On the Subunit Structure of the Cold Labile Adenosine Triphosphatase of Mitochondria*

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SUMMARY

Mitochondrial ATPase has been studied by equilibrium ultracentrifugation. Under virtually all conditions examined, the oligomeric structure (mol. wt. 2.8 x 10^6) is in equilibrium with smaller polypeptide chains. In dilute buffer, however, the proportion of dissociated subunits becomes appreciable only in the cold (5°C). At this temperature, the molecular weight of the subunits can be measured directly, and a value of 4.6 x 10^4 is obtained. Additional experiments in solutions of guanidine hydrochloride (6 to 9 M) indicate that no further dissociation beyond the 4.6 x 10^4 unit occurs. The results are evaluated in terms of a probably hexameric structure for the ATPase.

The ATPase of mitochondria is currently being studied intensively as a homogeneous coupling factor of oxidative phosphorylation and as one of the few well characterized enzymes exhibiting inactivation as a result of cold-labile subunit dissociation (1-3). These two aspects of the ATPase molecule are intimately related since the inactivation (and presumably dissociation into subunits) in the cold is repressed when the enzyme is integrated into the mitochondrial membrane. In addition, oligomycin inhibition observed with the enzyme in situ is not present with the isolated enzyme. In order to begin to relate the functional properties of ATPase to its quaternary interactions and conformation, we have undertaken a study to define more precisely the protein's subunit structure.

EXPERIMENTAL CONDITIONS

ATPase, prepared as described by Horstman and Racker (4), was stored as the ammonium sulfate precipitate at 4°C and 6 mg per ml, dry weight. Samples were prepared by centrifuging aliquots at 16,000 rpm at 5°C for 5 min and dissolving the protein in buffer to the desired concentration. The buffer was 20 mM potassium phosphate and 2 mM EDTA neutralized with KOH to pH 7.3. For a few experiments, the pellet was dissolved in a buffer containing 0.25 M sucrose, 10 mM Tris-sulfate, 4 mM ATP, and 2 mM EDTA, pH 7.4, and passed through Sephadex as described by Penefsky and Warner (3). Sedimentation equilibrium experiments were performed with the use of absorption or interference optics as described previously (5, 6). Experiments with interference optics were favored especially for solutions in the buffer containing ATP, since the absorption of the nucleotide restricted measurements with the scanner in the ultraviolet range. Molecular weight calculations were based on a partial specific volume of 0.74 as estimated from the amino acid composition (7).

RESULTS AND DISCUSSION

Experiments with ATPase under conditions that maintain the oligomeric structure (moderate temperature, low salt, and added ATP) confirmed the finding of Penefsky and Warner (3) of a 12 S species with a molecular weight of about 2.8 x 10^6. The results, as presented in Table I, however, were somewhat more variable (10 to 15%) than is generally found with homogeneous proteins. Two factors are likely to be responsible for this variation. First, the preparation may have contained small amounts of the inhibitor associated with mitochondrial ATPase (4, 8). The inhibitor, which has a molecular weight on the order of 4 x 10^4, would tend to raise the observed molecular weight to the extent that it is present. Second, a close examination of the sedimentation equilibrium data for 25°C, particularly with absorption optics, revealed that, at very low concentrations of protein, some dissociation occurs, especially when no ATP is added. The dissociation is revealed by upward curvature of log c versus r^2 near the meniscus (Fig. 1). The observed molecular weight for oligomeric ATPase will tend to be lowered by the dissociation into subunits.

The observation that dissociation of ATPase is noticeable at 25°C and pronounced at 5°C (Fig. 1) even in the absence of added excess salt permitted the subunit molecular weight to be determined directly. Since the lighter species is predominant at 5°C, its molecular weight can be determined from the sedimentation equilibrium data of log c versus r^2 from the region of the solution column near the meniscus. The line obtained from these points can then be extrapolated to the bottom of the cell and subtracted from the observed curve to provide an estimate of the heavier species present as well (6). This procedure is illustrated in Fig. 2. The results for the lighter species in a number of such experiments are presented in Table II. Considering that data are obtained from only a limited portion of the centrifuge cell, the data from different runs are in good agreement and suggest a molecular weight of 4.6 x 10^4 for the subunit formed in the cold.

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1 G. Ellis and S. J. Edelstein, unpublished results.
TABLE I

Molecular weights of oligomeric ATPase

Experiments were examined by interference or absorption optics or both (results are averaged). Determinations 1 to 5 were conducted with 20 mM potassium phosphate, 2 mM EDTA, and 4 mM ATP, pH 7.3. For Determinations 6 to 9, no ATP was added. Experiments were performed at 15,000 rpm at 25°.

| Determination | Initial concentration (mg/ml) | Molecular weight ($\times 10^4$) |
|---------------|------------------------------|-------------------------------|
| 1             | 0.34                         | 2.3                           |
| 2             | 0.68                         | 2.9                           |
| 3             | 1.32                         | 2.5                           |
| 4             | 0.40                         | 2.7                           |
| 5             | 0.46                         | 2.8                           |
| 6             | 1.16                         | 2.4                           |
| 7             | 1.18                         | 2.7                           |
| 8             | 1.18                         | 2.8                           |
| 9             | 1.00                         | 2.9                           |

Fig. 1. Sedimentation equilibrium of ATPase at 5°. The experiment was performed at 15,000 rpm in buffer without ATP. Data was recorded with the absorption optical system scanner and light of 280 nm. The samples were centrifuged overnight and equilibrium was verified by the invariance of traces recorded at an interval of 1 hour.

The molecular weight of the heavier species was also estimated from the bottom of the curve, although in this case only data at the lower speed (15,000 rpm) were suitable for the analysis. The results, which show some variation largely attributable to the nature of the estimation by subtracting the lighter species, nevertheless indicate a molecular weight of $2.8 \times 10^5 \pm 10\%$.

The results of the experiments presented above suggest a dissociation of oligomeric ATPase ($mol wt, 2.8 \times 10^5$) into subunits approximately one-sixth the size of the parent molecule ($mol wt, 4.6 \times 10^4$). The results of Fig. 2 also suggest that no species of intermediate molecular weights is present in significant amounts. Since Penefsky and Warner (3), from experiments with sodium dodecyl sulfate, reported a much lower subunit molecular weight of 29,000 and suggested a 10-subunit structure, the possibility of further dissociation beyond the $4.6 \times 10^4$ level was considered. To determine whether even smaller units are present in the molecule, sedimentation equilibrium experiments...

TABLE II

Molecular weights of lighter component

All experiments were conducted at 5° in 20 mM potassium phosphate, 2 mM EDTA. Determination 1 also contained ATP.

| Determination | Initial concentration (mg/ml) | Speed (rpm) | Molecular weight ($\times 10^4$) |
|---------------|------------------------------|-------------|-------------------------------|
| 1             | 1.00                         | 26,000      | 5.3                           |
| 2             | 1.00                         | 20,000      | 4.1                           |
| 3             | 1.35                         | 15,000      | 4.8                           |
| 4             | 1.00                         | 15,000      | 4.2                           |
| 5             | 0.50                         | 15,000      | 4.6                           |
| 6             | 1.35                         | 26,000      | 4.4                           |
| 7             | 0.50                         | 26,000      | 4.7                           |
were conducted with solutions of guanidine hydrochloride (6 M). As seen in Fig. 3, a linear dependence of log c versus \( r^2 \) was observed, as expected for a homogeneous system. Since the solution contains three components, the ATPase, guanidine, and water, the partial specific volume (\( \bar{v} \)) alone cannot be used in calculating molecular weight. Rather, it must be replaced by the term \( \phi' \) to correct for possible preferential interactions between the protein and either guanidine or water (9). Assuming that \( \phi' \) bears the same relationship to \( \bar{v} \) as is found for aldolase in 6 M guanidine hydrochloride, i.e. \( \bar{v} - \phi' = 0.01 \) to 0.02 (9), \( \phi' = 0.72 \) to 0.73 must be employed for the ATPase. Values corresponding to molecular weights of \( 4.5 \times 10^4 \) to \( 4.9 \times 10^4 \). Molecular weights in this range, which are consistent with the value \( 4.6 \times 10^4 \) obtained in the absence of guanidine (Table II), provide strong evidence that the minimum size polypeptide chain present. Subsequent experiments at 9 M guanidine also revealed no further dissociation. The simplest interpretation of the results of these sedimentation equilibrium experiments is that mitochondrial ATPase is an oligomer built up of six polypeptide chains of molecular weight \( 4.6 \times 10^4 \). No evidence is available at the present time concerning whether or not these chains are identical. If they are different in size, the differences would have to be relatively small to account for the homogeneity observed in guanidine hydrochloride (Fig. 3). The chains could be of similar size but quite different composition, however. In this regard, more precise chemical analysis and the determination of the number of binding sites for ATP would be of interest.

The results presented here also permit an interpretation of the 3 S, 9 S, and 12 S components of ATPase observed by Penefsky and Warner (3). The 12 S species is the oligomer of molecular weight \( 2.8 \times 10^5 \) present at 25°C. We observed that, at low temperature and in dilute buffer, only the 9 S and 3 S species are present, in roughly comparable amounts. Since only material with molecular weights of \( 2.8 \times 10^5 \) and \( 4.6 \times 10^4 \) is observed at sedimentation equilibrium under these conditions (Fig. 2), it is likely that the 9 S species is identical in molecular weight with the 12 S macromolecule but has undergone a conformational change to a more extended structure with a higher frictional coefficient. In effect, the 9 S species would be an intermediate on the way to the 3 S unit. These relationships may be summarized as:

\[
\text{12 S (mol wt = 2.8 \times 10^5)} \\
\text{11 \hspace{1cm} 9 S (mol wt = 2.8 \times 10^5)} \\
\text{9 \hspace{1cm} \hspace{1cm} \hspace{1cm} \hspace{1cm} \hspace{1cm} 6 \hspace{1cm} \hspace{1cm} S (mol wt = 4.6 \times 10^4)} \\
\]

The equilibrium may be shifted, by cold or salt (3), progressively, first to the 9 S species, then to the 3 S species. Although cold lability is often assumed to reflect hydrophobic bonding, the salt lability of ATPase (3) suggests that ionic interactions, which, like hydrophobic interactions, are predominantly entropic (10), may be the major factor involved.

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