J|A|C|S COMMUNICATIONS

Published on Web 12/07/2009

Site−Site Interactions Enhances Intramolecular Electron Transfer in *Streptomyces coelicolor* laccase

Ole Farver,*,† Armand W. J. W. Tepper,‡ Scot Wherland,§ Gerard W. Canters,‡ and Israel Pecht||

Institute of Analytical Chemistry, University of Copenhagen, 2100 Copenhagen, Denmark, Leiden Institute of Chemistry, Leiden University, 2300RA, Leiden The Netherlands, Department of Chemistry, Washington State University, Pullman, Washington 99164-4630, and Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

Received October 15, 2009; E-mail: of@farma.ku.dk

The multicopper oxidases (MCOs)[1] catalyze the four-electron reduction of O_2 to H_2O. Here we report that the intramolecular electron transfer (ET) rates in the small bacterial laccase isolated from *Streptomyces coelicolor* (SLAC)[2] markedly depend on the reduction state of the enzyme, possibly reflecting site−site interactions so far not observed in other MCOs. SLAC has recently been shown to have structural and reactivity features distinct from those of other laccases.[3,4] It was therefore of interest to investigate the intramolecular ET rates in SLAC.

The type 1 (T1) copper site transfers single electrons from substrates to the type 2/type 3 (T2/T3) trinuclear center (TNC) through efficient *through-bond* ET paths from T1 to *each* T3 Cu. SLAC is a trimer of identical, single peptide chain monomers.[4]

Pulse-radiolytically produced CO_2^- radicals reduce T1Cu(II) on a submillisecond time scale. The observed rate constant was found to linearly depend on protein concentration, characterizing a second-order process with a rate constant of $(1.9 \pm 0.2) \times 10^8$ M^{-1} s^{-1}. Subsequently, a partial T1Cu(I) reoxidation was observed with a concentration independent rate constant, ($k_{ET} = 6 \pm 1$ s^{-1}, 285 K in the initial reduction steps). Concomitant reduction of T3Cu(II) demonstrated that an intramolecular ET is taking place between the T1 and T3 sites (*cf.* Figure S2 in the Supporting Information (SI)). Both rates and amplitudes measured at 590 nm and 330 nm confirm this conclusion. Furthermore, reduction by 1-methyl nicotinamide radicals (1-MNA*) exhibited similar reaction patterns and identical rate constants.

The rate of intramolecular ET from T1Cu(I) to T3Cu(II) increased more than 10-fold as the SLAC reduction state increased with sequential pulses. Experiments were carried out on both fresh ("as isolated") SLAC solutions and on samples reoxidized by O_2 after full reduction by the radicals or by ascorbate ("cycled"). Both enzyme forms exhibited marked enhancement of the intramolecular ET rate constant; yet the change was larger for "cycled" samples (15−186 s^{-1}). Temperature dependence measurements of the internal ET rates enabled determination of the activation parameters (Table 1 and SI).

The suggested mechanism, independent of the enzyme form is

1. $R^* + T_1{}^OT_3{}^{OO} \rightarrow T_1{}^RT_3{}^{OO} \leftrightarrow T_1{}^OT_3{}^{RO}$ (internal ET with initial rate)
2. $R^* + T_1{}^OT_3{}^{RO} \rightarrow T_1{}^RT_3{}^{RO} \leftrightarrow T_1{}^OT_3{}^{RR}$ (internal ET with a higher rate)
3. $R^* + T_1{}^OT_3{}^{RR} \rightarrow T_1{}^RT_3{}^{RR}$ (R^* symbolizes reducing radicals, and the O and R superscripts indicate oxidized and reduced copper centers, respectively).

† University of Copenhagen.
‡ Leiden University.
§ Washington State University.
|| The Weizmann Institute of Science.

Table 1. Kinetic and Activation Parameters for the Intramolecular ET Equilibration in SLAC

enzyme form	reduction state	k_{ET}/s^{-1} (298 K)	$\Delta H^‡$ kJ mol^{-1}	$\Delta S^‡$ J K^{-1} mol^{-1}
"cycled"	initial	15 ± 3	9.5 ± 3.7	-189 ± 46
	final	186 ± 25	26.2 ± 6.0	-114 ± 18
"as isolated"	initial	8 ± 1	25.2 ± 3.2	-142 ± 15

According to this mechanism, as the reduction proceeds, the time-resolved absorption is expected to be biexponential. However, as also confirmed by simulations, the two exponentials could not be resolved with the available S/N level of the data and only a single weighted average for the ET rates could be extracted. Figure 1 illustrates two sets of data showing the observed rate constant increase as a function of electron equivalents added to the enzyme. This increase is not strictly linear but rather sigmoid for both "as isolated" and "cycled" SLAC. No further ET equilibration was observed after the uptake of 2 electron equivalents suggesting that fully reduced T3 species predominate at this stage and that T2Cu is not involved in this equilibration process. This is supported by the finding that ascorbate does not reduce T2Cu(II) in SLAC, indicating a low reduction potential for T2Cu (A. Tepper & G. W. Canters, unpublished results), and by simulations (SI).

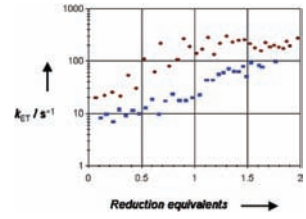

Figure 1. Observed rate constants of intramolecular T1Cu(I) to T3Cu(II) ET in SLAC as a function of sequentially introducing reduction equivalents (25 pulses, 0.3 μs each). (■) 22.7 μM "as isolated" laccase, (●) same solution after being "cycled"; 5 mM 1-MNA, 100 mM *tert*-butanol, 10 mM potassium phosphate, pH 7.3, and 25 °C. Ar saturated, anaerobic conditions.

The marked rate enhancement could be caused by changes in electron tunneling pathways, driving force, and/or reorganization energy. T1 and the two T3 Cu ions are linked, each by 11 covalent bonds, and the distances separating them are 12.2 and 12.7 Å, respectively.[4] Using an electronic decay factor of 1.0 $Å^{-1}$ [5] we calculate the activationless $k_{MAX} = 2.4 \times 10^7$ s^{-1}. The rate constant for initial reduction of the "as isolated" enzyme, 8 s^{-1}, is equivalent to an activation energy, $\Delta G^‡ = 0.378$ eV. We find the equilibrium constant for reaction 1 to be 0.41 at 298 K, equivalent to a driving force, $-\Delta G^0 = -0.023$ eV, and using the semiclassical Marcus

10.1021/ja908793d

equation,[6] a reorganization energy, $\lambda_{TOT} = 1.46$ eV, is calculated in agreement with all previously reported values.[5] The equilibrium constant for reaction 2 is 5.0 corresponding to $-\Delta G^0 = +0.041$ eV. This larger driving force contributes slightly, but since $|\Delta G^0| \ll \lambda_{TOT}$, the change in ET rate must be dominated by changes in λ_{TOT}. The ~20-fold increase in rate constant requires a decrease in $\Delta G^{\ddagger}$ to 0.32 eV that could occur by a small (0.2 eV) decrease in λ_{TOT} to 1.3 eV. Thus, upon reduction, structural changes in the TNC induced directly in the coordination sphere, in the SLAC quaternary structure, or in both must be the primary cause for lowering the reorganization energy.

Since ET from T1 to T3 proceeds in two separate steps, a semireduced T3 center is a necessary transient. In ascorbate oxidase, the reduced T3 Cu−Cu separation is 5.1 Å as compared to 3.8 ± 0.1 Å in the oxidized state.[7] Similar findings have been reported for T3 Cu proteins including hemocyanin (Hc),[8] tyrosinase (Ty),[9] and catechol oxidase.[10] No structural data are available for the intermediate reduction state of the MCOs. Still, the semireduced T3 site ("half-met form") has been studied for Hc's and Ty by spectroscopic techniques.[11,12] From EPR investigations on *Streptomyces antibioticus* Ty it has been concluded that the paramagnetic Cu in the half-met form still carries a hydroxy (or water) ligand, which is displaced when substrates bind.[12] The T1 to T3 transfer of the first electron in SLAC probably leads to uncoupling of the T3 pair and breaking of the OH^- bridge but leaves an ET path intact. The structure of this semireduced intermediate would thus be closer to that of the fully reduced than to the oxidized state and cause the observed decrease in λ_{TOT} for the second internal ET step.

No similar variation of internal ET rate with the extent of reduction has so far been observed in MCOs, neither in AO[13,14] nor in ceruloplasmin.[13] In SLAC, both of the T3 Cu−Cu distances indicate typical MCO structure in the oxidized state and do not suggest any particular structural origin for the rate enhancement as reduction proceeds. Thus, changes in the T3 coordination sphere may not be the only cause for the enhanced reactivity upon single electron reduction. Indeed, SLAC belongs to a structurally different MCO group;[4] hence the likely origin of the exceptional behavior could be its trimeric structure. Since the SLAC TNC is located at a monomer−monomer interface and each T3 Cu is bound to ligands from two *different* monomer chains,[4] it is conceivable that changes in the TNC reduction state cause changes in the quaternary structure of the trimer.[4,7,15] As stated by Skálova et al.,[4] "The trinuclear copper site tightly connects the two neighboring chains in the trimer" and thus provides a mechanism for quaternary structural change on reduction. In the analogous copper nitrite reductases, which are also homotrimers, evidence for marked interactions among the monomers has indeed been provided.[16]

The different rates of initial ET in "as isolated" and "cycled" forms of SLAC are reflected in a lower enthalpic contribution to the activation free energy and a more pronounced and compensating entropic contribution in the latter case. Uptake of the second electron and its transfer to the T3 probably coincide with the dissociation of this site's bridging ligand. The bond breaking in the transition state might cause the larger $\Delta H^{\ddagger}$ while loosening of the TNC structure could explain the $\Delta S^{\ddagger}$ increase between initial and final states of the "cycled" forms.

Control of internal ET rates by intrinsic site−site interactions is an intriguing functional feature, encountered in MCOs for the first time here. The only other known case of such control involving transition metal based redox catalysis is a negative site−site interaction between the hemes in *Pseudomonas aeruginosa* and *Pseudomonas stutzeri cd*$_1$ nitrite reductases.[17] The enhanced ET reactivity with increasing extent of reduction and the resultant preference for fully reduced T3 observed in SLAC have consequences for the O_2 reduction process: Binding of O_2 to $T_1^R T_3^{RR}$ or $T_1^O T_3^{RR}$ would yield a peroxy intermediate promoting additional electron uptake by the enzyme before the crucial steps of dioxygen bond splitting and water formation.[18] Moreover, by favoring the formation of the fully reduced T3 site over two half-reduced sites, this control provides a possible evolutionary advantage under conditions of limited reducing substrate.

Acknowledgment. O.F. thanks the Kimmelman Center for Biomolecular Structure and Assembly at the Weizmann Institute of Science for generous support. We thank Mr. Eran Gilad of the Hebrew University in Jerusalem for his technical support in running the accelerator. Dr. Miriam Eisenstein, Chemical Research Support, The Weizmann Institute of Science, is acknowledged for help in studying 3D structures and insightful ideas.

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Malmström B. G. In *Multicopper Oxidases*; Messerschmidt, A., Ed.; World Scientific: Singapore, 1997; pp 1−22.
(2) Machczynski, M.; Vijgenboom, E.; Samyn, B.; Canters, G. W. *Protein Sci.* **2004**, *13*, 2388–2397.
(3) Tepper, A. W. J. W.; Milikisyants, S.; Sottini, S.; Vijgenboom, E.; Groenen, E. J. J.; Canters, G. W. *J. Am. Chem. Soc.* **2009**, *131*, 11680–11682.
(4) Skalova, T.; Dohnalek, J.; Ostergaard, L. H.; Ostergaard, P. R.; Kolenko, P., P.; Duskova, J.; Stepankova, A.; Hasek, J. *J. Mol. Biol.* **2009**, *385*, 1165–1178, and references therein.
(5) Gray, H. B.; Winkler, J. R. *Q. Rev. Biophys.* **2003**, *36*, 341–372.
(6) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265–322.
(7) Messerschmidt, A.; Luecke, H.; Huber, R. *J. Mol. Biol.* **1993**, *230*, 997–1014.
(8) Magnus, K. A.; Hazes, B.; Ton-That, H.; Bonaventura, C.; Bonaventura, J.; Hol, W. G. J. *Proteins* **1994**, *281*, 302–309.
(9) Yasuyuki, M.; Kumagai, T.; Yamamoto, A.; Yoshitsu, H.; Sugiyama, M. *J. Biol. Chem.* **2006**, *281*, 8981–8990.
(10) Klabunde, T.; Eicken, C.; Sacchettini, J. C.; Krebs, B. *Nat. Struct. Biol.* **1998**, *5*, 1084–1090.
(11) Tepper, A. W. J. W.; Bubacco, L.; Canters, G. W. *J. Biol. Chem.* **2004**, *279*, 13425–13434.
(12) van Gastel, M.; Bubacco, L.; Groenen, J. J.; Vijgenboom, E.; Canters, G. W. *FEBS Lett.* **2000**, *274*, 228–232.
(13) (a) Farver, O.; Pecht, I. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 8283–8287. (b) Farver, O.; Wherland, S.; Pecht, I. *J. Biol. Chem.* **1994**, *269*, 22933–22936. (c) Farver, O.; Bendahl, L.; Skov, L. K.; Pecht, I. *J. Biol. Chem.* **1999**, *274*, 26135–26140.
(14) (a) Meyer, T. E.; Marchesini, A.; Cusanovich, M. A.; Tollin, G. *Biochemistry* **1991**, *30*, 4619–4623. (b) Tollin, G.; Meyer, T. E.; Cusanovich, M. A.; Curir, P.; Marchesini, A. *Biochim. Biophys. Acta* **1993**, *1183*, 309–314. (c) Kyritsis, P.; Messerschmidt, A.; Huber, R.; Salmon, G. A.; Sykes, A. G. *J. Chem. Soc., Dalton Trans.* **1993**, 731–735.
(15) Zaitseva, I.; Zaitsev, V.; Card, G.; Moshkov, K.; Bax, B.; Ralph, A.; Lindley, P. *J. Biol. Inorg. Chem.* **1996**, *1*, 15–23.
(16) (a) Li, H.-T.; Chang, T.; Chang, W.-C.; Chen, C.-J.; Liu, M.-Y.; Gui, L.-L.; Zhang, J.-P.; An, X.-M.; Chang, W.-R. *Biochem. Biophys. Res. Commun.* **2005**, *338*, 1935–42. (b) Lawton, T. J.; Sayavedra-Soto, L. A.; Arp, D. J.; Rosenzweig, A. C. *J. Biol. Chem.* **2009**, *284*, 10174–10180.
(17) (a) Farver, O.; Kroneck, P. M. H.; Zumft, W. G.; Pecht, I. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 7622–7625. (b) Farver, O.; Brunori, M.; Cutruzzolà, F.; Rinaldo, S.; Wherland, S.; Pecht, I. *Biophys. J.* **2009**, *96*, 2849–2856.
(18) Farver, O.; Goldberg, M.; Pecht, I. *Eur. J. Biochem.* **1980**, *104*, 71–77.

JA908793D