Oligomycin sensitivity conferral protein, in the absence of coupling factor 6 (F6), is able to bind the ATPase to mitochondrial membranes with an apparent association constant of 10^5 M^-1. The Fs-dependent ATPase binding has an apparent association constant 1 to 2 orders of magnitude lower than that obtained with oligomycin sensitivity conferral protein. The oligomycin sensitivity conferral protein-dependent, membrane-bound ATPase activity is sensitive to rutamycin while the Fs-dependent, membrane-bound ATPase activity is insensitive to rutamycin. F1-ATPase and Type II ATPase require F6 in addition to oligomycin sensitivity conferral protein and Fs to reconstitute 32P-ATP exchange activity in silicotungstic acid particles. This F6 requirement for the 32P-ATP exchange is not related to the Fs effect on the ATPase binding. The Type I ATPase and therefore the 26,500-dalton subunit associated with it requires Fs and F6 to reconstitute 32P-ATP exchange activity in silicotungstic acid particles. Oligomycin sensitivity conferral protein can be interchanged with the 26,500-dalton ATPase binding protein in the binding of the ATPase and the 32P-ATP exchange.

The mitochondrial H^+-translocating ATPase is composed of water-soluble proteins (F1) and water-insoluble, membrane sector proteins (F0). It has been suggested that the binding of F1-ATPase to the mitochondrial membrane requires both OSCP and F6 (=FC2) (2-5). The OSCP was postulated to bind the ATPase and the 32P-ATP exchange reaction of Type I and Type II ATPase binding has an apparent association constant 1-2 orders of magnitude lower than that obtained with OSCP and the bound ATPase activity is rutamycin-insensitive; (c) F1-ATPase and Type II ATPase require F6 in addition to OSCP and F6 to reconstitute 32P-ATP exchange activity, the F6 requirement for 32P-ATP exchange being unrelated to the effect of Fs in STA particles on the binding of the ATPase; (d) Type I ATPase and therefore the 26,500-dalton subunit associated with it requires Fs and F6 to reconstitute 32P-ATP exchange activity in STA particles; and (e) OSCP can be interchanged with the 26,500-dalton subunit in the stimulation of the ATPase binding and the 32P-ATP exchange reaction.

EXPERIMENTAL PROCEDURES

Materials

The following chemicals and enzymes were obtained from the indicated sources: Mops, Tris, Tes, EDTA, pyruvate kinase, lactate dehydrogenase, bovine serum albumin, and Coomassie blue B-250 from Sigma; NADH, ATP, and phosphoenolpyruvate from Boehringer Mannheim; SDS from BDH Chemical Ltd., Poole, England; ultrapure sucrose and (NH4)2SO4 from Schwarz/Mann; 2 M phenol reagent from Fisher; acrylamide, N,N'-methylenebisacrylamide, and Temed from Eastman; ammonium persulfate and AG 1-X2 resin from Bio-Rad, and digitonin from Pfaltz and Bauer. All these reagents were of reagent grade purity.

Adult male or retired breeder male albino rats (Sprague-Dawley Crl:CD(SD)BR) were obtained from the Charles River Breeding Laboratories, Wilmington, MA and were fed ad libitum Purina Rat Chow.

Methods

Preparation of Rat Liver Type I and Type II ATPase—Preparation of rat liver mitochondria, submitochondrial particles, the extraction of Type I and Type II ATPase from submitochondrial particles with chloroform, and their purification by a zone sedimentation was reported previously (1).

Preparation of Beef Heart OSCP, F6, F1-ATPase, and Factor B—Beef heart OSCP was prepared according to Senior (7) with some slight modifications. The (NH4)OH extract is treated with 238 mg of

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** The abbreviations used are: F1, coupling factor 1; Fs, coupling factor 6; F6 or FC2, coupling factor 6; OSCP, oligomycin sensitivity conferral protein; STA-STA particles, submitochondrial particles treated with trypsin (T) and urea (U) to remove Fs, sonicated at alkaline pH (A) to remove OSCP, and treated with silicotungstic acid (STA) to remove F6; Temed, N,N',N",N"'-tetramethylethylenediamine; SDS, sodium dodecyl sulfate; Tes, N-tris(hydroxymethyl)methyl-2-aminothanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid.

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Role of OSCP and Fs in ATPase Binding and P_i ATP Exchange

(NH_4)_2SO_4 at 0 °C for 16 h. The (NH_4)_2SO_4 precipitate was collected and dissolved in a minimal volume of buffer containing 15 mM Tris acetate (pH 8.0), 2 mM EDTA, and 2 mM dithiothreitol (buffer 1). This material was absorbed to a column of CM-Sephadex (C-25-120 mesh) and washed with buffer 1 containing 180 mM KCl until no more protein eluted. At this point, the KCl was increased to 320 mM which eluted the OSCP. The fractions containing OSCP were pooled and the OSCP precipitated with (NH_4)_2SO_4 (238 mg/ml).

Beef heart F_1 was prepared as described by Kanner et al. (8). Beef heart F_1-ATPase was prepared as described by Horstmann and Racker (9) and was generously provided by S. Joshi (Boston Biomedical Research Institute). Factor C, CM-cellulose stage, was prepared as described by Joshi et al. (10) and was liberally provided by J. B. Hughes (Boston Biomedical Research Institute).

Preparation of Submitochondrial Particles Depleted in ATPase-
Rat liver submitochondrial particles were treated sequentially with trypsin and urea and then passed through a French pressure cell at pH 10 to remove OSCP. These submitochondrial particles were then treated with 1.5% silicotungstic acid to remove Fs (3). Beef heart TUA-STA particles and STA particles were prepared exactly as described (3, 5). The ATPase-depleted submitochondrial particles were stored under liquid N_2 until used.

Measurement of the Binding of ATPase to the TUA-STA Particles-
Binding of Type I ATPase was measured by incubating the TUA-STA particles (1 mg/ml) with various amounts of Type I ATPase in a medium containing 20% ethylene glycol, 10 mM Tris/Tes, pH 7.5, and 2 mM EDTA; total volume was 50 or 100 μl. The incubations were carried out at room temperature for 15 min. At the end of the incubation, samples were centrifuged at 200,000 × g for 30 min in an 42.2 Ti rotor at 4 °C. Aliquots of the supernatant fluid were withdrawn and ATPase activity was measured. The pellet residues were rinsed 3 times with ATPase assay medium, then suspended in 40 μl of the same medium for ATPase activity and rutamycin sensitivity measurements.

For measuring the effect of OSCP and Fs on the binding of ATPase, rat liver Type II ATPase was incubated with OSCP (pretreated with 50 mM dithiothreitol at room temperature for 30 min) or Fs in a medium containing 20% ethylene glycol, 10 mM Tris/Tes, pH 7.5, and 2 mM EDTA; total volume was 50 or 100 μl. The incubations were carried out at room temperature for 15 min. At the end of the incubation, samples were centrifuged at 200,000 × g for 30 min in an 42.2 Ti rotor at 4 °C. Aliquots of the supernatant fluid fed withdrawn and ATPase activity was measured. The pellet residues were rinsed 3 times with ATPase assay medium, then suspended in 40 μl of the same medium for ATPase activity and rutamycin sensitivity measurements.

Assay of ATPase Activity—ATPase was assayed with a coupled enzymatic reaction as described previously (1). Rutamycin sensitivity was measured by adding 10 μg of rutamycin in the 3-ml assay mixture; the subsequent ATPase activity was determined.

32P-ATP Exchange—The 32P-ATP exchange was measured as described (10) after extractions of unesterified phosphate (as phosphomolybdic acid) with isobutyl alcohol/benzene (11).

Determination of Protein—The submitochondrial particle protein was determined in the presence of 0.5% SDS (12) and the soluble protein was determined according to Lowry et al. (13).

RESULTS

The results shown in Fig. 1 confirm the data in the literature (2, 3) that F_0 (FC_0) causes binding of F_1-ATPase to TUA-STA particles. However, in contrast to Ref. 2, OSCP alone was sufficient to bind F_1-ATPase. The bound ATPase activity was oligomycin-sensitive with OSCP-induced binding and oligomycin-insensitive with Fs-induced binding. Moreover, we found a rather large difference in the association constants for binding F_1-ATPase to TUA-STA particles. The K_a for Fs-induced binding of F_1-ATPase was at least 1 to 2 orders of magnitude less than the K_a found for OSCP-induced binding of F_1-ATPase. When Fs and OSCP were used in combination, the stoichiometry and K_a of ATPase binding were exactly the same as OSCP alone. We must conclude from these data that Fs does not participate in the binding of F_1 to TUA-STA particles. In order to show that this conclusion is valid for the rat liver ATPase preparation, we made hybrid ATPase complexes with beef heart OSCP, rat liver Type II ATPase, beef heart F_1-ATPase, F_0, and rat liver TUA-STA particles. The extent of ATPase binding varied from 0.2-0.8 nmol of ATPase bound per mg of TUA-STA particle. The variation in stoichiometry was due not to the TUA-STA particles but undefined differences in the ATPase preparations. As shown in Table I, Fs-induced binding was always rutamycin-resistant and low affinity binding, while OSCP-induced binding was always high affinity binding and the bound ATPase activity was rutamycin-sensitive. Again we must conclude that Fs is not involved in the specific binding of the ATPase to TUA-STA particles regardless of source of either the ATPase or particles. The percent ATPase bound is expressed as a function of the results shown in Fig. 1 and under "Methods" using the appropriate preparations.

Table 1

| Preparations | Nano moles ATPase bound/mg TUA-STA particles (rat liver) | K_a (M⁻¹) | Sensitivity of bound ATPase activity to rutamycin (%) |
|--------------|--------------------------------------------------------|--------|---------------------------------|
| Rat liver crude Type I ATPase | 0.6 | 1.4 x 10⁶ | 78 |
| Rat liver crude Type II ATPase | 0.3 | 3.5 x 10⁶ | 69 |
| Rat liver OSCP | 0.7 | 3.0 x 10⁶ | 83 |
| Rat liver OSCP | 0.8 | 3.6 x 10⁵ | 99 |
| Rat liver crude Type I ATPase and beef heart OSCP | 0.6 | 2.6 x 10⁶ | 86 |
| Rat liver crude Type II ATPase and beef heart OSCP | 0.3 | 3.3 x 10⁶ | 50 |
| Rat liver F_1-ATPase and beef heart OSCP | 0.3 | 1.7 x 10⁵ | 19 |

Fig. 1. Binding of beef heart F_1-ATPase to TUA-STA submitochondrial particles derived from beef heart mitochondria. Beef heart F_1 (8.1 μg) and beef heart TUA-STA particles (49.3 μg) were incubated at room temperature with various amounts of Fs or OSCP. The total volume of the reaction mixtures was 50 μl and it contained, in addition to the protein components, 10 mM Tris/Tes, pH 7.5, 2 mM EDTA, 20% ethylene glycol, and 13.5 mM dithiothreitol when OSCP was present. The Fs and F_1 and OSCP and Fs were incubated for 15 min and then, after addition of the TUA-STA particles, were incubated 15 min longer. The incubation mixtures were then centrifuged at 220,000 × g for 20 min at 23 °C. The supernatant fluid was assayed for ATPase activity and the pellet was assayed for rutamycin-sensitive ATPase activity. The units remaining in the supernatant fluid were designated as free ATPase. The bound ATPase was derived by subtracting the free ATPase from a control to which no particles had been added. Using a one-binding site model, the data were directly fitted using a nonlinear regression, iterative technique. The experimental points (O) corresponded well with the calculated points (C). The calculated binding parameters are shown.
oligomycin sensitivity conferral protein provides all the binding capacity to the membrane and functions indistinguishably from the 26,500-dalton ATPase binding protein.

If F$_6$ does not function in binding of the ATPase to the membrane, does it have any other function? It has been demonstrated that F$_6$ is required for the $^{32}$P-ATP exchange reaction (8). The data in Table II confirm this observation. Unlike the binding of ATPase to the membrane, the $^{32}$P-ATP exchange required F$_6$ in the presence of the ATPase binding protein (Type I ATPase) or OSCP. The only exception was with crude Type II ATPase where F$_6$ was not required for the exchange activity. The F$_6$ dependence was regained after purification of the crude Type II ATPase by zone sedimentation (data not shown). A requirement for F$_6$ in the $^{32}$P-exchange reaction catalyzed by beef heart F$_1$-ATPase in STA particles has been reported previously (8). These data demonstrate that the F$_6$ prepared in our laboratory by published procedures is active in the $^{32}$P-ATP exchange reaction.

The ability of the ATPase binding protein to enhance the $^{32}$P-ATP exchange reaction was evident by the ability of the Type I ATPase to catalyze the exchange reaction. The Type II ATPase was unable to catalyze $^{32}$P-ATP exchange unless supplemented with OSCP. Beef heart OSCP is therefore functionally interchangeable with the rat liver ATPase binding protein in the $^{32}$P-ATP exchange reaction. The hybrid ATPase complex formed from rat liver Type II ATPase and beef heart OSCP behaved similarly to the homologous enzyme (Type I ATPase) in both the ATPase binding to TUA-STA particles and the $^{32}$P-ATP exchange reaction in STA particles. We used beef heart particles prepared with 1% silicotungstic acid to measure the $^{32}$P-ATP exchange reaction (5) because, as others have also found (3), the TUA-STA particles are unable to carry out $^{32}$P-ATP exchange even when supplemented with coupling factors, despite their ability to reconstitute oligomycin-sensitive ATPase activity.

Since F$_6$ has enhancing effects on both the $^{32}$P-ATP exchange reaction.

### Table II

| Gradient Type | Crude Type | F$_6$-ATPase |
|---------------|------------|--------------|
| Exchanged nmoles P$_i$ x mg protein$^{-1}$ |
| Type I ATPase | 37.1 | 79.4 | 97.3 |
| -OSCP | 48.6 | 86.2 | 9.3 |
| -F$_6$ | ND$^*$ | 89.3 | 31.5 |
| -OSCP, F$_6$ | 89.3 | 31.5 |
| -OSCP, F$_6$ | 39.5 |
| -OSCP, F$_6$, F$_6$ | 14.2 | 2.4 |
| -OSCP, F$_6$, F$_6$ | 15.5 | 3.8 |

* ND, not determined.

FIG. 2. Lack of correlation between F$_6$-dependent P$_i$-ATP exchange and ATPase binding to STA particles. The P$_i$-ATP exchange reaction was measured as described in Table II and the ATPase binding to STA particles was measured as described in Fig. 1.

change reaction and on low affinity binding of the ATPase to submithochondrial particles, it was important to ascertain if this effect of F$_6$ on the $^{32}$P-ATP exchange was due to promotion of the binding of the ATPase to the membrane by F$_6$. We undertook the experiment shown in Fig. 2, where part of the sample was used to measure $^{32}$P-ATP exchange and the other part was used to measure the amount of ATPase bound to the membrane. It was evident that there is no correlation between the $^{32}$P-ATP exchange reaction and binding of F$_1$-ATPase to STA particles. There was a 3-fold increase in the $^{32}$P-ATP exchange and essentially no change in the binding of F$_1$-ATPase to the STA particles. Moreover, as was shown in Fig. 1, in order to see any effect with F$_6$ on ATPase binding, one needs 10-20 nmol of F$_6$ per mg of TUA-STA particles while in the $^{32}$P-ATP exchange, 1 nmol of F$_6$ per mg of STA particles gives maximum effect.

Similar results were obtained with rat liver Type I ATPase (data not shown).

### DISCUSSION

The present studies imply that F$_6$ does not play a significant role in the binding of ATPase to the mitochondrial membrane. F$_6$ is, however, involved in the $^{32}$P-ATP exchange. It is OSCP or the 26,500-dalton protein subunit which is responsible for both the ATPase binding and the conferring of the rutamycin sensitivity to the membrane-bound ATPase activity. Experimental evidence against the involvement of F$_6$ in the binding of ATPase include the fact that the F$_6$-dependent binding of ATPase was an apparent association constant 1-2 orders of magnitude lower than that of the OSCP-dependent or the 26,500-dalton subunit-dependent binding of ATPase. The $K_A$ of ATPase for F$_6$ is therefore 1-2 orders of magnitude weaker than that for OSCP or the 26,500-dalton subunit-dependent binding of ATPase. The $K_A$ of ATPase for OSCP and F$_6$ (at a concentration of F$_6$ approximately 7-10-fold higher than OSCP) is similar to that seen with OSCP alone. The presence of excess F$_6$ does not affect the binding parameters with respect to OSCP (data not shown). This observation again demonstrates that ATPase binds preferentially to OSCP sites in the membrane.

The lack of agreement on the role of F$_6$ in the binding of the ATPase to TUA-STA particles as suggested in the literature may arise from at least two uncertainties: qualitative...
observations and heterogeneous F₆ preparations. The F₆ effect had not previously been quantitatively analyzed except by Vandineau et al. (2). Our observation that the Kₐ for the OSCP-induced F₆-ATPase binding is 1-2 orders of magnitude greater than the binding in the presence of F₆ alone may be due to some differences between our preparations and that of Vandineau et al. (2). The OSCP used in their experiments was isolated from F₆X (F₆,OSCP) complex and is not homogeneous. We have isolated OSCP by the method published by Senior (7) and this preparation is homogeneous in gradient polyacrylamide gel (14-23%) in the presence of SDS.

The reported observation that OSCP does not increase the F₁ binding but only increases the rutamycin sensitivity (6) can be interpreted in light of our findings. In the absence of OSCP, F₁ binds to the unextracted F₆ sites (or some other yet unidentified F₁ binding site) in TUA particles (2), urea-F₆ (6), or NaBr F₆ (6, 14, 15); the bound ATPase therefore is insensitive to rutamycin. Upon addition of OSCP, F₁ now binds preferentially to OSCP sites instead of F₆ sites (or unidentified ATPase binding sites) in the membrane. The resultant particle ATPase activity may not be changed significantly, but the bound ATPase becomes rutamycin-sensitive. It is the presence of F₆ sites or yet undefined other ATPase binding sites on the membrane that marks the later binding effect of OSCP. By using TUA-STA particles which are depleted of OSCP as well as to some differences between our preparations and that of the same site in STA particles.

Rat liver TUA-STA particles appear to recognize OSCP and the 26,500-dalton protein equally well, since the binding of the Type II ATPase to rat liver particles in the presence of OSCP shows similar binding characteristics to that of the Type I ATPase. On the other hand, rat liver ATPase also recognizes OSCP and the 26,500-dalton protein equally well (data shown in the following paper). Further observations on the ³²P-ATP exchange in beef heart STA particles reconstituted with rat liver Type I, rat liver Type II and OSCP, or beef heart F₁ and OSCP indicate that as far as rat liver and beef heart mitochondria are concerned, the soluble ATPase, binding protein component, and the membrane components are completely interchangeable. Beef heart OSCP and yeast OSCP have been reported to be not interchangeable in yeast submitochondrial particles (16) with regard to the rutamycin sensitivity conferring activity, but partially interchangeable with regard to the ATPase-binding activity.

REFERENCES

1. Fisher, R. J., Liang, A. M., and Sundstrom, G. C. (1981) J. Biol. Chem. 256, 707-715
2. Vandineau, A., Berden, J. A., and Slater, E. C. (1976) Biochim. Biophys. Acta 449, 468-479
3. Knowles, A. F., Guilory, R. J., and Racker, E. (1971) J. Biol. Chem. 246, 2672-2679
4. Russel, L. K., Kirkley, S. A., Kleyman, T. R., and Chan, S. H. P. (1976) Biochim. Biophys. Res. Commun. 73, 434-443
5. Racker, E., Horstman, L. L., Kling, D., and Fessenden-Raden, J. M. (1969) J. Biol. Chem. 244, 6668-6674
6. Galante, Y. M., Wong, S.-Y., and Hatefi, Y. (1981) Arch. Biochem. Biophys. 211, 664-665
7. Senior, A. E. (1971) Bioenergetics 2, 141-150
8. Kanner, B. L., Serrano, R., Kandrich, M. A., and Racker, E. (1976) Biochem. Biophys. Res. Commun. 69, 1050-1056
9. Horstmann, L. L., and Racker, E. (1970) J. Biol. Chem. 245, 1336-1344
10. Joshi, S., Hughes, J. B., Shaikh, F., and Sanadi, D. R. (1979) J. Biol. Chem. 254, 10145-10152
11. Avron, M. (1960) Biochim. Biophys. Acta 40, 257-272
12. Bonsall, R. W., and Hunt, S. (1971) Biochim. Biophys. Acta 249, 266-280
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
14. MacLennan, D. H., and Tzagoloff, A. (1968) Biochemistry 7, 1603-1610
15. Glaser, E., Norling, B., and Emster, L. (1980) Eur. J. Biochem. 110, 225-235
16. Tzagoloff, A. (1970) J. Biol. Chem. 245, 1545-1551