JBIC Journal of Biological Inorganic Chemistry (2020) 25:233–238
https://doi.org/10.1007/s00775-020-01754-7

ORIGINAL PAPER

Stability/activity tradeoffs in *Thermus thermophilus* HB27 laccase

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Received: 4 November 2019 / Accepted: 25 December 2019 / Published online: 22 January 2020

Abstract
We report the temperature dependence of the formal potential of type 1 copper (Cu_{T1}) in *Thermus thermophilus* HB27 laccase. Employing $[Ru(NH_3)_4(bpy)](PF_6)_2$ (0.505 vs. NHE) as the redox titrant, we found that the $Cu_{T1}^{2+/+}$ potential decreased from approximately 480 to 420 mV (vs. NHE) as the temperature was raised from 20 to 65 °C. Of importance is that the ΔS_{rc}° of − 120 J mol^{-1} K^{-1} is substantially more negative than those for other blue copper proteins. We suggest that the highly unfavorable reduction entropy is attributable to Cu_{T1} inaccessibility to the aqueous medium. Although the active site residues are buried, which is critical for maintaining thermostability, the flexibility around Cu_{T1} is maintained, allowing enzyme activity at ambient temperature

Keywords Electron transfer · Thermodynamics · Thermophile

Abbreviations

Tth-lac, PDB ID: 2YAE	*Thermus thermophilus* HB27 Laccase
CPB, 2CBP	*Cucumis sativus* Cucumber basic protein
SBP, 1F56	*Spinacea oleracea* Spinach basic protein
UmCy, 1X9R	*Armoracia laphatifolia* Umecyanin
StCy, 1JER	*Cucumis sativus* Stellacyanin
PlCy, 1AG6	*Spinach oleracea* Plastocyanin
AfAz, 2IAA	*Alcaligenes faecalis* Azurin
PaAz, 5AZU	*Pseudomonas aeruginosa* Azurin
Trv-lac, 1GYC	*Trametes versicolor* Laccase

Introduction

The effects of temperature on enzyme survival and function have greatly influenced the studies of enzyme evolution [1, 2]. The enzyme of interest in this study is a laccase from a thermophilic bacterium, *Thermus thermophilus* HB27 (*Tth*-lac). Laccases, members of the multicopper oxidase (MCO) family, have four copper sites involved in electron transfer and enzyme catalysis. According to the consensus mechanism [3, 4], substrate oxidation occurs near a type 1 copper (Cu_{T1}), followed by long-range electron transfer to a trinuclear copper cluster, where, in a fully reduced enzyme, dioxygen is converted to water ($O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$). Of special interest is that laccases are capable of degrading lignin in recalcitrant lignocellulosic substrates as their primary function [5], even though the Cu_{T1} potentials in the bacterial enzymes [4] are as much as 0.5 V lower than that required for one-electron oxidation of polyphenols [6].

Tth-lac is optimally active at 65 °C for catalysis of aerobic oxidation of small polyphenolic substrates as well as ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic) acid], a common substrate for oxidative enzymes [7, 8]. In contrast to many other thermophilic enzymes [2, 9], *Tth*-lac is active at ambient temperature, although the catalytic efficiency (defined by the turnover number divided by the Michaelis constant, k_{cat}/K_m) for ABTS oxidation increases substantially with increasing temperature (Fig. 1). We anticipate that investigations of thermodynamic parameters will

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00775-020-01754-7) contains supplementary material, which is available to authorized users.

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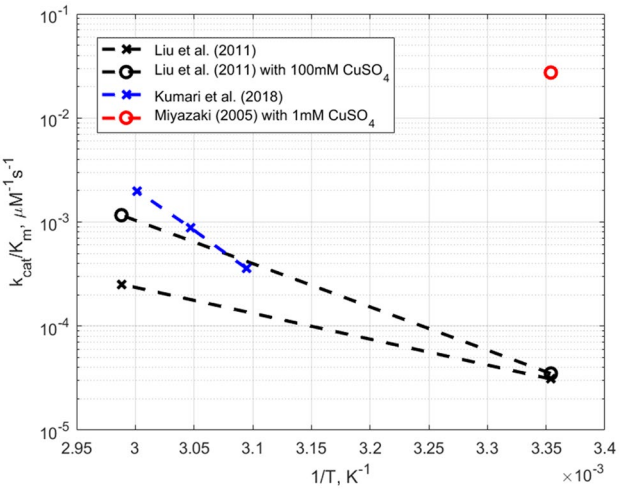

Fig. 1 Temperature dependence of the efficiency of *Tth*-lac catalyzed oxidation of substrate ABTS [7, 8, 29]

enhance the understanding of the remarkable stabilities and activities of extremophiles at elevated temperatures.

Surprisingly, the effects of temperature on the potentials of both the substrates and the active-site coppers have largely been neglected. As reduction entropies of $M^{(ox/red)}$ redox couples are proportional to ($Z_{ox}{}^2 - Z_{red}{}^2$), where Z_{ox} and Z_{red} are the charges of oxidized and reduced states [10], $\Delta S_{rc}°$ for $ABTS^{-/2-}$ is predicted to be negative, as observed for other $M^{-/2-}$ redox couples. It follows that the $ABTS^{-/2-}$ potential, which is about 670 mV (vs. NHE) at ambient temperature [11], is expected to decrease with increasing temperature. Based only on the decrease in ABTS potential, the activity of the enzyme likely would be higher at elevated temperatures. However, the charge dependence of $\Delta S_{rc}°$ is a small-molecule phenomenon; for a protein, as many other factors contribute, $\Delta S_{rc}°$ is unpredictable. Since the enzymatic activity also depends on the Cu_{T1} potential [12], we have investigated the temperature dependence of the Cu_{T1} potential in *Tth*-lac.

Methods

Sample preparation

Thermus thermophilus HB27 laccase was expressed in *E. coli* and purified following published methods [7] with slight modifications. (See the Supplementary Material (SM) for details on protein expression and purification protocols.) A two-step procedure with slight modifications was employed to ensure full metalation of the enzyme [8, 13]. The enzyme was metalated in 20 mM Tris buffer, pH 8 with 1 mM $CuSO_4$; and it was metalated again in 25 mM sodium acetate buffer, pH 6 with 1 mM $CuSO_4$ for at least 48 h. The enzyme was stored with excess copper at 4 °C until use, and the excess copper was removed by gradual buffer exchange to 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at pH 5.3 with multiple rounds of centrifugal spin filtration. The presence of a type 2 copper which has the smallest binding constant in the protein was confirmed with EPR and ICP-MS. $[Ru(NH_3)_4(bpy)](PF_6)_2$ was synthesized and characterized following published protocols (with slight modifications) [14].

Circular dichroism spectra

Circular dichroism (CD) spectra of the protein samples under N_2 were recorded from 260 to 190 nm to monitor conformational changes and/or thermal denaturation over the temperature range 20 to 65 °C. Measurements were made on 3 μM protein in 20 mM sodium phosphate buffer, pH 6. CD spectra reflecting the combined profiles of β-sheets, α-helices and random coils were obtained, and the spectra at different temperatures looked very similar (Figure S1), consistent with prior observations [8].

Estimation of the Cu_{T1} potential from redox equilibria

$Cu_{T1}{}^{2+/+}$ reduction potentials over the range 20–65°C were estimated by monitoring changes in UV–vis spectra of a deoxygenated sample containing the wild type protein and $[Ru(NH_3)_4(bpy)](PF_6)_2$. The formal $Ru^{3+/2+}$ potential of $[Ru(NH_3)_4(bpy)](PF_6)_2$ was reported to be 0.505 V [15], and $\Delta S_{rc}°$ is approximately + 56 J mol^{-1} K^{-1} [10]. A previous report estimated the *Tth*-lac $Cu_{T1}{}^{2+/+}$ potential to be approximately 0.5 V vs NHE at pH 5 and 6.5 [16].

Wild type *Tth*-lac (60 μM) and four equivalents (240 μM) of $[Ru(NH_3)_4(bpy)](PF_6)_2$ were deoxygenated by gentle evacuation/Ar-backfill cycles and then mixed together in a sealed quartz cuvette. UV–vis spectra of the sample were monitored at temperatures from 20 to 65 °C (Fig. 2). The spectra of $[Ru(NH_3)_4(bpy)]^{2+}$ and the wild type $Cu_{T1}{}^{2+}$ protein were monitored separately as functions of temperature. We assume that the $Cu_{T1}{}^{+}$ protein and $[Ru(NH_3)_4(bpy)]^{3+}$ do not make substantial contributions to spectra in the 400–700 nm range. Equilibrium concentrations of $Cu_{T1}{}^{2+}$ and Ru^{2+} in the mixed sample were determined by least squares decomposition of the mixed spectrum (480–650 nm) into a linear combination of the two component spectra.

Results and discussion

Temperature dependence of the Cu_{t1} formal potential

Based on redox equilibria determined by titration with $[Ru(NH_3)_4(bpy)](PF_6)_2$, the Cu_{T1} formal potential

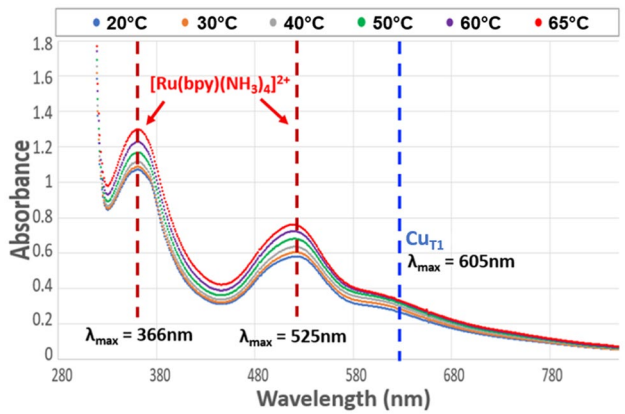

Fig. 2 UV–vis spectra (20–65° C) of a deoxygenated sample containing both wild type *Tth*-lac and four equivalents of $[Ru(NH_3)_4(bpy)](PF_6)_2$

decreased by approximately 60 mV (480 to 420 mV) from 20 to 65 °C. As temperature variations also affect buffer pH (the temperature coefficient for the pH of MES buffer is approximately − 0.011 per °C [17]), the pH is predicted to decrease from 5.3 to 4.8 with a temperature increase from 20 to 65 °C; and a pH decrease of 0.5 could produce an apparent increase in the $Cu_{T1}^{2+/+}$ potential at elevated temperature, even though solvent accessibility to the deeply buried copper site is low. It follows that the decrease in $Cu_{T1}^{2+/+}$ potential extracted from redox titration data at the higher temperature would be slightly greater if corrected for the pH change.

$Cu_{T1}^{2+/+}$ formal potentials in blue copper proteins range from approximately 200 to 800 mV and usually tend to decrease with increasing temperature (i.e., $\Delta S_{rc}^{\circ} < 0$) [18, 19]. The enthalpic contribution ($-\Delta H_{rc}^{\circ}/F$) to the potential is largely determined by ligand interactions with Cu_{T1}. A copper site with a weak axial bond has a higher formal potential, owing mainly to destabilization of the oxidized state [20]. The Cu_{T1} sites in high-potential laccases have a trigonal planar Cu_{T1} geometry with noncoordinating Phe or Leu residues in axial positions; and low-potential laccases have an axial Met residue in the inner coordination sphere. The entropic contribution ($T\Delta S_{rc}^{\circ}/F$) to the potential, on the other hand, is influenced by interactions with the protein scaffold and the surrounding aqueous medium.

The *Tth*-lac ΔH_{rc}° is comparable to that of other blue copper proteins [18, 19], but the standard reduction entropy change is much more negative (Fig. 3). It is notable that the *Polyporous versicolor* laccase ΔH_{rc}° (− 73.1 kJ mol^{-1}) is close to that of *Tth*-lac (− 81 kJ mol^{-1}), but ΔS_{rc}° for *P. versicolor* laccase (+ 7.1 J mol^{-1} K^{-1}) is much more positive, accounting for its high potential (780 mV vs. NHE) [18]. As a consequence of an unfavorable ΔS_{rc}° (− 120 J mol^{-1} K^{-1}), the potential of *Tth*-lac is much lower (Table 1).

The reduction entropy change for small molecules depends primarily on the ionic charge, owing to the entropy associated with solvent polarization for more highly charged ions. For redox centers buried inside proteins, the contribution to ΔS_{rc}° from solvent polarization will likely be attenuated, and the response of the polypeptide matrix to a change in oxidation state will become more important. We anticipate, then, that the large negative ΔS_{rc}° value for *Tth*-lac may be attributable to a combination of reduced solvent exposure and a more hydrophobic environment around the Cu_{T1} active site [18]. Hydrophobicity [21] and polarity [22] indices for all residues within 8 Å of Cu_{T1} were summed to characterize redox-center environments in nine different proteins, including *Tth*-lac. Of interest is that ΔS_{rc}° is not strongly correlated with either parameter (see Tables S2–S3, Figures S4–S6).

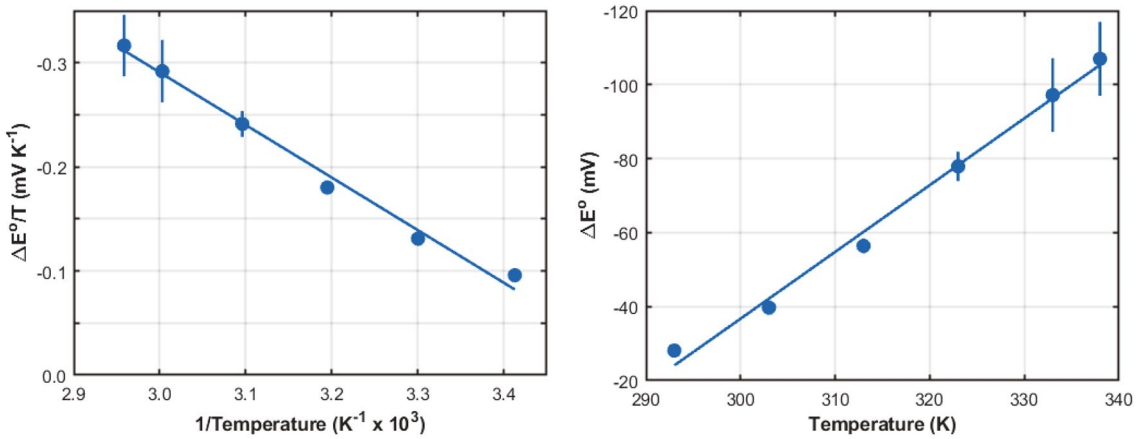

Fig. 3 Plots of $\Delta E^{\circ}/T$ vs 1/T (left) and ΔE° vs T (right) provide values of $\Delta H^{\circ} = -48$ kJ mol^{-1} and $\Delta S^{\circ} = -175$ J mol^{-1} K^{-1} for the reaction of *Tth*-lac Cu_{T1}^{2+} with $[Ru(NH_3)_4(bpy)]^{2+}$. The standard reduction enthalpy ($\Delta H_{rc}^{\circ}(Cu^{2+/+}) = -81$ kJ mol^{-1}, NHE reference) and entropy ($\Delta S_{rc}^{\circ}(Cu^{2+/+}) = -120$ J mol^{-1} K^{-1}) changes associated with Cu_{T1}^{2+} reduction are estimated by adding $\Delta H_{rc}^{\circ}(Ru^{3+/2+})$ and $\Delta S_{rc}^{\circ}(Ru^{3+/2+})$ [10] to ΔH° and ΔS°, respectively

Table 1 Effect of temperature on the reaction between Cu_{T1}^{2+} and $[Ru(NH_3)_4(bpy)]^{2+}$

T (K)	293	303	313	323	333	338
K_{eq} [a]	0.33(1)[b]	0.218(7)	0.123(5)	0.061(3)	0.034(4)	0.025(7)
$\Delta G°$ (kJ mol^{-1})	2.72(9)	3.8(1)	5.5(2)	7.5(4)	9.4(9)	10(2)
$\Delta E°$ (mV)	−28(1)	−40(1)	−56(2)	−78(4)	−100(10)	−110(10)
$E°(Ru^{3+/2+})$ (mV)[c,d]	505	511	517	522	528	531
$E°(Cu^{2+/+})$ (mV)[c]	477(1)	471(1)	460(2)	445(4)	430(10)	420(10)

[a]Equilibrium constant for the following reaction: $Cu_{T1}^{2+} + Ru^{2+} \overset{K_{eq}}{\rightleftharpoons} Cu_{T1}^{+} + Ru^{3+}$, $K_{eq} = \frac{[Cu^+][Ru^{3+}]}{[Cu^{2+}][Ru^{2+}]}$

[b]Estimated uncertainties in the last digit appear in parentheses. Error estimates provided by least-squares analyses of the data [see the SM for details.]

[c]Formal potential vs. NHE

[d]Reference [10]

Table 2 Reduction entropy change ($\Delta S_{rc}°$, J mol^{-1} K^{-1}) and measures of solvent accessibility of copper ligands in blue copper proteins (values of $\Delta S_{rc}°$ except for *Tth*-lac are from [18, 19] measured in the 5–45 °C temperature range)

	$\Delta S_{rc}°$ (J mol^{-1} K^{-1})	SASA (Å^2)	RSA (%)
CPB	31	39.8	18
SBP	7	41.8	19
UmCy	−17	47.8	22
StCy	−21	52.4	24
Trv-lac	−29	18.6	9
PlCy	−36	28.9	13
AfAz	−58	2.4	1.1
PaAz	−68	1.9	0.9
Tth-lac	−120	2.1	1.4

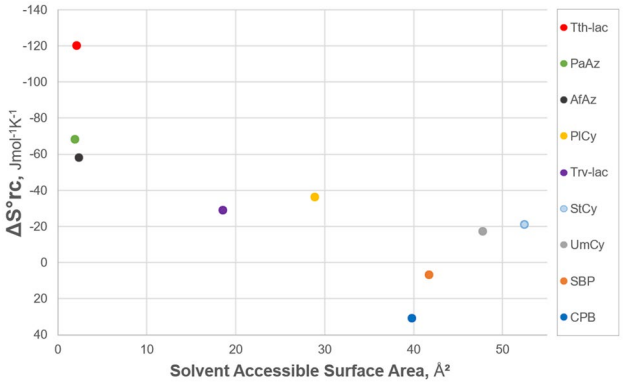

Fig. 4 Plot of the $Cu_{T1}^{2+/+}$ reduction entropy ($\Delta S_{rc}°$, J mol^{-1} K^{-1}) versus the solvent accessible surface area (Å^2) of active-site binding residues in blue copper proteins

Calculations of solvent accessible surface areas (SASA) of Cu_{T1} ligands provide quantitative estimates of redox-site exposure to aqueous solvent (Table S4). The relative solvent accessibility (RSA) for each residue is defined as the SASA normalized by a maximum allowed SASA [23]. Values of SASA and RSA for Cu_{T1} sites in nine proteins with known thermodynamic properties are given in Table 2. Notably, there is an apparent correlation between reduction entropy ($\Delta S_{rc}°$) and SASA: copper proteins with smaller SASA tend to exhibit more negative entropy changes upon reduction (Fig. 4). From our analysis, we have confirmed that reduced solvent accessibility around the metal site is one of the critical parameters affecting $\Delta S_{rc}°$. Note that in *Tth*-lac, only 0.04% of His393 and 1.4% of Cys445 are exposed to the aqueous medium. It is clear that the solvent accessibility of Cu_{T1} ligands in *Tth*-lac is lower than in other blue copper proteins: ligand exposures are approximately 20% in proteins with more positive reduction entropies, a group including CBP, SBP, UmCy, and StCy. RSA values exhibit a similar correlation with $\Delta S_{rc}°$ as shown in the SM (Figure S7). Analogous trends were found in a prior investigation of cytochrome redox thermodynamics; in this study, the values of $\Delta S_{rc}°$ correlated with heme solvent exposure [24].

The limited exposure of *Tth*-lac Cu_{T1} active site minimizes the solvent contribution to the reduction entropy change. The large negative $\Delta S_{rc}°$ observed for *Tth*-lac Cu_{T1}, then, likely arises from a substantial decrease in conformational entropy of the protein upon reduction. Acrylamide quenching of Trp fluorescence in oxidized *Tth*-lac (10–35 °C), an indicator of protein flexibility, is much greater than expected for a thermophilic enzyme [2, 9, 25]. We suggest that the structural flexibility of oxidized *Tth*-lac balances the molecular motions required for enzyme turnover while maintaining sufficiently low reorganization to support intraprotein electron transfer, thereby accounting for the observed oxidase activity at 25 °C [25].

If the Cu_{T1} active site ($[Cu(N_{His})_2(S_{Cys})(S_{Met})]^{+/0}$) were free in aqueous solution, $\Delta S_{rc}°$ would be positive, owing to the positive charge on the oxidized complex [10]. When the polypeptide of *Tth*-lac folds around this active site, the value of $\Delta S_{rc}°$ drops precipitously. A thermodynamic cycle indicates that the folding entropy change for the reduced protein is substantially more negative than that of the oxidized enzyme. The two primary contributions to the folding entropy change are a polypeptide conformational component (ΔS_{conf}) and a

hydration entropy change (ΔS_{hyd}) arising from encapsulation of hydrophobic residues in the protein [26]. The conformational entropy change upon folding is negative, whereas hydration makes a positive contribution to the total folding entropy change. We anticipate little difference in ΔS_{hyd} between the oxidized and reduced enzymes, suggesting that ΔS_{conf} must be substantially more unfavorable for folding around the reduced enzyme. The more negative value of ΔS_{conf} is consistent with less flexibility in the reduced enzyme.

It is notable that *Tth*-lac exhibits characteristics of both hot and cold adaptations: thermal stability (Figure S1) and flexibility [25]. The robust global structure of *Tth*-lac disfavors protein unfolding and copper loss at elevated temperatures [25]. We infer from the small value of SASA that Cu_{T1} in Tth-lac is buried inside the protein, and that this burial provides stability at the cost of a highly unfavorable reduction entropy. The large negative ΔS_{rc}° value for Cu_{T1} *Tth*-lac likely will lead to highly negative ΔS° values in substrate oxidation reactions, owing to entropically disfavored formation of cationic substrates [10]. At low reaction driving forces, the activation entropy for electron-transfer (ET) reactions is [27]:

$$\Delta S^{\dagger} = \frac{\Delta S^{\circ}}{2} - R\beta d,$$

where R is the gas constant, β is the exponential distance-decay factor for electron transfer (1.1 $Å^{-1}$, [28]), and d is the electron donor–acceptor separation. Negative values of ΔS° will have the same impact on ET rates as increasing the donor–acceptor distance by about 0.05 Å per entropy unit (J mol^{-1} K^{-1}), corresponding to a factor of 10 decrease in rate constant for each − 40 J mol^{-1} K^{-1} of reaction entropy change. Conversely, oxidation of *Tth*-lac Cu_{T1}^{+}, as occurs during enzyme turnover, is likely to be accompanied by a favorable entropy change, which could compensate for the weak coupling associated with the long distance between Cu_{T1}^{+} and the trinuclear Cu active site.

Concluding remarks

Thermophilic metalloenzymes tend to have active sites buried in a matrix of hydrophobic residues. We suggest that a tightly packed polypeptide scaffold limits solvent access to the active site, stabilizing *Tth*-lac at elevated temperatures. The reduced exposure of Cu_{T1} in *Tth*-lac, which minimizes the solvent contribution to the reduction entropy change, accounts for the large negative contribution by the polypeptide. The unfavorable entropy change upon reduction likely is a consequence of the unexpected flexibility of the oxidized protein. Regardless of its structural origins, the large negative Cu_{T1} redox entropy will have a substantial impact on electron-transfer kinetics and, hence, on enzyme activity. Our studies indicate that the remarkable capacity of thermophilic enzymes to remain active at extremely high temperatures is attributable to a subtle balance of many competing dynamic and thermodynamic factors. Understanding that interplay will be helpful in broadening the scope and utility of enzymes in industrial applications.

Acknowledgements Research reported in this publication was supported by the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health under award number R01DK019038. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Additional support for this research was provided by the Arnold and Mabel Beckman Foundation.

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