# Comment on:
# "Protein assemblies ejected directly from native membranes yield complexes for mass spectrometry"

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Abstract

Chorev et al. (*1*) describe mass spectrometry on mitochondrial membrane proteins ionized directly from their native environment. However, the assignments made to measured masses are incorrect or inconclusive, and lack experimental validation. The proteins are not in their 'native' condition: they have been stripped of tightly-bound lipids, and the complexes are fragmented or in physiologically irrelevant oligomeric states.

Main text

Native mass spectrometry has been applied extensively to analyze membrane proteins in detergent solutions. In Chorev et al. (*1*), Robinson and co-workers describe a technical breakthrough that enables proteins and complexes to be ionized directly from their native membrane environments. They present examples from bacterial and mitochondrial biology, and propose that physiologically relevant insights can be obtained. Here, we investigate the claims Robinson and co-workers have made from their analyses of mitochondrial membranes.

Robinson and co-workers (*1*) assigned masses of 809 and 782 kDa to complex I, a 975 kDa enzyme with known composition and structure (*2-4*). To match their masses to two fragments of complex I they summed masses of subsets of the enzyme's subunits, then augmented the combined masses with the masses of substrates and phospholipids, to thus rationalize the measured values. We thank the authors for publishing an Erratum to their manuscript, to correct many errors in the subunit masses used in their original calculations. Although minor inaccuracies still remain (see Supplementary Tables), they do not affect the subunit compositions of the two revised fragments proposed in the Erratum. Nevertheless, as Figure 1 shows, these two revised complex I fragments do not constitute functional or physiologically relevant forms of the enzyme. Furthermore, by using a flexible combinatorial approach to calculating masses it is possible to rationalize any mass value, and so there is little direct evidence to support the claim that the two observed masses arise from complex I at all.

For complex III, two masses of 169 and 339 kDa were assigned to the monomeric and dimeric states. However, complex III is a 468 kDa functional dimer in which the Rieske subunit in each monomer acts across the dimer interface in the other monomer (*5*). In other words, monomers are neither functional nor native. Here, the subunit masses used by Robinson and co-workers largely agree with published data (*6*) although the masses of some cofactors and post-translational modifications were omitted (see Supplementary Tables). Then, subunits were subtracted to provide a fit between

measured and expected masses but without clear rationale, resulting in structurally impossible fragments (see Figure 1). Therefore, without additional evidence, the assignment of these fragments to complex III must be questioned. For complex IV, two incorrect isoforms were used (the correct isoforms in heart are COX6A2 and COX7A1) and cofactors and modifications were again omitted (see Supplementary Tables). However, the calculated mass, including one cardiolipin and one phospholipid, is within error of the 208 kDa mass assigned to the monomeric form. A mass 'twice as big' was assigned to the dimeric form, but it was not specified and so we cannot evaluate this assignment. However, the existence of dimeric complex IV in the membrane is disputed, and previous results from Robinson's group indicate that about six tightly-bound phospholipids or cardiolipins are required to stabilize the dimer interface (*7*). Finally, many of the masses used by Robinson and co-workers did not agree with the experimentally determined masses of the subunits of ATP synthase (*2*) but, by good fortune, they obtained a value close to the correct total of 583 kDa (see Supplementary Tables). By comparison to an observed mass of 581 kDa, they propose that mammalian ATP synthase exists as a monomer. However, it is well established in a large number of species that native ATP synthase is a dimeric complex that is localized in long rows of dimers along the tips of cristae, defining their ultrastructure (*8*). Furthermore, the mass assigned to monomeric ATP synthase contains no contributions from bound phospholipids or cardiolipins. It is especially remarkable that no lipids occupy the internal cavity of the $c_8$-ring in the enzyme's rotor, detected previously by Robinson and co-workers in purified c-rings (*9*).

Finally, Robinson and co-workers assigned masses to monomeric and dimeric species of adenine nucleotide translocase (ANT1). First, their expected mass of 33,187 Da is incorrect. Amino acid sequencing and mass spectrometry have shown that native ANT1 lacks Met1, has an acetylated Ser2 and trimethylated Lys52, and is not succinylated (*10, 11*). The correct mass (32,921 Da) does not match the measured mass (33,195 Da) within experimental error and no control experiments with tight-binding inhibitors were performed to confirm the assignment. Second, the observed mass does not account for the three tightly-bound cardiolipins (37,271 Da) that stabilize the native carrier by bridging its domains and cannot be removed even by extensive detergent washes (*12*). The monomer has all the elements required for function and there is no robust experimental evidence that it forms dimers (*13*). To transport adenine nucleotides, ANT1 cycles back and forth between cytoplasmic and matrix states, both of which have been characterized structurally (*12, 14*), showing profound changes in shape that are incompatible with a stable dimerization interface.

Robinson and co-workers provide no confirmatory evidence for any of their assignments of masses to proteins and protein complexes. This inadequacy is especially evident in cases where measured masses do not match expected masses, so, in order rationalize the data, the expected masses have been adjusted by poorly justified subtractions of subunits and/or additions of modifications, substrates and phospholipids. But, even if the mass assignments of Robinson and co-workers had been confirmed, they would only have demonstrated that their methods have torn complexes I and III apart, broken the functional ATP synthase dimer, and removed tightly bound cardiolipins from ANT1. Thus, their methods are not 'more mild' than detergent extraction — a procedure that has supported extensive, long-standing and sophisticated structural, biochemical and physiologically-relevant studies on all these systems. The harsh and inadequately described pre-treatments of the membranes devised by Robinson and co-workers have not been validated for their ability to retain either native structure or function of protein complexes, and together with the ionization processes applied, they have damaged the proteins extracted from the membranes. Therefore, the work of Robinson and co-workers does not define the native states of any of the extensively characterized proteins present in mitochondria, and offers no new insights into mitochondrial biology.

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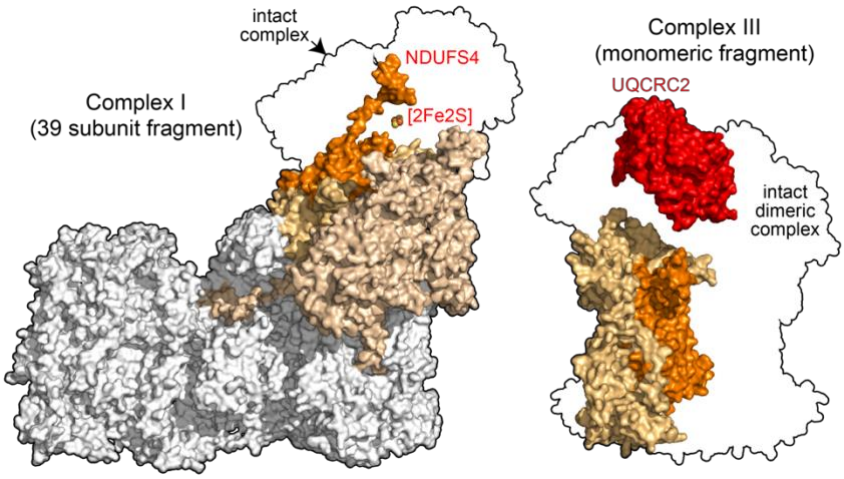
**Figure 1**




**The degraded fragments of complexes I and III proposed by Robinson and co-workers (*1*).** Subunits are colored according to how much of the contact area between the subunit and the rest of complex is retained in the proposed fragment: red, 0 to 25% retained; orange, 25 to 50%; pale orange, 50 to 75%; wheat, 75 to 100%; white 100%. The outlines depict the intact complexes. Created from 6G2J.PDB (*15*) and 1BGY.PDB (*5*).

## COMPLEX I

| Subunit | Alternative nomenclature | Protein mass (Δimport) | Known modifications | Calculated native mass | Chorev mass | Difference in Chorev mass | Comments on Chorev mass |
|---|---|---|---|---|---|---|---|
| ND1 | | 35,670 | N-formyl-Met | 35,698 | 35,699 | 1 | |
| ND2 | | 39,254 | N-formyl-Met | 39,282 | 39,284 | 1 | |
| ND3 | | 13,055 | N-formyl-Met | 13,083 | 13,082 | 0 | |
| ND4 | | 52,099 | N-formyl-Met | 52,127 | 52,130 | 3 | |
| ND4L | | 10,797 | N-formyl-Met | 10,825 | 10,825 | 0 | |
| ND5 | | 68,287 | N-formyl-Met | 68,315 | 68,320 | 5 | |
| ND6 | | 19,078 | N-formyl-Met | 19,106 | 19,107 | 1 | |
| NDUFV1 | 51 kDa | 48,499 | FMN, [4Fe-4S] | 49,306 | 48,503 | -803 | FMN, [4Fe-4S] not included |
| NDUFV2 | 24 kDa | 23,815 | [2Fe-2S] | 23,988 | 23,815 | -174 | [2Fe-2S] not included |
| NDUFV3 | 10 kDa | 8,437 | | 8,437 | 8,438 | | |
| NDUFS1 | 75 kDa | 76,961 | 2 x [4Fe-4S], [2Fe-2S] | 77,834 | 76,969 | -865 | 2 x [4Fe-4S], [2Fe-2S] not included |
| NDUFS2 | 49 kDa | 49,175 | Dimethyl-Arg | 49,203 | 49,199 | -4 | |
| NDUFS3 | 30 kDa | 26,432 | | 26,432 | 26,434 | 2 | |
| NDUFS4 | 18 kDa | 15,337 | | 15,337 | 15,338 | | |
| NDUFS5 | 15 kDa | 12,668 | ΔMet, 2 x SS | 12,532 | 12,534 | 2 | |
| NDUFS6 | 13 kDa | 10,536 | Zn | 10,599 | 10,534 | -65 | Zn missing |
| NDUFS7 | PSST | 20,078 | Hydroxyl-Arg, [Fe-4S] | 20,443 | 20,094 | -350 | [4Fe-4S] not included |
| NDUFS8 | TYKY | 20,196 | 2 x [4Fe-4S] | 20,896 | 20,194 | -702 | 2 x [4Fe-4S] not included |
| NDUFA1 | MWFE | 8,105 | | 8,105 | 8,106 | | |
| NDUFA2 | B8 | 11,080 | ΔMet, N-acetyl | 10,991 | 10,990 | 0 | |
| NDUFA3 | B9 | 9,349 | ΔMet, N-acetyl | 9,260 | 9,261 | 1 | |
| NDUFA5 | B13 | 13,316 | ΔMet, N-acetyl | 13,227 | 13,226 | 0 | |
| NDUFA6 | B14 | 15,054 | ΔMet, N-acetyl | 14,964 | 14,965 | 1 | |
| NDUFA7 | B14.5a | 12,677 | ΔMet, N-acetyl | 12,587 | 12,586 | -1 | |
| NDUFA8 | PGIV | 20,091 | ΔMet, 4 x SS | 19,952 | 19,959 | 7 | |
| NDUFA9 | 39 kDa | 39,115 | NADPH | 39,860 | 39,123 | -737 | NADPH missing |
| NDUFA10 | 42 kDa | 36,707 | Pi | 36,787 | 36,705 | -82 | Pi missing |
| NDUFA11 | B14.7 | 14,758 | ΔMet, N-acetyl | 14,669 | 14,668 | -1 | |
| NDUFA12 | B17.2 | 17,090 | N-acetyl | 17,132 | 17,131 | 0 | |
| NDUFA13 | B16.6 | 16,674 | ΔMet, N-acetyl | 16,584 | 16,584 | 0 | |
| NDUFAB1 | SDAP | 10,110 | 4'-phosphopantethine + 3-hydroxyundecanoate | 10,676 | 10,674 | -2 | |
| NDUFAB1 | SDAP | 10,110 | 4'-phosphopantethine + 3-hydroxyundecanoate | 10,676 | 10,674 | -2 | |
| NDUFB1 | MNLL | 7,097 | ΔMet | 6,966 | 6,966 | | |
| NDUFB2 | AGGG | 8,493 | | 8,493 | 8,493 | | |
| NDUFB3 | B12 | 11,141 | ΔMet, N-acetyl, 2 x Me | 11,080 | 11,038 | -42 | without N-acetyl |
| NDUFB4 | B15 | 15,184 | ΔMet, N-acetyl | 15,095 | 15,095 | 0 | |
| NDUFB5 | SGDH | 16,726 | | 16,726 | 16,728 | | |
| NDUFB6 | B17 | 15,524 | ΔMet, N-acetyl | 15,435 | 15,437 | 2 | |
| NDUFB7 | B18 | 16,398 | ΔMet, myristoyl, 2 x SS | 16,473 | 16,478 | 5 | |
| NDUFB8 | ASHI | 18,737 | | 18,737 | 18,738 | 1 | |
| NDUFB9 | B22 | 21,789 | ΔMet, N-acetyl | 21,700 | 21,699 | -1 | |
| NDUFB10 | PDSW | 20,965 | ΔMet, 2 x SS | 20,830 | 20,833 | 3 | |
| NDUFB11 | ESSS | 14,453 | | 14,453 | 14,452 | -1 | |
| NDUFC1 | KFYI | 5,829 | | 5,829 | 5,829 | | |
| NDUFC2 | B14.5b | 14,096 | N-acetyl | 14,138 | 14,096 | -43 | without N-acetyl |
| FMN | | | | 456 | 456 | 0 | |
| [2Fe-2S] | | | | 172 | 178 | 6 | |
| [4Fe-4S] | | | | 348 | 356 | 8 | |
| Total mass for essential components of complex I | | | | 974,867 | 973,800 | -1,067 | Above plus incorrect FeS (should be 2 x [2Fe-2S] and 6 x [4Fe-4S]) |
| Total mass for complex I minus NDUFV1, NDUFV2, NDUFV3, NDUFA2, NDUFS1 | | | | 804,311 | 803,384 | -927 | Above plus incorrect FeS (should be 3 x [4Fe-4S]) |
| Total mass for complex I minus NDUFV1, NDUFV2, NDUFV3, NDUFA2, NDUFS1, NDUFS3 | | | | 777,880 | 776,772 | -1,107 | Above plus incorrect FeS (should be 3 x [4Fe-4S]) |
| NADH | | | | 663 | 663 | 0 | |
| Ubiquinone-10 | | | | 863 | 836 | -27 | Typographical error? |
| CDL | | | | 1421 | 1421 | 0 | |
| Total mass for complex I plus NADH, UQ10 and 3 x CDL | | | | 980,657 | 979,563 | -1,094 | 980,986 calculated by Chorev et al from the same masses |
| Total mass for first subcomplex plus UQ10 and 3 x CDL | | | | 809,438 | 808,484 | -954 | 809,728 calculated by Chorev et al from the same masses |
| Total mass for second subcomplex plus UQ10 and 3 x CDL | | | | 783,006 | 781,872 | -1,134 | 783,116 calculated by Chorev et al from the same masses |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## COMPLEX III

| Subunit | Protein mass (Δimport) | Known modifications | Calculated native mass | Chorev mass | Error in Chorev mass | Comments on Chorev mass |
|---|---|---|---|---|---|---|
| UQCRC1 | 49,212 | | 49,212 | 49,212 | | |
| UQCRC2 | 46,524 | | 46,524 | 46,523 | | |
| CYB | 42,591 | 2 x b-haem, N-formyl-Met | 43,845 | 42,591 | -1,254 | 2 x b-haem, N-formyl-Met missing |
| CY1 | 27,287 | c-haem | 27,902 | 27,987 | 85 | Unexplained error |
| UQCRFS1 (Δ1-78) | 21,610 | [2Fe-2S], SS | 21,781 | 21,609 | -172 | [2Fe-2S], SS missing |
| UQCRB | 13,476 | ΔMet, N-acetyl | 13,387 | 13,345 | -42 | N-acetyl missing |
| UQCRQ | 9,720 | ΔMet | 9,589 | 9,589 | | |
| UQCRH | 9,175 | | 9,175 | 9,175 | | |
| UQCRFS1 (1-78) | 7,955 | N-acetyl, SS | 7,995 | 7,955 | -40 | N-acetyl, SS missing |
| UQCR10 | 7,458 | ΔMet | 7,326 | 7,326 | | |
| UQCR11 | 6,520 | | 6,520 | 6,520 | | |

| | | |
|---|---|---|
| Total mass for complex III monomer | 243,257 | 241,832 |
| Total mass for complex III dimer | 486,514 | 483,664 |
| Total mass for complex III monomer minus UQCRC1, UCCRH, UQCRFS1 (1-78), UQCR11 | 170,355 | 168,970 |
| Total mass for complex III dimer minus UQCRC1, UCCRH, UQCRFS1 (1-78), UQCR11 | 340,709 | 337,940 |

Notes:
Uniprot codes identified from structure of cytochrome bc1 complex from bovine heart mitochondria (1BGY.PDB)

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## COMPLEX IV

| Subunit | Protein mass (Δimport) | Known modifications | Calculated native mass | Chorev mass | Error in Chorev mass | Comments on Chorev mass |
|---|---|---|---|---|---|---|
| COX1 | 57,033 | N-formyl-Met, haem a3, H2O, haem a, CuB, OH- | 58,839 | 58,744 | -95 | N-formyl-Met, CuB missing |
| COX2 | 26,022 | N-formyl-Met, Y240-H244 link, CuA, Mg | 26,195 | 26,021 | -174 | N-formyl-Met, Y240-H244 link, CuA, (Mg) missing |
| COX3 | 29,933 | | 29,933 | 29,933 | | |
| COX4I1 | 17,153 | | 17,153 | 17,152 | | |
| COX5A | 12,436 | | 12,436 | 12,433 | -3 | Unexplained error |
| COX5B | 10,670 | Zinc | 10,734 | 10,670 | -64 | Zinc missing |
| COX6A2 | 9,533 | | 9,533 | 9,538 | 5 | Incorrect isoform |
| COX6B1 | 10,156 | ΔMet, N-acetyl, 2 x SS | 10,063 | 10,025 | -38 | N-acetyl, 2 x SS missing |
| COX6C | 8,610 | ΔMet, N-acetyl | 8,521 | 8,478 | -43 | N-acetyl missing |
| COX7A1 | 6,674 | | 6,674 | 6,609 | -65 | Incorrect isoform |
| COX7B | 6,357 | | 6,357 | 6,357 | | |
| COX7C | 5,441 | | 5,441 | 5,441 | | |
| COX8B | 4,962 | | 4,962 | 4,961 | | |
| | | Total monomer mass | 206,840 | 206,362 | | |
| | | Total dimer mass | 413,679 | 412,724 | | |
| Cardiolipin (CDL) | | | 1,448 | 1,448 | | |
| Phosphatydilethanolamine (PE) | | | 760 | 760 | | |
| | | Total monomer mass | 209,048 | 208,570 | | |
| | | Total dimer mass | 418,095 | 417,140 | | |
| | | Total monomer mass reported in Chorev et al. | | 208,299 | | |

Notes:
The mass of Mg has been added to COX2; it is shared between COX1 and COX2
Uniprot codes identified from structure of cytochrome c oxidase from bovine heart mitochondria at 1.5 A (5B1A.PDB)

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The Mg2+-containing water cluster of mammalian cytochrome c oxidase collects four pumping proton equivalents in each catalytic cycle. J.Biol.Chem. 291, 23882-23894.

## ATP synthase

| Subunit | Protein mass (Δimport) | Known modifications | Calculated native mass | Multiplied by stoichiometry | Chorev mass | Multiplied by stoichiometry | Error in Chorev mass | Comments on Chorev mass |
|---|---|---|---|---|---|---|---|---|
| α | 55,264 | N-pyro-Glu, ADP, Mg | 55,698 | 167,094 | 55,263 | 165,789 | -435 | N-pyro-Glu missing |
| β | 51,705 | | 51,705 | 155,116 | 51,563 | 154,689 | -142 | Unexplained error |
| γ | 30,141 | | 30,141 | 30,141 | 30,256 | 30,256 | 115 | Unexplained error |
| δ | 15,065 | | 15,065 | 15,065 | 15,065 | 15,065 | | |
| ε | 5,783 | ΔMet | 5,652 | 5,652 | 5,793 | 5,793 | 141 | Unexplained error |
| a | 24,816 | | 24,816 | 24,816 | 24,788 | 24,788 | -28 | Unexplained error |
| b | 24,669 | | 24,669 | 24,669 | 24,669 | 24,669 | | |
| c | 7,608 | TriMe-Lysine | 7,650 | 61,200 | 7,608 | 60,864 | -42 | TriMe-Lysine missing |
| d | 18,693 | ΔMet, N-acetyl | 18,603 | 18,603 | 18,692 | 18,692 | 89 | Met remaining, N-acetyl missing |
| e | 8,321 | ΔMet | 8,190 | 8,190 | 8,321 | 8,321 | 131 | Met remaining |
| f | 10,297 | ΔMet, N-acetyl | 10,208 | 10,208 | 10,297 | 10,297 | 89 | Met remaining, N-acetyl missing |
| g | 11,418 | ΔMet, N-acetyl | 11,328 | 11,328 | 11,417 | 11,417 | 89 | Met remaining, N-acetyl missing |
| A6L | 7,965 | | 7,965 | 7,965 | 7,973 | 7,973 | 8 | Unexplained error |
| F6 | 8,958 | | 8,958 | 8,958 | 8,958 | 8,958 | | |
| 6.8PL | 6,834 | | 6,834 | 6,834 | 6,834 | 6,834 | | |
| DAPIT | 6,435 | ΔMet | 6,303 | 6,303 | 6,453 | 6,453 | 150 | Unexplained error |
| OSCP | 20,930 | | 20,930 | 20,930 | 20,930 | 20,930 | | |
| | | | | | | | | |
| Total mass for ATP synthase monomer | | | | 583,071 | | 581,788 | | |
| Total mass for ATP synthase dimer | | | | 1,166,142 | | 1,163,576 | | |
| | | | | | | | | |
| Total mass for ATP synthase monomer reported in Chorev et al. | | | | | | 581,948 | | |


Reference

Carroll, J. Fearnley, I. M., Wang, Q. & Walker, J. E. (2009) Measurement of the molecular masses of hydrophilic and hydrophobic subunits of ATP synthase and complex I in a single experiment. Anal. Biochem. 395, 249-255.

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