Inhibition of the Oxidation of Glutamate and Isocitrate in Liver Mitochondria at a Specific NADP+‐Reducing Site

(antibiotic X-537A/chlorotetraacycline/membrane)

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ABSTRACT The cation‐complexing carboxylic‐acid antibiotic X-537A, at concentrations far below that required for ionophorous activity, selectively inhibits the oxidation of glutamate and isocitrate by liver mitochondria in steady‐state 3. The site of inhibition has been localized specifically at the reduction of NADP+. Glutamate and isocitrate dehydrogenases, the oxidation of NADP+‐dependent substrates, pyridine nucleotide transhydrogenations, and the respiratory chain between NADH (or NADPH) and O2 are unaffected by the antibiotic X-537A. Kinetic evidence, i.e., competition between chlorotetraacycline (a fluorescence probe for membrane‐bound bivalent cation) and X-537A, indicates that the NADP+‐reducing, antibiotic‐sensitive site is most probably associated with the inner mitochondrial membrane.

The cation‐binding carboxylic‐acid antibiotic X-537A (see formula in Fig. 1) was found to inhibit phosphorylative respiration of liver mitochondria with glutamate as substrate at concentrations that were about two orders of magnitude lower than required for the induction of the efflux of Mg2+ and Ca2+ (1). It was concluded from these studies that the inhibitory site of low concentrations of X-537A is most probably localized in the inner mitochondrial membrane, and that this site may contain a membrane‐bound bivalent cation. The participation of a membrane‐bound site for bivalent cations in mitochondrial electron transfer has hitherto not been considered. For this reason, we extended our previous work in order to ascertain the existence and function of this postulated regulatory system. The experiments reported in the present communication demonstrate that the antibiotic‐sensitive inhibitory site is specifically concerned with the reduction of NADP+ by either glutamate or isocitrate. It is shown, furthermore, that in coupled mitochondria, glutamate and isocitrate reduce mitochondrial NADP+, hence the inhibitory action of X-537A is restricted to the oxidation of these two cellular substrates. The antibiotic‐sensitive mitochondrial site represents a specific, hitherto unrecognized, inhibitory or potentially regulatory system of mitochondrial electron transfer. Discovery of this site opens the possibility of the further exploration of the cellular biochemical significance of this antibiotic‐sensitive membrane system.

MATERIALS AND METHODS

Rat‐liver mitochondria were isolated according to Schnaitman and Greenawalt (2) from male Sprague‐Dawley rats (200 g) which were starved for 18 hr before the experiments. The specific inhibitor of glutamate:oxalacetate aminotransferase, α,α′-difluoro‐oxalacetic acid (F2OAA) (3), was synthesized by a described method (4). NAD+, NADP+, and mitochondrial substrates were commercial products of highest purity. The antibiotic X-537A was a gift from Dr. J. Berger (Hoffman La Roche Co., N.J.). Mitochondrial O2 uptake was recorded in a Gilson recording polarograph. The reaction chamber (1.4‐ml volume) was kept at 30° by a surrounding water jacket. The fluorometric assay for membrane‐bound bivalent cations was carried out according to Caswell and Hutchinson (5). Fluorometric analyses for NAD+ and NADP+ were performed in perchloric‐acid filtrates according to Estabrook and Maitra (6). For fluorometric assays, a Zeiss spectrofluorometer (PMQ II‐ZFM 4) was used that was equipped with a scale expander (1:10) and a variable zero adjuster (Research Development Lab, University of California, San Francisco) and attached to a Texas Recorder. All mitochondrial experiments were performed in a medium composed of 100 mM sucrose, 25 mM KCl, 15 mM Tris[N‐tris(hydroxy‐methyl)methyl‐2‐aminooethanesulfonic acid]‐Tris buffer (pH 7.2), and 0.25 mM EGTA [ethylene glycol bis(β‐amino ethyl ether)‐N,N′‐tetraacetic acid] at 30°. For details, see legends of table and figures.

RESULTS

The substrate specificity of the inhibition of mitochondrial respiration by the antibiotic X-537A is demonstrated in Table 1. State‐4 respiration is unaffected by concentrations of the antibiotic which profoundly inhibit the oxidation of glutamate and of D,L‐isocitrate in steady‐state 3. With both L‐malate and glutamate substrate, the inhibitory effect of X-537A was greatly reduced. It is known from studies with site‐specific fluorocarboxylic‐acid enzyme inhibitors (3, 7), that the metabolic pathway of glutamate in liver mitochondria is regulated by the availability of oxalacetate, which if present in substrate quantities will preferentially shunt glutamate through the transaminative pathway. In the case of both glutamate and malate substrate, predominance of the transaminative glutamate pathway is expected. The lack of inhibition of the glutamate–malate system by X-537A, therefore, strongly suggests that the glutamate dehydrogenase‐dependent or oxidative metabolism of glutamate is the inhibitory site of the antibiotic. Variable contribution of oxalacetate from endogenous mitochondrial substrates results in partly oxidative, partly transaminative initial rates of glutamate metabolism when liver mitochondria are exposed to external

Abbreviation: F2OAA = α,α′-difluoro‐oxalacetate.
glutamate only. Addition of F4OAA as specific inhibitor of glutamate:oxalacetate transaminase (3) to mitochondria-metabolizing glutamate eliminates the sometimes variable contribution of the transaminative pathway to O2 consumption. The remaining respiration, which is difluorooxalacetate insensitive, is inhibited by X-537A to the same extent as the predominantly oxidative metabolism of glutamate by fresh mitochondria (the median inhibitory dose of X-537A is 20–50 pmol/mg of mitochondrial protein).

Since the specificity of the inhibitory effect of X-537A was restricted to the oxidation of glutamate and isocitrate under state-3 conditions, the nature of this inhibition was studied in detail. As shown in Fig. 2, the dose-response relationship between the concentration of the antibiotic and its effect on glutamate or isocitrate oxidation was found to be identical. A comparison of the effects of X-537A on O2 uptake and on the redox state of intramitochondrial pyridine nucleotides during state 4→3 transition is illustrated in Fig. 3. As expected, addition of ADP causes a rapid change of the redox state of pyridine nucleotides from a more reduced to a more oxidized (Fig. 3A) form, coincident with a large increase in the rate of O2 uptake. In the presence of inhibitory concentrations of the antibiotic, despite the inhibited respiration, the oxidized form of pyridine nucleotides was significantly increased (Fig. 3B). These changes in the redox state of pyridine nucleotides indicate that the inhibitory site of X-537A was not in the electron-transfer chain between NADH or NADPH and O2 but rather on the substrate side of pyridine nucleotides. In agreement with this conclusion, neither the oxidation of NADH or NADPH nor transhydrogenations between pyridine nucleotides, both reactions catalyzed by submitochondrial particles (prepared by sonication), were affected by X-537A. Since neither glutamate nor isocitrate dehydrogenases were inhibited by the antibiotic, it was apparent that the inhibitory effect of X-537A had to be at the site of the reduction of either NAD+ or NADP+ (i.e., between the primary dehydrogenase and the electron-transfer system of the inner membrane). The mode of inhibition by X-537A was studied by direct kinetic analysis of both mitochondrial pyridine nucleotides. First, mitochondrial reduced pyridine nucleotides were allowed to be oxidized in the presence of ADP + Pi for 3 min; thereafter, specific substrates were added together with rotenone to inhibit electron transfer to O2. In order to minimize the interference of the transaminative pathway of glutamate with the oxidative path, F4OAA was added in one series. The following conclusions can be drawn from the experiments shown in Fig. 4. It is apparent that glutamate and isocitrate rapidly reduce NADP+ but not NAD+, and that the rate of this reduction is specifically inhibited by X-537A (Fig. 4a and b). When transaminase was inhibited by F4OAA, only NADP+ was reduced by glutamate, and again this reduction was inhibited by the antibiotic (Fig. 4c). In contrast, the rates of reduction of pyridine nucleotides by β-hydroxybutyrate were not significantly affected by X-537A (Fig. 4d).

According to previous results (1), the inhibition of glutamate oxidation by X-537A coincided with the quenching of dichlorotetracycline fluorescence. Since this fluorescence probe is indicative of membrane-bound bivalent cations (5), a competitive relationship between the antibiotic X-537A and fluorescence (of dichlorotetracycline) may indicate that the antibiotic binds to the same membrane site as the fluorescence probe. As illustrated in a typical Dixon plot (Fig. 5), there is indeed a competition between X-537A and dichlorotetracycline.

DISCUSSION

The mode of action of X-537A indicates the existence of an unusual inhibitory site in mitochondrial electron transfer. Operation of this site depends on intact mitochondrial structure and function; thus it is a characteristic property of the organelle. No effect of X-537A on submitochondrial particles could be observed. As deduced from previous experiments (1), and further supported by the competitive kinetics between X-537A and the bivalent-cation complexing dichlorotetracycline probe, an inner membrane-bound bivalent cation (probably Mg2+) is a likely inhibitory site. Whereas the nature of this new membrane site is as yet unknown, the consequence of its inhibition by X-537A can be analyzed with greater precision. The inhibition by X-537A occurs at the pyridine nucleotide level; neither succinate nor cytochrome c oxidation is affected. The monitoring of the redox state of intramitochondrial pyridine nucleotides provides even more discrete localization of the inhibitory site of X-537A in electron transfer. Exposure of mitochondria to the antibiotic results in an increase of the rate and extent of oxidation of reduced pyridine nucleotides. Upon addition of glutamate or isocitrate and rotenone, X-537A decreases the rate of reduction of pyridine nucleotides. Specific analyses for pyridine nucleotides show that only the reduction of NADP+ is inhibited by X-537A.
This explains why X-537A is without the slow step rate exhibited.

Only state-4 respiration is insensitive to X-537A when initial rates of O2 consumption are monitored. The steady-state 4 is an equilibrium state (8), where rates of reduction and oxidation of pyridine nucleotides are equal. It follows that the rate-limiting step of respiration under state-4 conditions is not the rate of reduction of pyridine nucleotides by substrates. This explains why X-537A is without observable effect on the slow rate of O2 uptake in state 4. On the other hand, state-3 respiration, which is not limited by substrate or phosphate acceptor, becomes dependent on the rate of electron transfer from substrate to pyridine nucleotides. Since X-537A inhibits the reduction of NADP+ by glutamate and isocitrate in steady-state 3, the characteristic increase of respiration that is supported by NADP+-reducing substrates is necessarily inhibited.

The most unusual aspect of the effect of the antibiotic is its specificity for the reduction of NADP+, identifying both glutamate and isocitrate as NADP+-requiring substrates in liver mitochondria. It was postulated earlier (7) that NADP+ is probably the real mitochondrial coenzyme of glutamate de-
hydrogenase as it functions in the organized mitochondrial enzyme system. This conclusion, partly based on the results of Klingenberg, Slenczka, and Pette (9, 10), is directly confirmed by our present experiments. Similar conclusions were drawn independently by Tager et al. (11). The observed NADP+ specificity of isocitrate oxidation is also in agreement with earlier results (9) which showed that isocitrate rapidly reduced intramitochondrial NADP+. In the absence of a specific inhibitor for the intramitochondrial reduction of NADP+ by glutamate or isocitrate, the apparently anomalous coenzyme specificity of mitochondrial glutamate or isocitrate dehydrogenases may be explained by various alternative mechanisms (e.g., pyridine nucleotide transhydrogenations). In view of the greater affinity of crystalline glutamate dehydrogenase towards NAD+ (12) and the generally known existence of two isocitrate dehydrogenases (one NAD+, the other NADP+ specific), the reasons for the intramitochondrial preferential reduction of NADP+ by these substrates remained ambiguous. Our present experiments, which show that the reduction of mitochondrial NADP+ by glutamate or isocitrate involves an antibiotic-sensitive site associated with the inner membrane, largely eliminates previous uncertainties because our results suggest that the NADP+ specificity of glutamate and isocitrate dehydrogenase in liver mitochondria can be explained by a distinct membrane-associated system. It is proposed that the target site of X-537A—a membrane-bound bivalent cation system—is required for precise pyridine nucleotide-dehydrogenase interactions, and that glutamate and isocitrate dehydrogenases are obligatorily linked to membrane-associated NADP+. This working hypothesis implies an organized macromolecular association of certain matrix enzymes with the inner membrane. The ineffectivity of the antibiotic X-537A in inhibiting either pyridine nucleotide-linked dehydrogenases or transhydrogenases in submitochondrial systems is evidence in favor of the proposed mechanism. It is generally known that the oxidation-reduction of NAD+-linked systems are fairly well explained by equilibria and substrate-mediated coupling (13, 14), whereas, the reasons for the predominance of NADPH over NADP+ in mitochondria are not well understood. Regulation (associated with the inner membrane) of the redox state of NADP+ by the X-537A-sensitive site is most probably contributory to the maintenance of the specific state of mitochondrial NADP+.

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