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

John J. Wright a, Enrico Salvadori a,b, Hannah R. Bridges c, Judy Hirst c, Maxie M. Roessler a,*

a School of Biological and Chemical Sciences, Queen Mary University of London, Mile End Road, London E1 4NS, United Kingdom
b London Centre of Nanotechnology, University College London, 17-19 Gordon Street, London WC1H 0AH, United Kingdom
c Medical Research Council Mitochondrial Biology Unit, Wellcome Trust/MRC Building, Hills Road, Cambridge CB2 0XY, United Kingdom

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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, \( E_{m7} = -158 \text{ mV} \), 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.

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 oxidised 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 centres, 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 μL and a spin concentration of at least 20 μM, 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 oxidising 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 characterisation 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 antiferromagnetically 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) \([2Fe-2S]\) cluster the Fe\(^{2+} (S = 2)\) and Fe\(^{3+} (S = 5/2)\) subsites couple antiferromagnetically to produce an \( S = 1/2 \) ground state [4]. In reduced \([2Fe-2S]\) clusters, the type investigated here, the coupling between Fe\(^{2+}\) and mixed-valence Fe\(^{2+}/^{3+}\) 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 \([2Fe-2S]\) and \([4Fe-4S]\) clusters (Fig. 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 oxidised forms, whereas reduction of the enzyme by NADH (to approximately \(-0.4 \text{ V}\)) typically reduces only five clusters – in mammalian complex I their EPR signals are named N1h, N2, N3 and N4; N5 is only observed at very low temperatures (Fig. 1) [9,10]. The implication that

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* Corresponding author.
E-mail address: m.roessler@qmul.ac.uk (M.M. Roessler).

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the other three clusters remain oxidised in the NADH-reduced enzyme has been confirmed by Mössbauer spectroscopy [11]. EPR-based poten-
tiometric titrations have been used to investigate the reduction poten-
tials 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 [4Fe-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 prop-
erties of cluster N2 is essential for establishing its role in energy trans-
duction. Furthermore, the ability to study N2 alone (without interference from the other clusters) is a prerequisite for its detailed in-
vestigation by pulse EPR methods. The N2 EPR signal cannot be ob-
served 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 and demonstrated that N2 can be reduced selectively to enable future

2. Methods

2.1. Complex I preparation and assays

Complex I from *B. taurus* was purified at 4 °C using a modified ver-
sion of the protocol of Sharpley et al. [15]. 30 mL of mitochondrial mem-
brane suspension were solubilised by addition of 1% n-dodecyl-β-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 N2. The indicator dyes bromoresol 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 previ-
ously employed 50% [16] did not affect the temperature independence of the pH 7.0 buffer, which is henceforth referred to as the TIP (temper-
ature independent) buffer.

2.2. EPR sample preparation

EPR samples were prepared under anaerobic conditions in a Braun UniLab-plus glovebox (O2 < 0.5 ppm, N2 atmosphere) at room temper-

ature (22 °C). With the exception of the NADH-reduced sample (Fig. 3),

complex I samples were titrated to the desired reduction potential as first described by Dutton [17]. Fig. 2 shows a diagram of the custom-
made electrochemical glass cell (Scientific Glassblowing Service, Uni-
versity 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-cup joints with rubber O-
ring seals, were connected to an EmSTAT3+ potentiostat (PalmSens)
to monitor the potential of the cell solution continuously throughout the

The potential of the base of the cell, the potential of the solu-
cation 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 µL of cell solution were transferred into a 1.6 O.D. suprasil quartz EPR

tube (Wilmad, inner diameter 1.10 ± 0.05 mm) and

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ferred into a 1.6 O.D. suprasil quartz EPR tube (Wilmad, inner diameter 1.10 ± 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 re-

moved from the glovebox and stored in liquid nitrogen.

For titration 1, 150 µL of 20–25 µM complex I solution in pH 7 TIP buffer was transferred to the electrochemical cell and stirred in the
glovebox for 1 h at room temperature to remove O2. 9 µL of the cell solu-
tion were mixed with 1 µL 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

violagen were each added to the remainder of the cell solution to a

final concentration of 30 µM. To make each titration sample the reduc-
tion potential of the solution was adjusted through successive additions

(0.1–0.5 µL) 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 ferri-cyanide (in TIP buffer). Once the desired reduction potential was reached, 9 $\mu$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 $\mu$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 Elexys 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-MS2), 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-MS2 resonator (8 G/W½). For comparison, the standard Bruker cavity (ER 4102ST) operating at 9.8 GHz has a conversion factor of 1.48 G/W½. The measurement time for each of the CW EPR spectra presented in Figs. 3 and 4 was ca. 12.5 min. The two-pulse echo-detected field sweep (Fig. 5) was acquired with the pulse sequence $\pi/2-\tau-\pi-\tau$-echo ($\pi/2 = 16$ ns, $\tau = 132$ 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 (Fig. 4A). The fit in Fig. 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’ (Fig. 4C), the double integrals (‘areas’) of the N2 spectra derived from the full simulations (Fig. 4A) were plotted as a function of the reduction potential of individual samples and normalized, with 0 corresponding to fully reduced N2 and 1 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 Fig. 4C.

3. Results

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

Fig. 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 Fig. 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 Fig. 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 finally 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 Fig. 4A. The potential vs. time trace accompanying the titration (Fig. 4B) reveals that confidence in the reduction potential of each sample is very high (±1 mV). The measurement temperature was chosen so that only N2 and N1b contribute significantly to the spectra (see Fig. 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 Fig. 3). Simulations for N2 and N1b are included in Fig. 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 (Fig. 4C, black circles) and fitted to the one-electron Nernst equation, giving a reduction potential 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 ($R^2 = 0.9972$).

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 μL 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 (Fig. 4B).

Despite the small sample volumes used, the ER 4118X-MS2 resonator produces spectra with good signal-to-noise ratio (Fig. 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 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 N2 [12]) a poorly resolved anisotropy at X-band.

The reduction potential we have determined for cluster N2 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 N2 is known to be pH dependent [9,22]. The observed inconsistencies between the previously published data and our values 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.

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