Published in final edited form as:

Anal Biochem. 2006 December 15; 359(2): 262–264. doi:10.1016/j.ab.2006.08.039.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of cardiolipin extracted from detergent-solubilized mitochondrial electron transfer complexes

Tiffany McDonald-Marsh, Christopher A. Carroll, Neal C. Robinson, and Andrej Musatov*

Department of Biochemistry, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229–3900, USA

A major difficulty encountered in the study of isolated mitochondrial electron-transfer complexes is the lack of a sensitive method for analyzing bound phospholipid components, such as cardiolipin. We have utilized matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS)¹ for analysis of cardiolipin, which had been extracted from two isolated and detergent-solubilized complexes: NADH:ubiquinone oxidoreductase (complex I, EC 1.6.5.3) and cytochrome *c* oxidase (complex IV, EC 1.9.3.1). Analysis of cardiolipin bound to electron-transfer complexes is typically carried out by thin-layer chromatography or high-performance liquid chromatography (HPLC). The principal advantages of MALDI-TOF/MS over these methods are its picomole-to-femtomole sensitivity and the ability to give molecular mass values with a reported accuracy of $\pm 0.1\%$ or better. The critical step required for successful analysis of cardiolipin by MALDI-TOF/MS is the removal of coextracted detergents. Normal-phase silicic acid HPLC effectively removes detergents from cardiolipin extracted from detergent-solubilized Complex I or cytochrome *c* oxidase. The resulting purified cardiolipin can be successfully analyzed by MALDI-TOF/MS.

The inner mitochondrial membrane includes two populations of cardiolipin (1,3-diphosphatidyl-*sn*-glycerol): (1) bilayer cardiolipin that is not protein bound and is free to diffuse within the mitochondrial inner membrane and (2) cardiolipin that is tightly bound to mitochondrial inner membrane proteins, such as ADP/ATP carrier proteins, NADH dehydrogenase, succinate dehydrogenase, cytochrome *bc*₁, and cytochrome *c* oxidase (for review, see [1]). Each of these complexes is surrounded by inner membrane phospholipids (phosphatidylcholine, phosphatidylethanolamine, and cardiolipin), but only cardiolipin is tightly bound at specific sites on these enzymes. Based on our studies with cytochrome *c* oxidase and cytochrome *bc*₁ and on data available for complexes I and II, it is clear that cardiolipin is bound to these membrane proteins to maintain their structural and functional stability since removal of cardiolipin destabilizes these complexes and decreases functional activity. Furthermore, the high degree of unsaturation in the four acyl groups of cardiolipin makes it especially susceptible to oxidative damage. For example, oxidative damage to cardiolipin appears to be a significant factor in aging and programmed cell death [2]. Moreover, oxidative modification of cardiolipin bound to cytochrome *c* oxidase is one of the factors leading to peroxide-induced enzyme inactivation [3]. Therefore, highly sensitive methods are needed for the detection of cardiolipin bound to mitochondrial complexes. However, isolation and purification of inner mitochondrial membrane complexes are normally done in the presence of a detergent. Commercially available detergents bind to the complexes and consequently contaminate extracted phospholipids. The presence of these detergents in a sample strongly affects MALDI-TOF/MS analysis [4]. The extent of interference most likely depends on the

*Corresponding author. Fax: +210 567 6595. E-mail address: musatov@uthscsa.edu (A. Musatov).

nature of the sample, choice of detergent, matrix/sample preparation method, and experimental conditions. For example, high concentrations of dodecyl maltoside are useful in increasing the MALDI-TOF/MS peak intensity of cytochrome *bc*₁ subunits [5]. The presence of dodecyl maltoside also does not interfere with MALDI-TOF/MS analysis of cytochrome *c* oxidase subunits [6]. However, the presence of the same detergent in the solvent extracted cardiolipin results in complete loss of MALDI-TOF/MS signals (data not shown). Dodecyl maltoside has a molecular mass of 510 Da but can form singly charged dimer and trimer ions, which produce mass spectrometry peaks with *m/z* values as high as 1531 and therefore can interfere with the analysis of species with molecular masses under 2000 Da [7].

Cytochrome *c* oxidase was isolated from bovine heart muscle mitochondrial particles as previously described [6,8]. Purified enzyme was solubilized in 20 mM Tris-SO₄, pH 7.4, buffer, containing 2 mM dodecyl maltoside. Most of the phospholipids, except for tightly bound cardiolipin and residues of phosphatidylethanolamine, were removed by HiTrapQ ion-exchange column chromatography [8]. Bound phospholipids were extracted from the 2.5–5.0 μM dodecyl maltoside-solubilized cytochrome *c* oxidase using chloroform/methanol/water extraction [9]. Essentially all dodecyl maltoside was coextracted with phospholipids, i.e., extracted phospholipids also contained up to 2 mM detergent.

Two different silicic acid HPLC procedures were successfully used to purify cardiolipin from coextracted phospholipids and dodecyl maltoside. First is a normal-phase silicic acid HPLC with a gradient elution according to Nissen and Kreysel [10] with minor modifications. Briefly, the extracted phospholipid-detergent mixture was dried under nitrogen and the resulting film dissolved in chloroform:methanol mixture (2:1, v/v). The silicic acid HPLC column (5 μm Radial Pac Resolve Silica cartridge, 0.8 cm × 10 cm) was purchased from Waters Corp., Inc. Mobile phase component *A* was acetonitrile-water (80:20) and component *B* was acetonitrile. A linear solvent gradient from 87.5 to 25% *B* between 3 and 15 min was used, delivering a gradient of water running from 2.5 to 15%. HPLC elution was performed at room temperature, at a flow rate of 1 mL per min, with Waters 515 HPLC pumps and a Waters pump control module. Detection of phospholipid elution was monitored at 203 nm with a Waters 2487 Dual λ Absorbance Detector (Fig. 1). The identity of eluted phospholipids at particular time intervals was confirmed by chromatography of a standard phospholipid mixture (cardiolipin, phosphatidylethanolamine, and phosphatidylcholine). Standard phospholipids were purchased from Avanti Polar Lipids, Inc. The cardiolipin was collected, dried under N₂, dissolved in chloroform, and analyzed by MALDI-TOF/MS. The resulting mass spectrum (Fig. 2B) is fully consistent with the presence of a single cardiolipin molecular species with four linoleate side chains (*m/z* 1448.0). MALDI-TOF mass spectra were acquired on an Applied Biosystems Voyager-DE STR, operated in positive ion reflectron mode. The instrument was calibrated with a standard peptide calibration mixture consisting of des-Arg-Bradykinin (*m/z* 905.05), Angiotensin I (*m/z* 1297.51), Glu-Fibrinopeptide B (*m/z* 1571.61), and Neurotensin (*m/z* 1673.96) obtained from Applied Biosystems. Matrices (dissolved in 50% acetonitrile/0.1% trifluoroacetic acid) include 2,5-dihydroxybenzoic acid (50 mg/ml)/citric acid (100 mM). Spectra represent the average of 100 laser shots.

The second silicic acid HPLC procedure was performed using an isocratic normal-phase HPLC with cyclohexane:isopropanol:5 mM phosphoric acid in water 50:50:2.9, v/v/v as a mobile phase [11]. This method is designed to resolve cardiolipin without interference from other phospholipids. However, in most experiments up to two additional peaks were detected, indicating the presence of noncardiolipin species (data not shown, but see Fig. 1 in [10]). Nevertheless, elution of cardiolipin at a particular time was also confirmed using standard cardiolipin. Samples for MALDI-TOF/MS were prepared as described above and cardiolipin was successfully detected by MALDI-TOF/MS with results similar to that presented in Fig. 2.

A similar approach was used for the analysis of cardiolipin extracted from dodecyl maltoside-solubilized complex I, isolated from bovine heart mitochondria, by the method of Sazanov et al. [12]. Cardiolipin was extracted, purified by silicic acid HPLC, and analyzed by MALDI-TOF/MS as described above. Again, the majority of the ion abundance at m/z 1448 is cardiolipin with four linoleate side chains (Fig. 2C).

A number of studies have demonstrated the use of mass spectrometry for characterization of phospholipids and particularly cardiolipin (for review, see [13]). Most recently, for example, Hsu and Turk [14] described a multiple-stage ion-trap mass spectrometric approach with electrospray ionization for structural characterization of cardiolipin. In the present work, methodology has been developed for MALDI-TOF/MS rapid screening of cardiolipin extracted from two detergent-solubilized electron-transfer complexes. The conditions were optimized for removal of dodecyl maltoside, therefore it may be necessary to use a different strategy if a different detergent must be removed from cardiolipin. However, we believe that the described approach can be applied to analyze cardiolipin extracted from detergent-solubilized complexes in general. This procedure is a straightforward option for cardiolipin research, particularly in studies of the role that cardiolipin has in membrane proteins, and could potentially be used for detection of cardiolipin oxidation products.

Acknowledgments

This work was supported by the American Heart Association–Texas Affiliate (0465099Y to A.M.), the Pilot Grant from Barshop Institute for Longevity and Aging Studies (AG013319-11 to A.M.), National Institutes of Health (GMS 24795 to N.C.R.), and Robert A. Welch Foundation (AQ1481 to N.C.R.). The authors thank Patrizia Lemma-Gray for isolating bovine heart complex I and Dr. Susan T. Weintraub for helpful suggestions. MALDI-TOF mass spectra were acquired in the Institutional Mass Spectrometry Laboratory of the University of Texas Health Science Center.

Abbreviation used:

MALDI-TOF/MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

References

1. Robinson NC. Functional binding of cardiolipin to cytochrome *c* oxidase. *J. Bioenerg. Biomembr* 1993;25:153–163. [PubMed: 8389748]
2. Iverson SL, Orrenius S. The cardiolipin-cytochrome *c* interaction and the mitochondrial regulation of apoptosis. *Arch. Biochem. Biophys* 2004;423:37–46. [PubMed: 14989263]
3. Musatov A. Contribution of peroxidized cardiolipin to inactivation of bovine heart cytochrome *c* oxidase. *Free Radical Biol. Med* 2006;41:238–246. [PubMed: 16814104]
4. Funk J, Li X, Franz T. Threshold values for detergents in protein and peptide samples for mass spectrometry. *Rapid Commun. Mass Spectrom* 2005;19:2986–2988. [PubMed: 16178049]
5. Weintraub, ST.; Musatov, A.; Carroll, CA.; Robinson, NC. Analysis of mitochondrial electron transport complexes by MALDI-TOF mass spectrometry. A 50th American Society Mass Spectrometry Conference; Orlando, (USA). 2002.
6. Musatov A, Carroll CA, Liu Y-C, Henderson GI, Weintraub ST, Robinson NC. Identification of bovine heart cytochrome *c* oxidase subunits modified by the lipid peroxidation product 4-hydroxy-2-nonenal. *Biochemistry* 2002;41:8212–8220. [PubMed: 12069614]
7. Distler AM, Hiser C, Qin L, Hilmi Y, Ferguson-Miller S, Allison J. Mass spectrometric detection of protein, lipid and heme components of cytochrome *c* oxidase from *R. sphaeroides* and the stabilization of non-covalent complexes from the enzyme. *Eur. Mass Spectrom* 2004;10:295–308.
8. Sedláč E, Robinson NC. Phospholipase A(2) digestion of cardiolipin bound to bovine cytochrome *c* oxidase alters both activity and quaternary structure. *Biochemistry* 1999;38:14966–14972. [PubMed: 10555978]

9. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol* 1959;37:911–917. [PubMed: 13671378]
10. Nissen HP, Kreysel HW. Analysis of phospholipids in human semen by high-performance liquid chromatography. *J. Chromatogr* 1983;276:29–35. [PubMed: 6672022]
11. Gomez B Jr, Robinson NC. Quantitative determination of cardiolipin in mitochondrial electron transfer complexes by silicic acid high-performance liquid chromatography. *Anal. Biochem* 1999;267:212–216. [PubMed: 9918673]
12. Sazanov LA, Peak-Chew SY, Fearnley IM, Walker JE. Resolution of the membrane domain of bovine complex I into subcomplexes: implications for the structural organization of the enzyme. *Biochemistry* 2000;39:7229–7235. [PubMed: 10852722]
13. Pulfer M, Murphy RC. Electrospray mass spectrometry of phospholipids. *Mass Spectrom. Rev* 2003;22:254–332.
14. Hsu F-F, Turk J. Characterization of cardiolipin from *Escherichia coli* by electrospray ionization with multiple stage quadrupole ion-trap mass spectrometric analysis of $[M - 2H + Na]^+$ ions. *J. Am. Soc. Mass Spectrom* 2006;17:420–429. [PubMed: 16442306]

Fig. 1. Silicic acid HPLC purification of standard cardiolipin and cardiolipin extracted from dodecyl maltoside-solubilized cytochrome *c* oxidase or complex I. (A) Separation of mixed phospholipid standards (2 nmol of cardiolipin (CL), 4 nmol of phosphatidylethanolamine (PE), and 4 nmol of phosphatidylcholine (PC)). (B) HPLC analysis of phospholipids extracted from HiTrapQ-purified 1.0 nmol of cytochrome *c* oxidase. (C) HPLC analysis of phospholipids extracted from 1 nmol of isolated complex I. Detection of phospholipids elution was monitored at 203 nm with a Waters 2487 Dual λ Absorbance Detector.

Fig. 2. MALDI-TOF/MS of standard cardiolipin and cardiolipin extracted from cytochrome *c* oxidase or complex I. (A) MALDI-TOF mass spectra of standard cardiolipin. Beef heart standard cardiolipin was obtained from Avanti Polar Lipids. (B) MALDI-TOF mass spectra of HPLC-isolated cardiolipin extracted from HiTrapQ-purified cytochrome *c* oxidase. (C) MALDI-TOF mass spectra of HPLC-isolated cardiolipin extracted from complex I. In each case cardiolipin was dissolved in chloroform and ~15 pmol/ μ L spotted on the MALDI target using dihydroxybenzoic acid as the matrix. Each spectrum represents an average of 100 laser shots. Observed major peaks correspond to tetra-linoleoyl cardiolipin (1448 ± 1) modified by sodium ions.