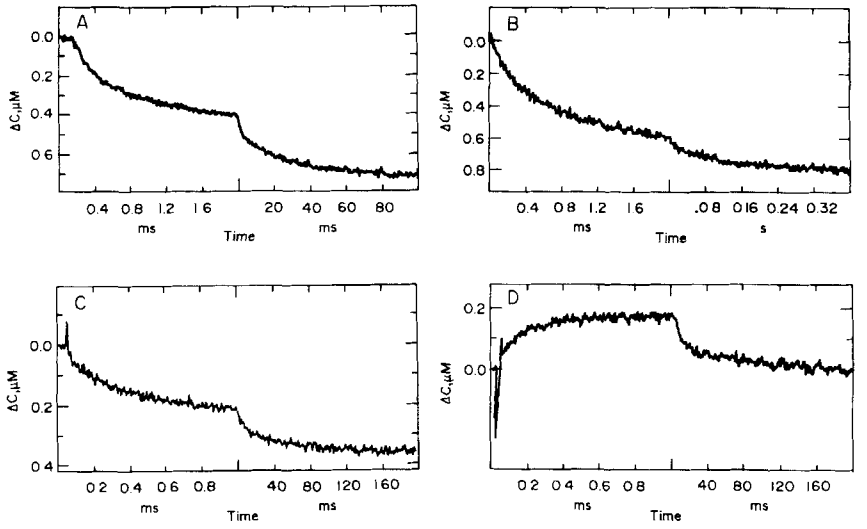

FIGURE 2. Time-resolved absorption changes of azurin following pulse radiolysis in N_2O saturated 0.1 M sodium formate, pH 7.0. (A) Ps. aeruginosa. 9.5 μM azurin; 625 nm; 25.0°C. (B) Alc. faecalis. 12.0 μM azurin; 625 nm; 30.1°C; (C) Ps. fluorescens, biotype B; 6.5 μM azurin; 625 nm; 18.2°C. (D) Same as (C) only at 410 nm.

In a slower time domain, a second reduction step is observed (Fig. 2), established as a first order process, independent of both protein and of reductant concentration (i.e. pulse width). The specific rates determined for the four different azurins at 25°C are listed in Table 1.

TABLE I. Rate constants at 298 K and activation parameters for the intramolecular reduction of Cu(II) by RS-SR^- in azurins at pH 7.0

Azurin	k, s^{-1}	ΔH^*, kJ mol^{-1}	$\Delta S^{\neq}$, J K^{-1} mol^{-1}	E', mV
P. aer.	44 ± 7	44.5 ± 4.0	-56.5 ± 7.0	304
A. spp.	8.5 ± 1.5	16.7 ± 1.5	-171 ± 18	260
P. fluor.	22 ± 3	36.2 ± 2.9	-36.2 ± 2.9	-
A. faec.	11 ± 2	54.4 ± 4.8	-45.0 ± 5.2	266

At 410 nm where azurin has a very small absorption in both the oxidized and reduced states, a strong transient band (Fig. 2D) is rapidly formed upon reduction by CO_2^-. This transient has been identified as a RS-SR^- radical ion[7]. Since all azurins contain an exposed single disul-

fide bridge (Fig. 1) we concluded that this radical ion is formed. The 410 nm absorption was found to decay in a slowd first order process, the rate constant of which was independent of both protein and CO_2^- concentration. The specific rates of the transient decay at 410 nm observed for all four azurins are, within experimental error, the same as the respective rates determined for the slow unimolecular Cu(II) reduction steps. This implies a slow intramolecular e.t. from $RS\text{-}SR^-$ to the Cu(II) center, a conclusion further supported by the finding that the yields of the transient $RS\text{-}SR^-$ correspond (± 10%) to those of Cu(I) formed in this step. The temperature dependencies of the slow intramolecular e.t. rates were measured for the four azurins over a 4-40°C range, monitored both at 625 nm (Cu(II) reduction) and at 410 nm ($RS\text{-}SR^-$ reoxidation). The activation parameters derived from these measurements are presented in Table 1.

We have earlier[7] estimated the reorganization energy, λ_v, of the slow intramolecular e.t. in P. aeruginosa to be 117 kJ mol^{-1}. This value includes the energy required for reorganization of the copper center, the disulfide site, and water molecules around it. λ is not likely to vary markedly among the four azurins examined here. Also, their redox potentials are not very different either. The LRET reactivity seems to be governed by the entropy of activation (Table 1). This term includes a contribution from the transition probability, κ[18]:

$$\Delta S^{\neq} = \Delta S^{*} + \ln \kappa$$

κ depends on the distance separating the electron donor from the acceptor[18]. The direct "through space" distance between the sulfurs of Cys3 and 112 (one of the copper ligands) is ~ 20 A in P. aer. azurin[14]. The three dimensional structure of the three other azurins studied are still unknown, but in view of their marked sequence homology, the distance is expected to be similar. However, this may not be the decisive factor. Rather, if the electron is mediated by coupling of donor and acceptor by a combination of covalent and hydrogen bonding, as well as non-covalent contacts, such a pathway may depend on very specific interactions within each protein. For P. aeruginosa azurin, the method of Beratan and Onuchic[10] was applied for calculating the tunneling matrix element for the donor/acceptor state at the appropriate nuclear configuration, assuming that the decay of the wave function can be expressed as a product of decays per bond[10]. In this way the putative shortest pathway was determined. It consists of a total of 25 bonds, including 2 hydrogen bonds and a single through-space jump (Fig. 3), with a total distance of 41 A. It is noteworthy that Trp48 is part of the pathway. We have earlier discussed the possible enhancement of e.t. rates through aromatic residues[11]. In the present calculations no specific parameters were assigned to this aromatic residue. Another point that has not yet been addressed, is the indole residue orientation which could be more or less advantageous. Two of the azurins studied here lack Trp48: In A. faecalis it is substituted by a Val, and in P. fluorescens residue 48 is a Leu. Significantly, this substitution causes only a limited decrease in reactivity, reducing the rate of intramolecular e.t. by a factor 2 - 4. Presently, no similar calculations were performed for these azurins, since their three dimensional structures are not yet known. However, model calculations based on sequence homology may be

very helpful. Finally, it should be reitterated here that the intramolecular e.t. studied in azurins, does not serve any physiological role, and hence, the path is not an an optimal one. This would probably be different for functional intramolecular e.t. in multi sited oxidases.

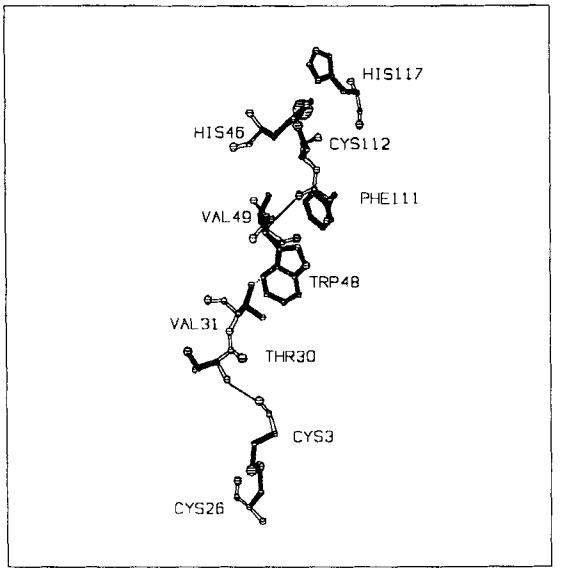

FIGURE 3. Calculated e.t. pathway from RS-SR$^-$ to Cu(II) in Ps. aeruginosa azurin. The pathway consists of 23 covalent bonds, 2 hydrogen bonds (thin line), and one through space jump (stipled line). The calculations were performed by Brad Jacobs, Chemistry Division, California Institute of Technology, using the procedure of Beratan and Onuchic[10].

Laccase.

The reaction of laccase with CO_2^- was studied under anaerobic conditions. At the T1 copper(II) absorption band (614 nm) a fast reduction step is observed with a first order rate constant of 120 $\pm$ 10 s^{-1} (Fig. 4), independent of both protein and reductant concentration. A transient with absorption maximum at 410 - 420 nm is formed within 1 ms and decays in a first order process (k = 100 $\pm$ 16 s^{-1}). This transient is most likely due to the formation of an RS-SR$^-$ radical anion. Thus, Type 1 Cu(II) is reduced by an intramolecular e.t. from the RS-SR$^-$, in a process analogous to that occurring in azurin.

Following the Type 1 Cu(II) reduction a slower reoxidation is observed (Fig. 4). This occurs with a rate constant of 1.0 s^{-1} independent of the extent of reduction of the type 1 Cu(II) and of the CO_2^- concentration. At 330 nm a decrease in absorption takes place on the same time scale. Since both rate and concentration changes are the same ($\pm$ 10%) as those observed at 614 nm, we conclude that this slow process is an intramolecular e.t. from T1 copper(I) to T3 Cu(II). In earlier studies[19] we found

that the rate limiting step for the T1 Cu(I) oxidation under turnover is not the reaction of the reduced enzyme with O_2, but rather an intramolecular e.t. step

$$v_{\text{steady state}} = k_{ox}\,[\text{T1 Cu(I)}]$$

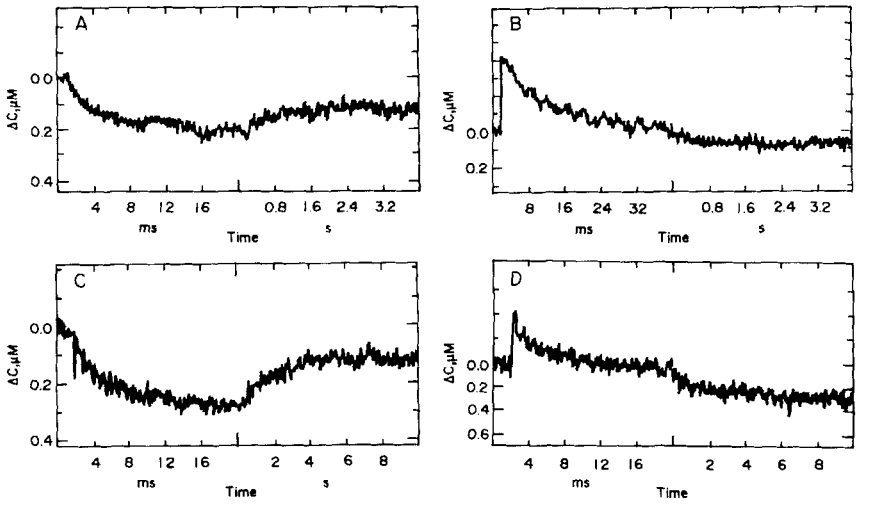

FIGURE 4. Time-resolved absorption changes of 10 μM laccase solutions following pulse radiolysis at pH 7.0. (A) Reduction and reoxidation of laccase monitored at 614 nm. 0.1 M formate; 22.0°C. (B) Same conditions as (A) but fast and slow reaction phases followed at 330 nm. (C) Reduction and reoxidation of laccase; 614 nm. 0.1 M pentaerythritol; 12.3°C. (D) Same conditions as (C) except that the reaction was followed at 330 nm at 10.8°C.

In an extensive series of experiments, k_{ox} was found = 4.3 $\pm$ 1.5 s^{-1}, fourfold the above T1 to T3 intramolecular e.t. rate. This raises the question to what extent the slow T1 Cu(I) to T3 Cu(II) e.t. reported here is physiologically significant. To further pursue this point, we studied the effect of fluoride ions which are known to inhibit the enzymatic activity of laccase by binding to the T2 copper site. In a series of experiments, where laccase was treated with 5 mM fluoride, inhibition of both the fast reduction of T1 Cu(II) and the slow intramolecular T1 to T3 e. t. (k_{red} = 23 s^{-1} and k_{reox} = 0.25 s^{-1} 25°C, pH 7.0) was observed. In order to exclude the possibility of anionic inhibition by $HCOO^-$ of the intramolecular e.t., we have also examined the reactivity of laccase under anaerobic conditions using the pentaerythritol radical as reducing agent (cf. Experimental section). This uncharged, rather bulky molecule is not likely to get access to the T2 site and its redox potential is more positive than that of CO_2^-; hence it is unable to reduce the disulfide. Absorption changes observed at 614 nm and 330 nm using pentaerythritol radicals as reductant are illustrated in Figure 4. Reduction of

the T1 Cu(II) is no longer a first order process, but depends on the radical concentration. Still, the slower reoxidation of T1 Cu(I) is also observed under these conditions, and has the same rate as that observed when CO_2^- is the reductant (± 10%). Furthermore, it corresponds to the rate of the slow reduction of Type 3 Cu(II) (1.1 s^{-1}, 25°C, pH 7.0). Thus, the slow intramolecular T1 to T3 e.t. is unaffected by formate ions.

An alternative rationale for the intramolecular e.t. in laccase being slower than the turnover rate is that the oxidase should be first activated by going through one or more catalytic cycles. We have therefore compared the reactivity of freshly thawed laccase with enzyme solutions that have gone through 24 hours turnover with ascorbate under aerobic conditions. However, no difference in reduction behavior by CO_2^- radicals of the two batches could be resolved. Another interesting possibility is that singly reduced laccase molecules react slower than those molecules containing more reduction equivalents. The low yield of reduced laccase pr pulse does not lead to a sigificant amount of multiply reduced molecules. Future pulse radiolysis studies of laccase reduced to different extents could resolve this possibility.

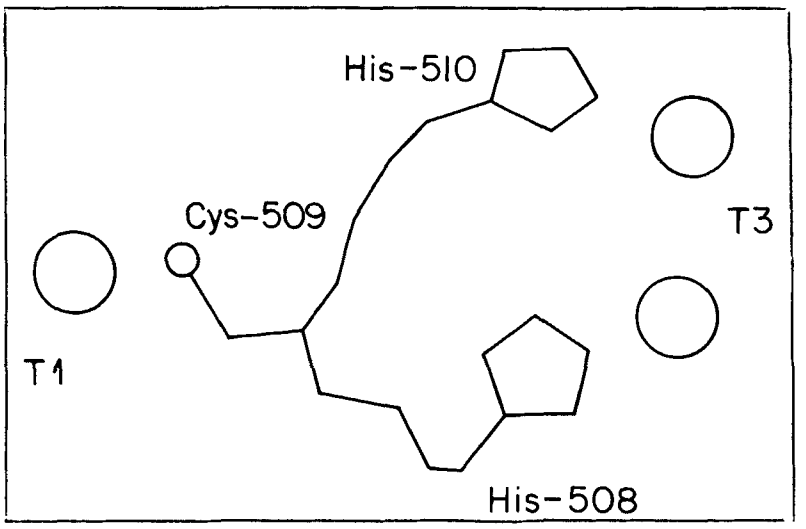

FIGURE 5. A proposed intramolecular e.t. pathway from Type 1 to Type 3 copper in laccase. The drawing is based on the crystal structure of ascorbate oxidase[15].

The three dimensional structure of ascorbate oxidase has recently been determined by x-ray crystallography[15]. Based on sequence homology, it may be assumed that the spatial folding of A.O. and laccase is quite similar. Thus, both enzymes contain a polypeptide strech with a -His-Cys-His- sequence[12], which in the A.O. structure provides the T1 and T3 copper ligands[15]. In laccase it most likely serves the same function. Using the

structure of A.O. we have drawn an outline of the T1 - T3 region shown in Fig. 5. The through-space distance from S_γ of Cys509 to N_ε of either His508 or His510 is ~ 9 A, and both paths include 9 covalent bonds. The through bond distance, using average bond lengths, is approximately 13.5 A. The question then arises why the intramolecular e.t. rate between T1 and T3 copper over a distance that is only a third of that betwen RS-SR^- to Cu(II) in azurin, is 40 times slower. One reason is that for the latter reaction the driving force is 69 kJ mol^{-1}, while in laccase the T1 - T3 e.t. driving force is only 7 kJ mol^{-1}.

We have examined the temperature dependence of this intramolecular e.t. The enthalpy of activation is 47.5 kJ mol^{-1} and the entropy of activation is -82 J $K^{-1}mol^{-1}$. The activation free energy, ΔG^*, the standard free energy of activation, ΔG^o, and the reorganization energy, λ, are related[18]:

$$\Delta G^* = \lambda/4(1 + \Delta G^o/\lambda)^2$$

λ = 120 kJ mol^{-1} for azurin[7] and we calculate a ΔG^* of 5 kJ mol^{-1}. In laccase, with an evolutionary optimized e.t. pathway, the reorganization energy is probably much lower than along the non-physiological e.t. path of azurin. Gray and co-workers[20] have estimated a reorganization energy, $\lambda/4$, for the T1 site in azurin to be less than 30 kJ mol^{-1}. Using an overall λ = 70 kJ mol^{-1}, we calculate a ΔG^* for laccase of 14 kJ mol^{-1}. This difference in activation free energy between azurin and laccase corresponds to the observed factor of 40 in the rate constants at 25°C. This marked difference in reactivity should therefore be further pursued in order to establish its functional significance.

Acknowledgements:
O.F wishes to acknowledge a short-term EMBO fellowship, (ASTF 6317) and financial support from the Danish Natural Science Research Council. We are grateful to professor R.P. Ambler for generously supplying the P. fluorescens, biotype B., and to Dr. D.N. Beratan and Mr. Brad Jacobs, California Institute of Technology, for calculations of e.t. pathways in azurins.

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