Video Article

EPR Monitored Redox Titration of the Cofactors of *Saccharomyces cerevisiae* Nar1

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Abstract

Electron Paramagnetic Resonance (EPR) monitored redox titrations are a powerful method to determine the midpoint potential of cofactors in proteins and to identify and quantify the cofactors in their detectable redox state.

The technique is complementary to direct electrochemistry (voltammetry) approaches, as it does not offer information on electron transfer rates, but does establish the identity and redox state of the cofactors in the protein under study. The technique is widely applicable to any protein containing an electron paramagnetic resonance (EPR) detectable cofactor.

A typical titration requires 2 ml protein with a cofactor concentration in the range of 1-100 μ M. The protein is titrated with a chemical reductant (sodium dithionite) or oxidant (potassium ferricyanide) in order to poise the sample at a certain potential. A platinum wire and a Ag/AgCl reference electrode are connected to a voltmeter to measure the potential of the protein solution. A set of 13 different redox mediators is used to equilibrate between the redox cofactors of the protein and the electrodes. Samples are drawn at different potentials and the Electron Paramagnetic Resonance spectra, characteristic for the different redox cofactors in the protein, are measured. The plot of the signal intensity versus the sample potential is analyzed using the Nernst equation in order to determine the midpoint potential of the cofactor.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51611/>

Introduction

It is striking that the most fundamental chemical processes sustaining life on this planet, photosynthesis, nitrogen fixation and respiration, are catalyzed by large protein complexes containing a wide range of organic and inorganic redox cofactors. It has been estimated that approximately 30% of all proteins contain one or more metal cofactors.^{1,2} Identifying and characterizing the redox cofactors can be established using direct electrochemistry (e.g., protein film voltammetry) or redox titrations. The two techniques are complementary in their nature and applicability. Voltammetry offers fast determination of midpoint potentials and electron transfer kinetics of cofactors that can react with an electrode surface.^{3,4} Usually this works well for electron transfer proteins, such as cytochrome c or ferredoxin. And it sometimes works for more complex proteins that have been immobilized to an electrode surface. Detailed knowledge of the nature of cofactors in the protein has to be available, as the voltammogram will not give any direct information on the identity of the cofactor. Redox titrations are more laborious to execute and require mg quantities of protein. However, they offer information on the midpoint potential and the identity of the cofactor.⁵ Furthermore in a single titration multiple cofactors in a protein can be monitored.

The principle of a redox titration is that the redox active protein or enzyme is chemically reduced or oxidized. In order to make sure that the cofactors react with the reductant or oxidant redox mediators are used. These redox mediators also react with an electrode so that the potential of the solution can be measured. The mediators act as a redox buffer and equilibrate between the cofactors in the protein and the electrode. After chemically poisoning the potential to the desired value, a sample is drawn and quickly frozen in liquid nitrogen to await further analysis with spectroscopic techniques. EPR spectroscopy is particularly useful in this respect as it can be used to quantitatively measure paramagnetic metal centers or organic radicals.

The redox titration can be performed in two directions: from low to high or from high to low midpoint potential. The choice is dependent on the stability of the cofactors under study. Often it is wise to start at low potential and add oxidant to stepwise increase the potential. This is called an oxidative titration and is described here. Here we show the redox titration of the iron-sulfur cluster containing protein Nar1 from *Saccharomyces cerevisiae*. This protein is involved, likely as a scaffold protein, in the cytosolic iron-sulfur cluster biosynthetic machinery (CIA pathway).^{6,7}

Protocol

1. Preparation of Setup and Solutions

1. Prepare buffer solution containing 100 mM Tris, 250 mM NaCl and 10% glycerol at pH 8.5. Deaerate the buffer by flushing with argon. Introduce the buffer into an anaerobic chamber.
2. Divide the buffer in two portions. To one portion (buffer A) add 1 mM DL-dithiothreitol (DTT, M_r 154.25 g/mol) and 1 mM L-cysteine. The other portion (buffer B) does not contain reducing agents.
3. Prepare oxidant and reductant solutions. Make 1 ml solutions of 2, 20, and 200 mM potassium ferricyanide (FIC, M_r 329.26 g/mol) and of sodium dithionite (SD, M_r 174.11 g/mol) in anaerobic buffer B.
4. Prepare mediator mix as follows:
 1. Add 160 μ M of each of the following redox mediators and 50 mM NaCl to buffer B: *N,N,N',N'*-tetramethyl-p-phenylenediamine (TMPD, M_r 237.17 g/mol, E^0 +0.276 V), 2,6-dichlorophenol indophenol (DCIP, M_r 290.08 g/mol, E^0 +0.217 V), phenazine ethosulphate (PES, M_r 334.4 g/mol, E^0 +0.055 V), Methylene blue (M_r 319.85 g/mol, E^0 +0.011 V), Resorufin (M_r 235.17 g/mol, E^0 -0.051 V), Indigodisulphonate (M_r 466.35 g/mol, E^0 -0.125 V), 2-hydroxy-1,4-naphthaquinone (M_r 174.15 g/mol, E^0 -0.145 V), Anthraquinone-2-sulfonate (M_r 328.28 g/mol, E^0 -0.225 V), Phenosafranin (M_r 322.8 g/mol, E^0 -0.252 V), Safranin O (M_r 350.84 g/mol, E^0 -0.280 V), neutral red (M_r 288.8 g/mol, E^0 -0.340 V), Benzyl viologen (M_r 409.4 g/mol, E^0 -0.350 V), and Methyl viologen (M_r 257.16 g/mol, E^0 -0.440 V).
 2. Wrap the bottle in aluminum foil to protect the mediators from light.
5. Prepare 10 mg/ml *S. cerevisiae* strep-tagged Nar1 protein (181 μ M, M_r 55.2 kDa) in buffer A. Express *S. cerevisiae* Nar1 in *E. coli* as a strep-tagged protein using a pET24A expression vector. Purify the protein in an anaerobic chamber using Strep-tactin chromatography media (IBA) and purify using a protein purification system such as Akta purifier (GE Healthcare).
6. Prepare the redox titration setup in an anaerobic chamber as follows:
 1. Connect a Ag/AgCl reference electrode and a platinum wire electrode to the titration vessel. Connect both electrodes to a voltmeter that can measure from mV up to V.
 2. Insert a small stirring bar.
 3. Place the titration vessel above a magnetic stirrer.
 4. Fill the titration vessel with Buffer B, mediator mix solution and protein solution to up to 2 ml, i.e. to final concentrations of 72 μ M protein and 38 μ M of each mediator. Note: depending on the protein concentration and the required mediator concentrations the amounts of the three solutions should be adjusted.
 5. Stir for 30 min and switch on the voltmeter.
7. Prepare 10 clean quartz glass EPR tubes. Introduce the tubes in the anaerobic chamber.
8. Fill a small (circa 200 ml) liquid nitrogen dewar with liquid nitrogen.

2. Perform Redox Titration

1. Wait until the potential of the solution is stable. Note: In practice this means that the potential drifts less than 1 mV per minute.
2. Note down the potential given by the voltmeter.
3. Draw the first sample of 200 μ l and inject into an EPR tube.
4. Cap the tube with a rubber stopper and remove the tube from the anaerobic chamber.
5. Freeze the tube in liquid nitrogen as follows: Caution! This has to be done slowly, otherwise the tube will break.
 1. First insert the tip of the tube in the liquid nitrogen.
 2. Wait until a hissing sound can be heard. Note: This may take up to 10 sec.
 3. Slowly insert the rest of the tube so that the sample part is fully submerged.
6. Add a small aliquot 1-2 μ l of the 2 mM SD solution to the solution in the titration vessel to lower the potential of the solution to -0.6 V. Note: If more than 10 μ l has to be added, switch to the 20 mM solution. As it is very difficult to poise to a specific potential value, the actual values recorded on the voltmeter are written down and used to construct the titration curve.
7. Draw a sample as described in step 2.3-2.5.
8. Note down the actual potential given by the voltmeter.
9. Add oxidant until the potential has increased by circa 50 mV. Start by adding a small aliquot 1-2 μ l of the 2 mM FIC solution. If more than 10 μ l has to be added, switch to the 20 mM solution.
10. Note down how much of the solutions has been added. Note: This is necessary to correct for sample dilution during the titration.
11. Draw a sample as described in the steps 2.3-2.5.
12. Note down the actual potential given by the voltmeter.
13. Repeat steps 2.9 and 2.12 until the potential range from -0.6 V to +0.2 V has been covered and all 10 EPR tubes are filled each with 200 μ l. Note: As the volume with every withdrawal is reduced the positioning of the electrodes may need to be adjusted have good contact with the solution without interference of the stirring. It may be difficult to measure the potential after withdrawal of the last sample and therefore the potential of that sample may be less accurate.
14. Optionally, store the frozen EPR samples in liquid nitrogen until the EPR measurements can take place.

3. Measure Titration Samples Using EPR Spectroscopy and Data Analysis

1. Record EPR spectra of the different titration samples as follows:

1. Use a high-vacuum pump to evacuate a quartz cryostat to $<10^{-5}$ bar.
 2. Switch on the water cooling of the EPR magnet. Open the flow of dry air through the EPR cavity.
 3. Switch on the power supply to the magnet and the computer of the EPR.
 4. Run a frequency calibration program. Set the EPR measurement parameters. Connect the cryostat to the liquid helium dewar. Cool the cavity to 9-16 K.
 5. Introduce the EPR sample in the cavity. Record the EPR spectrum.
2. Identify the different EPR species and use the best spectra to quantify the signals. Achieve EPR quantification by double integration of the spectra and comparison with the spectrum of an external copper standard solution 10 mM CuSO_4 /10 mM HCl /2 M NaClO_4 .⁸
 3. Make the titration curve as follows:
 1. Plot the EPR signal amplitude at a g-value that is characteristic for the paramagnetic species of interest against the potential.
 2. Change the EPR signal amplitude scale to a spins/molecule scale by using the EPR quantification from step 3.2.
 3. Correct the signal amplitude of each sample for the dilution that has occurred due to the additions of oxidizing or reducing agent.
 4. Correct the potential for the midpoint potential of the Ag/AgCl reference electrode.
 Note: The Ag/AgCl reference electrode (with saturated KCl) has a midpoint potential of +0.194 V versus the Standard Hydrogen Electrode (SHE) at 25 °C. Therefore, if the potential on the voltmeter reads *circa* -0.6 V, the potential with reference to the SHE is -0.4 V.
 4. Fit the signal to the Nernst equation as follows:
 For a species that is paramagnetic in the reduced state:

$$\text{EPR signal} = \frac{\text{Maximum EPR signal}}{1 + e^{\frac{(E - E_m)F}{RT}}}$$

For a species that is paramagnetic in the oxidized state:

$$\text{EPR signal} = \frac{\text{Maximum EPR signal}}{1 + e^{\frac{(E_m - E)F}{RT}}}$$

For a species that is paramagnetic in an intermediate state:

$$\text{EPR signal} = \frac{\text{Maximum EPR signal}}{1 + e^{\frac{(E - E_{m1})F}{RT}} + e^{\frac{(E_{m2} - E)F}{RT}}}$$

These equations are valid for redox reactions in which one electron is transferred, so $n = 1$.

E = potential in Volt

E_m = midpoint potential in Volt

F = Faraday constant = $96,480 \text{ C} \cdot \text{mol}^{-1}$

R = Gas constant = $8.314 \text{ J} \cdot \text{K}^{-1} \cdot \text{mol}^{-1}$

T = Temperature in Kelvin

Representative Results

The redox titration of the iron-sulfur cluster containing protein Nar1 from *Saccharomyces cerevisiae* resulted in the identification of three different cofactors: Nar1: a $[\text{3Fe-4S}]$ cluster, a $[\text{4Fe-4S}]$ cluster and a mononuclear Fe center (**Figure 1**). The EPR signals are characterized by their g-values: for the $[\text{3Fe-4S}]^+$ g_z 2.01, $g_{\text{crossover}}$ 2.00 (**Figure 1B**); for the $[\text{4Fe-4S}]^+$, g_z 2.02, g_y 1.93, g_x 1.82 (**Figure C**), and for the mononuclear Fe^{3+} g 4.3 and 9.7 (**Figure 1A**). The EPR signal of the $[\text{4Fe-4S}]^+$ cluster is very similar to what has been reported for Nar1 previously.^{6,9} The EPR signal of the mononuclear iron is characteristic for a rhombic high-spin ferric species (**Figure 1A**).

Quantification of the EPR signals indicated that the $[\text{4Fe-4S}]$ cluster and mononuclear Fe are equimolar at approximately 60% of the protein concentration, and that the $[\text{3Fe-4S}]$ cluster content is only 5% (**Figure 1**). The $[\text{3Fe-4S}]$ cluster is most likely a degradation product and incomplete cluster. The $[\text{3Fe-4S}]$ cluster signal disappeared above +0.05 V, possibly due to oxidative degradation.

The following midpoint potentials were determined for the different cofactors:

+0.003 V for the $\text{Fe}^{3+}/\text{Fe}^{2+}$ couple of the mononuclear Fe center (**Figure 2A**), -0.125 V for the $[\text{3Fe-4S}]^+ / [\text{3Fe-4S}]^0$ couple (**Figure 2B**) and between -0.45 and -0.50 V for the $[\text{4Fe-4S}]^{2+} / [\text{4Fe-4S}]^+$ couple (**Figure 2C**). Due to the low value of the $[\text{4Fe-4S}]$ cluster no precise midpoint potential could be determined. The low potential of the $[\text{4Fe-4S}]$ cluster indicates that it does not have a redox role in the protein as it will be very difficult to reduce the cluster. The mononuclear iron center can be reduced or oxidized in the cytoplasm. A redox role of this center is therefore possible.

Independent iron determination on the purified Nar1 protein was performed using the ferene method.¹⁰ This afforded 3.2 Fe/molecule. The mononuclear Fe signal represents 0.6 Fe per molecule, and the $[\text{3Fe-4S}]$ cluster is only 0.05 per molecule. This leaves 2.45 Fe/molecule, and therefore 0.61 $[\text{4Fe-4S}]$ cluster per protein molecule. The quantification of the EPR signal of the $[\text{4Fe-4S}]^+$ cluster at -0.45V resulted in 0.26 spins/molecule. So only 42% of the $[\text{4Fe-4S}]$ cluster could be reduced at -0.45 V. Based on these quantifications a midpoint potential of the $[\text{4Fe-4S}]^{2+} / [\text{4Fe-4S}]^+$ couple of -0.46 V was determined.

Figure 1: EPR spectra of the different cofactors of Nar1. (A) Mononuclear high spin ferric $S=5/2$ signal of Nar1 poised at +0.031 V. EPR conditions: microwave frequency, 9.386 GHz; microwave power, 20 dB; modulation frequency, 100 kHz; modulation amplitude, 12.5 G; temperature, 16 K. (B) $[3\text{Fe-4S}]^+$ cluster $S=1/2$ signal of Nar1 poised at -0.004 V. EPR conditions: microwave frequency, 9.386 GHz; microwave power, 16 dB; modulation frequency, 100 kHz; modulation amplitude, 12.5 G; temperature, 9.5 K. (C) $[4\text{Fe-4S}]^+$ cluster $S=1/2$ signal of Nar1 poised at -0.45 V. EPR conditions: microwave frequency, 9.385 GHz; microwave power, 16 dB; modulation frequency, 100 kHz; modulation amplitude, 12.5 G; temperature, 9.2 K. Below -0.25 V a sharp $S=1/2$ signal (*) appeared, which is due to the cation radical species of methyl viologen and benzyl viologen.

Figure 2: Redox titration of the cofactors of Nar1. (A) Titration curve of the mononuclear high spin ferric signal monitored at $g = 4.3$; (B) Titration curve of the $[3\text{Fe-4S}]^+$ signal monitored at $g = 2.01$. (C) Titration curve of the $[4\text{Fe-4S}]^+$ signal monitored at $g = 1.82$. The solid lines are fits for $n = 1$ redox transitions with the midpoint potentials $+0.003$ V for the $\text{Fe}^{3+}/\text{Fe}^{2+}$ couple of the mononuclear Fe center, -0.125 V for the $[3\text{Fe-4S}]^+/[3\text{Fe-4S}]^0$ couple -0.46 V for the $[4\text{Fe-4S}]^{2+}/[4\text{Fe-4S}]^+$ couple.

Figure 3: Nernst curves of the different redox mediators that constitute the mediator mix. The order is from right to left as given in the Protocol and in Table 1.

Name	Cas number	E^0 (V) vs SHE	M_w (g/mol)
<i>N,N,N',N'</i> -tetramethyl-p-phenylenediamine (TMPD) · (HCl) ₂	637-01-4	+0.276	237.17
2,6-dichlorophenol indophenol (DCIP), sodium salt	620-45-1	+0.217	290.08*
Phenazine ethosulfate (PES)	10510-77-7	+0.055	334.4
Methylene blue	122965-43-9	+0.011	319.85*
Resorufin, sodium salt	34994-50-8	-0.051	235.17
Indigodisulfonate (indigo carmine)	860-22-0	-0.125	466.35
2-hydroxy-1,4-naphtaquinone	83-72-7	-0.145	174.15
Anthraquinone-2-sulfonate Na ⁺ H ₂ O	153277-35-1	-0.225	328.28
Phenosafranin	81-93-6	-0.252	322.8
Safranin O	477-73-6	-0.280	350.84
Neutral red	553-24-2	-0.340	288.8
Benzyl viologen	1102-19-8	-0.350	409.4
Methyl viologen	1910-42-5	-0.440	257.16*

*anhydrous

Table 1: Redox mediators that constitute the mediator mix.

Discussion

The series of 13 redox mediators allows a redox equilibrium in the solution in the range from -0.45 to +0.3 V versus SHE (Table 1 and Figure 3). Well above and below these potentials all mediators are either completely reduced or completely oxidized and hence no further equilibration with the redox active centers of the protein can occur. This is an important notion, as in literature sometimes redox titrations are performed with only two or three mediators, allowing only a narrow range to be measured¹¹. Such redox titrations will only give reliable results if the redox cofactor has a midpoint potential within a range of *circa* ± 50 mV of the midpoint potential of the mediators. The temperature dependence of the midpoint potentials of the different mediators has been determined and it was shown that redox titrations can be performed at temperatures as high as 80 °C.¹² Evaporation of the titration solution can be prevented by adding a layer of Nujol mineral oil, which is compatible with most protein containing solutions.

The anaerobicity of the solutions and the titration vessel is very important. The titration should preferably be performed in an anaerobic glovebox. However, it is possible to use a sealed titration vessel connected to an argon gas supply. EPR tubes can be connected to an argon/vacuum

manifold using small pieces of rubber tubing. The titration samples can be injected into the tubes via the rubber tubing using gastight syringes with 13 cm long needles.

During the titration of Nar1 anaerobicity was very important as the protein precipitates under aerobic conditions (not shown). No precipitation occurred during the titration.

EPR monitored redox titrations are complementary to direct electrochemistry (voltammetry), although both approaches can be used to determine midpoint potentials of cofactors. The first one offers identification and quantification of the cofactor(s) in their EPR detectable redox state. The latter provides information on electron transfer kinetics and requires smaller amounts of protein. The protein samples for redox titrations do not require special pre-treatments or immobilization on a surface, as is often required for direct electrochemical approaches.

Direct electrochemistry has only been successful in specific cases and requires intensive experimentation in order to find optimal conditions. EPR monitored redox titrations offer a relatively straightforward approach to characterize redox cofactors, regardless of the complexity of the enzyme system. This technique is indispensable in the characterization of large protein complexes with multiple cofactors. In combination with structural information and characterization of enzyme kinetics, the catalytic mechanism can be studied in great detail.

In this study the cofactors of *S. cerevisiae* Nar1p were characterized. Three different types of iron cofactors were found: mononuclear iron, a [3Fe-4S] cluster and a [4Fe-4S] cluster. The quantity of the [3Fe-4S] cluster was only 5% of the protein concentration and likely represents an oxidative degradation product of the [4Fe-4S] cluster. Both the mononuclear iron and the [4Fe-4S] cluster were present in stoichiometric quantities. The midpoint potential of the [4Fe-4S] cluster is very low, which indicates that the physiological oxidation state of the cluster is 2+ and that it is not likely that the cluster has a redox function. The mononuclear iron, however, has an intermediate midpoint potential and possibly undergoes a redox change under physiological conditions.

Biocatalytic applications of redox enzymes are emerging in response to demands for more sustainable synthetic routes from renewable sources. Characterization of the redox properties of these enzymes is important to understand the mechanism and catalytic scope of these enzymes. For example, the midpoint potential of the T1 copper center of laccase is considered to be an important characteristic of these enzymes. The catalytic efficiency of laccases has been shown to be directly dependent on the thermodynamic driving force of the electron transfer, *i.e.* the difference in midpoint potential of the T1 copper center and the substrate¹³. Redox titrations offer a very robust way to obtain midpoint potentials of different redox active cofactors in enzymes and proteins. The technique is relatively demanding in terms of protein, generally requiring a few mg of protein, but it will very likely be successful.

Disclosures

The authors have nothing to disclose.

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