REVERSAL OF OLIGOMYCIN INHIBITION OF MITOCHONDRIAL ATPase BY IONOPHORIC COMPOUNDS

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1. Introduction

Oligomycin is a potent inhibitor of mitochondrial ATPase activity when the enzyme is bound to the membranes or when it is solubilized from yeast as a multi-subunit complex by extraction with Triton X-100 [1,2]. In contrast, oligomycin has no effect on the soluble F_1 ATPase which has been stripped of the most tightly associated membrane components of the complex [3]. We have recently established that oligomycin can be specifically bound to subunit 9 (nomenclature of Tzagoloff and Meagher [4]) of the enzyme complex by reductive alkylation [5]. This subunit is part of the 'membrane sector' of the ATPase complex and has a primary structure dominated by amino acids with nonpolar side chains [6]. It is soluble in chloroform/methanol (2:1) and is considered to be buried within the mitochondrial membrane structure.

A large number of mutants of the yeast *Saccharomyces cerevisiae* have now been reported that are resistant to oligomycin inhibition [7,8]. Most of these result from changes in mitochondrial DNA. The mitochondrial mutants have recently been divided into three classes based on mapping of genetic loci and on cross resistance with other inhibitors [8]. Mutants of Class B, but not Classes A or C appear to result in changes in subunit 9 of the ATPase. Mutants of this type thus allow analysis of the interaction of oligomycin with the enzyme [9].

Oligomycin has frequently been proposed to inhibit by blocking proton translocation across mitochondrial membranes [10]. Thus, its association with subunit 9 in the ATPase complex suggests that this small proteolipid may somehow be involved as a proton acceptor or with the movement of protons through the membrane. If this is the case, then it might be postulated that ionophoric substances may have a major effect on this function and that this may be examined by enzymic analysis of the oligomycin sensitive ATPase under appropriate conditions. The experiments reported here test this postulate and show that valinomycin can reverse oligomycin inhibition. This reversal is altered quantitatively by mutations causing modifications in subunit 9. Nigericin, but not CCCP has a similar effect in reversing the oligomycin block.

2. Materials and methods

The yeast strains used were D243-4A and a mitochondrially inherited oligomycin resitant strain OR-4 derived from D243-4A [11].

Cells were grown to early stationary phase in 1% Difco peptone, 1% yeast extract and 1% glucose. Cells were broken using a Braun homogenizer and mitochondria were isolated as described previously [12]. The mitochondria were lysed in 0.02 M Tris–HCl, 0.01 M EDTA buffer, pH 7.8 and homogenized in a glass-Teflon homogenizer at a protein concentration of 25 mg/ml. Submitochondrial particles were prepared by sonication [3] and Triton X-100 solubilized oligomycin sensitive ATPase was prepared by the method of Tzagoloff et al. [4] except that the final step was chromatography on Sepharose 6B rather than glycerol-gradient centrifugation [5].

ATPase was assayed photometrically by coupling to pyruvate kinase and lactic dehydrogenase using the method of Monroy and Pullman [13]. Inhibitors

were added as methanolic solutions. Oligomycin was obtained from Calbiochem, valinomycin from Sigma and nigericin was a gift from Dr I. H. Segel. Venturicidin was a gift from Dr A. W. Linnane. Phosphoenolpyruvate, pyruvate kinase, lactic dehydrogenase, ATP and NADH were purchased from Sigma Chemical Company.

3. Results

Inhibition of ATPase activities in lysed, homogenized mitochondrial particles from strains D243-4A and the oligomycin resitant mutant OR-4 prepared from this strain are illustrated in fig.1. It may also be seen in this figure that valinomycin has no measureable effects on this activity at the concentration-range tested. However, when oligomycin concentration was maintained constant at a level which caused about 90% inhibition of oligomycin sensitive ATPase while valinomycin was added in increasing amounts, inhibition due to oligomycin was reversed. This data is presented in fig.1 as ATPase activity versus [valinomycin]/[oligomycin] ratio. For strain D243-4A the ratio giving 50% recovery of ATPase activity is close to one. When strain OR-4, which has a decreased binding constant for oligomycin, was examined under identical conditions, the midpoint for reversal of inhibition was found near a [valinomycin]/oligomycin] ratio of 0.33.

While this method of analysis is convenient for comparing resistant and oligomycin sensitive strains, reversal of oligomycin inhibition by valinomycin is actually dependent upon the absolute concentration of valinomycin rather than on the ratio. This may be seen in fig.2, for example, in which the rate of ATPase reaction is followed in the presence of oligomycin at two different levels while maintaining valinomycin constant. The oligomycin and valinomycin do not appear to be competing for some common binding site on the enzyme. Addition of potassium ions to the lysed mitochondrial preparations was not necessary to obtain the valinomycin effect. However, in

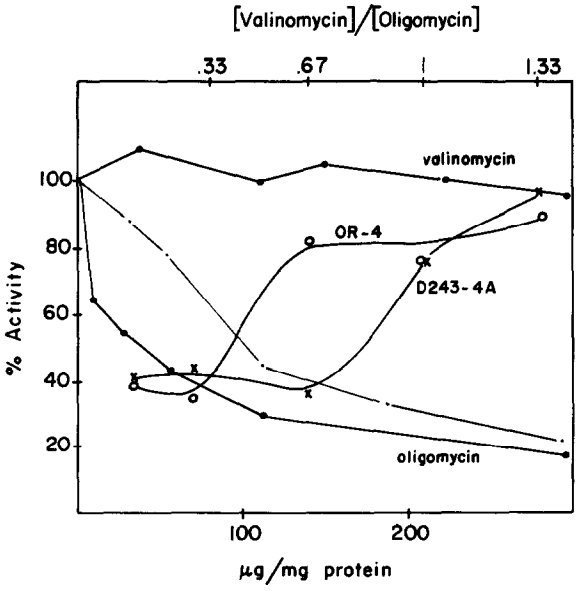

Fig.1. Reversal of oligomycin inhibition of mitochondrial ATPase by valinomycin. The inhibition of D243-4A (-●-●-) and OR-4 (-·-·-) by oligomycin is shown. Valinomycin has no inhibitory effect on ATPase activity (-o-o-). When oligomycin concentration was held at 15 µg/ml and valinomycin concentration was increased, the inhibition was reversed as shown by plotting activity against the ratio [valinomycin]/[oligomycin] for strain D243-4A (-x-x-) and for OR-4 (-o-o-).

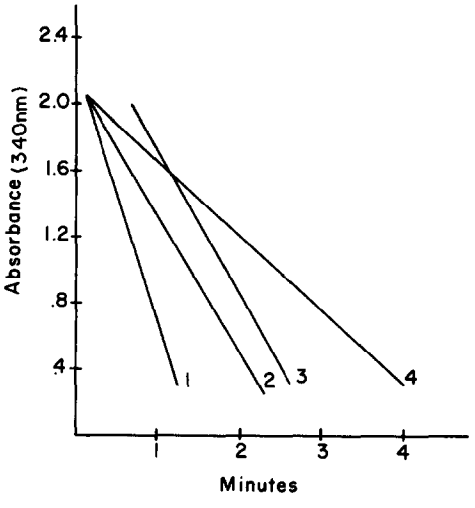

Fig.2. Rate of ATPase reaction in the absence of oligomycin (1), with 5 µg/ml oligomycin (4), with 5 µg/ml oligomycin plus 7.5 µg/ml valinomycin (2) and with 10 µg/ml oligomycin plus 7.5 µg/ml valinomycin. The protein concentration of lysed mitochondrial particles used in the assay was 0.75 mg/ml. The rate was measured by following the absorption charge of NADH in the coupled enzyme assay procedure of Monroy and Pullman [13].

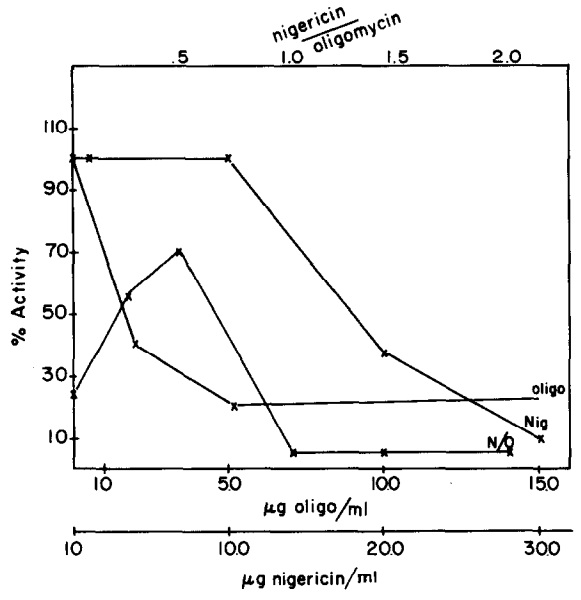

Fig.3. Reversal of oligomycin inhibition by nigericin. ATPase activity is shown as a function of oligomycin concentration (oligo), as a function of nigericin concentration (nig) and as a function of the nigericin/oligomycin ratio (N/O) with oligomycin held at a concentration of 10 μg/ml.

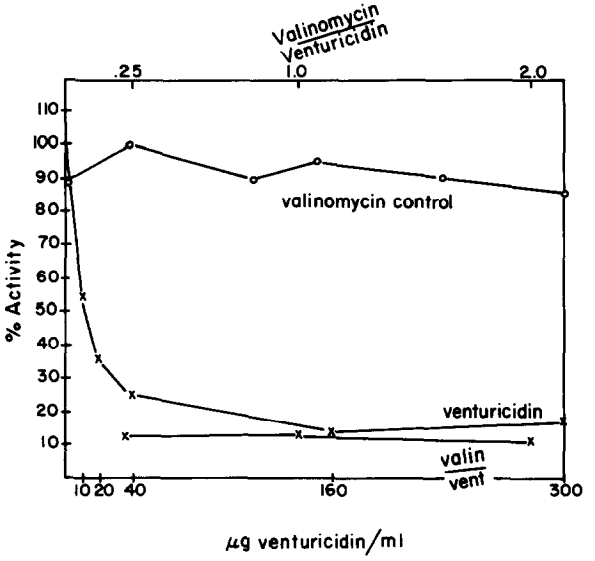

Fig.4. Failure of valinomycin to reverse venturicidin inhibition of ATPase activity. Venturicidin and valinomycin inhibition curves are shown as indicated on the figure. The activity in the presence of 10 μg/ml. venturicidin and varying levels of valinomycin is shown as the [valinomycin]/[venturicidin] ratio (Val/Ven).

the presence of 0.5 mM KCl the ability of valinomycin to reverse inhibition was slightly enhanced. On the other hand, addition of 0.5 mM KCl without valinomycin causes increased oligomycin inhibition.

Reversal of oligomycin inhibition by valinomycin is observed only in studies with mitochondrial particles. Preparations of Triton X-100 solubilized oligomycin sensitive ATPase, while strongly inhibited by oligomycin, are not subsequently reactivated by valinomycin.

The ionophoric compound nigericin also reverses the oligomycin inhibition (fig.3). However, at higher concentrations, nigericin itself becomes inhibitory. The level of nigericin required for reversal is again considerably decreased in mitochondrial particles from strain OR-4 with altered ATPase subunit 9.

Both valinomycin and nigericin have been reported to transport K^+-ions and protons across membranes. The ionophore carboxyl cyanide *m*-chlorophenylhydrazone (CCCP), which is exclusively a proton transporter had no measurable effect on the level of oligomycin inhibition.

Venturicidin is an ATPase inhibitor with properties similar in many ways to oligomycin. A number of yeast mutants resistant to valinomycin are simultaneously cross resistant to venturicidin. However, recent analyses have suggested that venturicidin acts at subunit 6 of the oligomycin sensitive ATPase complex rather than at subunit 9 [14]. It can be seen in fig.4 that venturicidin inhibition of D243-4A cannot be reversed by valinomycin.

4. Discussion

The results of these experiments can most readily be fit into current hypotheses regarding the mode of action of the various inhibitors and ionophores by proposing first that oligomycin blocks ATPase activity by binding to subunit 9 directly. This inhibits activity by preventing proton translocation. The ionophores valinomycin or nigericin provide a mechanism for relieving this block, allowing an alternate pathway for ion transport. With this proposal, however, it is some-

what surprising that CCCP does not also reverse inhibition. The ability to bind K^+ is apparently a requirement in ionophores reversing the oligomycin block. Also, mutations of subunit 9 (Class B oligomycin resistant mutants) which cause a decreased sensitivity to oligomycin simultaneously decrease the levels of ionophores required to reverse the inhibition of ATPase. These latter results would suggest either some specific mode of interaction between the ionophores and subunit 9 or that the ionophoric activity takes place in a membrane environment with properties greatly influenced by the structure of subunit 9. Direct competition of ionophore for oligomycin binding sites is ruled out by the experiments shown in figure 2 and intact membrane vesicles appear required for reversing the inhibition. Thus, ionophoric activity occurring in the immediate vicinity of the ATPase subunit 9 protein and influenced by its properties seems most likely.

This conclusions is somewhat at variance with the commonly postulated mode of function of valinomycin as a general ionophore. However, it is difficult to rationalize the data presented here with a mechanism postulating no specific interaction with proteins of the ATPase system. Support for the conclusion that very specific interaction may be involved comes from the report of Griffiths [7] that cytoplasmically inherited valinomycin resistant mutants of yeast may be obtained. While further analysis of these mutants is required, the observed cytoplasmic inheritance makes it unlikely that these mutants result from permeability changes or enhanced detoxification reactions.

The differences noted for ionophore effects in reversing oligomycin but not venturicidin inhibition give another indication that subunit 9 may be quite directly involved in some protonophoric function. Both these inhibitors act on the 'membrane sector' of oligomycin sensitive ATPase but not on the soluble F_1-ATPase. Inhibition by venturicidin at subunit 6 is not reversed by the ionophores, suggesting that this peptide is probably not involved directly with ion translocating functions. On the other hand, reversal of oligomycin inhibition at subunit 9 by a mechanism that probably does not displace the oligomycin but can substitute in ion-translocation does suggest this role for subunit 9 or a component attached to subunit 9.

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