Supporting Information

Optimizing the Conditions of a Multiple Reaction Monitoring Assay for Membrane Proteins: Quantification of Cytochrome P450 11A1 and Adrenodoxin Reductase in Bovine Adrenal Cortex and Retina

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Part A. Expression and Purification of ^{15}N-CYP11A1 and ^{15}N-AdR.

To obtain ^{15}N-labeled proteins, Escherichia coli cells GC5 were co-transformed with expression constructs for CYP11A1 and AdR and the Gro7 vector containing chaperones GroEL and GroES. Subsequent protein expression was according to Marley et al., *J. Biomol. NMR* **2001**, *20*, 71-71. Briefly, overnight cultures were diluted 100-fold with 3L of Terrific Broth medium and grown by shaking (250 rpm) at 37°C until $O.D_{600}$ reached 0.6. Cultures were then washed twice with M9 minimal salt solution containing 1 g/L of $^{15}NH_4Cl$ as the sole nitrogen source and incubated in 3L of M9 minimal media containing 0.5 mM δ-aminolevulinic acid. Expression of ^{15}N-CYP11A1 and ^{15}N-AdR and chaperones was induced by 1 mM isopropyl β-D-thiogalatopyranoside and 0.18 g/ml L arabinose, respectively. Cell cultures containing ^{15}N-CYP11A1 were grown at 28°C for 72 hours at 210 rpm shaking, and those with ^{15}N-AdR were shaken at 150 rpm at 30°C for 30 hours. Expression of ^{15}N-CYP11A1 and ^{15}N-AdR was monitored by a reduced CO difference spectrum and SDS-PAGE, respectively.

Purification of ^{15}N-CYP11A1 was as follows. Spheroplasts were prepared by suspending cells in 100 mM potassium phosphate buffer (KP_i), pH 7.2, containing 20% glycerol. The cell suspension was incubated with 0.5 mg/ml lysozyme for 30 min on ice and subjected to centrifugation at 3000 *g* for 20 min. The pellet was re-suspended in

100 mM KP_i, pH 7.2, containing 20% glycerol and complete EDTA free protease inhibitor cocktail (Roche Diagnostics). DNAse (0.5 µg/ml), sodium cholate (0.8%), β-mercaptoethanol (BME, 5 mM) were added, and spheroplasts were sonicated on ice using six 20 s pulses at 1 minutes intervals followed by a high speed centrifugation at 106000 *g* for 60 min. The supernatant after ultracentrifugation was diluted 2-fold with 10 mM KP_i, pH 7.2, containing 0.2% sodium cholate, 100 mM NaCl, and 20% glycerol and applied to DEAE-cellulose equilibrated 50 mM KP_i, pH 7.2, containing 0.5% sodium cholate, 50 mM NaCl, and 20% glycerol. The flow through fraction was applied to Ni-agarose column equilibrated with 50 mM KP_i, pH 7.2, containing 0.5% sodium cholate, 2 mM imidazole, 1 mM BME, and 20% glycerol. The column was washed with 5 column volumes of the equilibration buffer, and then with 5 column volumes of the equilibration buffer containing 10 mM imidazole. ^{15}N-CYP11A1 was eluted from the column with the equilibration buffer containing 300 mM imidazole. The P450 was diluted 5-fold with 10 mM KP_i, pH 7.2, containing 0.3% sodium cholate, 1 mM EDTA, and 20% glycerol and applied to hydroxyapatite column equilibrated with the same buffer. The column was washed with 10 column volumes of the equilibration buffer followed by elution of ^{15}N-CYP11A1 with the same buffer containing 400 mM KP_i, pH 7.2.

To purify ^{15}N-AdR, spheroplasts were prepared and treated as described above for ^{15}N-CYP11A1. The supernatant after high speed centrifugation was applied to DEAE-cellulose equilibrated with 10 mM KP_i, pH 7.2, and the flow through fraction was then applied to adrenodoxin-Sepharose column equilibrated with 10 mM KP_i, pH 7.2. The column was washed with 10 column volumes of the equilibration buffer, and ^{15}N-AdR was eluted with 50 mM KP_i, pH 7.2, containing 0.1 mM EDTA, 1 M NaCl, and 20% glycerol. The protein was dialyzed against 50 mM KPi, pH 7.2, 0.1 mM EDTA, and 20% glycerol and applied to 2',5'-ADP-Sepharose column equilibrated with the same buffer. The column was washed with 10 column volumes of the equilibration buffer, and ^{15}N-AdR was eluted with 50 mM KP_i, pH 7.2, containing 0.1 mM EDTA, 0.3 M NaCl, 1 mM adenosine, and 20% glycerol.

Part B. OrgMassSpecR

The OrgMassSpecR (Organic Mass Spectrometry with R) software program is an extension to the R programming language (http://www.r-project.org/). OrgMassSpecR was used to calculate the theoretical isotopic distributions of the ^{15}N-labeled peptides, and the theoretical precursor ion and product ion m/z values of the labeled and unlabeled peptides during the selection of the MRM transitions. The homepage for OrgMassSpecR (http://orgmassspecr.r-forge.r-project.org/) gives instructions for getting started (it assumes R is installed). The source code is also available through the

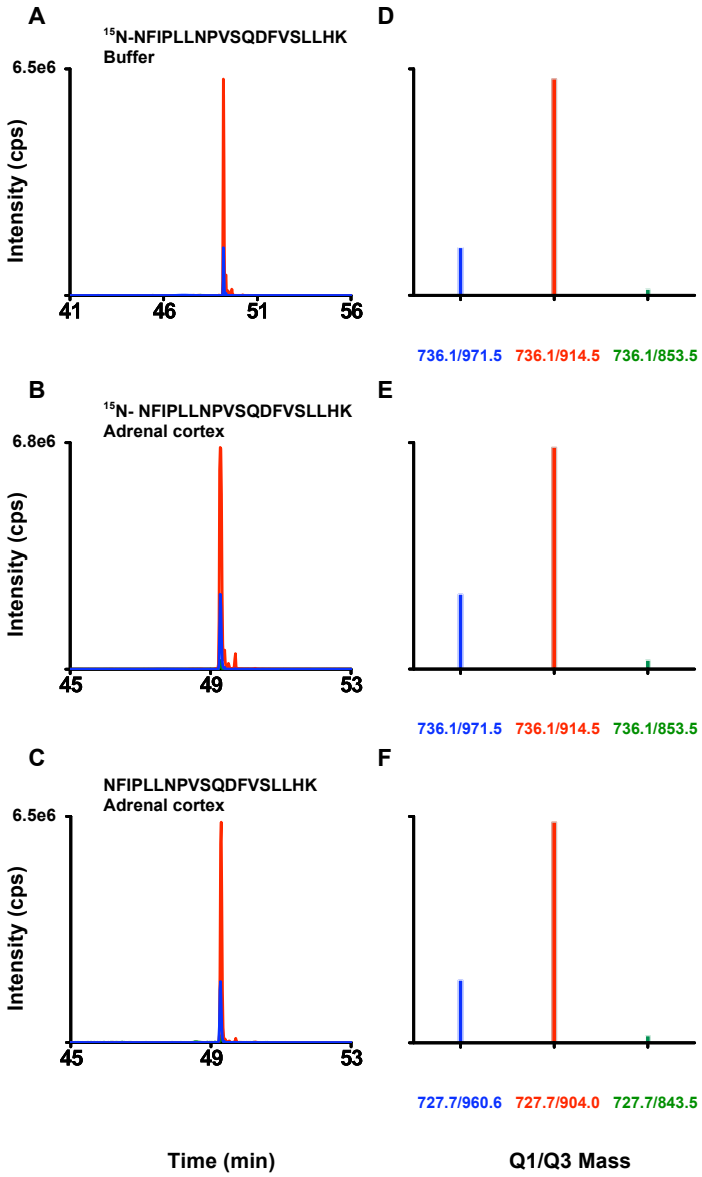
homepage. The theoretical isotopic distributions were determined using the `IsotopicDistributionN` function. For example the command,

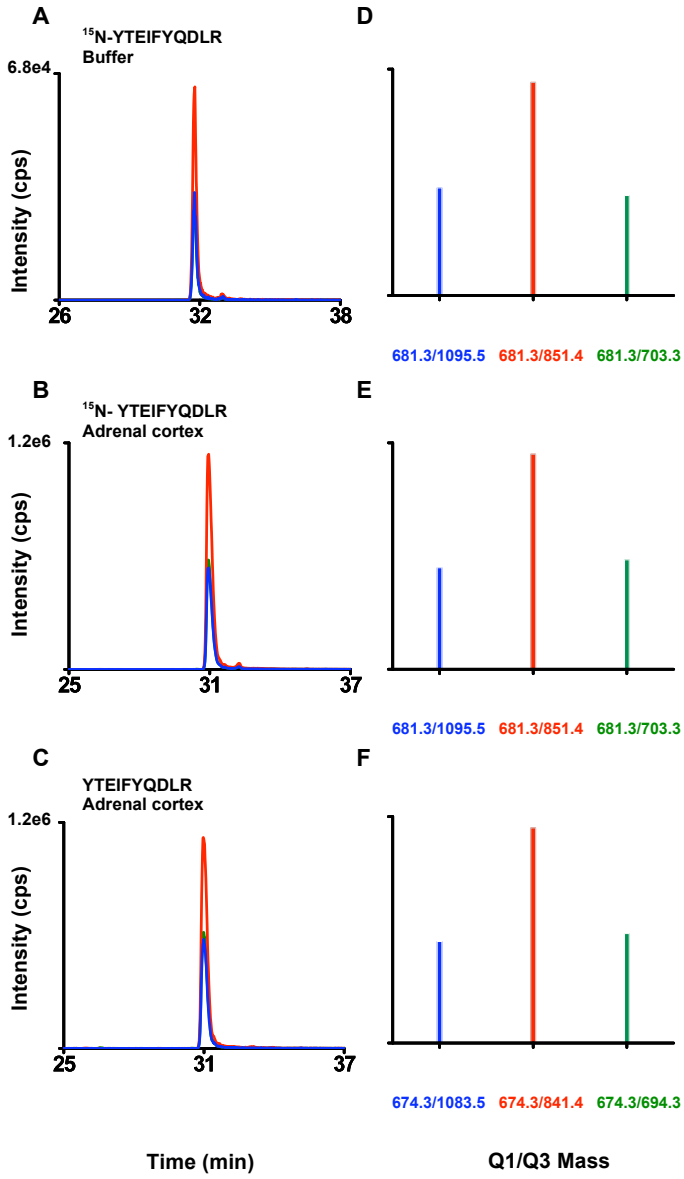
```
IsotopicDistributionN("NFIPLLNPVSQDFVSLLHK", incorp = 0.90)
```

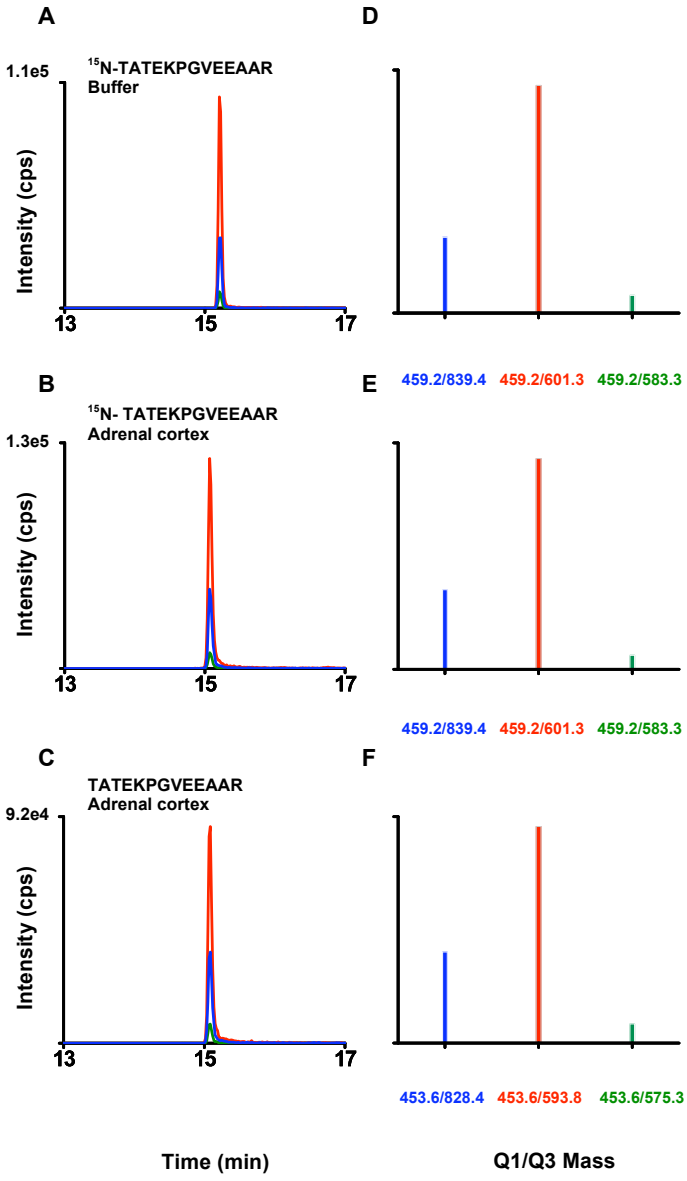
produces the isotopic distribution of the given peptide with 90% ^{15}N incorporation. The `Digest` function was used to determine theoretical tryptic peptides of the target proteins. The `FragmentPeptide` function was used to determine the b- and y-ions of selected peptides. The OrgMassSpecR help files contain usage information and examples for each function.

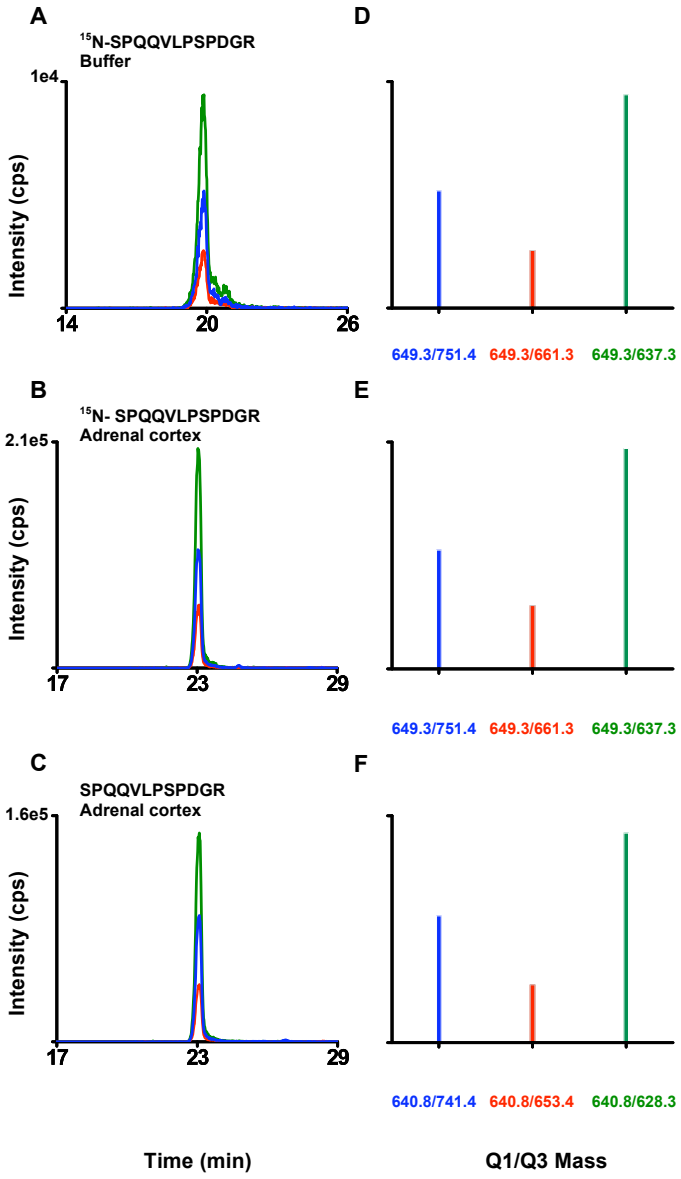
Part C. Extracted Ion Chromatograms (A-C) and MRM Spectra (D-F).

Data are shown for peptides NFIPLLNPVSQDFVSLLHK and YTEIFYQDLR from CYP11A1 and for peptides TATEKPGVEEAAR and SPQQVLPSPDGR from AdR. Transitions in the overlaid extracted ion chromatograms and the MRM/MS spectra have matching colors.




A
15N-YTEIFYQDLR
Buffer
6.8e4
Intensity (cps)
26
32
38
D
681.3/1095.5
681.3/851.4
681.3/703.3
B
15N- YTEIFYQDLR
Adrenal cortex
1.2e6
Intensity (cps)
25
31
37
E
681.3/1095.5
681.3/851.4
681.3/703.3
C
YTEIFYQDLR
Adrenal cortex
1.2e6
Intensity (cps)
25
31
37
F
674.3/1083.5
674.3/841.4
674.3/694.3
Time (min)
Q1/Q3 Mass


A
D
1.1e5
^{15}N-TATEKPGVEEAAR
Buffer
Intensity (cps)
13
15
17
B
E
459.2/839.4
459.2/601.3
459.2/583.3
^{15}N- TATEKPGVEEAAR
Adrenal cortex
1.3e5
Intensity (cps)
13
15
17
459.2/839.4
459.2/601.3
459.2/583.3
C
F
TATEKPGVEEAAR
Adrenal cortex
9.2e4
Intensity (cps)
13
15
17
453.6/828.4
453.6/593.8
453.6/575.3
Time (min)
Q1/Q3 Mass


A
^{15}N-SPQQVLPSPDGR
Buffer
1e4
Intensity (cps)
14
20
26
D
649.3/751.4
649.3/661.3
649.3/637.3
B
^{15}N- SPQQVLPSPDGR
Adrenal cortex
2.1e5
Intensity (cps)
17
23
29
E
649.3/751.4
649.3/661.3
649.3/637.3
C
SPQQVLPSPDGR
Adrenal cortex
1.6e5
Intensity (cps)
17
23
29
F
640.8/741.4
640.8/653.4
640.8/628.3
Time (min)
Q1/Q3 Mass