Analytical Biochemistry 423 (2012) 129–132

Contents lists available at SciVerse ScienceDirect

Analytical Biochemistry

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

Spectroscopic characterization of ^{57}Fe-enriched cytochrome *c*

Bogdan M. Leu [a,b,*], Tom H. Ching [a], Cuong Tran [a], J. Timothy Sage [a]

[a] Department of Physics and Center for Interdisciplinary Research on Complex Systems, Northeastern University, Boston, MA 02115, USA
[b] Advanced Photon Source, Argonne National Laboratory, Argonne, IL 60439, USA

ARTICLE INFO

Article history:
Received 26 September 2011
Received in revised form 12 December 2011
Accepted 13 December 2011
Available online 21 December 2011

Keywords:
Cytochrome *c*
^{57}Fe
Metal substitution
Ferrous sulfate
Resonance Raman

ABSTRACT

Investigation of the heme iron dynamics in cytochrome *c* with Mössbauer spectroscopy and especially nuclear resonance vibrational spectroscopy requires the replacement of the natural abundant heme iron with the ^{57}Fe isotope. For demetallization, we use a safer and milder ferrous sulfate–hydrochloric acid method in addition to the harsher commonly used hydrofluoric acid-based procedure. The structural integrity of the ^{57}Fe-reconstituted protein in both oxidation states is confirmed from absorption spectra and a detailed analysis of the rich resonance Raman spectra. These results reinforce the application of metal-substituted heme *c* proteins as reliable models for the native proteins.

Published by Elsevier Inc.

A better understanding of the processes of living organisms can be obtained through chemical modification of their various components such as the replacement of the heme iron in heme proteins [1]. Metal-substituted derivatives of cytochrome *c* (cyt *c*)[1] [2] have been investigated with a wide variety of techniques that provided information on the protein dynamics and functionality. The value of such applications depends on how faithfully modified cyt *c* reflects the structure of the native protein. For example, whereas Co–cyt *c* preserves the axial coordination of the metal, as shown from spectroscopic and X-ray diffraction experiments [3], Mn–cyt *c* is believed to be five-coordinate [4], Cu–cyt *c* at neutral pH was found to be either five-coordinate [5] or six-coordinate [6,7], and published work on Zn–cyt *c* reached differing conclusions regarding the presence [8,9] or absence [10] of the Zn–methionine link. The harsher conditions in common methods for metal extraction from the heme involving hydrogen fluoride (HF) raise additional concerns about the structural integrity of the reconstituted proteins. We are unaware of any published investigations comparing iron-reconstituted cyt *c* with the native protein that would confirm its structural integrity following harsh metal extraction procedures.

The native protein ligates the heme iron by a methionine (Met80) and a histidine (His18). The prosthetic group connects to the protein via two additional bonds: the covalent thioether linkages between the vinyls on the porphyrin ring and cysteines 14 and 17 (Fig. 1). Because of these additional linkages, the removal of the natural abundant heme iron from cyt *c* is more difficult than in proteins whose hemes are not covalently attached to the polypeptide backbone (e.g., myoglobin, hemoglobin). In the latter cases, heme *b* can be readily removed by acidification [11], and reconstitution with another metalloprotoheme is rapid and quantitative [12].

In this article, we describe the preparation of ^{57}Fe-enriched horse heart cyt *c*, both reduced and oxidized. Detailed spectroscopic analysis of the reconstituted proteins compares well with that of the native proteins. In particular, we observe spectroscopic markers for all four covalent links between heme and protein. Similar results follow either from the traditional metal extraction procedure or from a milder procedure that avoids the use of HF. These results confirm metal substitution as a valuable probe for investigating the structure and dynamics of proteins containing heme *c*.

Materials and methods

Materials

Horse heart cyt *c*, Sephadex G-25 gel filtration resins, ferrous sulfate heptahydrate, acetic acid, sodium phosphate mono- and dibasic, sodium acetate buffer salts, hydrochloric acid, and L-ascorbic acid were purchased from Sigma–Aldrich. Anhydrous hydrogen fluoride was obtained from Matheson Gas, potassium ferricyanide from ICN Biomedicals, ^{54}Fe metal from Cambridge Isotope Laboratories, and ^{57}Fe metal from Techsnabexport.

* Corresponding author at: Advanced Photon Source, Argonne National Laboratory, Argonne, IL 60439, USA. Fax: +1 630 252 0161.
E-mail address: leu@aps.anl.gov (B.M. Leu).

[1] *Abbreviations used:* cyt *c*, cytochrome *c*; HF, hydrogen fluoride.

0003-2697/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.ab.2011.12.025

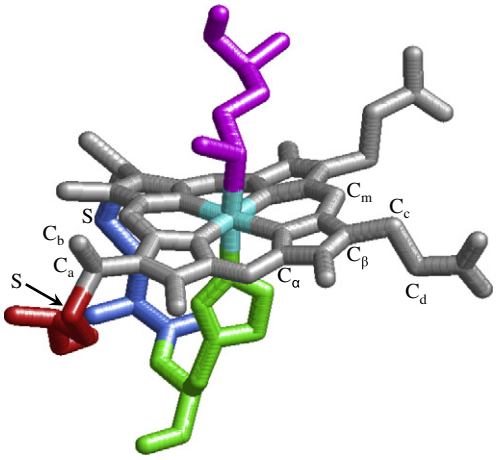

Fig.1. Cytochrome *c* heme moiety (1HRC [29]). Color scheme: cyan, iron; gray, heme; magenta, Met80; green, His18; red, Cys14; blue, Cys17. Hydrogen atoms are omitted for clarity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Methods

Resonance Raman spectra were recorded at room temperature with a beam power of 15 mW at the sample. The excitation wavelength was 413.1 nm, in resonance with the heme Soret band. The frequency calibration was verified using the rich Raman spectrum of fenchone as a standard. The instrument resolution was 2.8 cm^{-1}. Fluorescence was excited with the 413.1-nm line of a Kr^+ laser. No correction for grating or detector wavelength response was performed.

Cyt c reconstitution with ^{57}Fe

^{57}Fe(II) acetate synthesis

For the synthesis of isotopically enriched ferrous acetate, we followed a modified version of a method described previously [13]. In a Soxhlet extractor (Ace Glass), we refluxed 8 mg of ^{57}Fe (or ^{54}Fe) powder with 15 ml of glacial acetic acid for approximately 2 h. We transferred the dark brown acetate solution to a condenser (Ace Glass) and placed it over a heater for several hours to evaporate the acetic acid and extract the iron(II) acetate powder.

Removal of natural abundance iron

Cyt *c* demetallation is usually carried out according to the HF-based method of Robinson and Kamen [14]. In a fume hood, we placed 10 to 15 mg of lyophilized horse heart cyt *c* in a dry Teflon tube (to avoid contamination by metals) and cooled it in a Dewar flask containing liquid nitrogen. HF gas was introduced for a few seconds and allowed to condense on the Teflon tube, which was then transferred to a Dewar flask of ice and water. After removing the HF under nitrogen and adding a few milliliters of 0.05 M ammonium acetate buffer (pH 5.0), we passed the resulting bright purple solution through a Sephadex G-25 column equilibrated with the same buffer at 6 °C and collected the leading fraction representing porphyrin cyt *c*. Due to the relative instability of porphyrins under illumination [15], the Sephadex column was wrapped in aluminum foil and the room lights were turned off. HF is a corrosive acid whose unique properties make it extremely hazardous when it comes in contact with the eyes or skin or when it is inhaled.

Prior to the HF method, numerous procedures for the removal of iron from hemins have been described [16–22]. We used a modified version of the mildest and most convenient one [15], the ferrous sulfate–hydrochloric acid method of Morell and Stewart [21]. This approach takes longer to complete but avoids the hazards of working with HF. We dried 2 to 3 g of $FeSO_4$ heptahydrate through heating in a glass beaker until its color turned uniformly mustard and dissolved 700 mg in 5 ml of HCl at room temperature. In parallel, we prepared a solution consisting of cyt *c* (100 mg) and glacial acetic acid (95 ml). After degassing the flask, we bubbled argon through the protein solution for 30 min, added the $FeSO_4$ solution, and continued to flush argon gently for another 30 min. The cloudy liquid at the top of the flask was discarded, whereas the clear bright purple solution at the bottom was carefully removed and passed through a Sephadex G-25 column equilibrated with 0.05 M ammonium acetate buffer (pH 5.0) at 6 °C. We collected the leading fraction.

Removal of the heme iron by either method results in the appearance of an intense vibronically structured fluorescence band with a maximum at approximately 16,000 cm^{-1}, which diminishes on reconstitution with iron (Fig. 2, top). The absorption spectrum of porphyrin cyt *c* exhibits four characteristic visible bands at 505, 539, 569, and 620 nm (Fig. 2, middle).

^{57}Fe insertion

Inside an anaerobic glove box, we added 20-fold excesses of ascorbic acid and ^{57}Fe (or ^{54}Fe) ferrous acetate to freshly prepared porphyrin cyt *c* at pH 5.0 in an airtight screw-cap glass vial and placed this mixture in a water bath at 70 °C for 30 min. The resulting bright red solution was purified through a Sephadex G-25 column equilibrated with 0.05 M sodium phosphate buffer (pH 7.4). The yield was approximately 85% from the HF-based method and 15% to 20% from the $FeSO_4$–HCl procedure.

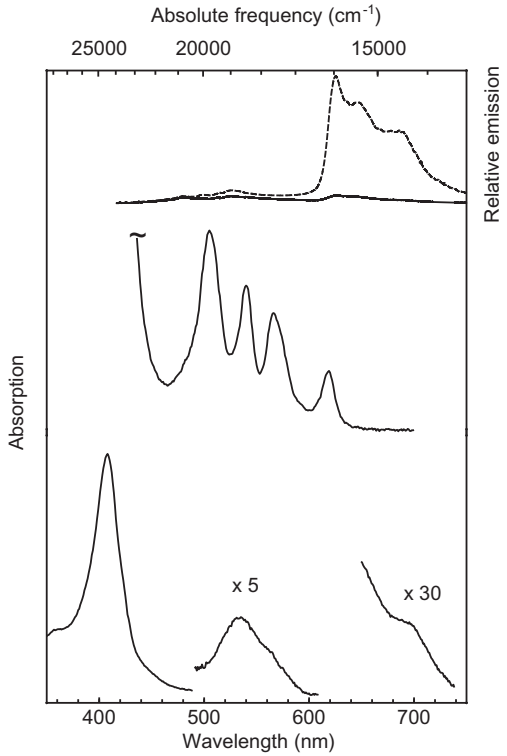

Fig.2. Top: removal of Fe from cyt *c* produces an intense fluorescence band (dashed line), which diminishes when Fe is reinserted (continuous line). No correction for detector wavelength response was performed. Middle: after Fe removal, the porphyrin exhibits a distinctive four-line visible absorption spectrum. Bottom: the absorption spectrum of reconstituted Fe(III) cyt *c* resembles that of the native protein, including a charge transfer band at 695 nm that indicates an intact Fe–S_{Met} bond in addition to the $\pi \rightarrow \pi^*$ excitations of the heme at shorter wavelengths.