Analytical Biochemistry 419 (2011) 110–116

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

# Electrochemical titrations and reaction time courses monitored in situ by magnetic circular dichroism spectroscopy

Justin M. Bradley [a], Julea N. Butt [a,b,*], Myles R. Cheesman [a,*]

[a] Centre for Molecular and Structural Biochemistry, School of Chemistry, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, UK
[b] Centre for Molecular and Structural Biochemistry, School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, UK

ARTICLE INFO

*Article history:*
Received 23 April 2011
Received in revised form 24 July 2011
Accepted 25 July 2011
Available online 30 July 2011

*Keywords:*
MCD
Magnetic circular dichroism
Electrochemistry
Metalloprotein

## ABSTRACT

Magnetic circular dichroism (MCD) spectra, at ultraviolet–visible or near-infrared wavelengths (185–2000 nm), contain the same transitions observed in conventional absorbance spectroscopy, but their bisignate nature and more stringent selection rules provide greatly enhanced resolution. Thus, they have proved to be invaluable in the study of many transition metal-containing proteins. For mainly technical reasons, MCD has been limited almost exclusively to the measurement of static samples. But the ability to employ the resolving power of MCD to follow changes at transition metal sites would be a potentially significant advance. We describe here the development of a cuvette holder that allows reagent injection and sample mixing within the 50-mm-diameter ambient temperature bore of an energized superconducting solenoid. This has allowed us, for the first time, to monitor time-resolved MCD resulting from in situ chemical manipulation of a metalloprotein sample. Furthermore, we report the parallel development of an electrochemical cell using a three-electrode configuration with physically separated working and counter electrodes, allowing true potentiometric titration to be performed within the bore of the MCD solenoid.

Magnetic circular dichroism (MCD)[1] spectroscopy, at ultraviolet–visible and near-infrared wavelengths (185–2000 nm), has proved to be invaluable in the study of metalloproteins containing cofactors such as heme [1–3], non-heme iron [4], iron–sulfur clusters [5–8], cobalt [9,10], nickel [11–13], and copper [14,15]. The technique measures the apparent circular dichroism (CD) induced by a magnetic field [16]. Despite similarities in the instrumentation used, the observation of MCD is not dependent on the chirality of the protein; the method is equally applicable to racemic model complexes [17]. The magnetic field will always induce signals across wavelengths at which the substance absorbs. Thus, MCD spectra contain the same electronic transitions observed in conventional absorbance spectroscopy, but the bisignate nature of the spectrum provides enhanced resolution and greater detail. This spectral detail offers an unmatched fingerprinting capability that can, for example, identify the spin and oxidation states of heme groups [1] and distinguish among the variety of iron–sulfur centers found in biological molecules [18–20].

Metalloprotein MCD can be measured at ambient or cryogenic temperatures. The latter requires adulteration with glassing agents [16] but has generally been preferred because MCD intensity from paramagnetic centers increases dramatically at low temperature. Thus, most metalloprotein MCD reported has been measured in glasses at temperatures of approximately 4.2 K. Hemoproteins represent the significant exception, giving rise to appreciable MCD at high temperatures [1]. Thus, ambient temperature MCD is used to diagnose spin state, oxidation state, and (in the case of low-spin Fe(III) hemes) axial ligation [21].

As with many techniques, much valuable information is to be gained from the ability to follow spectral perturbations that report on changes at the metal center. This includes equilibrium studies (chemical or redox titrations) as well as kinetic measurements. For technical reasons, such use of MCD has been limited almost exclusively to the measurement of series of static samples. Limited time domain studies have been achieved using a freeze–quench approach; samples can be frozen in approximately 30 s after preparation [22,23]. Alternatively, reacting aqueous samples can be quenched in cold liquids such as isopentane and, with considerable effort, packed into quartz tubes for electron paramagnetic resonance (EPR) [24]. However, this method presents additional challenges for MCD because achieving an optically transparent

---

* Corresponding authors. Fax: +44 1603 592003.
*E-mail addresses:* j.butt@uea.ac.uk (J.N. Butt), m.cheesman@uea.ac.uk (M.R. Cheesman).

[1] *Abbreviations used:* MCD, magnetic circular dichroism; CD, circular dichroism; MOTTLE, MCD-monitored optically transparent thin layer electrochemistry; T, Tesla; RT, room temperature; $cd_1$, cytochrome $cd_1$; Paz, pseudoazurin; Tyr$^-$, tyrosinate; SHE, standard hydrogen electrode.

0003-2697/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2011.07.030

sample requires mixing with glycerol at −30 °C to form a slurry. Although these multiple-sample methods can be used to chart changes at metal sites by MCD, they suffer from significant disadvantages. Large amounts of material are consumed, and they are extremely low throughput with, at best, two or three samples processed per day. Thus, it is difficult to monitor time-dependent events with useful resolution.

Similar problems beset MCD-monitored redox titrations. Multiple samples can be electrochemically poised and frozen for measurements. But again sample consumption is high, throughput is low, and drifts in potential can occur during the freezing process. We previously developed a method by which MCD could be used to probe the nature of a metalloprotein sample poised in situ at defined electrochemical potential [25]. The method, MCD-monitored optically transparent thin layer electrochemistry (MOTTLE), uses a three-electrode cell in a quartz cuvette, including an optically transparent working electrode. The shortest dimension of the cell by far is in the direction of the light path (0.5–1.0 mm). Diffusion allows the sample to come to pseudo-equilibrium with applied potential because the distance between transparent faces of the cell and the working electrode (0.25–0.5 mm) is far shorter than the separation of working and counter electrodes (~5.0 mm). This configuration of MOTTLE has already made significant contributions to the understanding of hemoproteins, especially those containing multiple cofactors [26,27].

The ability to use ambient temperature MCD to follow reactions initiated by in situ mixing of reagents would represent a significant advance. This requires reagent injection lines and a non-magnetic stirring mechanism sited inside the bore of a superconducting solenoid. A stirring mechanism would also require larger volume and longer pathlength cuvettes. However, this is also a prerequisite for physical separation of counter and working electrodes, a modification of MOTTLE that would allow true equilibrium to be reached at times minimized by the stirring. Longer pathlengths, especially if combined with stronger magnetic fields, would also increase MCD signal magnitude, facilitating the study of cofactors other than heme.

Such developments have previously been precluded by the solenoid bore diameter (25 mm). Stirring and injection mechanisms cannot be constructed in this limited space. Consequently, we have commissioned a superconducting solenoid that gives magnetic fields of up to 8 Tesla (T) and is fitted with a room temperature (RT) bore of 50 mm diameter. We report here the design, construction, and performance of apparatus that allows realization of the twin aims of in situ triggering of reactions followed by time-resolved RT MCD and enhanced potentiometric performance with stirred electrochemical cells.

## Materials and methods

MCD spectra were recorded using a Jasco J-810 spectropolarimeter with the sample located in an 8-T magnetic field within the ambient temperature bore of an Oxford Instruments Special Spectromag 1000 superconducting solenoid. Absorbance spectra were recorded using a Hitachi U-2900 spectrometer. All potentiometric and voltammetric measurements were performed using an Autolab PGSTAT12 instrument controlled by GPES software. Calibration of the Ag/AgCl reference electrode was as described previously [25].

### Redox cycling of *Paracoccus pantotrophus* $cd_1$

Cytochrome $cd_1$ ($cd_1$) was expressed and purified using established protocols [28]. Protein concentration was determined by electronic absorbance spectroscopy of the oxidized form using $\varepsilon_{406\ \mathrm{nm}} = 143{,}000\ \mathrm{M^{-1}\ cm^{-1}}$ per monomeric unit [29]. In situ injection of aliquots of reagent into a cuvette positioned in the bore of the superconducting solenoid was achieved using flexible cannulas. These were loaded with anaerobic reagents while in an $N_2$-filled chamber ($O_2 < 5$ ppm). Aliquots of reagent were loaded into the cannulas from a gas-tight syringe and were alternated with 5-μl aliquots of buffer. Sufficient nitrogen gas was drawn into the cannulas between reagent aliquots such that depression of the plunger to expel a total of 50 μl of gas and liquid from the barrel would deliver an accurately known volume (typically 1–3 μl) of reagent into the MCD cell. Cannulas were threaded through access channels in a purpose-built cuvette holder, and the tips were introduced into the cuvette through a Teflon lid (apparatus described below). The entire assembly was then removed from the $N_2$ chamber and immediately inserted into the bore of the solenoid. Insertion of a cannula provided a continuous flow of $N_2$ gas (humidified to prevent evaporation from sample) to drive a stirring mechanism and to remove dioxygen from the bore. Any trace oxygen inadvertently introduced during transfer was removed by stirring the sample under a flow of $N_2$ while the solenoid was charged (~10 min). The dimensions of the cell holder and cannulas were such that, once in place in the magnet bore, the entire length of the injection lines containing reagent were located within the region purged with nitrogen gas to maintain their anaerobicity.

### Potentiometry of *P. pantotrophus* pseudoazurin and horse heart cytochrome *c*

Horse heart cytochrome *c* (Acros Organics) was used without further purification. Concentrations were determined by electronic absorbance spectroscopy using $\varepsilon_{410} = 106{,}100\ \mathrm{M^{-1}\ cm^{-1}}$ for the Fe(III) state [30]. MCD-monitored potentiometric titrations were performed on cytochrome *c* samples of 6 μM concentration in 20 mM Hepes and 50 mM KCl (pH 7.4) containing the following mediators (each at 4 μM concentration): potassium ferricyanide, phenazine methosulfate, tetramethyl-*p*-phenylene diamine, anthraquinone-2,6-disulfonic acid, 2-anthraquinone sulfonic acid, 2-methyl-1,4-naphthoquinone, and tetramethyl-*p*-benzoquinone. Pseudoazurin (Paz) from *P. pantotrophus* was obtained as a by-product of the purification of $cd_1$ [28].

Concentrations were determined by electronic absorbance spectroscopy of the oxidized form using $\varepsilon_{590\mathrm{nm}} = 3000\ \mathrm{M^{-1}\ cm^{-1}}$ [31]. CD-monitored potentiometric titrations with Paz were performed on samples of 50 μM concentration in 50 mM potassium phosphate and 50 mM KCl (pH 8.0) and the following mediators (each at 4 μM concentration): potassium ferricyanide, phenazine methosulfate, tetramethyl-*p*-phenylene diamine, methyl-1,4-naphthoquinone, and tetramethyl-*p*-benzoquinone.

For both CD- and MCD-monitored potentiometric titrations, the sample was equilibrated at a set potential until both the spectral response at a fixed wavelength (550 nm for cytochrome *c* and 470 nm for Paz) and the current flowing in the cell became invariant with time. A full-wavelength scan was then recorded while monitoring the stability of the potential in the cell using the galvanostat mode of the Autolab PGSTAT.

## Results

### Construction of cell assembly

The apparatus was designed to position variable-pathlength cuvettes in the center of the solenoid, incorporating reagent delivery and stirring mechanisms while maintaining the bore anaerobic. For operation in the bore of the solenoid, the stirring mechanism must be metal free. A system for stirring cuvettes of 10 mm