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Chemistry

Temperature dependence of the reduction potential of blue copper in fungal laccase

(spectroelectrochemistry/thermodynamic parameters)

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ABSTRACT **Thin-layer spectroelectrochemical methods have been employed to measure the reduction potentials of the blue copper in *Polyporus versicolor* laccase (EC 1.10.3.2) between 7°C and 41°C (0.2 M sodium phosphate, pH 5.4). Thermodynamic parameters are: $\Delta S^\circ = -13.9 \pm 2$ cal/mol·K; $\Delta H^\circ = -22.1 \pm 0.5$ kcal/mol; E° (25°C) = 780 ± 3 mV vs. the normal hydrogen electrode. Comparison of the ΔS° and ΔH° values with those for single-site proteins suggests that the high potential of the blue copper in fungal laccase is attributable mainly to stabilization of the copper (I) center by enhanced ligand binding interactions and that protein solvation effects play a lesser role.**

Our interest in biological oxidation–reduction processes has led us to investigate the thermodynamics of metalloprotein electron transfer reactions. In previous experiments we have determined electron transfer enthalpies and entropies for several single-site metalloproteins (1, 2). We have now extended our work to include measurements of the thermodynamic parameters associated with electron transfer to the blue copper center in *Polyporus versicolor* laccase, a four-copper enzyme that catalyzes the reduction of molecular oxygen to water (3, 4).

MATERIALS AND METHODS

Fungal laccase A (*p*-diphenol:O_2 oxidoreductase, EC 1.10.3.2) from *Polyporus versicolor* was prepared according to the method of Fåhraeus and Reinhammar (5). Enzyme concentrations were determined at 610 nm, using a molar extinction coefficient (ε) of 4,900 M^{-1} cm^{-1} and a $\Delta\varepsilon$ (oxidized − reduced) of 4,600 M^{-1} cm^{-1} (6). Concentrated enzyme solutions were stored frozen at −60°C and dialyzed into 0.2 M sodium phosphate buffer (pH 5.4) prior to use.

Potassium octacyanomolybdate (IV) dihydrate was prepared according to the method of Leipoldt *et al.* (7). Sodium phosphate buffer (0.2 M, pH 5.4) (0.172 M NaH_2PO_4/0.0921 M Na_2HPO_4) was prepared from analytical grade reagents. The preparation of all protein and buffer solutions employed deionized water from a Barnstead NANOpure water purifier.

Formal reduction potentials at different temperatures for fungal laccase were determined by using an optically transparent thin-layer electrolysis (OTTLE) cell in a nonisothermal electrochemical cell configuration (1). The OTTLE cells were similar in construction to those described by Anderson *et al.* (8), employing gold minigrid as the working electrode material and optical path lengths on the order of 0.04–0.06 cm. Potentials were applied across the thin-layer cell by using a Princeton Applied Research model 174A polarographic analyzer and were accurately measured with a Keithley model 177 digital multimeter. The cell temperature was varied by using a variable temperature cell holder and measured directly (±0.2°C) with an Omega Engineering precision microthermocouple and a Fluke model 2175A digital thermometer. The thermocouple was situated in the protein solution in close proximity to the thin-layer cavity. All UV–visible spectra were obtained with a Cary 219 recording spectrophotometer.

Formal reduction potentials were determined by sequentially applying a series of potentials, E(applied), to the gold minigrid electrode. Each potential was maintained until electrolysis ceased so that the equilibrium value of the ratio of concentrations of oxidized to reduced forms of all redox couples in solution, [O]/[R], was established as defined by the Nernst equation. A "small molecule," electrochemically reversible redox couple was included in the protein solution to facilitate electron transfer between metalloprotein redox couples and the working electrode (9). Redox couples were incrementally converted from one oxidation state to the other by the series of applied potentials, for which each corresponding value of [O]/[R] was determined from the spectra. Formal reduction potentials and n values were determined from plots of E(applied) vs. log([O]/[R]).

All solutions were deoxygenated prior to use by vacuum/argon cycling on a vacuum/purified argon double manifold and loaded into the OTTLE cell by using rubber septum caps and syringe techniques. The platinum wire auxiliary electrode was situated in a compartment (containing deoxygenated supporting electrolyte) that was isolated from the protein solution by a porous glass frit (10). Octacyanomolybdate standards and solutions containing this reagent were always prepared immediately prior to use and protected from light to minimize photodecomposition of the Mo(V) species (11).

RESULTS AND DISCUSSION

The formal reduction potential of the redox mediator couple $[Mo(CN)_8^{3-}/Mo(CN)_8^{4-}]$ was determined to be 782 ± 2 mV vs. the normal hydrogen electrode (NHE) (ionic strength 0.2 M, pH 5.4, 25.0°C) by using the spectroelectrochemical procedure described above and by cyclic voltammetry at a platinum electrode. Molar extinction coefficients at 386 nm [λ_{max} for Mo(V)] for the oxidized and reduced octacyanomolybdates were calculated to be 1,503 and 126 M^{-1} cm^{-1}, respectively, under these conditions.

A typical thin-layer spectroelectrochemical experiment with fungal laccase is shown in Fig. 1. The additional absorbance in the 350- to 400-nm region is due to octacyanomolybdate, which

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Abbreviations: NHE, normal hydrogen electrode; eu, entropy units (cal/mol·K); OTTLE, optically transparent thin-layer electrolysis.

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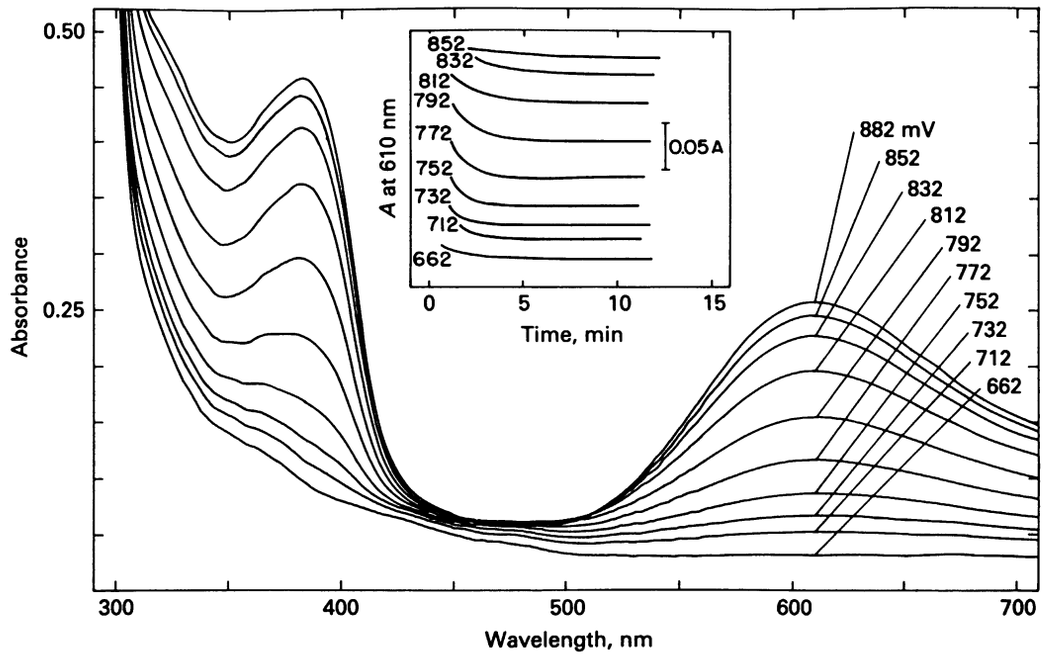

FIG. 1. Thin-layer spectroelectrochemical experiment with 0.9 mM fungal laccase, ionic strength 0.2 M, pH 5.4, sodium phosphate, at 25.4°C, redox mediator 4.1 mM octacyanomolybdate. The applied potentials are in mV vs. NHE. (*Inset*) Equilibration of the absorbance at 610 nm at each applied potential as a function of time.

was present in a 5-fold excess over protein. Because both $Mo(CN)_8^{3-}$ and $Mo(CN)_8^{4-}$ are transparent above 500 nm, all absorbance changes at 610 nm are due entirely to the blue copper chromophore in fungal laccase (4) and can be analyzed directly.

A least-squares analysis of the 610-nm Nernst plot [E(applied) vs. log([O]/[R])] gives a midpoint potential of 781 mV vs. NHE and a slope of a 62 mV (Fig. 2). This is in excellent agreement with results obtained by Reinhammar (775 mV) (12) under similar conditions of ionic strength, pH, and concentration of mediator relative to enzyme. All $E°$ values were determined from data that yielded linear correlation coefficients of at least 0.99 (several data sets were obtained that yielded linear correlation coefficients >0.999, but slight curvature in the plots was more commonly observed). Equilibration of the blue copper center at each applied potential was apparent from the stabilization of the absorbance at 610 nm (cf. Fig. 1 *Inset*).

The enzyme could be reversibly cycled between its fully oxidized and fully reduced forms, and the value of $E°$ calculated for the blue copper center was independent of the direction of the experiment. In a typical experiment the enzyme solution was put through two reductive cycles and two oxidative cycles and the mean of the four resulting $E°$ values was 781 mV with a SD of ±3.3 mV (Fig. 1).

The temperature dependence of the formal reduction potential of the blue copper center in fungal laccase is shown in Fig. 3. A least-squares fit of these data gives an $E°$ at 25°C of 780 mV and $(dE°/dT)_{25°C}$ = 73.9 μV/°C. The partial molal ionic entropy difference between the reduced and oxidized halves of the redox couple of interest (i.e., the electron transfer reaction entropy, $\Delta S°_{et}$) is directly proportional to the temperature coefficient of the nonisothermal cell (1, 13):

$$\Delta S°_{et} = S°_{red} - S°_{ox} = F(dE°/dT)_{25°C},$$

in which F is the Faraday. The $\Delta S°_{et}$ for the blue copper center in fungal laccase is therefore calculated to be 1.7 ± 2 entropy units [eu; cal/mol·K (1 cal = 4.18 J)]. The entropy for the com-

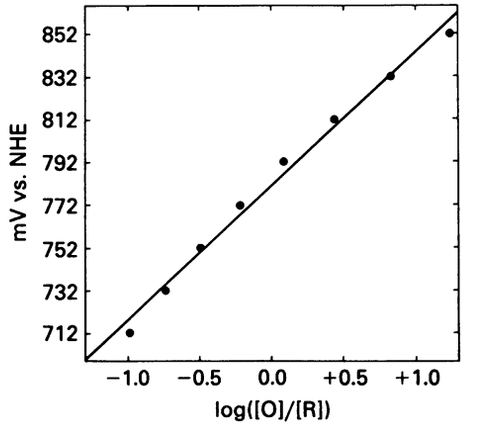

FIG. 2. Nernst plot for the blue copper in fungal laccase (610 nm, data in Fig. 1). The solid circles represent experimental points and the line is a least-squares fit of the E(applied) vs. log([O]/[R]) data. $E°$ = 781 mV, Nernst slope 62 mV, and linear correlation coefficient 0.994.

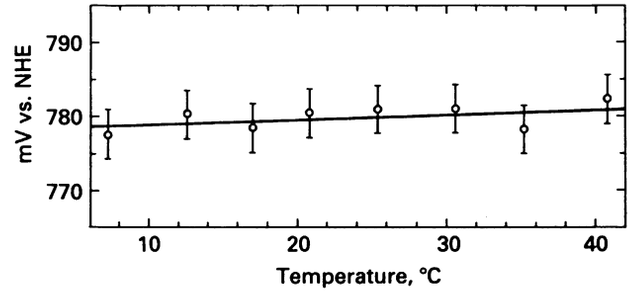

FIG. 3. Temperature dependence of the formal reduction potential for the blue copper in fungal laccase over the range 7–41°C (0.2 M pH 5.4 sodium phosphate). Bars indicate SD.

plete cell reaction adjusted to the NHE scale, $\Delta S°$, is -13.9 ± 2 eu when a value of -15.6 eu is assumed for $\Delta S°_{et}$(NHE) (i.e., $S°_{H^+}$ is assigned a value of zero) (1). The corresponding reaction enthalpy, $\Delta H°$, is -22.1 ± 0.5 kcal/mol with $E°(25°C) = 780 \pm 3$ mV.

The value of $\Delta S°_{et}$ for the blue copper in fungal laccase falls at the high end of the range (≈ 0 to -20 eu) established from measurements on several single-site metalloproteins, and it is not very different from that measured previously for bean plastocyanin (1). This suggests that the blue copper in the fungal enzyme is partially exposed to water molecules in the medium, in agreement with kinetic data (3). Thus it is reasonable to conclude that the anomalously high reduction potential is not solely the result of solvation effects. Indeed, the strikingly negative $\Delta H°$ for the fungal blue copper indicates that the copper(I)-ligand binding interactions are particularly strong. Our thermodynamic data, therefore, reinforce the conclusion based on spectroscopic analyses (14, 15) that the ligand environment of the blue copper in fungal laccase is perturbed significantly from that of the $Cu[N(His)]_2S(Cys)S(Met)$ site (16) of plastocyanin: the copper(II) ligand field is stronger (14), but, importantly, the binding is enhanced further in the copper(I) state. The reason for such impressive binding-site stabilization of copper(I) [recall that $\Delta H°$ for plastocyanin is only -14 kcal/mol (1)] is not known, but one intriguing possibility is that the copper–thioether(Met) interaction is much stronger in the fungal enzyme than it is in plastocyanin. Replacement of a weak interaction [structural studies (16) have revealed that the Cu—S(Met) "bond" is particularly weak in plastocyanin] with a normal Cu—S(Met) bond would tune the blue copper reduction potential to a relatively high value, as observed. Removal of this interaction entirely would produce a low-potential blue copper. Perhaps this is the reason that stellacyanin with $\Delta H° = -10$ kcal/mol (1) is in a (low-potential) class by itself.

An important question that remains is whether the critical role that enthalpic changes play in the variations of blue copper reduction potentials will extend to the type 3 coppers in the blue oxidases. It is known that the type 3 copper center in fungal laccase also possesses a high reduction potential (12), which may or may not be closely coupled in an enthalpic sense to the blue copper. Thus it becomes important to measure the electron transfer entropies and enthalpies for type 3 centers.

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