ATP synthase: From sequence to ring size to the P/O ratio

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There are tales of P/O battles at conferences in the 1950s. P/O is the number of ATP molecules synthesized by oxidative phosphorylation for each pair of electrons (hence, not P/O_2) passing from a particular substrate, typically NADH or succinate, via a respiratory chain, to O_2. Knowledge of the P/O ratio is fundamental for understanding the ATP yield from cell fuels and is a core metabolic parameter. The battles of old persisted because the P/O ratio is difficult to measure. Determination of O_2 consumed became easier as manometers were replaced by electrodes, but calibration is difficult because the dissolved O_2 concentration depends on temperature, ionic strength, history of the solution, and atmospheric pressure. Determining ATP made by suspensions of (usually animal) mitochondria is also error prone. Furthermore, mitochondria might be damaged, either homogeneously or heterogeneously, and thus provide underestimates of the P/O ratio because O_2 consumption can proceed without full coupling to ATP synthesis, e.g., owing to leakage of protons across membranes. The combatants in the P/O battles of the 1950s could never have imagined that P/O ratios might be assessed via structural biology, as illustrated by a study (1) in PNAS. Many biochemists also have not contemplated that a highly conserved enzyme has a variable relative subunit stoichiometry. The paper (1) unexpectedly shows variation in the stoichiometry of one critical subunit, c, among ATP synthases of mitochondria from different sources.

Protons and P/O Ratio

In view of the difficulties with P/O determination, there was a time when "the bigger the better" was the motto of some, but it became widely accepted that the P/O ratio was 3 or 2 depending on whether matrix NADH or succinate was the electron donor. These values thus gave rise to the notion of three "sites" of ATP synthesis associated with the mitochondrial respiratory chain. This notion has turned out to be incorrect because around 1960 Mitchell introduced his chemiosmotic mechanism, which envisaged that there was a separate ATP synthase (now F_1F_o), and that there were three sites of proton translocation rather than ATP synthesis. Each site was envisaged to translocate $2H^+/2e$, thus generating a proton electrochemical gradient, or protonmotive force in volts which would drive ATP synthesis by using $2H^+$ for each ATP made. Arithmetic shows that P/O ratios of 3 and 2 were retained. The idea of nonintegral P/O ratios was not envisaged although the chemiosmotic mechanism would allow such values.

During the 1970s, evidence accumulated that the stoichiometry of H^+ translocation was higher than originally envisaged. Ratios are also not easy to determine, but consensus $H^+/2e$ or H^+/O values of 10 and 6 for NADH and succinate were eventually accepted after another series of battles. The H^+/ATP ratio for the ATP synthase proved even harder to determine, but a variety of evidence started to point to a value of 3 for animal mitochondria; 10/3 suggests a P/O ratio for NADH oxidation of 3.3 (recall the 1950s battles). However, another dimension had been recognized, which is that the import of ADP and P_i into mitochondria in exchange for the export of ATP is associated with the net import of $^1H^+$ into the matrix; hence, P/O, for ATP delivered to the outside of mitochondria, would be $10/(3 + 1) = 2.5$ (NADH) and $6/(3 + 1) = 1.5$ (succinate), each considerably lower than then textbook values of 3 and 2. Subsequently, careful reevaluation of the P/O ratios for NADH and succinate indicated that the experimental values are close to 2.5 and 1.5 (2). Thus, with perhaps some lingering doubts, e.g., a report of well over 2.5 and 1.5 (3), the latter nonintegral P/O ratios became accepted. The charge translocation stoichiometries are not equal for the three sites of proton translocation; complexes I and IV of the respiratory chain each translocate double the charge/2e relative to complex III and thus the ATP/2e for the latter must be one half that for the other two complexes (4, 5).

P/O and ATP Synthase Structure

The F_1F_o ATP synthases in eukaryotes (mitochondria, thylakoids of chloroplasts) and eubacteria (but not archaea) are from sequence analyses very similar, and so a universal proton to H^+/ATP was expected. The first structure of the enzyme (6) showed that, as anticipated (7), there are three catalytic β chains, each separated by a related α subunit, on the globular F_1 part of the protein; at any one instant, each β is in a distinct conformation. The single γ chain is in the center of the $\alpha_3\beta_3$ assembly. The idea that this γ chain might rotate within the F_1 sector and, thus, drive the conformational interconversions of the β chains and, hence, ATP synthesis was confirmed by a seminal experiment (8). Rotation of γ must be driven by the passage of protons down the electrochemical gradient and through some of the polypeptides of the integral membrane sector, F_o, of the ATP synthase, which minimally comprises three polypeptides, a, b, and c. Their relative stoichiometries have been difficult to elucidate; hence, the stoichiometry was often given as ab_2c_{9-12}.

X-ray crystallography of yeast ATP synthase revealed a ring of c subunits attached to F_1 even in the absence of other subunits of the F_o sector (9). This work not only suggested that a and b were not interdigitated with c but also revealed the surprising stoichiometry of c_{10}. The C-terminal helix of the hairpin c subunit is at the outside of the ring and contains an essential aspartate or glutamate residue. This residue is believed to pick up an H^+ and rotate away from an interaction with the α subunit. Thus, 10 protons should drive a 360° rotation of the c ring, which is structurally connected to γ, giving synthesis of 3 ATPs as γ rotates through 360° within F_1; the H^+/ATP ratio would be $10/3 = 3.3$. The P/O ratio for NADH would be $10/(3.3 + 1) = 10/4.3 \approx 2.3$, thus moving below the range of values directly determined.

The apparently universal nature of the ATP synthase suggested that c_{10} would be widespread. However, structural analysis of stable assemblies of c rings from distinct sources has revealed not only c_{10} but also c_{11}, c_{13}, c_{14}, and c_{15} (1), with implication of H^+/ATP from 3.3 to 5. Nevertheless, it has been assumed that the stoichiometry would be c_{10} for all mitochondrial ATP synthases, but such a conclusion would be wrong.

The enzyme from bovine heart mitochondria has a c_8 ring (1). Thus, the H^+/ATP will be 2.67, and the predicted P/O will be $10/(2.67 + 1) = 2.7$ (recall extra "1" includes transport processes) for NADH and $6/(2.67 + 1) = 1.6$ for succinate, close to experimental values (2). Thus, provided that (*i*) the H^+/O ratios for NADH and succinate oxidation are indeed 10 and 6, respectively; (*ii*) the observed c ring stoichiometry is not some improbable peculiar artifact of protein purification/crystallization protocols; and (*iii*) the H^+/ATP can

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safely be obtained from the number of c subunits, P/O for NADH and succinate in the cell can be no higher than 2.7 and 1.6. The actual values might in any particular circumstance be lower than 2.7 and 1.6 for several reasons, including proton leakage or because the protonmotive force might be simultaneously driving more than one process.

What factors underpin the assembly of 8 rather than 10 c subunits per ring for animals rather than other mitochondrial sources? The c subunits adopt a hairpin helical structure with the N-terminal helix contributing to the smaller circumference inner ring. Structural and bioinformatic analysis conducted by Walker and colleagues (1) indicates that attempts to model a c_8 ring by using a yeast sequence and structure result in serious stereochemical clashes among the side chains of residues Ile-13, Leu-19, and Ile-23 of adjacent protomers in the hypothetical ring. Notably, each of these residues is replaced, with one exception, by alanine in all animalia species. On the other hand, this trio of alanines is not observed in lower eukarya and eubacteria (1), or in thylakoids, which have bigger rings. For the case of c_{13} in an alkalophilic bacillus species, a different variant stretch of sequence contributes to an enlarged ring diameter (10). A further interesting aspect described in ref. 1 is that the occurrence of trimethylated lysine in the c subunit of animal F_1F_o is correlated with possible stabilizing interaction with cardiolipin, the characteristic mitochondrial phospholipid.

Can bioinformatics and modeling produce rules for the number of c subunits and, thus, H^+/ATP in rings in different species? This issue is of particular interest for bacterial species because, although not always appreciated, determination of the P/O ratio is very difficult. It is impossible with intact cells because defined substrates cannot be added exogenously. Sequence analysis for an archaeal A-type synthase showed that the polypeptide equivalent to c occurs as a fusion of 13 closely related stretches of sequence. Thus, in this case, bioinformatics gives subunit stoichiometry and H^+/ATP ratio (11).

Traditional biochemical "intuition" led many to believe the bigger the better for the P/O. Clearly, the lower the H^+/ATP, then the larger the P/O, assuming that the respiratory chain proton translocating activity remains constant. On this basis, the higher H^+/ATP ratios predicted for yeast (3.3), thylakoids (4.66), or some thermophilic algae (5.0) make no sense because they result in lower ATP/2e ratios. However, a decrease in P/O allows for an increase in an important thermodynamic term. As explained in more detail elsewhere (12), the maximum ΔG of ATP synthesis in the stroma of chloroplasts or a cytoplasm of a bacterium is a function of the H^+/ATP ratio rather than of H^+/(ATP + 1) because, unlike for mitochondria, there is no electrogenic exchange of ATP for ADP and P_i across the membranes. The protonmotive force across many different membranes is similar, and so we need to explain how similar high ATP/ADP ratios (c 100) can be achieved. A higher H^+/ATP for thylakoid and bacteria ATP synthases in compensation for loss of the "+ 1 factor" is a possibility (12). An explanation for H^+/ATP = 2.7 in animalia and 3.3 in lower eukaryotes is not readily apparent, especially as the P/O can in any case be lower in the latter cases because the respiratory chain contains nonproton translocating pathways.

Determination of the H^+/ATP ratio from the c subunit stoichiometry is, however attractive, strictly speaking only a deduction; without independent evidence for the value then not only is the structure circular but so is the argument. Direct determination of the H^+/ATP ratio is difficult. One approach is to compare the magnitude of the ΔG for ATP synthesis with the protonmotive force. At equilibrium the relationship $\Delta G = H^+/ATP.F.\Delta p$ (where F is the Faraday constant and Δp is protonmotive force) should hold in cases where there is no transport step. Because ATP/ADP ratios can reach high values, errors in determining the low ADP concentration can be problematic, but nevertheless the errors are not very large. However, determining Δp is via indirect methods and the errors are uncertain. In a recent study (13), a range of pH gradients has been induced across membranes of phospholipids in which were embedded ATP synthases from either thylakoids or *Escherichia coli*. The suspending reaction medium contained different ratios of ATP/ADP.P_i and it was determined whether the induced gradient drove ATP synthesis or permitted net ATP hydrolysis. By extrapolation, the sizes of the gradients that allowed neither for various ATP/ADP ratios were determined and, hence, the H^+/ATP. This extremely thorough approach gave H^+/ATP = 4 for both enzymes, inconsistent with the c subunit stoichiometries of 14 and 10 for thylakoids and *E. coli* (latter supported by a report of a 36° stepping during rotation of the c ring; ref. 14) respectively. At present, most investigators are inclined to believe the H^+/ATP ratio deduced from the c subunit stoichiometry and to assume that the thermodynamic measurements must have a yet-to-be-identified flaw. However, there is more to do in this area. Not long ago, it was considered inconceivable by many that the H^+/ATP would not be a constant. The current structural evidence convinces most investigators that it is inconceivable that it is not given by the c subunit stoichiometry. "Inconceivable" is not always a permanent state.

The idea that a highly conserved protein such as the ATP synthase would not have a uniform subunit stoichiometry was not contemplated 10 years ago. Now it is accepted, and there is starting to be understanding, as discussed (1), of how what superficially appear to be minor sequence variations are the controllers of the stoichiometry. There may be other proteins where the same lesson is waiting to be learned.

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