Energy-dependent Uptake of N-Methyl-4-phenylpyridinium, the Neurotoxic Metabolite of 1-Methyl-4-Phenyl-1,2,3,6-tetrahydropyridine, by Mitochondria*

(Received for publication, January 30, 1986)

Rona R. Ramsay§§, and Thomas P. Singer§§§

From the §Molecular Biology Division, Veterans Administration Medical Center, San Francisco, California 94121, and the Departments of *Pharmacological Chemistry and §Biochemistry-Biophysics, University of California, San Francisco, California 94143

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), an impurity in certain batches of illicit heroin substitutes, is known to cause parkinsonian symptoms and degeneration of the nigrostriatal cells in drug abusers and primates. Neurotoxicity depends on oxidation of MPTP by monoamine oxidase in brain cells to the dihydropyridinium form, which is further oxidized to N-methyl-4-phenylpyridinium (MPP'), the 4-electron oxidation product. The latter is widely believed to be the compound responsible for neuronal destruction and the NADH dehydrogenase of the inner membrane has been postulated to be its target. This enzyme is inhibited, however, only at very high concentrations of MPP', while the steady-state concentration of MPP' in the nigrostriatal cells of MPTP-treated animals is several orders of magnitude lower. This paradox has now been resolved by the discovery of an energized uptake system for MPP' in mitochondria which rapidly concentrates MPP' to very high concentrations in the mitochondria at micromolar external concentrations. The process is dependent on the electrical gradient of the membrane, has a K_m of about 5 mM, and is completely blocked by respiratory inhibitors and uncouplers.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP1), an impurity in certain batches of the illicit drug "new heroin," has been identified as the compound responsible for the parkinsonian symptoms which appeared in the drug users (1). It was shown that neurotoxicity developed only after enzymic oxidation of MPTP in brain tissue (2). The enzyme responsible was monoamine oxidase, since its selective inhibitors blocked the oxidation of MPTP in vitro (2, 3) and its neurotoxicity in vivo (4, 5). Both the A and B types of monoamine oxidase process MPTP relatively rapidly, particularly the B type, initially to MPDP', which is then further oxidized by monoamine oxidase, as well as nonenzymatically to MPP' (3, 6). MPTP and its oxidation products are competitive inhibitors of both forms of monoamine oxidase; MPTP and MPDP' are also mechanism-based inhibitors of these enzymes (3, 6).

Most workers in the field believe that MPP' is the form responsible for the destruction of nigrostriatal cells and consequent dopamine depletion and neurological symptoms. Despite the presence of monoamine oxidases A and B in many tissues, only the cells of the substantia nigra are irreversibly destroyed in susceptible species (man and other primates). It has been shown that, following the enzymatic oxidation of MPTP, the MPP' diffuses from the astrocytes and is selectively bound by the dopamine uptake system in the synapses and transported to the stroma of dopaminergic neurons (7).

Cell death has been proposed to occur as a result of inhibition by MPP' of the oxidation of NADH-linked substrates in the mitochondria and consequent ATP depletion (8). We have shown that MPP' blocked NADH oxidation at moderate concentrations (~0.5 mM) only in intact mitochondria, not in inverted inner membranes (ETP) or Complex I (9). The carriers of the NADH-linked substrates (d- and tricarboxylate carriers) and the dehydrogenases which initiate the oxidation of these substrates were not inhibited by MPP'. NADH oxidation in inverted inner membranes (ETP) or Complex I could be inhibited by high concentrations (>10 mM) of MPP'. It appeared likely, therefore, that a concentration mechanism for MPP' might exist in intact mitochondria.

We report here on the existence of an energy-dependent process in rat liver mitochondria which rapidly concentrates MPP' against its concentration gradient. The uptake is energized by the oxidation of succinate or glutamate plus malate and by ATP itself. Respiratory inhibitors and uncouplers prevent the uptake and cause release of the accumulated MPP' from the mitochondria. The energy-dependent uptake system is saturable with a K_m of 5 mM at 25 °C, but major accumulation of MPP' in the mitochondria occurs even at the low concentrations of MPP' which have been measured in the nigrostriatal cells of MPTP-treated animals.

**EXPERIMENTAL PROCEDURES**

Liver mitochondria were isolated by the method of Schuttelman and Greenawalt (10). [3H]Methyl-MPP' was from New England Nuclear and unlabeled MPP' as in previous work (3, 6). Dopamine, antimony A, valinomycin, and nigericin were from Sigma. The uptake of labeled MPP' was measured as follows. Fresh mitochondria were suspended in 3.3 ml of 0.15 mM KCl, 50 mM EGTA, 20 mM MOPS, pH 7.4, at 25 °C to give 8 to 10 mg of protein/ml. [3H]MPDP' (112 cpm/nmol) was added to zero time to a concentration of 0.5 mM, unless otherwise indicated. Samples of 0.5 ml were removed and centrifuged for 1.2 min in a microcentrifuge. The pellets were resuspended in 0.2 ml of water and deproteinized with 50 μl of 28% (v/v) HClO_4. The radioactivity in the original supernatant and in the pellet extract were measured in a scintillation counter. Intramitochondrial space, measured as the difference between the [3H]H_2O and [3H]glucose penetration volumes (11), averaged 0.6 μl/mg in the experiments reported.
Mitochondrial Uptake of MPP⁺

**Fig. 1.** Effect of substrates, inhibitors, and uncoupler on the uptake of MPP⁺ by rat liver mitochondria. Experiments shown in A, B, and C used different preparations of mitochondria. Uptake was measured as described under "Experimental Procedures." In A the protein was 8.9 mg·ml⁻¹ and other additions were: ○, no addition (MPP⁺ alone); □, 5 mM glutamate + 2.5 mM malate; △, 5 mM succinate; ▲, 5 mM succinate + 60 µM 2,4-dinitrophenol; ●, 10.6 µM antimycin + 10 µg·ml⁻¹ rotenone; ■, 5 mM glutamate + 2.5 mM malate + 10.6 µM antimycin + 19 µg·ml⁻¹ rotenone; and X, 5 mM glutamate + 2.5 mM malate + 60 µM 2,4-dinitrophenol. In B the protein was 9.2 mg·ml⁻¹ and the additions were: circles, 5 mM glutamate + 2.5 mM malate + 2 mM ADP + 2 mM MgCl₂ + 2 mM phosphate either preincubated for 2 min (State 4, ○) or not (State 3, ●); squares, the same (without preincubation) plus 60 µM 2,4-dinitrophenol (□) or plus 10.6 µM antimycin + 10 µg·ml⁻¹ rotenone (■); ▲, 5 mM ATP alone. In C the protein was 9.3 mg·ml⁻¹ and the additions were: 5 mM glutamate, 2.5 mM malate, 2 mM ADP, 2 mM MgCl₂ and 2 mM phosphate. At 10 min, 60 µM 2,4-dinitrophenol was added.

**RESULTS AND DISCUSSION**

Fig. 1 illustrates the time course of the uptake of 0.5 mM MPP⁺ by rat liver mitochondria. In previous studies (8, 9) 0.5 mM MPP⁺ completely blocked the oxidation of NAD⁺-linked substrates in rat liver and brain mitochondria in State 3. As seen in Fig. 1A, significant uptake was observed without an added energy source but the reaction was less extensive, presumably because the available energy was exhausted. The presence of glutamate plus malate or of succinate greatly increased both the rate and amount of uptake, whereas respiratory inhibitors (rotenone plus antimycin A) or uncoupler (2,4-dinitrophenol) completely prevented uptake. The rate of uptake in the presence of substrates was the same with and without added ADP (Fig. 1B). Added ATP alone supported extensive uptake (Fig. 1B). Addition of an uncoupler in the course of uptake supported by glutamate plus malate caused immediate efflux of the accumulated MPP⁺ (Fig. 1C). These results demonstrate that an energy-dependent accumulation of MPP⁺ occurs against its concentration gradient.

Fig. 2 demonstrates that the driving force for the accumulation of MPP⁺ is the electrical potential. Valinomycin, which collapses the transmembrane potential (ΔΨ), completely abolishes uptake of MPP⁺. In contrast, nigericin, which equilibrates protons across the membrane, enhances uptake. It has been shown (12) that the transmembrane potential and pH gradient of energized mitochondria may be varied independently. The membrane potential is decreased by increasing the external concentration of K⁺ in the presence of valinomycin without affecting ΔpH. Conversely, the pH gradient has been shown to be altered by varying the concentration of inorganic phosphate. Using the conditions described (12), increasing the concentration of K⁺ progressively inhibited the uptake of MPP⁺, while increasing the concentration of phosphate was without effect. It would seem from these data that the driving force for the energy-dependent uptake of MPP⁺ by mitochondria is the membrane potential.

Fig. 3 shows the concentration dependence of MPP⁺ uptake in the presence of glutamate plus malate, with or without respiratory inhibitors. In the presence of respiratory inhibitors or uncoupler the “uptake” of MPP⁺ is constant with time and is linearly dependent on the concentration of MPP⁺ in the medium (Fig. 3). This phenomenon is probably due to binding of the lipophilic phenyl group to the mitochondrial membrane. Though passive equilibration of MPP⁺ across the membrane cannot be excluded, if it occurs, it must do so very rapidly, since this binding does not alter after 30 s. The amount of nonspecific binding is approximately 4 times that expected for passive equilibration. In actively respiring mitochondria a saturable accumulation of MPP⁺ is superimposed on this binding. From the double reciprocal plot (Fig. 3, inset) a Kₑₐₚ = 5.3 mM and V = 51 nmol·min⁻¹·mg⁻¹ were calculated. However, rapid and extensive accumulation was observed even at the low concentration of MPP⁺ (~30 µM) which has been measured² in the nigrostriatal cells of mice treated with MPP⁺. When mitochondria were incubated with 39 µM MPP⁺ for only 30 s, 16% of the total MPP⁺ added was found in the matrix. The internal concentration was calculated to be 0.9 mM, representing a 23-fold concentration from the external medium. In the experiment shown in Fig. 2, mitochondria

² T. Shinka and N. Castagnoli, Jr., unpublished data.
respiring in the presence of glutamate plus malate and ADP accumulated MPP\(^+\) to a matrix concentration of 24.4 mM in 10 min, leaving only 0.34 mM MPP\(^+\) outside of the mitochondria (a 72-fold concentration). It is clear that the energized uptake is rapid and extensive. It seems likely, therefore, that with low external concentration of MPP\(^+\), all of the inhibitor would eventually accumulate in the matrix.

The effect of temperature on the uptake was measured in mitochondria preloaded with ATP by preincubation with glutamate plus malate and ADP (Fig. 4). The process is highly temperature-dependent, as has been observed with other mitochondrial uptake systems (13). The energy of activation cannot be calculated from these data, however, since they may represent the summation of the effects of temperature on the rate of uptake and on the \(K_a\) for MPP\(^+\).

The experiments described posed two important questions. First, if a carrier-mediated uptake is involved, what is its physiological substrate? Since MPP\(^+\) is a quaternary nitrogenous compound, positively charged metabolites, such as choline and arginine, appeared to be possibilities. At 1 mM concentration, choline inhibited the uptake of 0.5 mM MPP\(^+\) marginally and arginine not at all. MPTP and 4-phenylpyridine (1 mM) also inhibited only slightly. The second question is whether this uptake system is part of the sequence of biochemical events which leads from MPTP to the destruction of nigrostriatal cells. Recent, unpublished experiments in collaboration with Dr. A. Trevor (University of California) have shown that MPP\(^+\) is taken up in mitochondrial preparations from rat brain under the same conditions as in liver. The uptake by midbrain mitochondria may be distinguished from MPP\(^+\) binding to synaptosomes (7) by virtue of the fact that the latter is inhibited by dopamine; the former is not: 1 mM dopamine was without effect on MPP\(^+\) uptake in liver. In midbrain preparations the effect of 1 mM dopamine was marginal, but in preparations containing cortical mitochondria the majority, but not all, of the uptake was sensitive to respiratory inhibitors and 1 mM dopamine gave partial inhibition, showing that in the cortical preparation both mitochondria and synaptosomes were present.

The experiments described reconcile the observations that in intact mitochondria brief preincubation with 0.5 mM or less MPP\(^+\) blocks respiration with NAD\(^+\)-linked substrates (8, 9) but in inverted mitochondria, inner membrane preparations, and in Complex I far higher concentrations of MPP\(^+\) are required to block NADH oxidation (14). Since inhibition of mitochondrial NADH dehydrogenase leads to cessation of oxidative phosphorylation and since central nervous system cells are known to depend on aerobic ATP synthesis for maintenance of cellular integrity, it is tempting to think that the energized MPP\(^+\) concentration mechanism described here is part of the series of biochemical events which lead from the penetration of MPTP across the blood-brain barrier to neuronal destruction. This suggestion must remain tentative, however, until the resistance of other cell types, e.g. liver cells which contain both monoamine oxidase and the MPP\(^+\) uptake system, is satisfactorily explained.

REFERENCES

1. Langston, J. W., Ballard, P., Tetrad, J. W., and Irwin, I. (1983) Science 219, 979–980
2. Chiba, K., Trevor, A., and Castagnoli, N., Jr. (1984) Biochem. Biophys. Res. Commun. 120, 574–578
3. Salach, J. I., Singer, T. P., Castagnoli, N., Jr., and Trevor, A. (1984) Biochem. Biophys. Res. Commun. 125, 831–835
4. Heikkila, R. E., Manzino, L., Cabbat, F. S., and Duvoisin, R. C. (1984) Nature 311, 467–469
5. Langston, J. W., Irwin, I., Langston, E. B., and Forno, S. (1984) Science 225, 1480–1482
6. Singer, T. P., Salach, J. I., and Crabtree, D. (1985) Biochem. Biophys. Res. Commun. 127, 707–712
7. Javitch, J. A., D'Amato, R. J., Strittmatter, S. M., and Snyder, S. H. (1984) Proc. Natl. Acad. Sci. U. S. A. 82, 2173–2177
8. Nicklas, W. J., Vyas, I., and Heikkila, R. E. (1985) Life Sci. 36, 2503–2508
9. Ramsay, R. R., Salach, J. I., and Singer, T. P. (1986) Biochem. Biophys. Res. Commun. 134, 743–748
10. Schnaitman, C., and Greenwall, J. W. (1968) J. Cell Biol. 38, 158–175
11. Pfaff, E., Klingenberg, M., Ritt, E., and Vogell, W. (1968) Eur. J. Biochem. 5, 222–233
12. Klingenberg, M., and Rottenberg, H. (1977) Eur. J. Biochem. 73, 125–130
13. Halestrap, A. P. (1975) Biochem. J. 148, 85–96
14. Ramsay, R. R., Salach, J. I., Dadgar, J., and Singer, T. P. (1986) Biochem. Biophys. Res. Commun. 135, 269–275