Anisotropic Inhibition of Energy Transduction in Oxidative Phosphorylation in Rat Liver Mitochondria by Tetraphenylarsonium*

(Received for publication, December 17, 1979)

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Tetraphenylarsonium (TPA+) inhibited energy transduction in oxidative phosphorylation in mitochondria but not in submitochondrial particles, which are inside-out relative to the membranes of mitochondria. TPA+ incorporated into the inside of submitochondrial particles inhibited ATP synthesis in the particles. TPA+ also inhibited the reduction of NAD by succinate coupled with oxidation of succinate by O2 and hydrolysis of ATP. Energization of mitochondrial inner membranes with succinate and with ATP induced binding sites on the membranes for TPA+. The amounts of energy-dependent binding sites for TPA+ on mitochondria energized with succinate and with ATP, respectively, were 90 and 13 nmol/mg of protein. TPA+ also caused shrinkage of mitochondria energized with succinate and with ATP in an energy-dependent fashion. The energy-dependent binding of TPA+, TPA+-induced H+-ejection, TPA+-induced shrinkage of mitochondria, and TPA+-induced inhibition of energy transduction occurred in parallel.

The present findings show that TPA+ inhibits energy transduction by binding to negative charges created on lipophilic domains near the surface of the outer side (C-side) of the mitochondrial inner membranes, and that it has no inhibitory activity on the inner side (M-side) of the membranes.

Recently, it has been shown in this laboratory (1–5) that positively charged anisotropic inhibitors (e.g. ethidium and acriflavine) inhibit energy transduction in oxidative phosphorylation in mitochondria by binding to, and neutralizing negative charges created on the outer side (C-side) of the membranes, and that these had no inhibitory activity on the inner side (M-side) of the membranes.

These findings suggest (1) that the negative charges appeared on the C-side of the energized mitochondria are part of and essential for energy-transducing mechanism, in good accord with the previous findings (6, 7). This idea suggests that some amphipathic cations inhibit energy transduction in oxidative phosphorylation by binding to, and neutralizing the negative charges created on the surface of the C-side. The present paper shows that TPA+ also inhibited energy transduction in oxidative phosphorylation by binding to negative charges created on lipophilic domains near the surface of the C-side of mitochondria, and that it had no inhibitory activity on the M-side of the membranes. A preliminary account of this work has appeared (4).

MATERIALS AND METHODS

Tetraphenylarsonium chloride and sodium tetraphenylboron were purchased from Tokyokasei, Tokyo, Japan. Silicone oil (SH-550) was a product of Nakarai Chemicals Co., Kyoto, Japan. Other reagents were as described previously (1).

Rat liver mitochondria were isolated by the method of Hogeboom (8), as described by Myers and Slater (9), except that 0.25 m sucrose containing 2 mM Tris (pH 7.4) was used for homogenization and two washings. Sondicated submitochondrial particles were prepared by a modification of the method of Hansen and Smith (10) as follows. Rat liver mitochondria were suspended at a concentration of 40 mg of protein/ml in medium (pH 7.4) containing 0.25 m sucrose, 10 mM Tris, 1 mM ATP, 1 mM MgCl2, 1 mM sodium succinate and maintained at −15°C overnight. The mitochondrial suspension was thawed just before preparation of the particles, diluted to a concentration of 20 mg of protein/ml with the same medium, and treated in a Kubota, model 200M, Inosanet at 180 wats for 30 s at about 2°C. The resulting suspension was centrifuged at 25,000 × g for 20 min and the supernatant was recentrifuged at 144,000 × g for 30 min. The final pellet was suspended in 0.25 m sucrose (containing 2 mM Tris) and was used as the preparation of submitochondrial particles.

Incorporation of TPA+ into submitochondrial particles was as follows. The mitochondrial (1 mg of protein/ml) were incubated for 5 min at 25°C in medium (pH 7.4) containing 400 μM TPA+, 10 mM succinate, 0.4 μg of rotenone/mg of protein, 5 mM MgCl2, 2 mM EDTA, 15 mM KCl, 50 mM sucrose, 25 mM Tris. The resulting suspension was rapidly cooled to about 0°C and then centrifuged at 18,000 × g for 10 min. The precipitate was used as the starting material for preparing submitochondrial particles as described above except that the mitochondria were sonicated in the medium for the preparation of submicronochondrial particles containing 500 nmol of TPA+/mg of protein.

TPA+-induced H+-ejection from mitochondria is measured as follows. Mitochondria (1 mg of protein/ml) were preincubated for 3 min at 25°C in the presence of 2 mM succinate or 2 mM ATP, 2 μg of rotenone, 5 mM MgCl2, 2 mM EDTA, 20 mM KCl, 183 mM sucrose, 1 mM Tris, in a final volume of 3 ml at pH 7.0. The reaction was started by adding TPA+ and was followed with a Hitachi-Horiba, model F-7, expanded scale pH meter equipped with a Horiba pH electrode (6028-107).

Fluorescence change of NAD was determined with a Hitachi, model MPF3, spectrofluorometer, using the wavelength of 360 nm for excitation and measuring fluorescence at 450 nm. A glass filter (V-Y43) was placed in front of the photomultiplier to eliminate the actinic light.

For measurement of binding of TPA+ to mitochondria, the mitochondria (1 mg of protein/ml) were incubated for 5 min with a known

*This work was supported by grants from the Ministry of Education, Science and Culture of Japan (Grants 488080 and 411309). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: TPA+, tetraphenylarsonium; TPB-, tetraphenylboron.
concentration of TPA+ in the presence of 10 mm succinate (+0.75 μg of rotenone) or 2 mm ATP (+0.15 μg of antimycin A), 5 mm MgCl2, 2 mm EDTA, 25 mm Tris, 180 mm sucrose at pH 7.4 in a final volume of 1.5 ml at 25°C. The incubation mixture was shaken during the reaction. Then the mixture was rapidly cooled to about 0°C, layered over 1 drop of silicone oil, and centrifuged at 8000 × g for 2 min in an Eppendorf, model 5000, microcentrifuge, and the remaining cation was determined spectrophotometrically by measuring the red shift in the absorbance maximum of ethidium on its reaction with TPB− using a Hitachi, model 556, two-wavelength double beam spectrophotometer, as described elsewhere (3).

Protein was estimated from the contents of cytochromes a + a₃ in mitochondria and submitochondrial particles as described previously (11). The amount of ³²P-labeled substances was determined by the method of Nielsen and Lehninger (12) as modified by Avron (13).

RESULTS

Anisotropy of the Mitochondrial Inner Membranes with Respect to Inhibition of Energy Transduction by TPA+—TPA+ at 75 μM inhibited the increased rate of oxygen uptake with a phosphate acceptor (State 3) in mitochondria with succinate (Fig. 1). With up to 75 μM TPA+, this inhibition was released completely by addition of the uncoupler 2,4-dinitrophenol (DNP), but with above 100 μM TPA+ the inhibition was not completely released by the uncoupler (Figs. 1 and 2). TPA+ had the same effect with glutamate and malate as substrates. Energy-dependent binding of TPA+ to the mitochondrial membranes energized with succinate occurred even with 300 μM TPA+, as mentioned later (Fig. 6). Therefore, these results show that TPA+ does not inhibit electron transport, but inhibits energy transduction in oxidative phosphorylation. At concentrations of above 50 μM, TPA+ slightly stimulated State 4 respiration, as reported previously by Mon- tal et al. (14). Fig. 1 also shows that TPB− reversed the inhibition of State 3 respiration by TPA+. This reversal was complete on addition of TPB− at the same concentration as TPA+.

TPA+ also inhibited ATP-Pi exchange in mitochondria (data not shown). Fig. 3 shows that 75 μM TPA+ completely inhibited ATP synthesis in mitochondria, but that concentrations of up to 550 μM TPA+ only slightly inhibited ATP synthesis in submitochondrial particles, which are inside-out oriented under "Materials and Methods," inhibited ATP synthesis in the particles, as shown in Table I.

These results indicate that like ethidium and acriflavine (1, 2), TPA+ inhibited energy transduction in oxidative phosphorylation by acting on the outer side (C-side) of mitochondrial inner membranes, and that it had no inhibitory activity on the inner side (M-side) of the membranes.

TPA+ also inhibited the reduction of NAD by succinate.
TABLE I

Effect of incorporation of TPA⁺ into submitochondrial particles on ATP synthesis in the particles

<table>
<thead>
<tr>
<th>Submitochondrial particles</th>
<th>ATP synthesis (nmol ATP/mg protein min⁻¹ %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control particles</td>
<td>21</td>
</tr>
<tr>
<td>Particles containing TPA⁺</td>
<td>0.8</td>
</tr>
</tbody>
</table>

TPA⁺ was introduced into the submitochondrial particles as described under "Materials and Methods." Control particles were obtained similarly, but without the addition of TPA⁺. Other conditions were as for Fig. 3.

Fig. 4. Inhibition by TPA⁺ of respiration (succinate — O₂)-linked reduction of NAD by succinate in mitochondria. Mitochondria (1 mg of protein/ml) were preincubated for 5 min at room temperature in the presence of 3 μg of oligomycin/mg of protein, 5 mM MgCl₂, 2 mM EDTA, 15 mM KCl, 50 mM sucrose, 25 mM Tris, in a final volume of 3.0 ml at pH 7.4. Succinate, antimycin A, and TPA⁺ were added as indicated. Fluorescence changes of NAD were measured as described under "Materials and Methods."

Fig. 5. Effects of TPA⁺ concentration on ATP-linked, and respiration (succinate — O₂)-linked reduction of NAD by succinate in mitochondria. Conditions were as for Fig. 4 except that the indicated concentrations of TPA⁺ were used. Complete inhibitions of reduction of NAD by succinate coupled with oxidation of succinate by O₂ and hydrolysis of ATP were taken as the decrease in the fluorescence at 450 nm caused by addition of antimycin A or oligomycin, respectively.

Fig. 6. Binding of TPA⁺ to mitochondria energized with succinate. Conditions were as described under "Materials and Methods."

Fig. 7. Dose-response curves of the ATP-dependent binding of TPA⁺ to mitochondria and of its inhibition of ATP synthesis in the membranes. Conditions were as described under "Materials and Methods." The amount of ATP-dependent binding of TPA⁺ was calculated by subtracting the amount of energy-independent binding (+ ATP and 3 μg of oligomycin/mg of protein) from the total binding. The percentage inhibition of ATP synthesis in mitochondria was obtained from Fig. 3.

coupled with oxidation of succinate by O₂ and hydrolysis of ATP in mitochondria (Figs. 4 and 5). Fig. 5 also shows that the dose-response curves for the inhibition of the reversal electron flow in the membranes closely coincided with the dose-response curves for the energy-dependent binding of TPA⁺ to the membranes energized with succinate and ATP, respectively (Figs. 6 and 7). These inhibitions are clearly different from those of well known inhibitors of energy transduction in oxidative phosphorylation, such as oligomycin (19, 20), dicyclohexylcarbodiimide (19, 20), and aurovertin (19), because the latter inhibit the reduction of NAD by succinate which is dependent on ATP, but not that which is dependent on respiration. These results are similar to guanidines, which are inhibitors of energy transduction (21–24).

Energy-dependent Binding of TPA⁺ to Mitochondria—Typical plots of the amount of TPA⁺ bound to mitochondria energized with succinate as substrate (A) and nonenergized with antimycin A (B) as a function of the concentration of added TPA⁺ are shown in Fig. 6. The amounts of succinate-dependent binding of TPA⁺ (A) and ATP-dependent binding of TPA⁺ (Fig. 7) increased to saturation levels at concentrations of about 300 μM and 75 μM, respectively. Fig. 7 also shows that, as in the case of ethidium (5),
Anisotropic Inhibition of Energy Transduction

The ratios of the amount of ejected H⁺ to the amount of TPA⁺ bound to mitochondria energized with succinate and ATP in an energy-dependent fashion were 0.4 to 0.7 and 0.9 to 1, respectively. TPA⁺ also caused shrinkage of mitochondria energized with succinate and with ATP in an energy-dependent fashion (Fig. 10).

Similarity of the Dose-Response Curves for the Various TPA⁺-induced Phenomena—Fig. 11(A) clearly shows that the dose-response curve of the ATP-dependent binding of TPA⁺ to mitochondria closely coincided with the dose-response curves for the inhibition by TPA⁺ of ATP synthesis in the membranes, TPA⁺-induced H⁺-ejection from the membranes energized with ATP, TPA⁺-induced shrinkage of the membranes energized with ATP, and the inhibition by TPA⁺ of the reduction of NAD by succinate coupled with ATP hydrolysis in the membranes. Similarly, the dose-response curves for four TPA⁺-induced phenomena in mitochondria energized with succinate coincided closely; namely, succinate-dependent binding of TPA⁺ to the membranes, TPA⁺-induced H⁺-ejection from the membranes energized with succinate, TPA⁺-induced shrinkage of the membranes energized with succinate, and inhibition by TPA⁺ of the reduction of NAD by succinate coupled with oxidation of succinate by O₂, as

the dose-response curve of the ATP-dependent binding of TPA⁺ to the membranes closely coincided with the dose-response curve for its inhibition of ATP synthesis in the membranes, indicating that the energy-dependent binding of TPA⁺ to the membranes inhibits energy transduction in oxidative phosphorylation.

Scatchard plots (25) of the data on succinate-dependent binding of TPA⁺ and ATP-dependent binding of TPA⁺ are shown in Fig. 8. The number of binding sites of TPA⁺ in mitochondria energized with succinate and ATP, represented as the intercepts of the linear portions of the plots, were 90 and 13 nmol/mg of protein, respectively. The apparent dissociation constants (Kd values) of the TPA⁺-mitochondrion complexes formed with succinate and ATP, evaluated from the slopes of the lines, were 33 and 18 μM, respectively. The amount of TPA⁺ bound to mitochondria energized with both succinate and ATP was the same as that bound to the membranes energized with succinate only. Fig. 9 shows that TPA⁺ bound competitively with ethidium in energized mitochondria, indicating that it combined with the same binding site as ethidium in the membranes in an energy-dependent fashion. Previously, we showed (1, 3) that ethidium binds to negative charges on the surface of the C-side in an energy-dependent fashion. Thus, TPA⁺ must also bind to these negative charges on the C-side.

The addition of TPA⁺ to mitochondria energized with succinate and ATP also caused H⁺-ejection into the medium, in good accordance with the results found by Hirose et al. (26).

Fig. 8. Scatchard plot of energy-dependent binding of TPA⁺ to mitochondria. Values were calculated from data in Figs. 6 and 7.

Fig. 9. Competitive inhibition by TPA⁺ of energy-dependent binding of ethidium to mitochondria energized with succinate. Conditions were as for Fig. 6 except that the amount of ethidium was estimated by measuring A600 with a Hitachi, model 556, two-wavelength, double beam spectrophotometer.

The amounts of ejected H⁺ to the amount of TPA⁺ bound to mitochondria energized with succinate and ATP in an energy-dependent fashion were 0.4 to 0.7 and 0.9 to 1, respectively. TPA⁺ also caused shrinkage of mitochondria energized with succinate and with ATP in an energy-dependent fashion (Fig. 10).

Similarity of the Dose-Response Curves for the Various TPA⁺-induced Phenomena—Fig. 11(A) clearly shows that the dose-response curve of the ATP-dependent binding of TPA⁺ to mitochondria closely coincided with the dose-response curves for the inhibition by TPA⁺ of ATP synthesis in the membranes, TPA⁺-induced H⁺-ejection from the membranes energized with ATP, TPA⁺-induced shrinkage of the membranes energized with ATP, and the inhibition by TPA⁺ of the reduction of NAD by succinate coupled with ATP hydrolysis in the membranes. Similarly, the dose-response curves for four TPA⁺-induced phenomena in mitochondria energized with succinate coincided closely; namely, succinate-dependent binding of TPA⁺ to the membranes, TPA⁺-induced H⁺-ejection from the membranes energized with succinate, TPA⁺-induced shrinkage of the membranes energized with succinate, and inhibition by TPA⁺ of the reduction of NAD by succinate coupled with oxidation of succinate by O₂, as
shown in Fig. 11(B). Therefore, it is concluded that these TPA'-induced phenomena occurred in parallel.

**DISCUSSION**

The present experiments showed that TPA' inhibited energy transduction of oxidative phosphorylation in mitochondria but not in submitochondrial particles, which are inside-out relative to the membranes of mitochondria (15-18).

This sidedness of the inhibition could be explained by supposing that TPA' penetrates the membranes, becoming concentrated inside the mitochondria (minus inside), and that it is extruded from the particles (plus inside) electrophoretically, i.e., in a manner dependent on the transmembrane potential (27-31). Accumulation of this cation in the mitochondrial matrix inhibited ATP synthesis by acting on phosphorylative enzymes on the side of the membranes facing the matrix.

However, as with ethidium and acriflavine (1, 2, 4), this explanation does not account satisfactorily for the following observations:

1. If the energy-dependent binding (uptake) of TPA' to the mitochondria is due to electrophoretic transport down the transmembrane potential, the amount of TPA' taken up into the membranes should be proportional to the amount of added TPA', as shown in Fig. 12. However, on increasing the amount of added TPA' the energy-dependent binding of TPA' to the mitochondria became saturated (Figs. 6 and 7). Scatchard plots of the data in these figures showed that the amounts of TPA' bound to membranes energized with succinate and ATP were 90 and 13 nmol/mg of protein, respectively (Fig. 8). Thus, these results clearly show that the energy-dependent binding of TPA' is not due to electrophoretic transport down the transmembrane potential.

2. TPA' incorporated into the submitochondrial particles inhibited ATP synthesis in the particles (Table 1). In this experiment TPA' was not present in the suspension medium and energization of the TPA'-containing particles with succinate did not cause release of TPA' from the particles into the medium. Therefore, these results and Fig. 3 show that TPA' inhibited ATP synthesis by acting on the C-side of the membranes. These conclusions are consistent with the facts that TPA' is freely soluble in water, but the inside of the membranes is composed of hydrophobic components.

3. TPA' competed with ethidium for its binding site in mitochondria (Fig. 9). Recently, we2 found in experiments on photoaffinity labeling of mitochondria by monoozide ethidium (not diazide ethidium (33)) that ethidium specifically binds to a protein of mitochondria (molecular weight about 10,000 in the presence of 0.1% sodium dodecyl sulfate) in a molar ratio

\[ \Delta \psi = 596 \log \left( \frac{[TPA']_{mem}}{[TPA']_{extr}} \right) \text{(mV at } 25^\circ\text{C)} \]

\[ \text{Amount of TPA' taken up into the mitochondrial matrix (nmol/mg protein)} \]

\[ \Delta \psi = 180 \text{ mV} \]

\[ \Delta \psi = 140 \text{ mV} \]
of 14 monoazide ethidium to the protein, in energy-dependent fashion. TPA' competitively inhibited the binding of monoazide ethidium to the protein. These findings will be described in detail in a later paper. Ethidium binds to negative charges created on the surface of the C-side (1, 3), and thus, TPA' must also bind to these sites. Therefore, it is concluded that TPA' inhibited energy transduction in oxidative phosphorylation by binding to negative charges created on the surface of the C-side, and that it had no inhibitory activity on the inner side (M-side) of the membranes.

4. TPA' caused shrinkage of mitochondria energized with succinate and with ATP in an energy-dependent fashion (Figs. 10 and 11). If the energy-dependent binding of TPA' is due to electrophoretic transport down the transmembrane potential, TPA' should cause swelling, rather than shrinkage of the mitochondria.

As seen from Table I, the inhibitory effect of TPA' on ATP synthesis in mitochondria is not due to modification of the transport systems for respiratory substrates, inorganic phosphate, and adenine nucleotide. This finding is supported by the facts that energy-dependent binding of TPA' to the mitochondria and TPA'-induced H' ejection from the membranes occur with either respiratory substrates or ATP (Figs. 6 to 11).

It is not clear at present how TPA' inhibits energy transduction. However, it may be important to note that the four phenomena occurred in parallel; namely, energy-dependent binding of TPA', TPA'-induced shrinkage, TPA'-induced H' ejection, and TPA'-induced inhibition of energy transduction, as clearly shown in Fig. 11.

In conclusion, TPA' inhibited energy transduction in oxidative phosphorylation by binding to negative charges created on lipophilic domains near the surface of the C-side of mitochondrial inner membranes, but it had no inhibitory activity on the inner side (M-side) of the membranes.

Acknowledgments—T. H. wishes to thank Professor Takekazu Hori (Institute for Protein Research, Osaka University) for his helpful suggestions and continuous encouragement.

REFERENCES