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The K^+-Ionophores Nonactin and Valinomycin Interact Differently with the Protein of Reconstituted Cytochrome *c* Oxidase

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Abstract

The K^+-ionophores valinomycin and nonactin induce a qualitatively identical change of the visible spectrum of isolated oxidized cytochrome *c* oxidase (red shift), but the amplitude is half with nonactin. Valinomycin, in the presence or absence of a protonophore, stimulates the respiration of the reconstituted enzyme to a higher extent than nonactin and results in a higher K_m for cytochrome *c*. In contrast, nonactin causes a fivefold rate of proton conductivity across a liposomal membrane, after induction of a K^+-diffusion potential. The data indicate that respiratory control by these antibiotics is not only due to degradation of a membrane potential, but rather to specific interaction with and modification of cytochrome *c* oxidase.

Key Words: Cytochrome *c* oxidase; valinomycin; nonactin; respiratory; proton conduction; protonophore.

Introduction

The phenomenon of respiratory control, discovered by Lardy and Wellman (1952), was originally understood as the stimulation of respiration of isolated mitochondria by the phosphate acceptor ADP. It implied a coupling between electron transport and ATP synthesis, which can be cleaved by uncouplers of oxidative phosphorylation. Later, after the advent of Mitchell's chemiosmotic hypothesis (Mitchell, 1961, 1966), the term was also used to describe the stimulation of electron flow in "proton pumps" by uncouplers, either in intact organelles (vesicles) or in reconstituted systems (Nicholls, 1982). According to the chemiosmotic hypothesis, protonophores and ionophores

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stimulate electron transport of proton pumps by degrading the proton and/or ion gradient across the membrane. Thus, respiratory control is generally understood as the limitation of electron flow by the membrane potential and/or pH gradient (Nicholls, 1982). Protonophores and ionophores are assumed to solely translocate protons and ions across the lipid bilayer of biological membranes, not to modify the proton pumps. Nevertheless, it has been repeatedly demonstrated that uncoupler of oxidative phosphorylation do also interact specifically with energy-transducing enzymes (Hanstein, 1976; Hanstein *et al.*, 1974).

Recently we demonstrated a specific and stoichiometric binding of valinomycin to isolated as well as reconstituted cytochrome *c* oxidase, as shown by a red shift in the Soret region of the visible spectrum (Steverding and Kadenbach, 1989). In addition, we showed that proton translocation in reconstituted cytochrome *c* oxidase is induced by valinomycin, because no proton pumping, but instead an uptake of protons, is found in the absence of the ionophore, whereas maximal proton pumping is obtained at 1 mol valinomycin per mole reconstituted cytochrome *c* oxidase (Steverding and Kadenbach, 1989). Here we show that also respiratory control of reconstituted cytochrome *c* oxidase by the K^+-ionophores valinomycin and nonactin must be due, at least in part, to a specific interaction with the enzyme protein, because the two ionophores have an opposite effect on induction of proton conductivity in pure liposomes via a potassium diffusion potential, and on the spectral change and stimulation of respiration in reconstituted cytochrome *c* oxidase.

Materials and Methods

Chemicals

L-α-Phosphatidylcholine (type II from soybean), cytochrome *c* (type VI from horse heart), and nonactin were obtained from Sigma, and valinomycin and CCCP from Boehringer. All other reagents were of the highest purity grade commercially available.

Preparation of COX and COX Vesicles

COX[2] from bovine heart was prepared from isolated mitochondria as described before (Errede *et al.*, 1979) and reconstituted into liposomes by the cholate dialysis method as described by Casey *et al.* (1979a). The orientation

[2]Abbreviations: COX, cytochrome *c* oxidase; val, valinomycin; non, nonactin; CCCP, carbonyl cyanide *m*-chlorphenylhydrazone; RCR, respiratory control ratio.

of the enzyme was measured as described by Casey *et al.* (1979b). It was found that more than 90% of the enzyme complex was orientated "right side out" as in intact mitochondria.

Measurements of Spectra

Difference spectra of isolated COX, with and without the ionophore, were recorded with an Uvikon 810 spectrophotometer (Kontron) from 480 to 380 nm at a concentration of 2.5 μM cytochrome aa_3 in 100 mM K-Hepes, pH 7.4, 50 mM KCl, and 0.05% laurylmaltoside.

Activity Measurements

Oxygen uptake was measured polarographically with a Clark-type electrode according to Ferguson-Miller *et al.* (1978) in 10 mM K-HEPES, pH 7.4, 40 mM KCl, 0.1 mM EDTA, 25 mM ascorbate, 0.02 μM COX, and 50 μM cytochrome *c* in the absence or presence of 1 μM antibiotics and/or 3 μM CCCP at 25°C.

Ferrocytochrome *c* oxidation was measured photometrically with an Uvikon 810 spectrophotometer (Kontron) at 550 nm in 10 mM K-HEPES, pH 7.4, 40 mM KCl, 0.014 μM COX, and 10 μM ferrocytochrome *c* in the absence or presence of 1 μM antibiotics and/or 3 μM CCCP at room temperature. The apparent K was calculated from the slopes of semilogarithmic plots (ln A vs. time) by linear regression. RCR is defined as the quotient of COX activity in the presence and absence of uncoupler.

Measurement of Proton Conductivity of COX Vesicles and COX-Free Liposomes

Proton conductivity was measured with a pH microcombination electrode (405-M5 from Ingold, Steinbach) connected to a Beckman Expandometric IV pH meter as described by Papa *et al.* (1987). 250 μl COX vesicles or 250 μl COX-free liposomes, respectively, were suspended in 1 mM Na-HEPES, 100 mM choline-Cl, and 0.1 mM EDTA, pH 7.0, to a final volume of 1.5 ml, thus resulting in a potassium ion gradient of about 30 mM K^+ inside and 5 mM K^+ outside of the vesicles. The proton conductivity was initiated by addition of 1 μM antibiotic. The system was calibrated with small aliquots of a standard solution of 10 mM HCl.

Results

Valinomycin and nonactin are known to exclusively translocate K^+ ions across lipid bilayers (Lardy *et al.*, 1967). Both ionophores induce the same

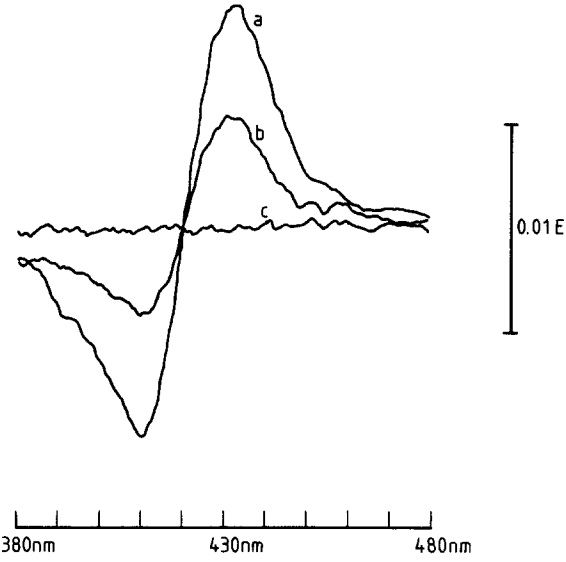

Fig. 1. Spectral change of soluble COX in the oxidized γ-band by antibiotics. 2.5 μM soluble COX in 100 mM K-HEPES, 50 mM KCl, and 0.05% laurylmaltoside, pH 7.4, was incubated with 10 μM antibiotic in 1 μl ethanol (sample cuvette) or with 1 μl ethanol (reference cuvette) and after 15 min the difference spectrum was recorded. (a) 10 μM valinomycin; (b) 10 μM nonactin; (C) without antibiotic.

spectral change in soluble oxidized bovine heart cytochrome *c* oxidase, which differs only in the amplitude, as shown in Fig. 1. This spectral change, however, is not accompanied by a change of the electron transport activity of the soluble enzyme, as measured by the polarographic assay. With the spectrophotometric method, less than 15% inhibition of the rate of ferrocytochrome *c* (10 μM final concentration) oxidation was obtained in the presence of 1 μM antibiotic (not shown).

Full stimulation of the respiratory rate of reconstituted cytochrome *c* oxidase requires the addition of both protonophores and ionophores, because the enzyme is assumed to generate both a proton gradient (proton pumping) and a membrane potential (vectorial uptake of protons from the matrix for the formation of H_2O). Although valinomycin and nonactin are assumed to degrade the membrane potential in the same manner, they stimulate the rate of respiration of reconstituted cytochrome *c* oxidase to a different extent, measured either by the polarographic or photometric assay, in the absence or presence of a protonophore (CCCP) (Table I). The different respiratory control ratio obtained with valinomycin and nonactin could be explained by a different lipid solubility of the two antibiotics, requiring more nonactin to obtain the same extent of respiratory control. Therefore a

Table I. Respiratory Control Ratio of COX Vesicles Measured with Antibiotics and/or CCCP[a]

Experiment	RCR	
	Polarographic assay[b]	Photometric assay[c]
1 μM val	1.9 ± 0.4 (5)	2.1 ± 0.4 (3)
1 μM val + 3 μM CCCP	9.8 ± 0.6 (7)	8.5 ± 0.3 (2)
1 μM non	1.4 ± 0.2 (5)	1.2 ± 0.4 (3)
1 μM non + 3 μM CCCP	6.7 ± 0.5 (7)	6.3 ± 0.7 (2)
3 μM CCCP	4.0 ± 0.4 (8)	4.9 ± 0.7 (3)
1 μM val + 1 μM non + 3 μM CCCP	8.9 ± 0.5 (12)	n.d.

[a]For experimental conditions, see Materials and Methods. The values presented are mean ± SEM. The number of experiments is given in parentheses.
[b]The turnover of COX vesicles in the absence of uncoupler was 27.3 ± 4.8 s^{-1}.
[c]The apparent K value of the ferrocytochrome *c* oxidation in the absence of uncoupler was 0.025 ± 0.009 s^{-1}.

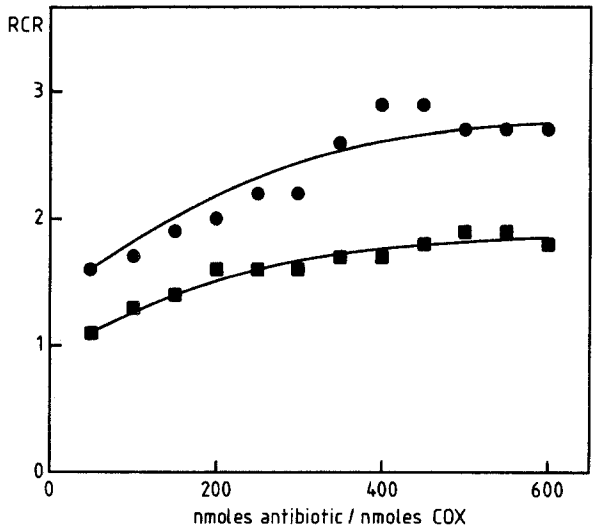

Fig. 2. Dependence of the respiratory control ratio on the antibiotic/COX ratio. Oxygen uptake of COX vesicles was measured polarographically as described under Material and Methods with increasing amounts of antibiotics. (●) Valinomycin; (■) nonactin. The turnover of the COX vesicles in the absence of antibiotics was 24.7 ± 1.5 s^{-1}.

titration of the two ionophores was performed as shown in Fig. 2. At higher concentrations both antibiotics stimulate the rate of respiration, presented as respiratory control ratio, but the final rate is higher with valinomycin than with nonactin.

The results suggest an interaction of the two ionophores with the enzyme protein. Therefore it was of interest to see if the kinetics of ferrocytochrome

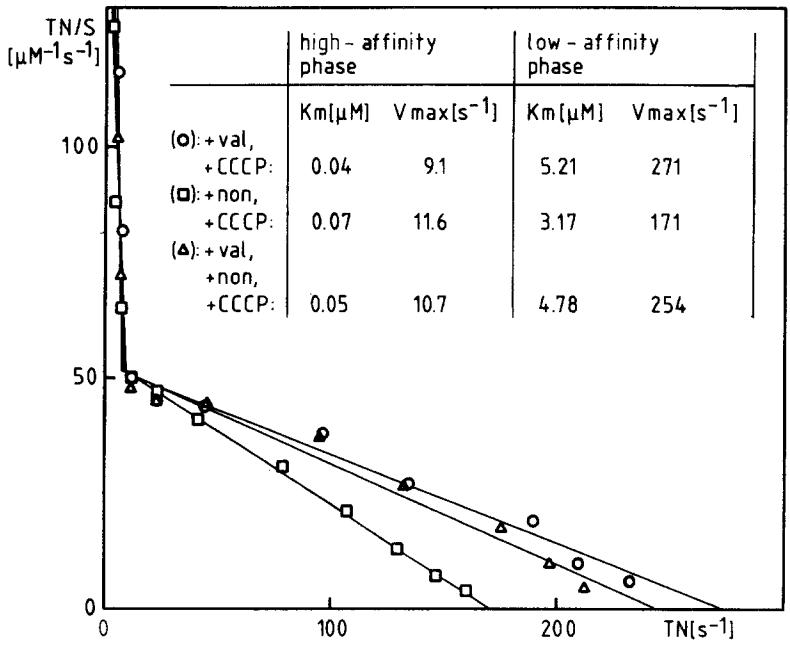

Fig. 3. Eadie–Hofstee-plot of the kinetics of cytochrome *c* oxidation by vesicles in the presence of antiobiotics and CCCP. Enzymatic activity was measured polarographically in 10 mM K-HEPES, pH 7.4, 0.1 mM EDTA, 25 mM potassium ascorbate, and 0.02–40 μM cytochrome *c* with 0.02 μM reconstituted COX. (○) 1 μM valinomycin and 3 μM CCCP; (□) 1 μM nonactin and 3 μM CCCP; (△) 1 μM valinomycin, 1 μM nonactin, and 3 μM CCCP.

c oxidation of the reconstituted enzyme was also influenced by the two ionophores in a different manner. In Fig. 3 is presented an Eadie–Hofstee plot of the kinetics of ascorbate oxidation of the reconstituted enzyme at various cytochrome *c* concentrations, measured polarographically. In the presence of CCCP nonactin results in a lower V_{max} and lower K_m for cytochrome *c* (at the low-affinity phase) than valinomycin.

The higher rate of respiration obtained with valinomycin, as compared to nonactin (Fig. 2), could be interpreted by a higher ionophoretic activity of valinomycin. Therefore the K^+ diffusion potential-driven proton conductivity of liposomes, with or without incorporated cytochrome *c* oxidase, was measured (Fig. 4). The applied ionophore concentration was sufficient to induce the full K^+ diffusion potential (Krishnamoorthy and Hinkle, 1984). Nonactin induced a 5–10 times higher proton conductivity than valinomycin in COX vesicles or in pure liposomes. This result, however, does not allow one to distinguish between translocation of protons through the lipid bilayer or via the ionophores, due to their nonspecific protonophoric activity. The difference in the proton conductivity suggests that protons are translocated,

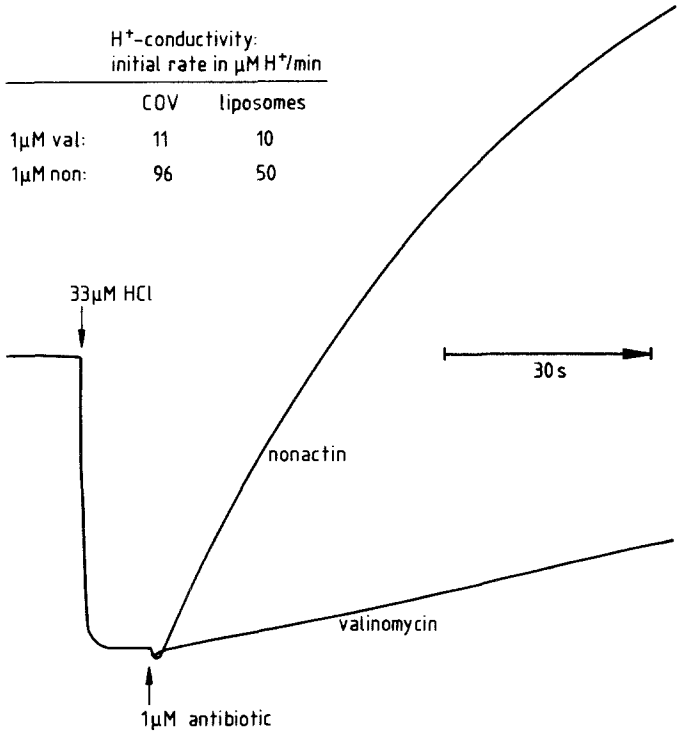

Fig. 4. Proton conductivity of COX vesicles and liposomes. The proton conduction of COX vesicles (COV), as shown in the figure, and of COX-free liposomes, presented in the table of the inset, was induced by addition of 1 μM valinomycin or 1 μM nonactin, respectively. For further details see Materials and Methods.

at least in part, via the ionophores and that nonactin represents a better protonophore than valinomycin. The data of Table I, however, demonstrate a higher uncoupling of respiration by valinomycin than by nonactin, which correlates well with the enlarged spectral change obtained with valinomycin, but not with the different ionophoretic activity of the two antibiotics.

Discussion

Recently we have shown a stoichiometric binding of valinomycin to reconstituted cytochrome *c* oxidase, accompanied by a conformational change, as indicated by a red shift of the visible spectrum (Steverding and Kadenbach, 1989). It was also found that the reconstituted enzyme, instead of proton pumping, takes up protons in the absence of valinomycin, whereas 1 mol of valinomycin per mole cytochrome aa_3 is sufficient to modify the

enzyme into a proton-pumping species. In the present publication we have asked the question: Is valinomycin-induced respiratory control of reconstituted cytochrome *c* oxidase due to degradation of a membrane potential via translocation of K^+ ions through the lipid bilayer, or to modulation of respiratory activity due to binding of the antibiotic to the enzyme?

The comparison of the effects of valinomycin and nonactin, both K^+ ion-specific ionophores (Lardy *et al.*, 1967), clearly indicates that respiratory control cannot be solely interpreted by membrane potential degradation, but rather reflects, at least in part, a modification of the catalytic properties of COX: (1) Valinomycin and nonactin bind to the soluble enzyme, but the latter causes only half of a qualitatively identical spectral change (Fig. 1). (2) Even at very high antibiotic concentrations, nonactin stimulates the rate of respiration of reconstituted cytochrome *c* oxidase only to half of the value obtained with valinomycin (Fig. 2). (3) The two antibiotics modify the K_m for cytochrome *c* of the reconstituted enzyme to a different extent (Fig. 3). Whereas these three observations could be interpreted by assuming a higher lipophily of valinomycin as compared to nonactin, the results of Fig. 4 contradict this interpretation.

In summary, we conclude that respiratory control of reconstituted cytochrome *c* oxidase by valinomycin cannot solely be interpreted by degradation of a membrane potential. The stimulation of respiration is at least due in part to binding and modification of the enzyme catalytic activity.

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References

Casey, R. P., Chappell, J. B., and Azzi, A. (1979a). *Biochem. J.* **182**, 149–156.
Casey, R. P., Thelen, M., and Azzi, A. (1979b). *Biochem. Biophys. Res. Commun.* **7**, 1044–1051.
Errede, B., Kamen, M. O., and Hatefi, Y. (1978). *Methods Enzymol.* **53**, 40–47.
Ferguson-Miller, S., Brautigan, D. L., and Margoliash, E. (1978). *J. Biol. Chem.* **253**, 149–159.
Hanstein, W. B. (1976). *Biochim. Biophys. Acta* **456**, 129–148.
Hanstein, W. G., and Hatefi, Y. (1974). *J. Biol. Chem.* **249**, 1356–1362.
Krishnamoorthy, G., and Hinkle, P. C. (1984). *Biochemistry* **23**, 1640–1645.
Lardy, H. A., and Wellman, H. (1952). *J. Biol. Chem.* **195**, 215–224.
Lardy, A. H., Graven, N. S., and Estrada-O, S. (1967). *Fed. Proc.* **26**, 1355–1360.
Mitchell, P. (1961). *Nature (London)* **191**, 144–148.
Mitchell, P. (1966). *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, Glynn Research, Bodmin.

Nicholls, D. G. (1982). *Bioenergetics. An Introduction to the Chemiosmotic Theory*, Academic Press, London, New York.
Papa, S., Capitanio, N., and DeNitto, E. (1987). *Eur. J. Biochem.* **164**, 507–516.
Steverding, D., and Kadenbach, B. (1989). *Biochem. Biophys. Res. Commun.* **160**, 1132–1139.