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Electrochemical Evidence that Pyranopterin Redox Chemistry Controls the Catalysis of YedY, a Mononuclear Mo Enzyme

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A longstanding contradiction in the field of mononuclear Mo enzyme research is that small-molecule chemistry on active site mimic compounds predicts ligand participation in the electron-transfer reactions, but biochemical measurements only suggest metal-centred catalytic electron transfer. With the simultaneous measurement of substrate turnover and reversible electron-transfer which is provided by Fourier transformed alternating current voltammetry we show that Escherichia coli YedY is a mononuclear Mo enzyme which reconciles this conflict. In YedY, addition of trimethylamine N-oxide (TMAO) (3) but the inaccessibility of the physiological substrate of YedY Mo(VI) state in experiments using isolated Mo(VI) oxidation state is needed to initiate the catalytic reduction of either dimethyl sulfoxide or trimethylamine N-oxide.

Based on comparison with earlier studies and our UV-vis redox titration data, we assign the reversible one-proton and one-electron reduction process centred around +174 mV vs SHE at pH 7 to a Mo(V) to Mo(IV) conversion but ascribe the two-proton and two-electron transition occurring at negative potential to the organic pyranopterin ligand system. We predict that a dihydro to tetrahydro transition is needed to generate the catalytically active state of the enzyme. This is a novel mechanism, suggested by the structural simplicity of YedY, a protein in which Mo is the only metal site.

Fourier transformed alternating current voltammetry | mononuclear molybdenum enzyme | protein film electrochemistry | pyranopterin | YedY

Introduction

Most living species require a Mo enzyme (1) and apart from nitrogenase all of these Mo-containing proteins are part of the large family of "mononuclear Mo" enzymes. The general ability of mononuclear Mo enzymes to catalyse two-electron oxygen atom transfer reactions has been attributed to the Mo(V)/Mo(VI) oxidation state cycling of the active site, and this mechanism is a common part of Undergraduate syllabuses (1,2). Escherichia coli YedY is a mononuclear Mo enzyme (3) and, based on sequence homology, the majority of sequenced Gram-negative bacterial genomes encode a YedY-like protein (3-5). Uniquely for a mononuclear Mo enzyme, it has not been possible to form the YedY Mo(VI) state in experiments using ferriyamine as an oxidising agent, and an unusually positive reduction potential for the Mo(V)/Mo(IV) transition (+132 mV vs SHE at pH 7) was determined from EPR experiments (6). Although the physiological substrate of YedY is unknown, a possible role at pH 7 to a Mo(V) to Mo(IV) conversion but ascribe the two-proton and two-electron transition occurring at negative potential to the organic pyranopterin ligand system. We predict that a dihydro to tetrahydro transition is needed to generate the catalytically active state of the enzyme. This is a novel mechanism, suggested by the structural simplicity of YedY, a protein in which Mo is the only metal site.

Fourier transformed alternating current voltammetry | mononuclear molybdenum enzyme | protein film electrochemistry | pyranopterin | YedY

The mononuclear Mo enzymes are ubiquitous throughout life and the notion that their activity arises from Mo(V)/Mo(IV) redox cycling is a central dogma of bioinorganic chemistry. We prove that YedY, a structurally simple mononuclear Mo enzyme, operates via a strikingly different mechanism: the catalytically active state is generated from addition of three-electrons and three-protons to the Mo(V) form of the enzyme, suggesting for the first time that organic-ligand based electron-transfer reactions at the pyranopterin play a role in catalysis. We showcase Fourier transformed alternating current voltammetry as a technique with powerful utility in metalloenzyme studies, allowing the simultaneous measurement of redox catalysis and the underlying electron-transfer reactions.

Significance

Reserved for Publication Footnotes
structure calculations were used to assign redox states to the pyranopterin ligands in all known mononuclear Mo enzyme structures (10). It was concluded that while enzymes from the sulfite oxidase family (such as YedY) contain pyranopterin ligands in the "dihydro" form, the xanthine dehydrogenase family of enzymes contain the two proton, two electron more reduced "trihydro" form of the pyranopterin (10).

Traditionally, redox-potential measurements of enzymes have required substrate-free conditions to either permit a solution equilibrium to be established (spectroscopic redox titrations) or to prevent catalytic signals from masking the non-catalytic response (film electrochemistry). Fourier transformed alternating current voltammetry (FT acV) is a technique which offers the ability to measure catalytic chemical redox reactions and reversible electron transfer processes in a single experiment (11, 12). In the FTacV measurement, a large amplitude sine wave of frequency f is superimposed on a linear voltage-time sweep (11, 13-15) and the resulting current-time response is measured and then Fourier transformed (FT) into the frequency domain to give a power spectrum of harmonic contributions at frequencies f, 2f, 3f etc. Band selection of the individual harmonics followed by inverse FT resolves the data back into the time domain. The higher harmonic components only arise from fast, reversible redox reactions, devoid of catalysis and baseline contributions, but the aperiodic (dc) component (f = 0) gives the same catalytic information as a traditional direct current cyclic voltammetry (dcV) experiment, and can therefore show catalytic turnover (13, 14, 16).

In this study we both discover novel mononuclear Mo enzyme redox chemistry as well as demonstrate the significant advantages of using FTacV to probe the mechanism of a redox active enzyme.

Results

Electrochemical Observation of Two Redox Transitions by YedY.

We prove that YedY can reversibly form three different oxidation states, i.e. the enzyme can undergo two different redox transitions. This is shown in Fig. 2A which contains dcV YedY electrochemistry data measured under conditions of pH 7, 25°C. The enzyme has been adsorbed onto the surface of the electrode and the signals which are observed are typical for non-catalytic redox enzyme "film" electrochemistry (SI Appendix (Fig. S1)) (17). In Fig. 2A, both enzyme-redox transitions are visible as "peak" Faradaic signals at around +170 mV and -250 mV (control experiments confirm that these signals are not present with a YedY-free electrode). The YedY signals were stable over at least 20 continuous 100 mV s⁻¹ cyclic voltammograms but only one scan is shown for clarity.

A significant limitation of the protein film dcV technique is that the Faradaic non-catalytic enzyme signals are very small in relation to the "background" signal from the non-Faradaic (double layer charging) electrode process. To permit analysis of the YedY-only redox chemistry we have computed the non-Faradaic response using a polynomial function and then subtracted this from the experimental data to give pure Faradaic data. The baseline-subtracted signals thus obtained are scaled by a 20-fold multiplication factor in Fig. 2A. From Fig. 2A the integrated area of the baseline-subtracted negative potential process, centred around -250 mV, is approximately 1.8 times larger than the integrated area of the baseline-subtracted positive potential process centred around +170 mV. We therefore conclude that almost twice as many electrons are passed in the negative potential redox transition relative to the positive potential redox transition.

As shown in the SI Appendix (Fig. S2B), the redox transition measured for YedY at positive potential is well modelled by a
Fig. 3. FTacV measurements of the YedY two-electron redox transition. (A) Gray solid lines show, in descending order, the 7th, 8th, 9th and 10th ac harmonic signals measured for YedY using FTacV with a frequency of 9 Hz. The response from a bare (YedY-free) graphite electrode under the same conditions is shown by light red dotted line.

Fig. 4. FTacV of YedY in the presence and absence of DMSO substrate. (A) 6th harmonic component of a 219 Hz FTacV experiment on YedY in the absence (black solid line) and presence (gray dashed line) of 200 mM DMSO. The response of a blank or bare (YedY-free) graphite electrode in the absence of DMSO, measured using the same FTacV parameters, is shown by the gray solid line. (B) The aperiodic dc component of the same FTacV experiment on YedY in the absence (black solid line) and presence (gray solid line) of DMSO. Other conditions: scan rate 15.83 mVs⁻¹, amplitude 150 mV, buffer solution of 50 mM MES and 2 M NaCl, pH 7, 25°C and stationary electrode.

Nernstian one-electron process (equivalent to a peak width at half height, δ, of 90 mV) with a pH 7 mid-point potential Ɛ_{m/2} = +174 ± 4 mV. We attribute this process to the Mo(V/IV) redox transition. Our assignment of the positive potential redox process as a metal-based transition is supported by UV-vis solution spectroelectrochemistry measurements made from 750 to 320 nm under an atmosphere of Ar and shown in the SI Appendix (Fig. S3). As-purified YedY, known to be in the Mo(V) state, exhibits two peaks in the optical spectrum (6, 18): a broad absorbance centred at 503 nm and another at approximately 360 nm. Upon lowering the solution potential from +0.21 V to ~0.09 V, i.e. passing through the positive potential redox transition, both spectral signals are bleached, indicating a metal-based reduction. These spectral changes can be reversed by raising the potential back to +0.21 V. No spectral changes accompany the negative potential redox reaction, i.e. no UV-vis changes are measured when the solution potential is stepped between -0.44 and -0.09 V.

Using film electrochemistry, there is no evidence of any further redox transitions at more positive potentials, even when the potential range is extended to the solvent/electrode limit (SI Appendix (Fig. S4)), so in agreement with other techniques, we also cannot observe a Mo(V/IV) redox transition.

Analysis of the negative potential baseline-subtracted dcV waveshape shown in the SI Appendix (Fig. S2C) suggests a cooperative, non-simultaneous two electron charge transfer process, i.e. one electron is transferred and then a second electron follows onto the same centre (19-22). We measure Ɛ_{m/2} = -248 ± 1 mV, but using dcV it is very difficult to derive more precise mechanistic...
information regarding the separate one-electron processes which combine to give the "envelope" signal.

Experiments at different pH reveal that the $E_m$ values for the Mo(V/IV)-assigned reaction and the negative potential redox processes change by $-53$ and $-55$ mV per pH unit, respectively (Fig. 2B), close to the $-59$ mV per pH unit expected for a one-proton per electron process at 25°C (23). "Trumpet plots" of the reductive and oxidative peak potentials vs. scan rate show greater peak separation at lower scan rates for the Mo(V/IV) signals compared to the negative potential transition, suggesting that the Mo-based redox process has a slower electron transfer rate (SI Appendix (Fig. S5)) (24). Combining all the dcV information, we suggest a $1^\text{H}^+ + 1^\text{e}^-$ process for the Mo(V/IV) transition and a faster $2^\text{H}^+ + 2^\text{e}^-$ process for the negative potential transition, over the pH range measured.

**Fourier Transformed ac Voltammetry (FTacV) of YedY.** Analogous to the dcV experiment shown in Fig. 2, FTacV was used to interrogate YedY redox chemistry over a wide potential range and this is shown in the SI Appendix (Fig. S6). There are two significant differences between the two results: firstly, relative to dcV, the signal-to-background response of YedY at around 250 mV is much larger in the higher harmonic components of the FTacV measurements; secondly, whereas in dcV the peak area for the negative potential YedY signal is approximately double the peak area of the Mo(V/IV) signal, in FTacV the Mo(V/IV)-assigned signal is not visible above the noise. The fact that very little Mo(V/IV) signal is observed means that the 9 Hz frequency applied in the FTacV outpaces Mo-based electron transfer processes and we can therefore state that YedY’s negative potential electron transfer processes is much faster than the Mo(V/IV) redox transition. Based on analysis of the FTacV signals we define the Mo(V/IV) process as a quasi-reversible electron transfer reaction with an apparent heterogeneous charge transfer rate $k_{\text{app}}$ of less than $10$ s$^{-1}$, consistent with analysis of dcV trumpet plot data which suggests $k_{\text{app}} = 3 - 6$ s$^{-1}$.

In order to learn more about the negative potential process, Fig. 3A shows 9 Hz FTacV measurements focussed on the YedY reversible redox transition centred at around -250 mV. At least 12 harmonic components are detected and, in stark contrast to dcV, no baseline subtraction is required prior to analysis of the higher harmonic signals because YedY-free controls confirm there is negligible baseline contribution from a bare electrode. The potentials of the central maxima of the odd harmonics and the central minima of the even harmonics provide a direct measure of the midpoint potential and these values agree with the baseline subtracted dcV $E_m$ data. Similar to changing the scan rate in dcV, changing the frequency in FTacV provides a qualitative means of assessing the electron transfer rate. As shown in Fig. 3B, when the same negative potential range is interrogated using a range of frequencies, well defined ac harmonics are observed up to 219 Hz, indicating that the low potential YedY redox reaction involves extremely fast electron transfer.

**Electrocatalytic Activity of YedY.** The ability of FTacV to separately resolve catalytic and electron transfer steps in a single experiment is shown in Fig. 4. Solution assays have shown that YedY catalyses the reduction of N- or S-oxides with concomitant oxidation of reduced benzyl viologen (3). The enzyme has the largest specificity constant, $k_{\text{cat}}/K_M$, for the substrates DMSO and TMAO, where $k_{\text{cat}}$ refers to the catalytic turnover rate and $K_M$ denotes the Michaelis constant (3). When YedY is adsorbed onto a graphite electrode and then placed in a solution of DMSO, the negative catalytic reduction current, revealed by the dc component of the data (Fig. 4B), steadily increases as the electrode potential is lowered below $-0.3$ V at pH 7 ("control" enzyme-free electrode experiments in the presence of DMSO show no reductive current, see SI Appendix). In contrast, the aperiodic component resembles a "blank" electrode in the absence of YedY but absence of substrate (Fig. 4B). The reduction potential for DMSO is +160 mV at pH 7 and work by Heffron et al. shows that the enzyme E. coli DMSO reductase is capable of reducing DMSO at more positive voltages than YedY (25). In Fig. 4B the "onset potential", i.e. the electrochemical voltage required to initiate YedY-catalysed DMSO reduction, is therefore an enzyme-specific property and not a substrate related behaviour.

The $6^\text{th}$ harmonic signal for YedY, shown in Fig. 4A, is unchanged in the presence or absence of 200 mM DMSO, showing that substrate has not affected the electron transfer properties of the negative potential redox process: it remains very clearly distinguishable, revealing the redox potential without any need for background subtraction. Comparison between the high harmonic data in the presence and absence of substrate therefore suggests that the negative potential two-electron, two-proton reduction process generates the catalytically active state of YedY, since catalysis does not commence until the potential is sufficiently negative for this reaction to have occurred. Experiments at different pH further support the hypothesis that the most reduced state of the enzyme reacts with substrate because the onset potential of YedY-catalysed DMSO-reduction changes between pH 5 and 8 in exactly the same way as the potential of the two-electron non-catalytic redox signal (SI Appendix (Fig. S7)). Data extracted from experiments at pH 7 and different DMSO concentrations also corroborate published solution assay measurements with $K_M = 35 \pm 5$ mM at pH 7, 25°C and $k_{\text{cat}} = 4.2 \pm 0.9$ s$^{-1}$ at $-359$ mV, pH 7, 25°C (SI Appendix (Fig. S8)) (3, 8, 26). Electrocatalytic experiments have also been conducted using TMAO as a substrate; these are shown in the SI Appendix (Fig. S9). With TMAO as a substrate we again observe that the onset potential of YedY catalysis is approximately -0.3 V at pH 7, far more negative than the equilibrium redox potential for the substrate (TMAO has reduction potential +130 mV at pH 7 (27)).

**Simulation of the FTacV data.** Simulation of the FTacV data makes it possible to harness the technique’s ability to provide a quantitative measure of electron transfer rates and deconvolution of separate redox potentials in a single experiment, insight that we cannot access with dcV (22). Fig. 3A shows simulations of the FTacV data using the $1^\text{e}^- + 1^\text{e}^-$ mechanism described in the SI Appendix. The same parameters were used to simulate all the harmonic signals. To minimise the parameter space used...
in simulations the value for uncompensated resistance, $R_u$, was derived from a separate impedance spectroscopy measurement and $R_{app}$, the apparent coverage of enzyme on the electrode, was estimated from dcV measurements. Both electron transfer steps exhibit fast kinetics as reflected by $k_{app}^2 = 2.0 \times 10^{12}$ s$^{-1}$ used in simulations. To achieve a close agreement to experimental data the apparent reversible potentials of the two sequential one-electron transfers must be similar and $R_{app}^2 = -239$ mV and $R_{app}^2 = -261$ mV are used to produce data in Fig. 3. The charge transfer coefficient $\alpha$ was always assumed to be 0.5 and its exact value could not be determined as the simulations are insensitive to $\alpha$ at these very high electron transfer rates. As expected, simulations using Marcus theory rather than Butler Volmer theory made no difference under these reversible conditions. As shown in the SI Appendix we confirmed that different redox reaction models will not simulate the data, confirming that the low potential YedY redox reaction is neither a 1e$^-$ reaction (Fig. S10) or a simultaneous 2e$^-$ transfer mechanism (as opposed to a stepwise 1 e$^-$ + 1 e$^-$ reaction, Fig. S11).

When simulating the FTacV data obtained from experiments at different frequency (Fig. 3B), all the same parameters were used except for the enzyme-electrode coverage value, $R_{app}$, which was lowered with increasing frequency from 1.15 pmol cm$^{-2}$ in the first 9 Hz measurement, to 0.85 pmol cm$^{-2}$ for 59 Hz (Fig. 3B and SI Appendix (Fig. S12)). This trend did not reflect true enzyme desorption because a final measurement at 9 Hz yielded data that was best simulated using $R_{app}$ of 0.9 pmol cm$^{-2}$. As described in a recent theoretical study (28), kinetic dispersion, meaning that different enzyme orientations on the electrode surface have different electron transfer rates ($k_{app}^2$), is believed to be the major reason that $R_{app}$ decreases as the frequency increases. Dispersion is a common observation in protein film electrochemistry measurements (29).

Discussion

We present dcV and FTacV data that prove that E. coli YedY forms three stable oxidation states. Relative to the well-characterised Mo(V) form of the enzyme, formation of the catalytically active state requires addition of three electrons and three protons and we summarise our proposed mechanism in Fig. 5. We assign the YedY redox transition which has $E_{m,7} = +174 \pm 4$ mV to the Mo(V/IV) process and ascribe the redox transition with $E_{m,8} = -239$ mV and $E_{m,9} = -261$ mV to a pyrroloporphin dinhydride-tetrahydrole interconversion.

Using spectroelectrochemistry it has been demonstrated that an oxidation of $-0.09$ V is sufficient to bleach the absorbance peaks observed in UV-vis spectra of the as-isolated d-Mo(V), enzyme. The disappearance of the absorbance centred at 503 nm is consistent with our assignment of the positive potential redox process being Mo-based; dithiole-S ligand to metal charge transfer processes give rise to this spectral feature so it should be a reporter signal for changes to the metal redox state (18). Both dcV trumpet plot data and low intensity FTacV harmonic currents indicate that the Mo(V/IV) redox reaction has a slow electron transfer rate, from $3 \rightarrow 0$ s$^{-1}$, which suggests structural reorganisation. This correlates with the XAS mechanism that six coordinate Mo(V) is reduced to a five coordinate Mo(V) species (9).

Varying the pH from 4 to 9 causes the electrochemically-determined $E_m(Mo(V/IV))$ value to decrease by 53 mV per pH unit which indicates a one-electron, one-proton transition in agreement with the proposed XAS mechanism: Mo(V)$^-$OH$^-$ + 1H$^+$ + 1e$^-$ $\rightarrow$ Mo(IV)$^-$OH$^-$(9). There is a discrepancy between the midpoint potentials we measure using electrochemistry and those reported from an EPR redox titration, with respective $E_{m,7}$ values of $+174$ mV and $+132$ mV (6). The EPR data is also pH-independent over a range of pH 6-8 (6). Whereas the dcV electrochemical data could be accurately simulated as a one-electron Nernstian process, the EPR Nernst plots were fit as physically impossible 1.3 and 1.63 electron processes for the oxidative and reductive titrations, respectively. As noted in the EPR study (6), the complex spectroscopic data is difficult to interpret and we suggest that the disparity in midpoint potential values may reflect this challenge. We also note that the electrochemical and EPR redox potential measurements are made on very different timescales as protein film electrochemistry affords the advantage of "wiring" the enzyme to the electrode, permitting rapid potential control, whereas achieving solution redox potential equilibration for EPR requires many minutes.

Our experiments at highly oxidising potentials confirm the unusual stability of the YedY Mo(V) state with respect to oxidation, setting a limiting value of $E_m(Mo(V/IV)) > +600$ mV. The Mo(VI) oxidation state is therefore defined as physiologically irrelevant and thus plays no direct role in a catalytic reaction mechanism.

FlacV permitted simultaneous measurement of the putative pyrroloporphin dinhydride transition and catalysis. The onset potential for enzymatic reduction of either DMSO or TMAO is more negative than the redox potential of either substrate (27) and instead correlates with the pyrroloporphin-assigned two-electron, two-proton reversible redox transition across the pH range 5 to 8. We assign this process to the pyrroloporphin cofactor because the structural simplicity of YedY is such that there are no other putative redox active centres apart from the Mo (3). In sulfite oxidase fold enzymes such as YedY, which is crystallised in the Mo(V) oxidation state, the geometry of the pyrroloporphin is consistent with a 10,10a-dihydro form (10); in Fig. 5 we show the three-electron, three-proton reduced catalytically active Mo(VI) form of the enzyme with a tetrahydride pyrroloporphin ligand. We have chosen to display the two-electron reduced pyrroloporphin ligand in a ring-closed tetrahydride state because this is consistent with the structure found most frequently at the active site of Mo-containing enzymes (10). However it should be noted that an alternative, ring-opened confirmation exists at the same oxidation state level (7). For simple pterins, reversible two-electron and two-proton transitions between tetrahydride and dihydride forms are well known, as is further oxidation of the dihydride state, so pyrroloporphin redox reactions would be expected on the basis of chemical analogues (7, 30). It has been proposed that a nitrate reductase undergoes reversible enzymatic inactivation under oxidising conditions because the pyrroloporphin converts from the tetrahydride to the dihydride state, however all the substrate oxidations make it seem unlikely that this is the case (31). Our experiments therefore provide the first evidence of catalytically relevant pyrroloporphin redox chemistry (Fig. 5).

It is not possible to conclude if the two electrons for substrate reduction are supplied directly by the pyrroloporphin or if reduction of the pyrroloporphin ligand activates the Mo in such a way as to promote changes to the metal redox state catalysis. In the reduced tetrahydride state, the dithiolene chelate of a pyrroloporphin has increased electron donating ability to Mo, which will decrease the Mo reduction potentials, because the oxidised dihydride form is more oxidising than the dithiolene chelate and the pterin ring (10). This could make the Mo(VI) state indirectly accessible. There is no conclusive structural information about how the substrate coordinates to YedY, XAS experiments on the Mo(V) state at pH 8 showed a possible long-range coordination of TMAO to Mo, but DMSO coordination was undetectable (8). To probe substrate binding, future experiments would need to be conducted under reducing conditions.

Comparison of Fig. 2 and Fig. 3 demonstrates how the complete absence of background current in the higher harmonic FTacV YedY signals results in much better defined non-catalytic
Materials and Methods

Samples and Solutions. Escherichia coli Yedd was prepared as described previously (3, 6) and stored in a buffer solution of 20mM 3-N-morpholino-propanesulfonic acid (MOPS), pH 7 which was also used for the UV-Vis spectrophotometric experiments, a protein concentration of approximately 10 mg mL

All film electrochemistry experiment solutions were prepared using deionised water. Enzyme was adsorbed onto the electrode surface by centrifugation at 10 min. All values quoted are the average of at least three experiments and the error bars are the standard errors calculated from all repeat data.

Electrochemical Simulations. Simulations of FTacV data were based on a B20-former form for heterogeneous electron transfer kinetics (22) and the mechanism described in the SI Appendix and were performed using the Monash Electrochemistry Simulator (MecSim) digital simulation software package (25). The charge transfer coefficient was assumed to be α = 0.5 in all simulations and all other simulation parameters were optimised to give a close fit between theoretical and experimental data using a heuristic approach.

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29. http://www.garethkennedy.net/MecSim.html