RESEARCH COMMUNICATION

Is Complex II involved in the inhibition of mitochondrial respiration by *N*-methyl-4-phenylpyridinium cation (MPP⁺) and *N*-methyl- β -carbolines?

Matthew J. KRUEGER,*† Anthony K. TAN,*† Brian A. C. ACKRELL*† and Thomas P. SINGER*†‡§

*Molecular Biology Division, Veterans Affairs Medical Center, San Francisco, CA 94121, †Department of Biochemistry and Biophysics and ‡Division of Toxicology, University of California, San Francisco, CA 94143, U.S.A.

It has been reported that *N*-methyl- β -carbolinium analogues of the neurotoxic *N*-methyl-4-phenylpyridinium cation (MPP⁺) inhibit NADH-linked mitochondrial oxidations, as well as mitochondrial respiration on succinate nearly to the same extent [Fields, Albores, Neafsey and Collins (1992) Arch. Biochem. Biophys. 294, 539–544]. Those authors further claimed that MPP⁺ itself also blocks respiration through succinate dehydrogenase, in addition to its well-known effect on NADH dehydrogenase (Complex I), and concluded that both effects may contribute to the development of Parkinsonian symptoms. Since *N*-methyl- β -carboliniums are thought to be endogenous metabolites, these findings, if verified, would have important implications on the etiology of idiopathic Parkinsonism. We have re-examined these observations, using mitochondria after full activation of succinate dehydrogenase, as well as submitochondrial particles, in which complexities due to membrane transport are not present. We report the following observations. (1) *N*-Methyl- β -carboliniums inhibit mitochondrial respiration on NAD⁺-linked substrates in a time-dependent manner, and the inhibition is potentiated by the presence of tetraphenylboron anion (TPB[−]), as expected for positively charged compounds. (2) Unlike MPP⁺

itself, however, these compounds are uncouplers at higher concentrations, so that the effects seen in State 3 cannot be assigned exclusively to inhibition of NADH oxidation. (3) The effects on succinate oxidation in mitochondria, in which the full activity of the enzyme is expressed, are 1–1.5 orders of magnitude lower than on respiration via Complex I and are thus unlikely to contribute significantly to the neurotoxicity. (4) The effect of MPP⁺ on mitochondrial respiration via succinate dehydrogenase is trivial, in accord with previous reports from several laboratories, but contradicting the findings of Fields et al. (cited above). (5) In submitochondrial particles the inhibition of NADH oxidation (via the complete respiratory chain) has been confirmed, but it differs markedly from the action of MPP⁺ in two respects. First, the enhancement by TPB[−] is very small; secondly, the inhibition of NADH oxidation measured using ubiquinone (Q) analogues is far lower, suggesting that Complex I is *not* the only target. (6) In submitochondrial particles the inhibition of succinate oxidation by either O₂ or Q analogues is incomplete, trivial or absent. (7) We thus conclude that we find no basis for assigning any potential biological effect of *N*-methyl- β -carboliniums to the blockade of succinate oxidation.

INTRODUCTION

Fields et al. (1992) recently reported that, in rat liver mitochondria, *N*-methyl-4-phenylpyridinium cation (MPP⁺) and *N*-methyl- β -carbolinium compounds inhibit not only the oxidation of NAD⁺-linked substrates, but also of succinate. Moreover, the β -carboliniums were said to inhibit NADH and succinate dehydrogenases to a similar extent. These observations on the action of MPP⁺ seemed to be in conflict with reports from other laboratories that no significant inhibition of succinate oxidation is detectable either in the absence or in the presence of tetraphenylboron anion (TPB[−]) (an agent which greatly potentiates the action of MPP⁺ and its derivatives) when State 3 respiration on malate + glutamate in rat liver or brain mitochondria is virtually completely blocked (Nicklas et al., 1985; Vyas et al., 1986; Ramsay et al., 1986, 1987; Heikkilä et al., 1990). In the hope of clarifying these contradictory observations we decided to compare the action of MPP⁺ and of typical *N*-methyl- β -carbolinium compounds on Complex I (NADH dehydrogenase) and Complex II (succinate dehydrogenase) in mitochondria and in submitochondrial particles.

EXPERIMENTAL

Rat liver mitochondria and submitochondrial particles from ox heart (electron-transport particles, ETP) were isolated as in previous work (Ramsay et al., 1989a). 2,3-Dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (decylubiquinone) was obtained from Sigma. The *N*-methylated β -carbolinium compounds 2,9-dimethylharmanium iodide (2,9-Me₂HA) and 2,9-dimethylnorharmanium iodide (2,9-Me₂NH) were the gifts of Dr. M. A. Collins, Loyola University, Chicago, IL, U.S.A. The sources of all other compounds were as described by Ramsay et al. (1989a).

Mitochondrial respiration was measured polarographically at 30 °C in 25 mM sucrose/75 mM mannitol/95 mM KCl/5 mM potassium phosphate/20 mM Tris/HCl/5 mM MgCl₂/50 μ M EDTA, pH 7.4. The oxidation of NAD⁺-linked substrates was measured in the presence of 2.4 mg of mitochondrial protein/ml. After 4 min incubation with or without inhibitor and 5 mM glutamate + 2.5 mM L-malate, the oxygen-electrode chamber was closed and State 4 respiration was recorded for 1 min; then ADP was added to give 0.6 mM final concentration, and State 3 respiration was recorded. With succinate as substrate, mito-

Abbreviations used: MPP⁺, *N*-methyl-4-phenylpyridinium cation; TPB[−], tetraphenylboron anion; decylubiquinone, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; 2,9-Me₂NH, 2,9-dimethylnorharmanium iodide; 2,9-Me₂HA, 2,9-dimethylharmanium iodide; ETP, electron-transport particles; Q, ubiquinone.

§ To whom correspondence should be sent, at the following address: (151-S), Molecular Biology Division, Veterans Affairs Medical Center, 4150 Clement Street, San Francisco, CA 94121, U.S.A.

chondria (30.1 mg/ml) in the buffer described above were pre-incubated with 20 mM succinate under argon at 37 °C for 7 min in a vessel equipped with a rubber diaphragm, then cooled in ice. This assures full activation of succinate dehydrogenase, since the enzyme is normally present in partly deactivated form in aerobic mitochondria (Singer, 1974). Aliquots (1.5 mg) of protein were added to the sucrose/mannitol/Tris/phosphate/KCl/MgCl₂/EDTA buffer in a 1.5 ml reaction chamber at 30 °C. Succinate was then added to 6.7 mM final concentration, and also inhibitor, if present. After 4 min, State 4 respiration was recorded for 1 min and, following the addition of ADP to 0.6 mM concentration, State 3 respiration was measured.

With ETP particles NADH oxidase activity was measured polarographically at 30 °C in 0.25 M sucrose/50 mM potassium phosphate, pH 7.4, with 0.045 mg of protein in 2 ml reaction volume. After pre-incubation with the inhibitor for 5 min, NADH was added to 0.28 mM concentration and respiration was recorded. Succinoxidase activity was assayed following activation of the particles (3 mg of protein/ml of sucrose/phosphate buffer) for 10 min at 30 °C with 1 mM malonate (Singer, 1974). The activated particles were then diluted with the appropriate inhibitor in sucrose/phosphate and incubated for 5 min at room temperature. An aliquot (15 μ l) containing 0.0225 mg of protein was then placed in the 2 ml reaction chamber containing 0.25 M sucrose/50 mM potassium phosphate, pH 7.4, and inhibitor at the same concentration as was present during pre-incubation. Succinate (20 mM) was added, and the activity at 30 °C was recorded. Residual inhibition by the malonate was corrected by treating ETP control samples not containing MPP⁺ or β -carbolines with malonate in the same way.

Succinate:ubiquinone (Q) oxidoreductase activity was assayed spectrophotometrically at 30 °C by monitoring the disappearance of the 274 nm band of oxidized decylubiquinone. The reaction mixture contained, in 1 ml total volume, 0.25 M sucrose/50 mM phosphate, pH 7.4, 0.015 mg of ETP protein (activated with malonate as described above), 1 mM KCN and 52.5 μ M decylubiquinone. Following 5 min incubation with the inhibitor being studied, the reaction was started by adding succinate (20 mM).

NADH:Q oxidoreductase was measured spectrophotometrically at 340 nm at 30 °C. The reaction mixture contained 0.05 M sucrose/0.025 mM phosphate, pH 7.4, 52.5 μ M decylubiquinone, 1 mM KCN (where present), 10 μ M TPB⁻ and 0.031 mg of ETP protein in 1 ml final volume. Following pre-incubation with the inhibitor, the assay was started by adding NADH to 0.28 mM concentration.

RESULTS AND DISCUSSION

Inhibition of mitochondrial respiration

Figures 1(a) and 1(b) show the inhibition of malate + glutamate oxidation in State 3 without (a) and with (b) 10 μ M TPB⁻ by 2,9-Me₂NH, a typical *N*-methylated carbolinium analogue of MPP⁺. It is known (Heikkilä et al., 1990; Ramsay et al., 1989a,b) that TPB⁻ greatly accelerates the development of inhibition by MPP⁺ and its positively charged analogues by facilitating the transport of MPP⁺ derivatives across the inner membrane and their penetration into the hydrophobic inhibition site (Ramsay et al., 1989a). In accord with a previous report (Albores et al., 1990), TPB⁻ similarly lowers the IC₅₀ of *N*-methylated β -carbolinium compounds by over an order of magnitude (Figures 1a and 1b). It should be noted that inhibitors were pre-incubated with mitochondria for 5 min in all our experiments in order to stimulate the conditions used by others (Fields et al., 1992; Albores et al., 1990).

Figures 1(c) and 1(d) illustrate the inhibition of succinate oxidation in State 3 by 2,9-Me₂HA in the absence and presence of 10 μ M TPB⁻ respectively. Comparison with Figures 1(a) and 1(b) shows that, in contrast with the virtually complete inhibition of oxidations via Complex I in the presence of TPB⁻ (Figure 1b), that of succinate oxidation is incomplete with both inhibitors, despite the presence of TPB⁻ (Figure 1d). Secondly, the IC₅₀ values in the presence of TPB⁻ are some 33 to near 50 times higher when respiration on succinate is measured than with NAD⁺-linked substrates (Figures 1b and 1d; Table 1). This conflicts with the report (Fields et al., 1992) that the inhibition of succinate oxidation required only 1- or 2-fold higher concentrations of these compounds than the inhibition of respiration on malate + glutamate. A further discrepancy is in the IC₅₀ values obtained in the absence of TPB⁻. In the presence of malate + glutamate the IC₅₀ for 2,9-Me₂HA after 5 min incubation was reported as 100 μ M and for succinate as 250 μ M (Table 1 in Fields et al., 1992) versus 29 μ M and 725 μ M respectively, found by us (Table 1). This translates to an IC₅₀ ratio of 25, not 2.5 (Table 1 in Fields et al., 1992).

We have also attempted to confirm the report (Fields et al., 1992) that MPP⁺ inhibits not only mitochondrial oxidations via Complex I, but also that of succinate, albeit at a concentration about 7 times higher. As shown in Table 1, we found an apparent IC₅₀ after 5 min pre-incubation of 1500 μ M in the absence of TPB⁻, which agrees satisfactorily with the value of 1200 μ M reported by Fields et al. (1992). Their value for the IC₅₀ for glutamate + malate oxidation (170 μ M) agrees with that usually found in this laboratory and reported by others (Nicklas et al., 1985; Heikkilä et al., 1990). Thus the difference in the extents of inhibition of Complexes I and II is sufficiently large that no

Figure 1 Inhibition of mitochondrial respiration by *N*-methylated β -carboliniums in State 3

Oxidation of malate-glutamate (a) in the absence and (b) in the presence of 10 μ M TPB⁻. Oxidation of succinate (c) in the absence and (d) in the presence of 10 μ M TPB⁻. Symbols in (c) and (d): Δ , 2,9-Me₂NH; $\circ$, 2,9-Me₂HA. Experimental conditions were as described in the Experimental section.

Table 1 Differential effects of *N*-methyl- β -carboliniums and of MPP^+ on the oxidation of NADH and succinate

TPB^- , when present, was at a concentration of 10 μM .

Assay system	Inhibitor	IC ₅₀ (μM)*	
		Without TPB [−]	With TPB [−]
Mitochondrial respiration on:			
Glutamate + malate	2,9-Me ₂ NH	80	6
	2,9-Me ₂ HA	29	4
	MPP ⁺	100	4
Succinate	2,9-Me ₂ NH	830	200
	2,9-Me ₂ HA	(725)¶	140
	MPP ⁺	1500	580
ETP			
NADH oxidase	2,9-Me ₂ NH	1100	600
	2,9-Me ₂ HA	1600	1100
Succinoxidase	2,9-Me ₂ NH	(10% max)†	(12% max)†
	2,9-Me ₂ HA	(5% max)‡	(7% max)‡
NADH:Q oxidoreductase	2,9-Me ₂ NH	(40% max)§	(45% max)§
	2,9-Me ₂ HA	(20% max)	(22% max)
Succinate:Q oxidoreductase	2,9-Me ₂ NH	(16% max)§	(18% max)§
	2,9-Me ₂ HA	0	0

* IC_{50} is the inhibitor concentration yielding 50% inhibition compared with the control.

$\dagger$ Maximum inhibition reached at 2.2 mM inhibitor.

$\ddagger$ Maximum inhibition reached at 2 mM inhibitor.

$\S$ Maximum inhibition reached at 2.8 mM inhibitor.

$\parallel$ Maximum inhibition at 1.2 mM inhibitor.

∇ Uncertain value, because 50% inhibition was only asymptotically approached.

inhibition of succinate oxidation would be detected at the concentrations normally used in studying the action of MPP^+ on intact mitochondria. Moreover, while TPB^- potentiates the effect of MPP^+ on malate + glutamate oxidation some 74-fold (Heikkilä et al., 1990; Ramsay et al., 1989a), it has a much smaller (2.6-fold) effect on the inhibition of succinate oxidation (Table 1). This is not surprising, since most of the potentiation by TPB^- of the inhibition of mitochondrial respiration on glutamate + malate is due to increased penetration of MPP^+ into the hydrophobic inhibition site on NADH dehydrogenase, not to increased uptake into mitochondria (Heikkilä et al., 1990; Ramsay et al., 1989a).

Uncoupling of mitochondrial respiration by *N*-methylated β -carbolines

In an attempt to understand why the inhibition of succinate oxidation in State 3 by β -carbolinium levels off at 50–60% even in the presence of TPB^- (Figures 1c and 1d), we compared the effect of 2,9- Me_2NH on respiration supported by succinate in States 3 and 4 (Figure 2, solid versus open symbols, respectively). It is seen that, at concentrations in excess of ~ 0.8 mM in the absence of TPB^- and ~ 0.25 mM with TPB^- present, 2,9- Me_2NH acts as an uncoupler, increasing O_2 uptake in State 4. These are the concentrations where the inhibition in State 3 tends to reach a plateau (Figures 1c and 1d). At higher concentrations the inhibition seems to be reversed, but this is due to the release of respiratory control, the inhibitor acting as an uncoupler.

Inhibition in submitochondrial particles

ETP, a submitochondrial particle, offers a much simpler system to localize and study in detail the interaction of inhibitors with respiratory-chain components, since it is devoid of complicating factors, such as respiratory control and its uncoupling and, being

Figure 2 Uncoupling of mitochondrial respiration on succinate by 2,9- Me_2NH

Solid symbols, State 3 respiration; open symbols, State 4 respiration.

inverted, the problem of penetration of the inhibitor across the inner membrane is also circumvented, although the electron-transport system is intact in all known respects. Figure 3 compares the inhibition of NADH and succinate oxidation by 2,9- Me_2NH without and with 10 μM TPB^- present. The IC_{50} data derived from such experiments are summarized in Table 1. The difference in the action of the inhibitor on the two systems is dramatic: without TPB^- , inhibition of NADH oxidation reached $\sim 65\%$ at the highest concentration tested, and with TPB^- present it exceeded 80% at 2 mM 2,9- Me_2NH , becoming virtually complete at higher concentrations (not shown), while succinate oxidation was only inhibited by 10–12% with or without TPB^- . Inhibition by 2,9- Me_2HA was also essentially negligible (Table 1). Such effects are relatively trivial and raised the question whether they

Figure 3 Inhibition of NADH (□) and succinate (Succ) (○) oxidation via the complete respiratory chain in submitochondrial particles

Experimental conditions were as described in the Experimental section.

reflect a direct action on Complex II, as has been suggested by Fields et al. (1992), or on a component further along the respiratory chain.

Assays of the NADH:Q and succinate:Q oxidoreductase activities (Table 1) seemed to favour the latter alternative, since the maximal inhibition of the former reaction by 2,9-Me₂HA + TPB⁻ was only 45%, and by 2,9-Me₂HA even less (22%). Succinate oxidation by Q was not inhibited at all by any concentration of 2,9-Me₂HA tested. However, any possibility that Complex III might be inhibited by β -carbolines was eliminated by showing that the oxidation of chemically reduced decylubiquinone by cytochrome *c* was not significantly inhibited by 2,9-Me₂NH or 2,9-Me₂HA. Fields et al. (1992) reported that, in mitochondria, respiration on ascorbate/tetramethylenephenylenediamine was also not inhibited by these compounds, thereby indicating no effect on Complex IV. The fact that NADH:Q oxidoreductase assay was inhibited far less than the NADH:O₂ reaction, therefore, could reflect a certain lack of specificity by the Q analogue or some influence on inhibitor

binding by the high concentrations of Q analogue used in the assays.

While the inhibition site responsible for the partial blockade of succinate-supported respiration in mitochondria by *N*-methylated β -carbolines thus remains unclear, a major effect on Complex II is obviated in view of the virtual absence of inhibition of the succinate oxidase system in submitochondrial particles. Noting that the concentration of MPP⁺ in the nigrostratum of MPP⁺-treated animals seems to be in the order of 30 μ M (Singer et al., 1987), and the very high concentrations of *N*-methylated β -carbolines required to effect even a partial block of mitochondrial respiration, inhibition of succinate oxidation is not likely to contribute to the neurotoxicity of MPP⁺. For similar reasons, we would question, in fact, the explicit suggestion (Fields et al., 1992) that the inhibition of Complex II might enhance the neurotoxicity of *N*-methylated β -carbolines.

This study was supported by the U.S. Public Health Service (Program Project HL-16251), the National Science Foundation (grant no. DMB 9020015) and the Department of Veterans Affairs.

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