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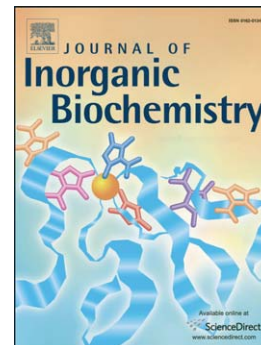
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## Small-volume potentiometric titrations: EPR investigations of Fe-S cluster N2 in mitochondrial complex I

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In memory of Professor Graeme Hanson and his contributions to Bioinorganic Chemistry and Electron Paramagnetic Resonance

### Abstract

EPR-based potentiometric titrations are a well-established method for determining the reduction potentials of cofactors in large and complex proteins with at least one EPR-active state. However, such titrations require large amounts of protein. Here, we report a new method that requires an order of magnitude less protein than previously described methods, and that provides EPR samples suitable for measurements at both X- and Q-band microwave frequencies. We demonstrate our method by determining the reduction potential of the terminal [4Fe-4S] cluster (N2) in the intramolecular electron-transfer relay in mammalian respiratory complex I. The value determined by our method,  $E_{m7} = -158$  mV, is precise, reproducible, and consistent with previously reported values. Our small-volume potentiometric titration method will facilitate detailed investigations of EPR-active centres in non-abundant and refractory proteins that can only be prepared in small quantities.

**Keywords**

electron paramagnetic resonance – iron-sulfur clusters – NADH:ubiquinone oxidoreductase  
– redox titrations

**1. Introduction**

Potentiometric titrations are instrumental for obtaining the reduction potentials of redox-active centres in proteins. They provide essential information for understanding the roles of these centres in electron and energy transfer processes. Various spectroscopic techniques can be used to monitor the presence of an oxidized or reduced state (or both) during a titration but electron paramagnetic resonance (EPR) spectroscopy, owing to its selectivity for unpaired electrons and its sensitivity to the different environments of otherwise identical centers, is particularly useful for investigating large and complex proteins with numerous redox-active sites. However, potentiometric titrations monitored by EPR have a major drawback: they require large amounts (tens of nanomoles) of protein that, in many cases, are not readily available. A typical EPR-based titration requires 10 to 20 samples, each with a volume of 100-250  $\mu\text{L}$  and a spin concentration of at least 20  $\mu\text{M}$ , so it is surprising that there have been so few attempts to address this limitation. Recently, Murray *et al.* described an *in situ* electrochemical method for oxidizing or reducing small-volume EPR samples for high-frequency EPR measurements [1], but the ability of this diffusion-limited method to set a precise reduction potential in a large and hydrophobic protein is questionable. Clearly, a robust and reliable method for accurately setting the potential in low-volume samples that are suitable for characterization by EPR is required.

Iron-sulfur (Fe-S) clusters are ubiquitous in nature. They lack convenient UV-visible spectroscopic handles but all have a paramagnetic ( $S \neq 0$ ) ground state in at least one oxidation state, making EPR a preferred method for investigating them. Within Fe-S clusters the Fe subsites couple ferro- and antiferro-magnetically to produce a ladder of spin states with different energies, but for the EPR-active oxidation state the ground state is often

$S = 1/2$  [2,3]. In the simplest example of a (reduced)  $[2\text{Fe-2S}]^+$  cluster the  $\text{Fe}^{2+}$  ( $S = 2$ ) and  $\text{Fe}^{3+}$  ( $S = 5/2$ ) subsites couple antiferromagnetically to produce an  $S = 1/2$  ground state [4]. In (reduced)  $[4\text{Fe-4S}]^+$  clusters, the type investigated here, the coupling between  $\text{Fe}^{2+}$  and mixed-valence  $\text{Fe}^{2.5+}$  pairs is complex but still commonly results in an  $S = 1/2$  ground state.

Respiratory complex I (NADH:ubiquinone oxidoreductase), a key enzyme in oxidative phosphorylation in mitochondria, contains a particularly intriguing electron-transfer relay formed from  $[2\text{Fe-2S}]$  and  $[4\text{Fe-4S}]$  clusters (Figure 1) [5]. This unusually long (80 Å) relay (comprising 8 clusters in most species) [6–8] channels electrons from the site of NADH oxidation to the site of quinone reduction, providing the redox energy required for proton translocation. Almost everything known about the properties of the Fe-S clusters in complex I stems from EPR spectroscopy. All the clusters are diamagnetic and thus EPR silent in their oxidized forms, whereas reduction of the enzyme by NADH (to approximately  $-0.4$  V) typically reduces only five clusters – in mammalian complex I their EPR signals are named N1b, N2, N3 and N4; N5 is only observed at very low temperatures (Figure 1) [9,10]. The implication that the other three clusters remain oxidised in the NADH-reduced enzyme has been confirmed by Mössbauer spectroscopy [11]. EPR-based potentiometric titrations have been used to investigate the reduction potentials of the five EPR-visible Fe-S clusters [9,10] and have contributed to establishing an alternating potential energy profile of low- and high-potential centres along the relay [11,12].

The terminal  $[4\text{Fe-4S}]$  cluster in the complex I relay, cluster N2, is the donor of electrons to bound ubiquinone and may play an important role in the unknown mechanism by which intramolecular electron transfer is coupled to proton translocation [5]. Improved knowledge of the properties of cluster N2 is essential for establishing its role in energy transduction. Furthermore, the ability to study N2 alone (without interference from the other clusters) is a prerequisite for its detailed investigation by pulsed EPR methods. The N2 EPR signal cannot be observed in isolation in the NADH-reduced enzyme by simply adjusting the measurement temperature – although Prisner and co-workers exploited the very different relaxation

properties of N1b and N2 to investigate N2 separately in *Yarrowia lipolytica* complex I using REFINE (relaxation filtered hyperfine) spectroscopy [13]. Alternatively, it may be possible to set the reduction potential of the enzyme solution to make N2 the only paramagnetic centre present, as N2 is known to have the highest reduction potential of all the Fe-S clusters in the mammalian complex [5]. Two reduction potential values have been reported for N2 in *Bos taurus* complex I, the subject of this study:  $-140$  mV at pH 7 and  $-154$  mV at pH 7.8 (vs the standard hydrogen electrode) [9,14]. Although these values differ by only 14 mV, the reduction potential of cluster N2 in the same enzyme has also been reported to shift by  $-60$  mV per pH unit [9,14] so the different values are not fully consistent.

Here, we describe a new method that minimizes the amount of protein required for an EPR-based redox titration. Our method requires 10-40 times less protein than established methods, and by taking advantage of the small sample volume requirements of the standard Bruker split-ring X-band EPR resonator ER 4118X-MS2, it enables continuous-wave and pulse EPR measurements at X- and Q-band frequencies on the same sample. We demonstrate the effectiveness of our method by determining the reduction potential of cluster N2 in mammalian complex I, and demonstrate that N2 can be reduced selectively to enable future pulse EPR measurements to interrogate its properties and role in energy transduction.

## 2. Methods

### 2.1. Complex I Preparation and Assays

Complex I from *B. taurus* was purified at  $4$  °C using a modified version of the protocol of Sharpley et al [15]. 30 mL of mitochondrial membrane suspension were solubilised by addition of 1% n-dodecyl- $\beta$ -D-maltoside (DDM, Glycon) and loaded onto a Q-sepharose ion-exchange column. The ion exchange buffers (set to pH 7.55 at  $20$  °C) contained 20 mM Tris-HCl, 2 mM EDTA, 10% (v/v) ethylene glycol and 0.2% (w/v) DDM (Buffer A). Buffer B also contained 1 M NaCl and complex I was eluted in 35% B. Complex I-containing fractions

were pooled, concentrated, applied to a Superose 6 Increase column and eluted in buffer (set to pH 7.0 at 20 °C) containing 30 mM HEPES, 20 mM sodium phosphate, 150 mM NaCl, 10% (v/v) glycerol and 0.03% (w/v) DDM. The complex I concentration was determined by the bicinchoninic acid assay (Pierce) and a further 20% glycerol was added before the sample was flash-frozen for storage in liquid N<sub>2</sub>. The indicator dyes bromocresol green and bromothymol blue (Sigma Aldrich) were used as described by Sieracki et al. [16] to establish that the pH of the buffer did not change upon freezing in dry-ice and acetone, the cooling bath used for freezing EPR samples (see Section 2.2.). Using 30% glycerol instead of the previously employed 50% [16] did not affect the temperature independence of the pH 7.0 buffer, which is henceforth referred to as the TIP (temperature independent) buffer.

## 2.2. EPR Sample Preparation

EPR samples were prepared under anaerobic conditions in a Braun UniLab-plus glovebox (O<sub>2</sub> < 0.5 ppm, N<sub>2</sub> atmosphere) at room temperature (22 °C). With the exception of the NADH-reduced sample (Figure 3), complex I samples were titrated to the desired reduction potential as first described by Dutton [17]. Figure 2 shows a diagram of the custom-made electrochemical glass cell (Scientific Glassblowing Service, University of Southampton) used to carry out the titrations. The cell was equipped with a 2 or 0.5 mm glass-encased platinum working electrode (total diameter at tip ~5 mm, Scientific Glassblowing Service, University of Southampton; Pt from GoodFellow) and an Ag/AgCl mini-reference electrode (2 mm diameter, DRI-REF-2, World Precision Instruments). The electrodes, held in place through screw-cap joints with rubber O-ring seals, were connected to an EmSTAT3+ potentiostat (PalmSens) to monitor the potential of the cell solution continuously throughout the titration. Due to the pointed base of the cell, the potential of the solution can be measured in volumes as low as 30 µL. A micro stirring bar (2 mm diameter) was used to stir the cell solution; the experiments discussed here were carried out at room temperature but the cell is fitted with a water jacket for experiments at different temperatures. A further un-capped joint enabled

addition of mediators and oxidising and reducing agents, and the removal of samples, using microliter glass syringes (SGE Analytical Science). To make an EPR sample, 9  $\mu\text{L}$  of cell solution were transferred into a 1.6 O.D. suprasil quartz EPR tube (Wilmad, inner diameter  $1.10 \pm 0.05$  mm) and flash-frozen in a cold trap containing ethanol, cooled from outside the glovebox by a dewar filled with dry ice and acetone. Frozen EPR samples were removed from the glovebox and stored in liquid nitrogen.

For titration 1, 150  $\mu\text{L}$  of 20-25  $\mu\text{M}$  complex I solution in pH 7 TIP buffer was transferred to the electrochemical cell and stirred in the glovebox for 1 hour at room temperature to remove  $\text{O}_2$ . 9  $\mu\text{L}$  of the cell solution were mixed with 1  $\mu\text{L}$  of 20 mM NADH (Sigma, in TIP buffer) to make the NADH-reduced sample. Then, the redox mediators methylene blue, indigotrisulfonate, 2-hydroxy-1,4-naphthoquinone and benzyl viologen were each added to the remainder of the cell solution to a final concentration of 30  $\mu\text{M}$ . To make each titration sample the reduction potential of the solution was adjusted through successive additions (0.1 - 0.5  $\mu\text{L}$ ) of 5, 10, 25 or 50 mM sodium dithionite (in 50 mM tetraborate buffer, pH 9.6 – these tiny additions did not change the pH of the enzyme solution significantly), or of 5 or 10 mM potassium ferricyanide (in TIP buffer). Once the desired reduction potential was reached, 9  $\mu\text{L}$  of the solution was transferred to an EPR tube and flash-frozen. Titration 2 was carried out in the same way, using a different sample from the same protein preparation, except the starting volume was 140  $\mu\text{L}$  and no NADH-reduced sample was made.

All reduction potentials are given relative to the potential of the standard hydrogen electrode (SHE). The reference electrode potential was determined to be +210 mV vs. SHE using quinhydrone (Sigma Aldrich) as an external standard, and found to be stable over the experimental timescale.

### 2.3. EPR spectroscopy

EPR measurements were performed using an X/Q-band Bruker Elexsys E580 Spectrometer (Bruker BioSpin GmbH, Germany) equipped with a closed-cycle cryostat (Cryogenic Ltd,

UK). The EPR measurement temperature was calibrated with an external Cernox thermometer. The magnetic field was calibrated at room temperature with a Bruker strong pitch sample ( $g = 2.0028$ ). All measurements were carried out in an X-band split-ring resonator module with 2 mm sample access (ER 4118X-MS-2W), operated in either continuous-wave (CW) or pulse mode. Baseline spectra of the empty resonator, of samples containing only buffer, as well as of oxidised complex I were found to be identical; all the spectra presented have been baseline-subtracted.

For CW measurements of the titration samples, the Q value, as reported by the built-in Q indicator in the *Xepr* programme (typically 800), was used as a guide to position each sample in the same position in the resonator. CW measurements were carried out at lower microwave powers and higher temperatures than usual [10,12,18] because of the high conversion factor of the ER 4118X-MS-2W resonator ( $8 \text{ G/W}^{1/2}$ ). For comparison, the standard Bruker cavity (ER 4102ST) operating at 9.8 GHz has a conversion factor of  $1.48 \text{ G/W}^{1/2}$ . The measurement time for each of the CW EPR spectra presented in Figures 3 and 4 was ca. 12.5 minutes. The two-pulse echo-detected field sweep (Figure 5) was acquired with the pulse sequence  $\pi/2-\tau-\pi-\tau$ -echo ( $\pi/2 = 16 \text{ ns}$ ,  $\tau = 132 \text{ ns}$ ) and measured at 12 K.

#### 2.4. EPR spectral analyses

All spectral simulations were carried out using EasySpin [19]. The parameters for cluster N2 were determined from CW spectra showing only N2 (Figure 4A). The fit in Figure 3 was obtained by keeping the parameters for N2 fixed and taking the values for N1b, N3 and N4 reported previously [12] as starting parameters.

In order to generate the 'Nernst plots' (Figure 4C), the double integrals ('areas') of the N2 spectra derived from the full simulations (Figure 4A) were plotted as a function of the reduction potential of individual samples and normalized, with 1 corresponding to fully reduced N2 and 0 to fully oxidised N2. The one-electron Nernst equation was fitted to the



experimental data points using the Matlab curve-fitting tool. A clear outlier from titration 1 (sample S8) was not included in the fitting of the Nernst curve in Figure 4C.

### 3. Results

#### 3.1. Temperature dependence of CW EPR spectra of reduced complex I in the MS-2 resonator

Figure 3 shows X-band CW EPR spectra of NADH-reduced *B. taurus* complex I measured in a split ring MS-2 resonator as a function of temperature. At 20 K four of the eight clusters (N1b, N2, N3, and N4, see Figure 1) are clearly visible, as expected, as  $S = 1/2$  species. As the temperature is increased clusters N4, N3 and N2 successively broaden and become indistinguishable from the background, in agreement with the reported trend in relaxation rate,  $N1 < N2 < N3 < N4 < N5$  [10,18,20,21]. The parameters for the simulated spectrum presented alongside the experimental data at 18 K (see Figure 3 legend) are in excellent agreement with those reported previously [10,12,18]. Note that resonators typically used for X-band CW measurements reveal N3 and N4 clearly only below 12 K; we attribute our ability to observe them at higher temperatures and lower microwave powers to the much higher power conversion factor of the split-ring MS-2 resonator (see Section 2.3.). Consistent with this, N5, usually detected only at 4-7 K [10,18], was faintly detectable at 10 K, though it became clearer at lower temperatures (spectra not shown).

#### 3.2. Small-volume titration of complex I

CW EPR spectra of complex I poised at decreasing potentials, and measured at 25 K, are summarised in Figure 4A. The potential vs time trace accompanying the titration (Figure 4B) reveals that confidence in the reduction potential of each sample is very high ( $\pm 1$  mV). The measurement temperature was chosen so that only N2 and N1b contribute significantly to the spectra (see Figure 3), and both the temperature and microwave power were optimised for N2. In agreement with previous data showing that N2 has a higher potential than N1b in *B. taurus* complex I, the almost axial EPR spectrum of N2 ( $g_{x,y,z} = 1.923, 1.927,$

2.056) becomes visible before N1b ( $g_{x,y,z} = 1.926, 1.942, 2.023$ ) as the potential becomes more negative. At  $-173$  mV and below, the broad and featureless signal developing below  $g = 1.9$  reveals that N3 and N4 are becoming reduced (see Figure 3). Simulations for N2 and N1b are included in Figure 4A and the N2 signal reaches its maximal intensity at approximately  $-310$  mV, when the cluster is fully reduced. The normalized double-integrals of the simulated N2 EPR signals were plotted as a function of potential (Figure 4C, black circles) and fitted to the one-electron Nernst equation, giving a reduction potential of  $-159$  mV. The titration was repeated and the reduction potential of N2 was determined to be  $-158$  mV (Figure 4C, open triangles; corresponding CW EPR spectra not shown) – demonstrating excellent reproducibility between independent data sets. Our value ( $-158$  mV) for the reduction potential of N2 is broadly in agreement with those determined previously in bovine complex I (Table 1). It differs by  $\sim 20$  mV with that determined in submitochondrial particles at the same pH but in a buffer mixture whose temperature dependence is not known. A direct comparison with the value previously determined in isolated complex I (in pH 7.8 bis-tris propane buffer) is difficult not only because the ( $-60$  mV per pH unit) pH dependence of the N2 reduction potential requires confirmation but also because of the large pH difference (1.6 pH units) between bis-tris propane solutions at ambient and cryogenic temperatures [16].

In order to confirm the successful selective reduction of cluster N2, an echo-detected field sweep of complex I reduced to  $-154$  mV (sample S7 from titration 1) was measured at 12 K using the MS-2 resonator (Figure 5). The numerical first derivative is equivalent to the CW field sweep obtained at 25 K, demonstrating that N2 is indeed the only reduced Fe-S cluster present. Measurements at lower temperatures (down to 5 K) also did not reveal any additional paramagnetic species.

#### 4. Discussion

The titration method described has been demonstrated to be a straightforward method for obtaining reliable and consistent reduction potential data on EPR- and redox-active centres

in large and complex proteins using only minute sample volumes. The total volume of enzyme solution required for each complete titration was 140  $\mu\text{L}$  whereas established EPR-based potentiometric methods typically require 1.5 to 5 mL. Moreover, by working under strictly anaerobic conditions we achieved remarkably stable potential readings, over the entire potential range (Figure 4B).

Despite the small sample volumes used, the ER 4118X-MS2 resonator produces spectra with good signal-to-noise ratio (Figure 4A) and our spectra are comparable to spectra obtained using standard CW instrumentation. Nonetheless, we note that the high microwave field in the MS2 resonator has disadvantages, although immaterial for the present study. In particular, given the high conversion factor, we found it difficult to record CW EPR spectra at lower temperatures ( $< 10\text{ K}$ ) under non-saturating conditions. This could hamper the reliable determination of spectral parameters (e.g. linewidths) for some species. Notably, our use of standard Q-band EPR tubes opens up the possibility of carrying out Q-band measurements on the same set of samples as used for CW and pulse X-band measurements. This constitutes a considerable advantage for spectra that display (unlike  $\text{N}_2$  [12]) a poorly resolved  $g$  anisotropy at X-band.

The reduction potential we have determined for cluster  $\text{N}_2$  in bovine complex I agrees well with an equivalent value from submitochondrial particles (Table 1), suggesting that this physical property of the buried Fe-S cluster is independent of the preparation. A larger difference might have been expected with the value obtained previously with isolated complex I at pH 7.8 (see Table 1) – given that the reduction potential of  $\text{N}_2$  is known to be pH dependent [9,22]. The observed inconsistencies between previously published data and our value may be attributable to the different buffers used: it is well known that some buffers change their pH significantly upon freezing [23,24] so reduction potentials determined in different buffers at nominally at the same pH value may not correspond. Here, we eliminated any pH-induced reduction potential shift by using a buffer known to retain constant pH even at cryogenic temperatures [16]. Although glycerol has been reported as having a profound

effect on the pH value upon freezing [24], we found that lowering the glycerol concentration from 50% (as reported by Sieracki *et al.* [16]) to 30% (in our preparations) had no detectable effect.

The work presented here enables future experiments to interrogate the reduction potentials of redox-active centres in a much wider range of proteins than previously possible, and it specifically enables further experiments to probe the properties and mechanistic contributions of cluster N2 in complex I. First, it will be important to confirm the extent and origin of the pH dependence of the N2 cluster reduction potential, which has been proposed to be due to a nearby histidine residue [22]. Second, our ability to reduce N2 selectively will allow future investigations by pulsed EPR (without interference from the other many Fe-S clusters present) to probe the local environment of the cluster further. Together, these strategies will advance knowledge of the properties of cluster N2 and lead to better understanding of its role in energy transduction.

### Abbreviations

EPR (electron paramagnetic resonance) – Fe-S (iron-sulfur) cluster – mw (microwave)

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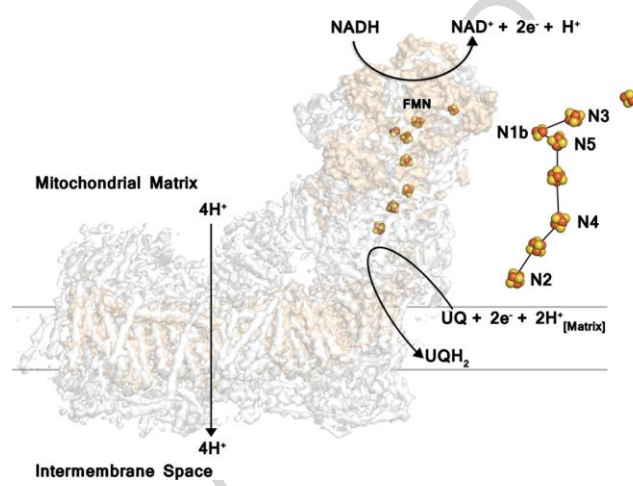
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**Table 1 – Experimentally determined midpoint potentials for cluster N2 in *B. taurus* complex I.** The estimated  $E_m$  value of  $-106$  mV was obtained by applying a shift of  $-60$  mV per pH unit [9].

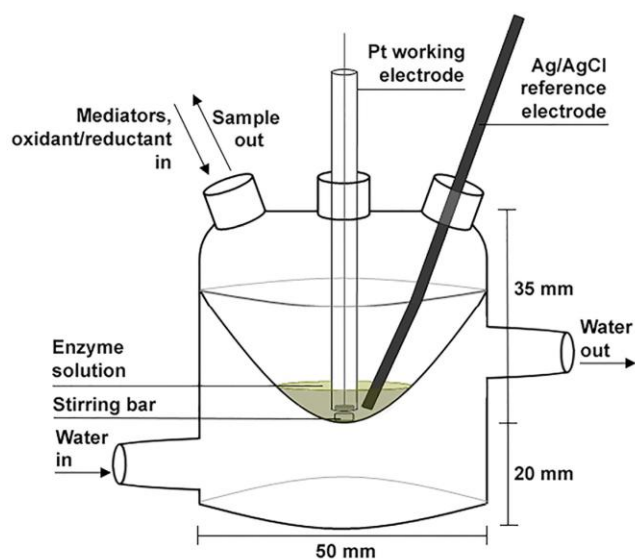
$E_m$ (mV)	Preparation	Buffer	Ref.
<b><math>-158</math> (pH 7)</b>	Isolated complex I	HEPES:phosphate (temperature-independent [16])	this work
<b><math>-140</math> (pH 7)</b>	Submitochondrial particles & mitochondria	MES/MOPS/HEPES/TAPS (temperature dependence not known)	[9]
<b><math>-154</math> (pH 7.8)</b> est. $-106$ (pH 7)	Isolated complex I	Bis-Tris Propane (temperature dependent [16])	[14]

**Single column Table**



**Figure 1 – The structure, reactions and Fe-S clusters in *B. taurus* complex I.** Two electrons are transferred from NADH to ubiquinone (FMN = flavin mononucleotide, UQ = ubiquinone, UQH<sub>2</sub> = ubiquinol). The Fe-S clusters are labelled according to their EPR signals; unlabelled clusters in the chain remain oxidised in the NADH-reduced enzyme. The figure was created using PyMOL from the electron cryo-microscopy density map EMD-2676 and the structural model 4UQ8.pdb.

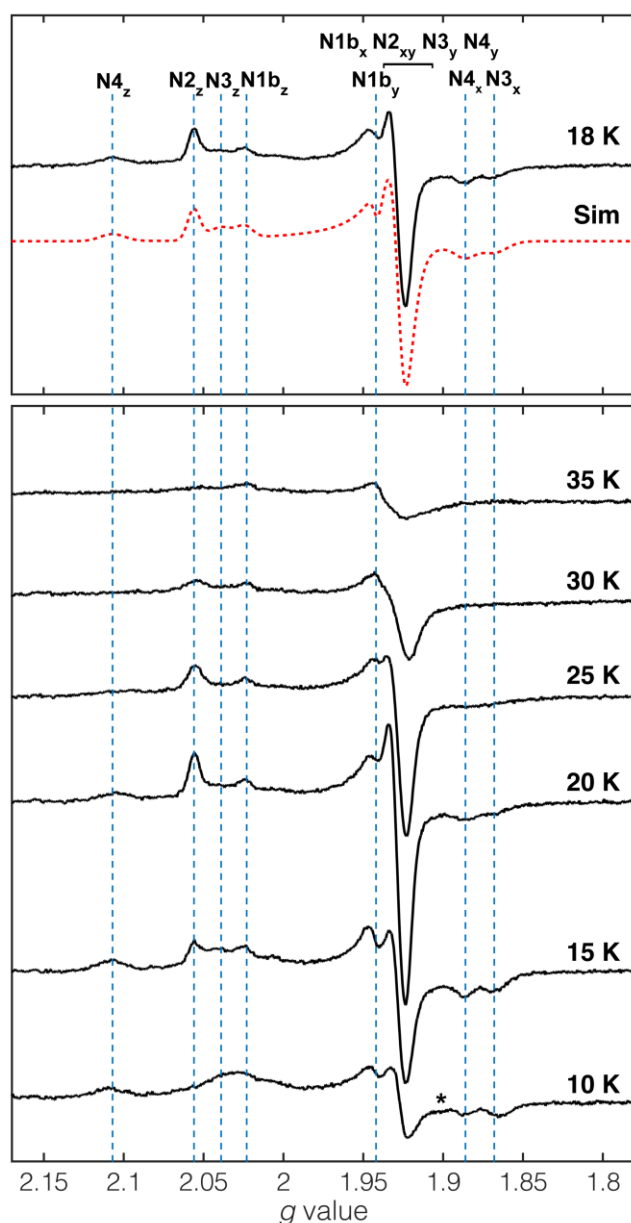
**Single-column Figure**



**Figure 2 – Schematic diagram of the glass cell and electrodes used for potentiometric titrations.** The temperature of the cell solution can be controlled using the water jacket.

**Single-column Figure**

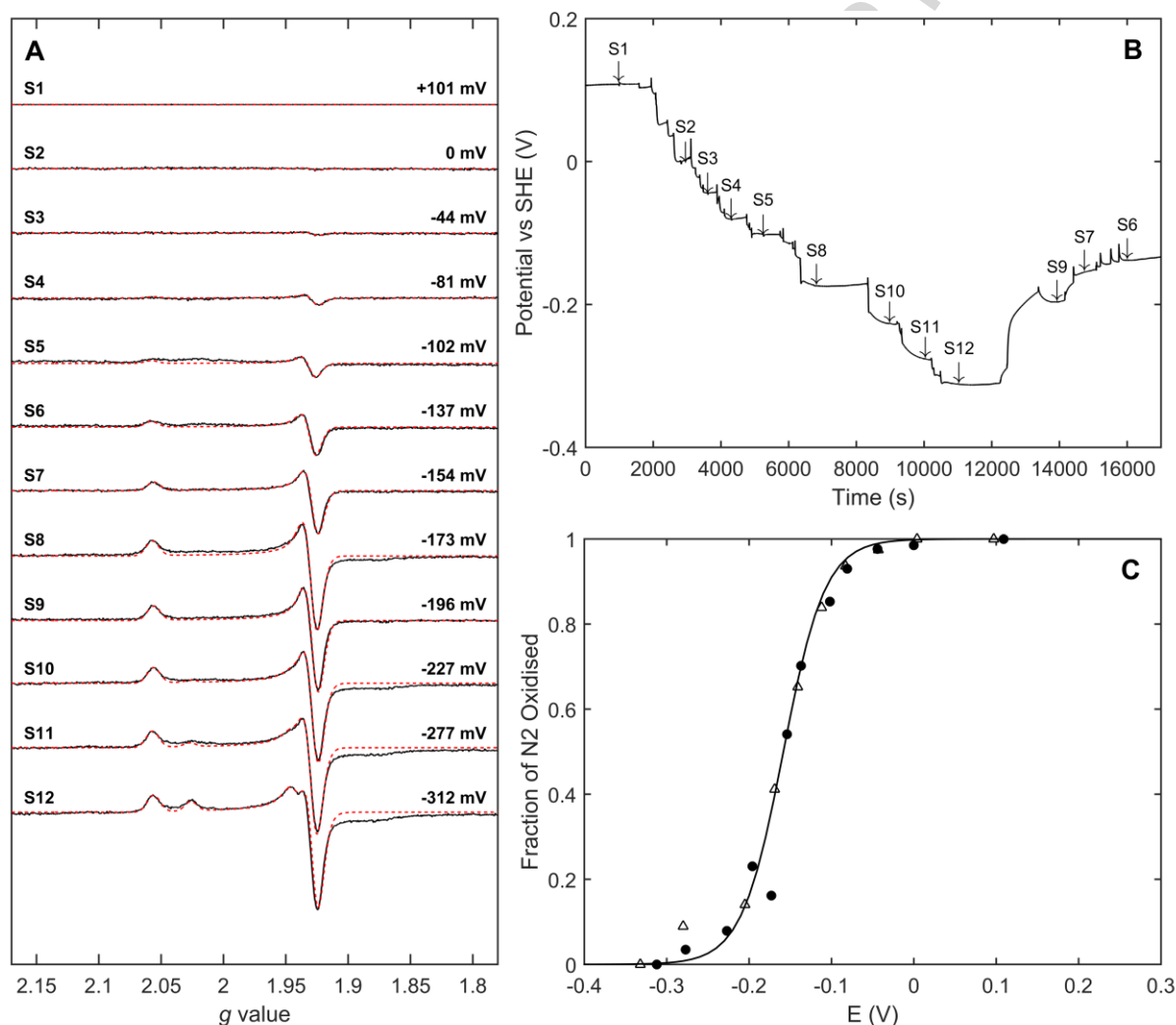




**Figure 3.** X-band CW EPR spectra of NADH-reduced *B. taurus* complex I recorded at different temperatures using the MS-2 resonator. 9  $\mu$ L of complex I ( $\sim$ 20  $\mu$ M) in TIP buffer (pH 7.0) was reduced with 2 mM NADH. EPR measurement conditions: 3.2 mW power, 100 KHz modulation frequency and 0.7 mT modulation amplitude. The gain and number of scans was the same throughout and the spectra were not normalized. Simulation parameters ( $g_{x,y,z}$ ): N1b = (1.926, 1.942, 2.023), N2 = (1.923, 1.927, 2.056), N3 = (1.868, 1.934, 2.039), N4 = (1.886, 1.928, 2.107). The vertical dotted lines correspond to  $g$  values

used in the simulations. The asterisk at 10 K indicates the  $g_x$  peak of N5 (expected  $g$  values: 1.898, 1.928, 2.064 [18]).

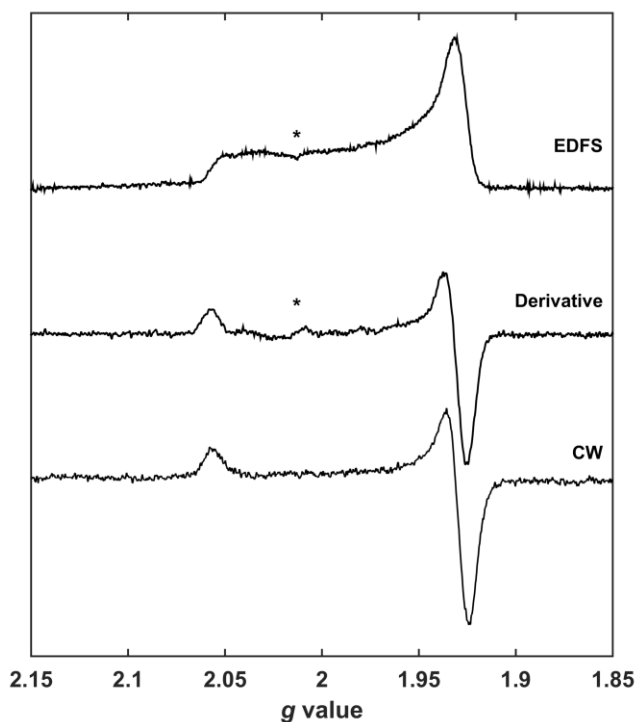
### Single-column figure



**Figure 4 – Small-volume potentiometric titration of the N2 cluster of *B. taurus* complex I.** (A) X-band (9.5 GHz) CW EPR spectra of *B. taurus* complex I reduced to selected reduction potentials (titration 1) with simulations (dotted lines). Measurements were performed at 25 K, 2 mW power, 100 KHz modulation frequency and 0.7 mT modulation amplitude. (B) Potentiometric titration trace of complex I (titration 1). Steps correspond to the addition of sodium dithionite or potassium ferricyanide. Sample extractions are indicated with arrows and numbered according to descending reduction potential. (C) Peak areas of the N2 spectra as a function of reduction potential (black circles for titration 1, white triangles

for titration 2), fitted to the one-electron Nernst equation (solid line) with midpoint potentials of  $-159$  mV (titration 1,  $R^2 = 0.9961$ ) and  $-158$  mV (titration 2,  $R^2 = 0.9988$ ). The combined fitting for the Nernst curve across both titrations was  $-158$  mV ( $R^2 = 0.9972$ ).

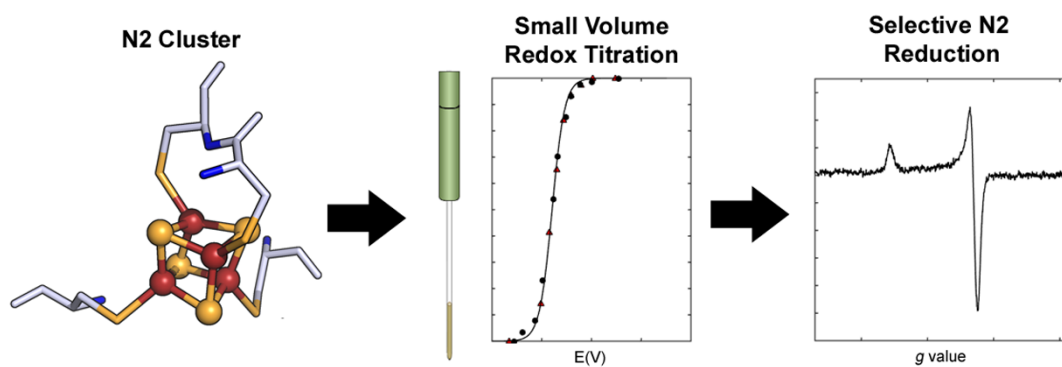
### ***Double column Figure***



**Figure 5 – Field sweep spectra of *B. taurus* complex I reduced to  $-154$  mV.** X-band echo-detected field sweep (EDFS, top) at 12 K, its numerical first derivative (middle) and CW EPR spectrum at 25 K (bottom, spectrum S7 from Fig. 4A). Small impurities in the resonator give rise to the peak marked with an asterisk.

### ***Single-column Figure***

***All Figures and can be in black and white in the printed version.***



### Graphical abstract

The graphical abstract (from left to right) shows the iron-sulfur cluster N<sub>2</sub> in NADH:ubiquinone oxidoreductase (complex I), the EPR-monitored small-volume redox titration by which its reduction potential was determined, and an EPR spectrum of N<sub>2</sub> that demonstrates that the cluster has been reduced selectively (no other paramagnetic species are present).

**Highlights**

- A new low-volume strategy for EPR-based potentiometric titrations
- The method requires up to 40 times less enzyme than conventional methods
- The reduction potential of cluster N2 in bovine complex I is -158 mV at pH 7
- Cluster N2 can be isolated from the other Fe-S clusters by selective reduction

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