Journal of Bioenergetics and Biomembranes, Vol. 14, Nos. 5/6, 1982

Inhibition of Mitochondrial Substrate Anion Translocators by a Synthetic Amphipathic Polyanion

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Received March 27, 1982; revised April 26, 1982

Abstract

A synthetic polyanion (a copolymer of methacrylate, maleate, and styrene in 1:2:3 proportion with an average molecular weight of 10,000 dalton) inhibits the tricarboxylate, oxoglutarate, dicarboxylate, and adenine nucleotide translocators of rat liver mitochondria. The activity versus inhibitor concentration curves are sigmoidal. The inhibition of the oxoglutarate and tricarboxylate translocators by the polyanion is competitive, while that of the adenine nucleotide translocator is of mixed-type. The K_I values of the polyanion are the following: for oxoglutarate translocator 4.0 μM, tricarboxylate translocator 1.2 μM, and adenine nucleotide translocator 1.3 μM with ADP and 0.8 μM with ATP. It is suggested that the polyanion acts primarily by increasing the negative charge of the inner membrane at the outer surface, and the sensitivity of the translocators toward the polyanion depends on the number of negative charges of their substrates.

Key Words: Anion transport; tricarboxylate; 2-oxoglutarate; adenine nucleotide; polyanion inhibitor; membrane transport.

Introduction

Some years ago we studied the interaction of a synthetic polyanion—a copolymer of methacrylate, maleate, and styrene in 1:2:3 proportion with an average molecular weight of 10,000 dalton—with rat liver mitochondria (König *et al.*, 1977). It was found that among other effects this polyanion strongly inhibited the adenine nucleotide translocator. Since other mitochondrial anion translocators, such as the phosphate, dicarboxylate, and tricarboxylate translocators, were not affected, this polyanion appeared to be a quite specific inhibitor of the adenine nucleotide translocator. Phosphate, dicarbox-

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0145-479X/82/1200-0297$03.00/0

ylate, and tricarboxylate transport was measured by the isoosmotic swelling method (Chappell and Haarhoff, 1967) which uses extremely high substrate concentrations. This latter fact prompted us to reinvestigate systematically the effect of the polyanion on the mitochondrial substrate anion translocators, including kinetic studies. In this paper it will be shown that the polyanion inhibits not only the adenine nucleotide translocator but other substrate anion translocators as well, albeit with different kinetics and different efficacy.

Materials and Methods

[1,5-^{14}C] citric acid, [5-^{14}C] 2-oxoglutaric acid, [U-^{14}C] malic acid, [2-^{3}H] ATP, [2-^{3}H] ADP, and [^{3}H] H_2O were purchased from the Radiochemical Centre (Amersham, England). The radiochemical purity of the anionic substrates, checked by thin layer chromatography, was greater than 98%.

The polyanion was prepared by polymerizing its components (methacrylic acid, maleic acid anhydride, and styrene) as described earlier (König *et al.*, 1977) and further purified by ammonium sulfate precipitation. Its composition was controlled as follows: acid content by back titration, styrene content by measuring the absorbance at 260 nm, and methacrylic acid:maleic acid anhydride ratio by determining the oxygen content. It contained 8% more styrene than the amount calculated on the basis of the composition of the polymerization mixture, and there was practically no difference between the calculated and determined ratios of methacrylic acid:maleic acid anhydride present in the polymer. The average molecular weight of the polyanion was determined after dialysis in a Knauer electronic osmometer using a Sartorius 11536 membrane and found to be 10,000. All other reagents were the purest commercially available.

Rat liver mitochondria were prepared in 0.25 M sucrose, 1 mM EGTA, and 20 mM Tris-HCl, pH 7.2, as described previously (König *et al.*, 1977). The mitochondrial protein was determined by a modified biuret method (Palmieri *et al.*, 1979).

The mitochondria (40–50 mg protein) were loaded with malate by incubating them in 10 ml medium consisting of 100 mM KCl, 1 mM EGTA, and 20 mM Tris-HCl, pH 6.8, in the presence of 2 μg/ml rotenone and 0.5 mM L-malate. After 2 min incubation at 20°C, ice cold KCl–EGTA–Tris buffer was added and the mitochondria separated by centrifugation at 8000 $\times$ *g* for 10 min at 0°C. In some experiments, the intramitochondrial malate was labeled by adding to the mitochondrial suspension carrier-free [^{14}C]malate (approx. 1 μC/ml).

Table I. Exchange Between Intramitochondrial Malate and Extramitochondrial Anions[a]

	External anion	% exchange		
		Without PA	With 10 μM PA	With 20 μM PA
Expt. 1:	citrate 0.5 mM	79	33	15
	citrate 1.0 mM	79	44	31
Expt. 2:	oxoglutarate 1.0 mM	61	42	28
	phosphate 1.0 mM	50	32	22

[a]The reaction mixture contained 100 mM KCl, 1 mM ethyleneglycolbis(2-aminoethylether)-*N,N'*-tetraacetic acid (EGTA), 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) neutralized with Tris, pH 7.2, 2 μg rotenone, 0.5 μg antimycin, 10 μg oligomycin, [^{14}C]malate-loaded mitochondria (3.0 mg protein in Expt. 1 and 3.3 mg protein in Expt. 2), and the polyanion at the concentrations indicated. The exchange was initiated by addition of citrate, oxoglutarate, or phosphate at the concentrations indicated. After 2 min (Expt. 1) or 1 min (Expt. 2) the reaction was terminated by centrifugation. Other conditions as described in Materials and Methods section. PA = polyanion.

The exchange between intramitochondrial [^{14}C] malate and externally added anions was measured as follows. [^{14}C] Malate-loaded mitochondria were preincubated in 1.0 ml medium under the conditions specified in the legend to Table I at 4°C for 4 min. The exchange was initiated by addition of the appropriate counteranions and terminated 1 or 2 min later by centrifugation. At these times of reaction, the exchange is not yet at equilibrium. The percentage exchange was calculated according to the equation: percentage exchange = $100(cpm_{control} - cpm_{assay})/cpm_{control}$, where cpm_{assay} and $cpm_{control}$ represent the radioactivity in the pellet extracts in the presence and absence of external anions, respectively (Palmieri and Klingenberg, 1979). Occasionally, malate was also assayed enzymatically (Hohorst, 1962) with malate dehydrogenase using the double-beam spectrophotometer and a wavelength pair of 350 and 375 nm. The enzymatic analyses were in good agreement with the radioactive data.

The kinetics of [^{14}C]citrate/malate exchange were studied using the inhibitor stop method essentially as described previously (Palmieri and Klingenberg, 1979). Malate-loaded mitochondria were preincubated in "Eppendorf cups" in 1.0 ml medium for 4 min at 0°C under the conditions detailed in the legends to Figs. 1–2. The exchange was started by the addition of [^{14}C]citrate and, after 30 sec incubation at 0°C, it was stopped by rapid addition of 10 mM 1,2,3-benzenetricarboxylate. The mitochondria with the trapped [^{14}C]citrate were separated from the incubation medium in a microcentrifuge, the supernatant was carefully removed, and the pellet was extracted with perchloric acid and assayed for radioactivity. All "Eppendorf cups" contained also [^{3}H]H_2O. Corrections were made for the [^{14}C]citrate present in the sucrose-permeable space by running parallel samples in which

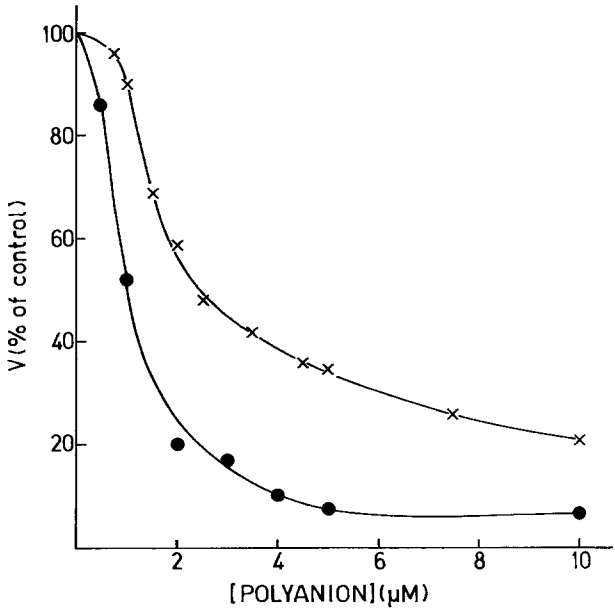

Fig. 1. Effect of increasing concentrations of the polyanion on the rate of [^{14}C]citrate–malate exchange. The reaction mixture contained 100 mM KCl, 1 mM ethyleneglycol bis(2-aminoethylether)-*N*,*N*'-tetraacetic acid (EGTA), 20 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonate (HEPES) neutralized with Tris, pH 7.2, 2 μg rotenone, 0.5 μg antimycin, 10 μg oligomycin, malate-loaded mitochondria (2.9 mg protein), and the polyanion at the concentrations indicated. The exchange was started by adding [^{14}C]citrate. Other conditions as described in Materials and Methods section. (●) 0.074 mM citrate (V = 1.75 nmol × min^{-1} × mg protein^{-1}); (×) 0.157 mM citrate (V = 2.06 nmol × min^{-1} × mg protein^{-1}).

the inhibitor was added to the incubation medium 30 sec before the radioactivity (Palmieri and Klingenberg, 1979).

The kinetics of [^{14}C]oxoglutarate/malate exchange were studied by the same technique described above. The exchange was started with [^{14}C]2-oxoglutarate and after 12 sec stopped by addition of 20 mM 2-phenylsuccinate (Palmieri and Klingenberg, 1979).

The kinetics of the adenine nucleotide uptake were studied by the method of Wojtczak and Zaluska (1967) with some modifications. Mitochondria were preincubated in 1.0 ml medium at 0°C for 4 min under the conditions specified in the legend of Fig. 4. The uptake was initiated by [2-^{3}H]ATP or [2-^{3}H]ADP and after 10 sec stopped by the addition of 50 μM carboxyatractylate. After rapid centrifugation the pellet was washed twice in 1 ml of the medium used for the isolation of the mitochondria. The final pellet was extracted with

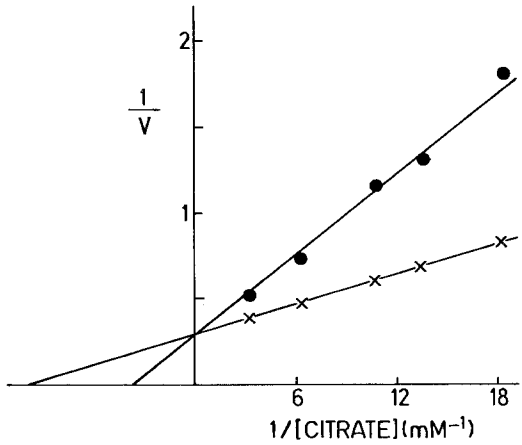

Fig. 2. Competitive inhibition of citrate uptake by the polyanion. Experimental conditions as in Fig. 1 except that [^{14}C]citrate was added at the concentrations indicated. Mitochondrial protein was 3.5 mg. V is expressed in nmol × min^{-1} × mg protein^{-1}. (×) control; (●) with 2 μM polyanion.

perchloric acid and assayed for radioactivity. Corrections were made by running parallel samples in which carboxyatractylate was added to the incubation medium 10 sec before the labeled substrate.

Since at 0–4°C (at which temperatures the activity of the substrate anion translocators was measured) the interaction of the polyanion with the mitochondria is rather slow (see König *et al.*, 1977), the mitochondria were preincubated with the polyanion for 4 min. In preliminary experiments this was found to be necessary to develop the maximal effect of the polyanion. However, at the concentrations used in the present investigation the polyanion did not cause leakage of adenine nucleotides or of any other anionic substrate tested from the mitochondria, and it did not affect the osmotic properties of the inner mitochondrial membrane. Furthermore, it did not significantly increase the leakiness of the outer membrane to an intermembrane space marker like adenylate kinase (see also König *et al.*, 1977).

All experiments were repeated at least twice.

Results

The exchanges between intramitochondrial [^{14}C]malate and externally added citrate, oxoglutarate, and phosphate reflect the activity of the tricarboxylate, the oxoglutarate, and the dicarboxylate translocator, respectively

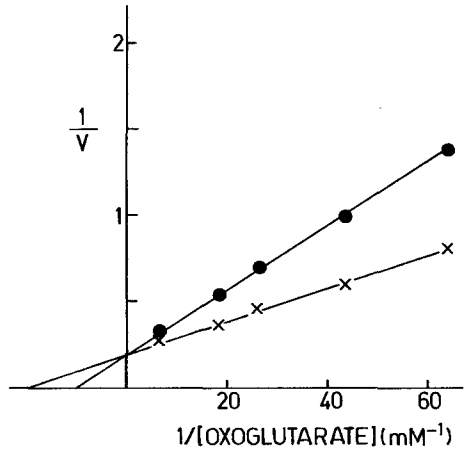

Fig. 3. Competitive inhibition of oxoglutarate uptake by the polyanion. Experimental conditions as in Fig. 1 except that [^{14}C]oxoglutarate was added at the concentrations indicated. Mitochondrial protein was 3.2 mg. V is expressed in nmol × min^{-1} × mg protein^{-1}. (×) control; (●) with 4 μM polyanion.

(Fonyò *et al.*, 1976). The data of Table I demonstrate that the polyanion inhibits all three exchanges. Furthermore, it can be seen that the inhibition depends on the concentration of both the polyanion and the external anionic substrate.

The effect of the polyanion on the rate of [^{14}C]citrate/malate exchange in malate-loaded mitochondria is shown in Fig. 1. From the data it appears that the polyanion is a powerful inhibitor of the tricarboxylate translocator. Fifty percent inhibition of the exchange is caused by about 1 μM polyanion at 74 μM citrate concentration and by 2.5 μM polyanion at 157 μM citrate concentration. Both curves, especially the one at 157 μM citrate, are sigmoidal. The kinetics of the inhibition by the polyanion were further analyzed by varying the concentration of citrate. The results of a typical experiment, shown as a Lineweaver–Burk plot in Fig. 2, indicate that the inhibition by the polyanion is competitive with respect to citrate. From these experiments a K_i of 1.2 μM for the polyanion was calculated.

The nature of the inhibitory effect of the polyanion on the oxoglutarate translocator was studied by measuring the rate of [^{14}C]oxoglutarate/malate exchange. The activity versus inhibitor concentration curve was sigmoidal again, but the polyanion was found to be less effective in this exchange than toward the citrate/malate exchange (data not shown). From Lineweaver–

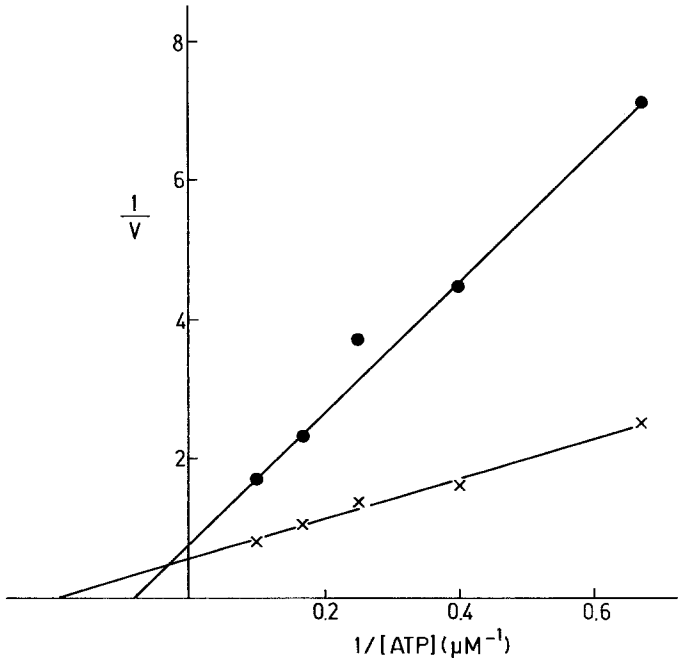

Fig. 4. Mixed-type inhibition of ATP uptake by the polyanion. The reaction mixture contained 100 mM KCl, 1 mM ethylenedi aminetetraacetic acid (EDTA), 20 mM HEPES-Tris, pH 7.2, 10 μg oligomycin, 1 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and mitochondria (2.9 mg protein). The uptake was started by adding [^{3}H]ATP at the concentrations indicated. Other conditions as described in Materials and Methods section. V is expressed in nmol $\times$ min^{-1} $\times$ mg protein^{-1}. (×) control; (●) with 2 μM polyanion.

Burk plots the inhibition was found to be competitive (Fig. 3), and a K_i of 4.0 μM for the polyanion was evaluated.

The kinetics of the inhibition by the polyanion of the adenine nucleotide translocator were studied by measuring the rate of [^{3}H]ATP uptake by the mitochondria. The activity versus inhibitor concentration curve was sigmoidal (not shown). In this case, however, the Lineweaver–Burk plots show that the inhibition is of the mixed-type (Fig. 4). The K_i was calculated to be 0.8 μM for the polyanion. If [^{3}H]ADP was used as a substrate instead of [^{3}H]ATP, similar results were obtained with a K_i of 1.3 μM, i.e., very close to the value found in the case of the tricarboxylate translocator.

Discussion

The results of this paper show that the polyanion inhibits the oxoglutarate, dicarboxylate, tricarboxylate, and adenine nucleotide translocators of rat

liver mitochondria, though with different sensitivity. As preliminary experiments indicate that the phosphate/H^+ translocator is also inhibited, it seems probable that all mitochondrial substrate anion translocators are inhibited unspecifically by the polyanion. In this respect the situation is similar to that of other known nonpermeant negatively charged amphipathic inhibitors of the mitochondrial substrate anion translocators such as palmityl-CoA (Halperin *et al.*, 1972; Morel *et al.*, 1974) and agaric acid (Chàvez and Clapp, 1975; Stipani *et al.*, 1977; Chàvez *et al.*, 1978), which were thought to be specific earlier. Anionic detergents at sublytic concentrations (Duszynski and Wojtczak, 1974) and tetraphenylboron (Meisner, 1973) also inhibit the adenine nucleotide translocator. In contrast, it was shown that mitochondrial substrate anion translocators were activated (lowering their K_m without changing V_{max}) by cations or by increasing H^+ concentration, i.e., by decreasing the negative charge of the mitochondrial inner membrane surface (Meisner *et al.*, 1972). It is likely that our amphipathic polyanion inhibits mitochondrial substrate anion translocators primarily by increasing the negative charge at the outer surface of the inner membrane. Thus, our results give additional support to the contention that alteration of the surface charge significantly influences the function of the mitochondrial substrate anion translocators (Quagliariello *et al.*, 1973). It is interesting that Wojtczak and Nalecz (1979) have recently shown that the activity of membrane-bound enzymes is also controlled by the surface charge of the membrane.

The most important conclusion can be drawn from the K_i values of the polyanion (oxoglutarate translocator 4 μM, tricarboxylate translocator 1.2 μM, and adenine nucleotide translocator 1.3 μM with ADP and 0.8 μM with ATP), namely that the sensitivity of the substrate anion translocators toward the polyanion depends on the number of negative charges of their substrate. This conclusion can be made because all translocators were assayed at the same pH (pH 7.2) and is supported by the finding that the adenine nucleotide translocator is more sensitive toward the polyanion with ATP as a substrate than with ADP.

The sigmoidal shape of the activity versus inhibitor concentration curves might be best interpreted by a positive cooperativity between the polyanion molecules. For explaining the mixed type inhibition of the adenine nucleotide translocator—contrary to the other translocators examined—we have to suppose that in this case the polyanion has a dual effect. It acts not only by repelling competitively the negatively charged substrate from the active center, but it also interacts either with other site(s) of the translocator itself or with the membrane lipids in the close vicinity of the translocator. By this latter effect it decreases the adenine nucelotide transport in a noncompetitive manner.

Acknowledgments

The visit of Dr. Tamàs König to Bari was supported by a joint grant from the University of Bari and the Semmelweis University.

References

Chappell, J. B., and Haarhoff, K. N. (1967). In *Biochemistry of Mitochondria* (Slater, E. C., Kaniuga, Z., and Wojtczak, L., eds.), Academic Press, New York, p. 75.

Chàvez, E., Chàvez, R., and Carrasco, N. (1978). *Life Sci.* **23,** 1423.

Chàvez, E., and Klapp, M. (1975). Biochem. Biophys. Res. Commun. **67,** 272.

Duszynski, J., and Wojtczak, L. (1974). *FEBS Lett.* **40,** 72.

Fonyò, A., Palmieri, F., and Quagliariello, E. (1976). In *Horizons in Biochemistry and Biophysics* (Quagliariello, E., *et al.,* eds.), Addison-Wesley, Reading, Massachusetts, p. 60.

Halperin, M. L., Robinson, B. H., and Fritz, I. B. (1972). *Proc. Natl. Acad. Sci. USA* **69,** 1003.

Hohorst, H. (1962). In *Methods of Enzymatic Analyses* (Bergmeyer, H., ed.), Verlag Chemie, Weinheim, p. 328.

König, T., Kocsis, B., Mészàros, L., Nahm, K., Zoltàn, S., and Horvàth, I. (1977). *Biochim. Biophys. Acta* **462,** 380.

Meisner, H., Palmieri, F., and Quagliariello, E. (1972). *Biochemistry* **11,** 949.

Meisner, H. (1973). *Biochim. Biophys. Acta* **318,** 383.

Morel, F., Lauquin, G., Lunardi, J., Duszynski, J., and Vignais, P. V. (1974). *FEBS Lett.* **39,** 133.

Palmieri, F., and Klingenberg, M. (1979). In *Methods in Enzymology* (Fleischer, S., and Packer, L., eds.), Vol. LVI, Academic Press, New York, p. 279.

Palmieri, F., Stipani, I., and Iacobazzi, V. (1979). *Biochim. Biophys. Acta* **555,** 531.

Quagliariello, E., Meisner, H., and Palmieri, F. (1973). In *Mechanisms in Bioenergetics* (Azzone, G. F., *et al.,* eds.), Academic Press, New York and London, p. 239.

Stipani, I., Francia, F., Palmieri, F., and Quagliariello, E. (1977). *Bull. Mol. Biol. Med.* **2,** 72.

Wojtczak, L., and Nalecz, M. J. (1979). *Eur. J. Biochem.* **94,** 99.

Wojtczak, L., and Zaluska, H. (1967). *Biochem. Biophys. Res. Commun.* **28,** 76.