MODE OF ACTION OF THE ANTIBIOTIC X-537A ON MITOCHONDRIAL GLUTAMATE OXIDATION

Diane C. Lin and Ernest Kun

Depts. of Biochemistry & Biophysics and Pharmacology and the Cardiovascular Research Institute, University of California, San Francisco, San Francisco, California 94122

Received December 19,1972

Summary:

At 0.05 to 0.01 μM concentrations the monocarboxylic acid antibiotic X-537A inhibits ADP or 2,4-dinitrophenol-activated oxidation of glutamate but has no appreciable effect on state 4 respiration. ATP synthetase activity is also inhibited. There is no efflux of Mg^{2+} or Ca^{2+} from the mitochondria under these conditions. Dissociation of membrane bound Mg^{2+} induced by X-537A is reversed and prevented by Mg^{2+} + ATP but inhibitory effects of the antibiotic are not. Half maximal effects of X-537A occur when the ratio of X-537A to mitochondrial non-diffusible Mg^{2+} is 1/800 to 1/400. It is proposed that this small fraction of membrane associated Mg^{2+} may be at the catalytic site of energy transfer and irreversible inhibition by X-537A is due to hydrophobic complexation of Mg^{2+} in situ.

Apart from known functions of bivalent cations in enzymatic catalysis, bivalent cations, especially Mg^{2+} and Ca^{2+} play an important but not well understood role in energy coupled processes of mitochondria (1) and chloroplasts (2). It was observed that ejection of non-diffusible mitochondrial Mg^{2+} coincides with the loss of the ability of mitochondria to respond with increased rates of electron transfer to DNP* or to ADP (1, 3, 15). Furthermore, ATP-ase activity, K^{+} retention and respiration dependent Ca^{2+} loading of the inner membrane (cf. 4) were also found to depend on non-diffusible, presumably membrane associated Mg^{2+} (3). Two modes of participation of membrane bound Mg^{2+} in mitochondrial bioenergetics can be envisaged. Bound Mg^{2+} may be part of the energy transducing catalytic system or Mg^{2+} binding itself may be an energy coupled process.

We have approached this problem by a study of the mechanism of action of the antibiotic X-537A (see chemical structure in Ref. 5) on typical energy coupled processes of rat liver mitochondria. It is known (6, 7) that X-537A forms hydrophobic complexes with mono and bivalent cations and it has been reported that this physical property enables this antibiotic to function as an ionophore for Ca^{2+}, as studied with sarcoplasmic reticulum (5, 8, 9).

Inhibition of the oxidation of glutamate by X-537A (cf. 17) has been pre-

*DNP = 2,4-dinitrophenol
TES-TRIS Buffer = N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) adjusted to pH 7.2 with tris (hydroxymethyl) aminomethane (TRIS).

viously observed but the mechanism of inhibition remained unexplored. Another antibiotic, A-23187, is a more specific chelator of bivalent cations and it was recently found (10) that ionophorous behavior of A-23187 explains most of its effects on mitochondria. As shown in the present report, X-537A at low concentrations inhibits mitochondrial energy coupled processes, and this effect occurs at concentrations of the antibiotic which are more than 2 orders of magnitude lower than required for the induction of flux of bivalent cations.

MATERIALS AND METHODS

Rat liver mitochondria were prepared according to Schnaitman and Greenawalt (11). Initial rates of ATP synthesis (within 60 seconds) were determined by ^{32}P incorporation into ATP following the method of Nielsen and Lehninger (12), as modified in this laboratory (13). Membrane bound bivalent cations were assayed by chlorotetracycline fluorescence according to Caswell and Hutchinson (14). Mitochondrial O_2 consumption was monitored in a recording polarograph, Mg^{2+} and Ca^{2+} efflux from mitochondria were determined by atomic absorption analysis with the aid of the Perkin Elmer 403 spectrophotometer as described earlier (15). The reaction medium in all experiments consisted of 0.15 M sucrose, 0.03 M TES-TRIS buffer (pH 7.2) and 0.01 M glutamate. All reactions were carried out at 30^{0}. (For details, see Legends of Figures).

RESULTS

The antibiotic X-537A had no appreciable effect on state 4 respiration (not shown), but markedly inhibited ADP or DNP activated respiration at 0.5 to 1 X 10^{-7} M, (equivalent to 0.025 to 0.05 n moles X-537A per mg protein). There is a characteristic dose-response relationship between the concentration of the antibiotic and its inhibitory effects. When activities were plotted against the concentration of X-537A, up to 2 to 3 X 10^{-7} M antibiotic, precipitous slopes were obtained, whereas above this concentration the inhibition increased only slowly and reached nearly 100% at 10^{-5} M antibiotic. The presence of 1 mM EGTA did not modify the inhibition below 2 X 10^{-7} M X-537A, therefore inhibitions are not due to any Ca^{2+} mediated effects. The same inhibitory effects of X-537A were obtained in K^{+} free medium or in the presence of 25 mM K^{+}. That X-537A acts as an ionophore for bivalent cations at 1 to 5 X 10^{-5} M antibiotic concentrations was confirmed by determinations of the efflux of both Mg^{2+} and Ca^{2+}, as shown in the Table.

Initial rates of ATP synthesis (Fig. 1), chlorotetracycline fluorescence (Fig. 2) and ADP and DNP stimulated respiration (Fig. 3a and b) were markedly inhibited at 1 to 2 X 10^{-7} M antibiotic. The sensitivity of these mitochondrial reactions to low concentrations (below 2 X 10^{-7} M) of the antibiotic decreased in the following order: chlorotetracycline fluorescence > DNP stimulated respiration > ADP stimulated respiration > ATP

TABLE

Induction of Bivalent Cation Efflux by X-537A

M of X-537A	Mg^{2+} Efflux*	Ca^{2+} Efflux*
0	6.10	6.72
1×10^{-6}	6.03	6.72
1×10^{-5}	11.82	8.62
5×10^{-5}	15.59	11.34

*n moles per mg protein in 3 minutes at 30^{0}. Total mitochondrial Mg^{2+} = 24.8 n moles/mg; total mitochondrial Ca^{2+} = 18.5 n moles/mg.

The inhibitory effects of X-537A are illustrated in Figs. 1 to 3.

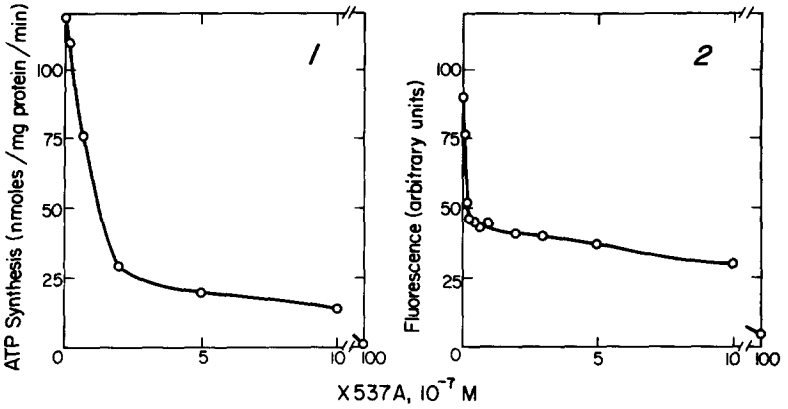

Fig. 1: Inhibition of ATP synthesis by X-537A. Mitochondria (2 mg protein/ml) are preincubated for 1 min. in the presence and absence of X-537A before 2 mM ADP and 2 mM Pi (labelled with ^{32}P) are added. Reaction time is 1 min.

Fig. 2. Inhibition of chlorotetracycline fluorescence by X-537A. Mitochondria (0.25 mg protein/ml) are preincubated in the presence and absence of X-537A for 1 min. before 10 μM chlorotetracycline is added and fluorescence measured at 365 nm excitation and 530 nm emission.

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synthesis. Addition of external Mg^{2+} + ATP prevented and reversed the inhibitory effect of X-537A on chlorotetracycline fluorescence but had no effect on the inhibition of ADP or DNP stimulated respiration. Other bivalent cations, e.g. Ca^{2+} or Mn^{2+} did not replace Mg^{2+}, in fact both Ca^{2+} and Mn^{2+} in the absence of X-537A induced a rapid decline of chlorotetracycline fluorescence. The effects of ATP + Mg^{2+} on fluorescence are illustrated in Fig. 4 (a & b). ATP alone had only a small and transient effect (4c), but the much larger effect of Mg^{2+} alone was augmented by ATP (4d).

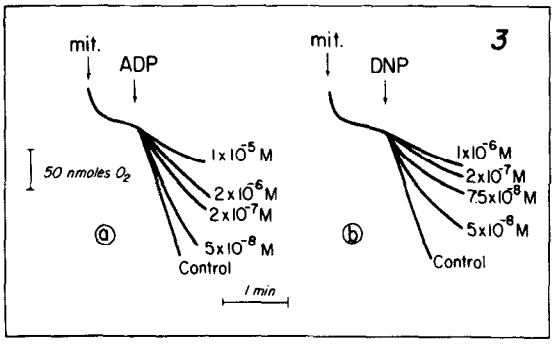

Fig. 3: Inhibition of activated respiration by X-537A. ADP + Pi (final concentration of both = 2 mM) or DNP (final concentration 50 μM) are added 1 min. after mitochondria have been suspended in the medium at 2 mg mitochondrial protein/ml.

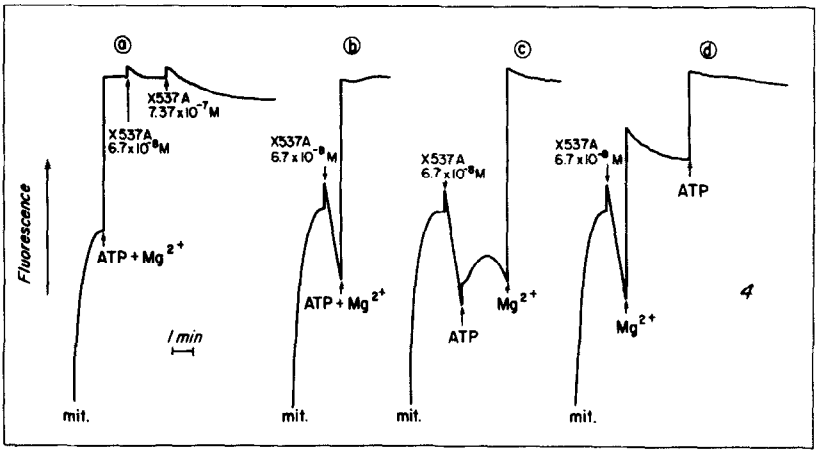

Fig. 4: Reversibility of the effect of X-537A on membrane bound Mg^{2+}. 10 μM chlorotetracycline was added to mitochondrial suspension (0.17 mg/ml) at the beginning. Further additions are shown with arrows: (a) 4.2 mM ATP + $MgCl_2$, then X-537A; (b) X-537A first, then ATP + Mg^{2+}; (c) first X-537A then ATP and Mg^{2+} in succession; (d) first X-537A then Mg^{2+} and ATP in succession.

DISCUSSION

It is apparent that X-537A has two separable effects on mitochondria. At low concentrations (below about 2 X 10^{-7} M) the antibiotic irreversibly inhibits DNP or ADP stimulated respiration and ATP synthesis. No ionophorous effects can be observed on bivalent cations at these low concentrations of X-537A. The second effect of X-537A is an induced Ca^{2+} and Mg^{2+} efflux which is detectable only at 100 to 500 times higher concentrations of the antibiotic than required to inhibit 70-80% of energy coupled reactions.

Inhibition of ATP synthesis-coupled respiration by X-537A is reminiscent of the effect of oligomycin (cf. 16) except that DNP stimulated respiration is not influenced by oligomycin. Apparently X-537A inhibits energy transfer at a different site than oligomycin. Since none of the known ionophorous antibiotics act in this manner (16, 17, 18 & 19), the specificity of action of X-537A on mitochondrial energy linked processes is best explained by hydrophobic complex formation with a specific membrane associated bivalent cation, most probably Mg^{2+}. Numerous analyses of liver mitochondria revealed that on the average the concentration of non-diffusible mitochondrial Mg^{2+} is 20 n moles Mg^{2+}/mg protein. It is readily calculated that the concentration of X-537A required to inhibit 50% of energy coupled mitochondrial reactions is sufficient to bind only 1/800 to 1/400 part of non-diffusible Mg^{2+}. Consequently this small fraction of mitochondrial Mg^{2+} must play a special, apparently catalytic role in mitochondrial energy transfer.

Based on experiments shown in Fig. 4 indicating reversibility of antibiotic-induced dissociation of membrane bound Mg^{2+}, in contrast to the irreversible inhibition of energy coupling by X-537A, two types of membrane associated Me^{2+} sites are proposed. The reaction of X-537A with the catalytic Me^{2+}-site ($\sim X\text{-}Me^{2+}$) is shown in Equ. 1.

Equ. 1

$$\sim X\text{-}Me + n(X\text{-}537A) \longrightarrow -X\text{-}Me(X\text{-}537A)n$$

Once this complexation occurs, this site loses its catalytic properties, i.e. $\sim X$ is converted to -X. The immediate consequence of reaction 1 is the dissociation of membrane bound Mg^{2+} from sites other than $\sim X\text{-}Me^{2+}$ (i.e. site Y), a reaction which is, however, reversible (by Mg^{2+} + ATP, see Fig. 4) and is described in Equ. 2.

Equ. 2

$$Y\text{-}Mg^{2+} \rightleftharpoons Y + Mg^{2+}.$$

According to this interpretation, the role of mitochondrial non-diffusible Mg^{2+} is more complex than previously assumed (see Refs. 1 & 3). A small fraction of membrane associated Mg^{2+} may function as part of a catalytic system of energy transfer ($\sim X\text{-}Me^{2+}$) whereas the bulk of non-diffusible mitochondrial Mg^{2+} ($Y\text{-}Mg^{2+}$) is held in mitochondria by processes which are energy dependent. Recently Reed and Lardy (20) observed that the specific bivalent cation ionophore A-23187 depletes mitochondrial Mg^{2+} to a concentration of 2 to 3 n moles per mg protein, yet oxidative phosphorylation in the presence of EGTA is still operative. This phenomenon, which appears to argue against the catalytic role of Mg^{2+} in energy transfer, is explained by our results, since the concentration of Mg^{2+} associated with energy transfer as shown by titration with X-537A, is far below the concentration of Mg^{2+} found in A-23187-treated mitochondria (20). It is probable that A-23187 acts on $Y\text{-}Mg^{2+}$.

ACKNOWLEDGMENTS

This work was supported by grants HD-01239 (USPHS), BC 47C (American Cancer Society) and HE-06285 (USPHS, Cardiovascular Research). A sample of X-537A was a gift of Dr. J. Berger, Hoffman La Roche Company, New Jersey; Diane C. Lin is a postdoctoral research fellow of the USPHS and Ernest Kun is a recipient of the Research Career Award of the USPHS. The skillful assistance of Mr. Allan S. Izen is acknowledged with thanks.

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