Removal of "Tightly Bound" Nucleotides from Phosphorylating Submitochondrial Particles*

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Phosphorylating submitochondrial particles from beef heart (ETPH) prepared here contained about 2.4 nmol of ATP and 1.9 nmol of ADP/mg of protein after repeated washing of the particles. Essentially all of the "tightly bound" ATP and ADP was removed by trypsin treatment. The trypsin-treated ETPH had increased ATPase activity, undiminished NADH oxidase and succinate oxidase activity, but energy-coupling activity (ATP-driven reversed electron transfer) was abolished. Removal of half the ATP and ADP occurred at low levels of trypsin and was associated with loss of half of the coupling activity. Gel filtration of ETPH in high ionic strength buffer also removed ADP and ATP from the particles, resulting in loss of energy-coupling activity, while ATPase activity was increased.

The results support the contention that the tightly bound ADP is essential in energy coupling in mitochondria. Tightly bound ATP may also play an essential role.

**Experimental Procedure**

**Materials**

ETPH were prepared from beef heart mitochondria as described by Beyer (Ref. 2, p. 189). The ETPH showed P/O ratios of around 1.5 with succinate as substrate. Protein content of ETPH samples was estimated by the biuret method (3). All other preparations and chemicals have been described in the accompanying preceding paper (1).

**Methods**

**Nucleotide Content of ETPH**—The ATP and ADP content of washed ETPH was assayed fluorometrically following procedures described in the preceding paper (1). Samples of ETPH were prepared for analysis by washing four times in 0.25 M sucrose, 10 mM Tris/acetate, 1 mM EDTA, pH 7.5, centrifuging at 105,000 × g for 30 min between each wash to sediment the particles. The particles (0.5-ml samples) were extracted by addition of 10% (w/v) cold HClO4 to a final concentration of 4% (w/v). About 5 to 6 mg of particles were assayed per experiment. The denatured protein was removed by centrifugation and the supernatants assayed for ATP and ADP as described in the preceding paper (1). Gel filtration of ETPH at 0°C was done in columns (24 × 1.5 cm). The column buffers are described in Tables IV and V. ETPH was applied to the columns in samples of 0.5-ml volume containing 8 to 9 mg. The effluent particles were collected by centrifugation at 165,000 × g for 30 min and were resuspended in column buffer.

**Trypsin Treatment of ETPH**—Samples of ETPH (1 mg/ml) in 0.25 M sucrose, 10 mM Tris/acetate, 1 mM dithiothreitol, 1 mM ATP, 4 mM MgCl2, pH 7.5, were incubated at room temperature with varying amounts of trypsin added as a 1 mg/ml of solution in 1 mM H2SO4. Soybean trypsin inhibitor was added to stop the reaction (5-fold excess by weight). Control experiments showed that trypsin had no effects at all if added after the soybean inhibitor. Treated ETPH were then centrifuged (165,000 × g for 30 min) and resuspended in either 0.25 M sucrose, 10 mM Tris/acetate, 1 mM EDTA, pH 7.5 (for analysis of tightly bound nucleotides) or in 0.25 M sucrose, 10 mM Tris/acetate, 1 mM dithiothreitol, 4 mM MgCl2, pH 7.5 (for coupling and ATPase assays).

**ATPase Assays**—ATPase activity was assayed colorimetrically as described in the preceding paper (1).

**Results**

**Nucleotide Content of ETPH**—The content of tightly bound ATP and ADP in ETPH prepared here was slightly variable. Values found are shown in Table I. Effects of Trypsin on ATPase Activity, Nucleotide Content,
"Tightly Bound" Nucleotides in ETPH

TABLE I

"Tightly bound" nucleotide in ETPH

ETPH were washed and nucleotide content was assayed as described under "Methods." Trypsin treatment of ETPH was carried out as described under "Methods." Experiment A, first sample of ETPH. Experiment B, two more samples of ETPH which had lower nucleotide content. Nucleotide content is expressed as nanomoles per mg of ETPH ± S.D. with range and number of observations in parentheses.

| Sample                              | Nanomoles ATP/mg ETPH | Nanomoles ADP/mg ETPH |
|-------------------------------------|------------------------|------------------------|
| Experiment A                        |                        |                        |
| ETPH                                | 2.4 ± 0.4 (2.37-2.49; 5) | 1.91 ± 0.45 (1.48-2.00; 5) |
| ETPH plus trypsin inhibitor plus trypsin (125 µg/mg) | 2.35 ± 0.39 (2.28-2.47; 3) | 1.87 ± 0.41 (1.38-2.00; 3) |
| ETPH plus trypsin (10 µg/mg)        | 1.40 ± 0.05 (1.35-1.44; 3) | 0.82 ± 0.06 (0.76-0.88; 3) |
| ETPH plus trypsin (125 µg/mg)       | 0.28 (0.0-0.56; 3)     | 0 (3)                 |
| Experiment B                        |                        |                        |
| ETPH                                | 1.55 (2)               | 1.25 (2)               |

TABLE II

Abolition of energy-coupling activity of ETPH by trypsin

Samples were treated with trypsin and ATP-driven reversed electron transfer assays were done as described under "Methods.

| Sample                              | Energy-coupling activity (nmol NADH formed/min/mg) | "Tightly bound" nucleotide (ATP + ADP) |
|-------------------------------------|---------------------------------------------------|----------------------------------------|
| ETPH                                | 50.4 ± 100%                                       | 100%                                   |
| ETPH plus trypsin inhibitor plus trypsin (125 µg/mg) | 50.4 (± 100%)                                      | 90%                                    |
| ETPH plus trypsin (10 µg/mg)        | 23.5 ± 43%                                       | 51%                                    |
| ETPH plus trypsin (25 µg/mg)        | 11.4 ± 23%                                       | 51%                                    |
| ETPH plus trypsin (125 µg/mg)       | 0 ± 0%                                            | 0-12%                                  |

TABLE III

Levels of trypsin which abolish energy-coupling have no effect on NADH oxidase or succinate oxidase activity in ETPH

Oxygen uptake was measured polarographically at 30° for 2 min. The incubation medium contained in a final volume of 2 ml, pH 7.6, 1 mg of ETPH, 12 µmol of ATP, 12 µmol of MgSO₄, 35 µmol of KF, 10 µmol of Tris/SO₄, 0.96 µmol of EDTA, 120 µmol of sucrose, 0.5 mg of cytochrome c. Three micromoles of potassium succinate or 3 µmol of NADH were added to initiate the reaction.

| Substrate     | Sample       | O₂ uptake |
|---------------|--------------|-----------|
| Succinate     | ETPH         | 0.202     |
|               | Trypsin-ETPH (10 µg/mg) | 0.207     |
|               | Trypsin-ETPH (125 µg/mg) | 0.196     |
| NADH          | ETPH         | 0.111     |
|               | Trypsin-ETPH (10 µg/mg) | 0.115     |
|               | Trypsin-ETPH (125 µg/mg) | 0.109     |

Strength Buffers on ATPase Activity, Nucleotide Content, and Coupling Activity of ETPH—In the preceding paper we showed that gel filtration of F₁ in a buffer of high ionic strength removed nucleotide and eliminated coupling activity of F₁, while leaving ATPase activity unreduced and stable. We wished therefore to see if parallel experiments yielded similar results in ETPH.

First we passed ETPH through Sephadex G-25 columns in TE buffer (50 mM Tris/SO₄, 1 mM EDTA, pH 8.0) or in TEK buffer (50 mM Tris/SO₄, 1 mM EDTA, 60 mM K₂SO₄, pH 8.0) at either 4° or 20°. Energy-coupling activity was completely lost in all cases. Subsequently, by trial and error, we alighted upon two low ionic strength buffer systems in which energy-
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coupling activity was retained after gel filtration of ETPH at 4°C and these were (a) 0.25 M sucrose, 10 mM Tris/acetate, 4 mM MgCl₂, 1 mM dithiothreitol, pH 7.5; and (b) 0.25 M sucrose, 10 mM Tris/acetate, 1 mM dithiothreitol, pH 7.5 or pH 8.0. The effect of ionic strength on the coupling activity and the nucleotide content of ETPH was then ascertained by adding K₂SO₄ in increasing amounts to these buffers. The effects are documented in Tables IV and V. A correlation was noted between degree of removal of total tightly bound nucleotide and loss of energy-coupling activity. ATP could be removed completely without complete loss of coupling activity, and seemed to be removed more readily than ADP. In control experiments it was shown that addition of K₂SO₄ to the

energy-coupling assay itself did not cause inhibition of NAD reduction.

Gel filtration of ETPH in these buffers increased ATPase activity markedly, and the activity remained very sensitive to inhibition by oligomycin (Table VI).

**DISCUSSION**

The results presented here extend the observations of the preceding paper (in which soluble F₄ was studied) to the membranous ETPH system. Clearly the two techniques which we found efficacious in removing tightly bound nucleotide from soluble F₄ are able to remove them from membrane-bound F₄. Higher concentrations of trypsin were required in the

**TABLE IV**

**Gel filtration of ETPH in magnesium-containing buffer at varying ionic strength: effects on coupling activity and nucleotide content**

The gel filtration was done at 0°C as described under "Methods." The column buffer was 0.25 M sucrose, 10 mM Tris/acetate, 1 mM dithiothreitol, 4 mM MgCl₂, pH 7.5. "Tightly bound" ATP and ADP, and coupling activity were assayed in the particles as described under "Methods." Results are given as mean ± S.D. with range and number of observations in parentheses.

| Sample | Coupling activity | ATP | ADP | ATP + ADP |
|--------|-------------------|-----|-----|----------|
| ETPH (buffer alone) | 63.8 ± 4.98 (5) | 79.5 | 1.31 (1.12-1.55; 5) | 55 | 1.88 (1.12-2.00; 5) | 98 | 74 |
| ETPH (buffer + 20 mM K₂SO₄) | 55.8 ± 10.6 (5) | 69.6 | 1.29 (1.20-1.68; 3) | 54 | 1.84 (1.09-2.15; 5) | 96 | 73 |
| ETPH (buffer + 40 mM K₂SO₄) | 54.7 ± 4.06 (5) | 68.3 | 1.24 (1.20-1.28; 3) | 52 | 1.79 (1.10-2.00; 3) | 93 | 70.5 |
| ETPH (buffer + 60 mM K₂SO₄) | 36.05 ± 6.95 (5) | 32.5 | 0.77 (0.42-1.15; 5) | 32 | 1.36 (0.72-1.96; 5) | 71 | 49.5 |
| ETPH (buffer + 150 mM K₂SO₄) | 20.1 ± 1.70 (5) | 25.0 | 0.16 (0.11-0.30; 5) | 6.7 | 1.14 (0.47-1.65; 5) | 59 | 30.5 |
| ETPH (buffer + 300 mM K₂SO₄) | 22.7 ± 0.88 (5) | 28.3 | 0 (3) | 0 | 1.05 (0.89-1.40; 5) | 55 | 24 |
| ETPH (buffer + 500 mM K₂SO₄) | 18.6 ± 0.34 (5) | 23.2 | 0 (3) | 0 | 0.95 (0.89-1.10; 3) | 48 | 22 |
| Native ETPH (no column treatment) | 80.1 ± 5.99 (7) | 100 | 2.4 (2.37-2.49; 5) | 100 | 1.91 (1.48-2.00; 5) | 100 | 100 |

**TABLE V**

**Gel filtration of ETPH in magnesium-free buffer at varying ionic strength: effects on coupling activity and nucleotide content**

The technique is described in Table IV. Results are given as mean ± S.D. with range and number of observations in parentheses. The column buffer in these experiments was 0.25 M sucrose, 10 mM Tris/acetate, 1 mM dithiothreitol, pH 7.5.

| Sample | Coupling activity | ATP | ADP | ATP + ADP |
|--------|-------------------|-----|-----|----------|
| ETPH (buffer alone) | 63.8 ± 4.98 (5) | 79.5 | 1.31 (1.12-1.55; 5) | 55 | 1.88 (1.12-2.00; 5) | 98 | 74 |
| ETPH (buffer + 60 mM K₂SO₄) | 55.8 ± 10.6 (5) | 69.6 | 1.29 (1.20-1.68; 3) | 54 | 1.84 (1.09-2.15; 5) | 96 | 73 |
| ETPH (buffer + 150 mM K₂SO₄) | 54.7 ± 4.06 (5) | 68.3 | 1.24 (1.20-1.28; 3) | 52 | 1.79 (1.10-2.00; 3) | 93 | 70.5 |
| ETPH (buffer + 300 mM K₂SO₄) | 36.05 ± 6.95 (5) | 32.5 | 0.77 (0.42-1.15; 5) | 32 | 1.36 (0.72-1.96; 5) | 71 | 49.5 |
| ETPH (buffer + 500 mM K₂SO₄) | 20.1 ± 1.70 (5) | 25.0 | 0.16 (0.11-0.30; 5) | 6.7 | 1.14 (0.47-1.65; 5) | 59 | 30.5 |
| ETPH (buffer + 300 mM K₂SO₄) | 22.7 ± 0.88 (5) | 28.3 | 0 (3) | 0 | 1.05 (0.89-1.40; 5) | 55 | 24 |
| ETPH (buffer + 500 mM K₂SO₄) | 18.6 ± 0.34 (5) | 23.2 | 0 (3) | 0 | 0.95 (0.89-1.10; 3) | 48 | 22 |
| Native ETPH (no column treatment) | 80.1 ± 5.99 (7) | 100 | 2.4 (2.37-2.49; 5) | 100 | 1.91 (1.48-2.00; 5) | 100 | 100 |

**TABLE VI**

**ATPase activity of ETPH after gel filtration**

The 0.1-mg samples of ETPH collected from the column, centrifuged, and resuspended were assayed for 2 min at 30°C as described under "Methods." Oligomycin when present was added at 50 μg/mg protein final concentration. The pH of all column buffers was 7.5.

| Column buffer | ATPase activity | ATTrase |
|---------------|-----------------|---------|
| 0.25 M sucrose, 10 mM Tris/acetate, 1 mM dithiothreitol | 2.6 | 0.86 | 67 |
| Buffer plus 150 mM K₂SO₄ | 2.55 | 0 | 100 |
| Buffer plus 300 mM K₂SO₄ | 2.29 | 0.02 | 99 |
| Buffer plus 4 mM MgCl₂ | 1.12 | 0 | 100 |
| Buffer plus 4 mM MgCl₂ plus 300 mM K₂SO₄ | 1.40 | 0.19 | 86 |
| ETPH (no column treatment) | 0.96 | 0.22 | 77 |
brane-bound system, but control experiments showed that other essential elements of the energy-coupling system (NADH oxidase, succinate oxidase) were unaffected by the trypsin treatment, and the F₄ remained membrane-bound and oligomycin-sensitive. Juntti et al. (4) have previously found that trypsin treatment inactivated transhydrogenase activity and oxidative phosphorylation in "MgATP" submitochondrial particles, but had no effect on NADH oxidase, succinate oxidase, or oligomycin-sensitive ATPase. Similarly, some modifications were required in the gel filtration system (higher ionic strength was required) in order to remove all the tightly bound nucleotide, but again the F₄ remained membrane-bound, retained full oligomycin sensitivity, and the salt alone (K₂SO₄) did not affect the ATP-driven succinate → NAD electron transfer reaction.

The removal of tightly bound nucleotides by either method was associated with loss of energy-coupling activity. Thus the results support the recent hypotheses of Slater et al. (5-7) and Bover et al. (8-10) that tightly bound nucleotides play an essential role in energy-coupling systems. As we have discussed in the preceding paper (1) while it seems clear that tightly bound ADP is required for energy coupling in mitochondria, there is some doubt in our minds that tightly bound ATP is required since the soluble F₄ which we prepare here has no tightly bound ATP. Further it may be noted that gel filtration of ETPH (Tables IV and V) removed all tightly bound ATP at certain levels of ionic strength which left some significant residual coupling activity. However a better correlation was seen between loss of coupling activity and loss of total nucleotide than with loss of ADP alone. This doubt regarding the requirement for tightly bound ATP may be decided by measuring ATP and ADP content of both F₄ and ETPH after reconstitution, or incubation, or both, in the energy-coupling assay medium and we are currently engaged in these studies. As we have indicated in the preceding paper, we favor the view that whereas trypsin probably causes a subtle structural lesion in the F₄, gel filtration in high ionic strength buffer probably acts by inducing a conformational change in F₄. Thus we may be able to reverse the loss of coupling in ETPH caused by gel filtration in high ionic strength buffer, and experiments aimed at such reversal are in progress. It will be of great importance to find out whether reloading of tightly bound ADP and ATP occurs as coupling is restored.

Finally, the fact that membrane-bound ATPase activity is retained, indeed enhanced, in the nucleotide-depleted ETPH preparations supports the suggestions made in the previous paper, namely that non-energy-linked ATPase activity and membrane-binding properties of F₄ do not require tightly bound ATP or ADP.

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REFERENCES

1. Leimgruber, R. M., and Senior, A. E. (1976) J. Biol. Chem. 251, 7103-7109
2. Beyer, R. E. (1967) Methods Enzymol. 10, 186-195.
3. Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) J. Biol. Chem. 177, 751-766
4. Juntti, K., Torndal, V. B., and Ernster, L. (1970) in Electron Transport and Energy Conservation (Tager, J. M., Papa, S., Quagliariello, E., and Slater, E. C., eds) pp. 257-271, Adiagtica Editrice, Bari
5. Slater, E. C., Rosing, J., Harris, D. A., van de Stadt, R. J., and Kemp, A. (1974) in Membrane Proteins in Transport and Phosphorylation (Azzone, G. F., Klingenberg, M. E., Quagliariello, E., and Siliprandi, N., eds) pp. 139-147, North-Holland Publishing Co., Amsterdam
6. Harris, D. A., and Slater, E. C. (1975) Biochim. Biophys. Acta 387, 325-348
7. Harris, D. A., and Slater, E. C. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C., and Siliprandi, N., eds) pp. 379-384, North-Holland Publishing Co., Amsterdam
8. Bover, P. D., Cross, R. L., and Momsen, W. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 2837-2839
9. Bover, P. D., Stokes, B. O., Wolcott, R. G., and Degani, C. (1975) Fed. Proc. 34, 1711-1717
10. Bover, P. D., Smith, D. J., Rosing, J., and Kayalar, C. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E. C., and Siliprandi, N., eds) pp. 361-372, North-Holland Publishing Co., Amsterdam
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J. Biol. Chem. 1976, 251:7110-7113.