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OXONOL RESPONSE AND ENERGY TRANSDUCTION IN AN ATP-P_i EXCHANGE COMPLEX

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Among preparations of oligomycin-sensitive ATPases, complex V stands out because it is capable of ATP-P_i exchange as isolated, without further manipulations[1]. Therefore, this complex should be especially useful for studying the final steps in the mechanism of ATP synthesis in mitochondria. Since charge separation is a logical step in the mechanism of electrogenic proton pumping, we have tested whether complex V is able to elicit an energy-dependent response of a potential-sensitive dye. Indeed, we found that in the presence of ATP and complex V, oxonol VI undergoes a spectral red shift similar to the one observed with submitochondrial particles[2]. In this contribution, the effect of substrates and energy transfer modifiers on the oxonol response of complex V will be presented.

Fig. 1 shows the effect of inhibitors, ionophores and uncouplers on the ATP-dependent red shift of oxonol VI in the presence of complex V as measured by the absorbance increase at 630 nm-602 nm. It is seen that valinomycin, CCCP and oligomycin abolish the oxonol response fast and completely, while the inhibition by DCCD takes 20-30 sec to fully develop. In contrast to submitochondrial particles (SMP) the effect of valinomycin does not require the presence of K^+ and nigericin, probably because complex V as pre-

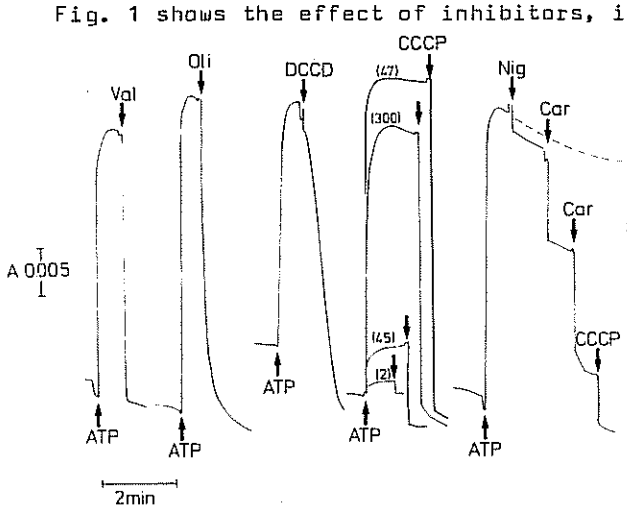

Fig. 1. ATP-dependent response of oxonol in the presence of complex V. Complex V: 0.25 mg/ml buffer (0.25 sucrose, 10 mM $MgSO_4$, 50 mM Tris-SO_4, pH 7.5); oxonol: 1 /uM; ATP: 5 mM; valinomycin: 0.2 /ug/ml, nigericin: 0.5 /ug/ml; oligomycin: 1 /ug/ml; cardiolipin: 65 /ug/ml; DCCD: 10 /uM; CCCP: 20 /uM, Temp.: 30° C.

pared contains ammonium sulfate. Nigericin alone is essentially ineffective. Cardiolipin which is known to dissociate F_1-ATPase from submitochondrial particles[3,4] decreases the oxonol response at 0.26 mg/mg protein. Fig. 1 shows also that large absorbance increases are only obtained with complex V preparations with good ATP-P_i exchange activities (numbers in parentheses: nmol P_i exchanged/min mg protein). Fig.2 shows the specificity of nucleoside triphosphates (NTP) in eliciting the oxonol response. Energization by ATP is rapidly and completely reversed by AMP-PNP at a concentration well below that necessary for nearly complete inhibition of ATP-P_i exchange[1]. At 50/uM, AMP-PNP decreases dramatically the rate of the ATP-induced response and limits the extent to about 50% of the control. At this concentration of AMP-PNP, ATP-P_i exchange is unaffected while the ATPase activity is nearly fully inhibited under the conditions used (Table I). UTP which is an ineffective substrate in complex V catalyzed NTP-P_i exchange[1] induces a slow absorption increase reaching maximally 25% of the ATP response, even at concentrations up to 15 mM. GTP (and

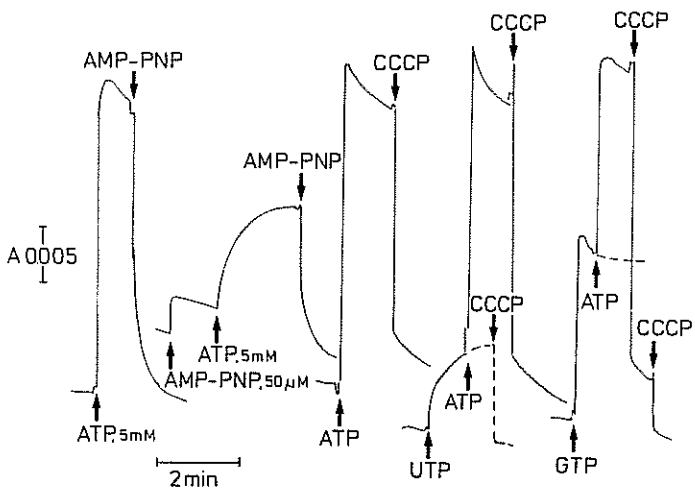

Fig. 2. Nucleoside triphosphate-induced oxonol response. Conditions as in Fig. 1. AMP-PNP, ATP, UTP, GTP: 1 mM each; CCCP: 10/uM.

TABLE I

EFFECT OF MODIFIERS ON ACTIVITIES OF COMPLEX V

Inhibitor	Concentration mM	nmol/mg	% ATPase activity	% ATP-P_i exchange	% ATP-induced spectral shift
AMP-PNP	0.05		10	110	50
NPA	0.03		100	10	75
Valinomycin		0.9	100	50	0
Aurovertin D		25	10		50

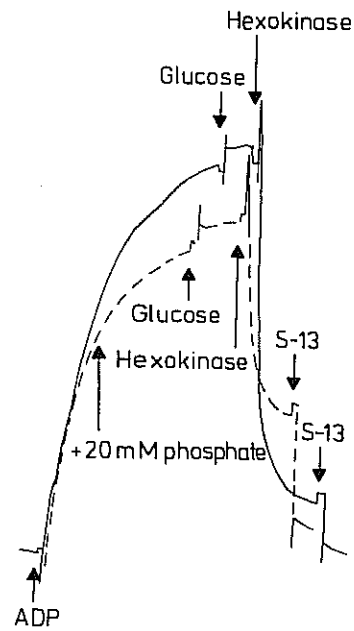

Fig. 3. ADP-induced oxonol response. Conditions as in Fig. 1. ADP: 1 mM; glucose: 25 mM; hexokinase: 20 U, S-13: 1 µM.

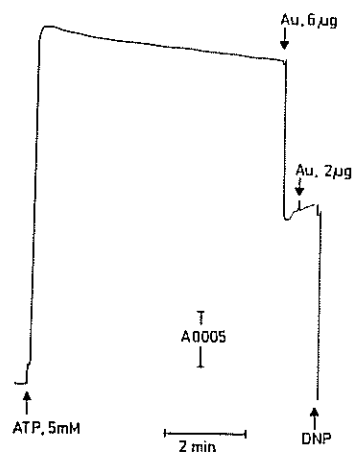

Fig. 4. Effect of aurovertin on the ATP-dependent oxonol response. Conditions as in Fig. 1. DNP: 0.2 mM.

ITP, not shown), although only marginally active in NTP-P_i exchange[1], elicits a rapid oxonol response limited to ~50% of the ATP effect. Similar to UTP and AMP-PNP + ATP, the effect of ADP develops slowly, reaching ~80% of the ATP response (Fig. 3). The ADP effect can be modulated by phosphate and, like all NTP responses shown above, is sensitive to uncouplers and hexokinase + glucose.

In contrast to the energy transfer inhibitors mentioned above, aurovertin inhibits the ATP-dependent spectral response only to ~50% even at 32 µg/ml protein (17 µM), i.e. at a concentration which is two orders of magnitude higher than that necessary for half-maximal inhibition of ATPase[5]. As seen in Fig. 4, the other half of the oxonol response is readily abolished by DNP. Similarly, some uncouplers are apparently unable to affect more than half of the oxonol response even at very high concentrations. Thus, as seen in Fig. 5, the effect of DNP and 6(2'-acetoxyethyl)-2,4-dinitrophenol (AE-DNP) appears to level off near 50% of the oxonol response. 6(2'-hydroxyethyl)-2,4-dinitrophenol (HE-DNP), an uncoupler which inhibits energy-dependent reactions half-maximally at 40-170 µM[6] is completely ineffective in this test at least at ≤400 µM. This situation is changed in the presence of aurovertin. Fig. 6 shows that the oxonol response remaining after treatment with 4 µM aurovertin is much more sensitive to uncouplers. Similarly, the effectiveness of aurovertin is potentiated by the presence of uncouplers at concentrations which by themselves have little

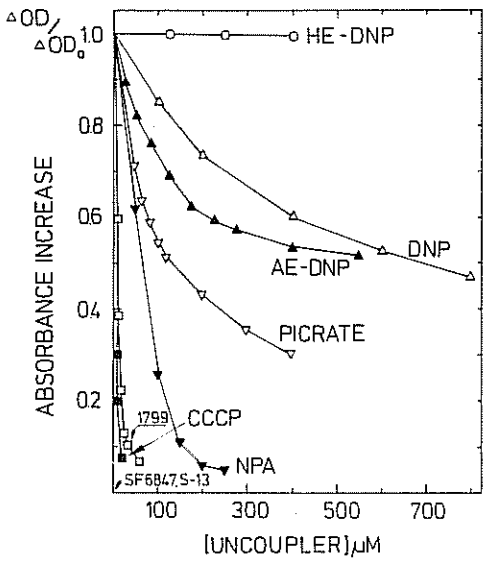

Fig. 5. Effect of uncouplers on the ATP-dependent oxonol response Conditions as in Fig. 1.

TABLE II

RATIO OF CONCENTRATIONS NECESSARY FOR 50% UNCOUPLING OF THE OXONOL RESPONSE IN COMPLEX V AND SMP

Uncoupler	$\frac{\mu M}{\mu M}$	$\frac{nmol/mg}{nmol/mg}$
CCCP	35.3	14.1
SF 6847	12.5	5.0
S-13	8.8	3.5
TTFB	8.8	3.5
DNP	3.1	1.2
1799	2.9	1.2
Picrate	2.9	1.2
TPB^-	2.0	0.8
PCB^-	2.0	0.8

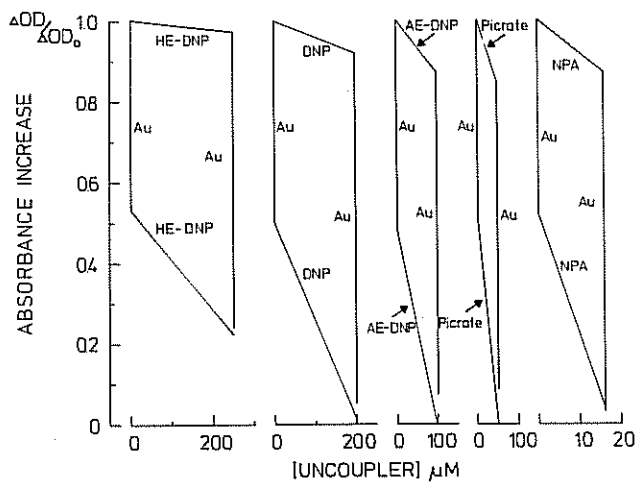

Fig. 6. Combined effects of uncouplers and aurovertin on the ATP-induced oxonol response. Conditions as in Fig. 1. Aurovertin: 4.2 μM.

effect.

The effect of uncouplers on the oxonol response of complex V differs quantitatively from the situation in submitochondrial particles. It is seen in Table II that a number of uncouplers are 3 to 35 times less effective on complex V than in SMP. In contrast, non-protonophoretic, penetrating anions[7] such as TPB^-, PCB^- and picrate are nearly equally effective in both systems.

DISCUSSION

Oxonol V and VI are potential-sensitive probes responding to charge separation in chloroplasts[8], submitochondrial particles and ATPase vesicles[9]. In this contribution we show that with oxonol VI, energization of complex V can be monitored, even though a test with ACMA (not shown) fails to indicate a pH gradient. The virtual absence of Δ pH in complex V is also suggested by the ability of valinomycin to fully abolish the oxonol response in the absence of nigericin, and by the failure of nigericin to increase the oxonol response (Fig. 1), in contrast to the situation in submitochondrial particles[2]. These data can be explained by the presence of NH_4^+ in equilibrium with diffusible NH_3 [10].

The nucleotide specificity (Fig. 2) shows that GTP and UTP induce oxonol responses which are, within a 5 to 15-fold concentration range, constant and smaller than the ATP, but relatively larger than the NTP-P_i exchange activities[1]. The effect of modifiers (Table I) and the specific activity data indicate a qualitative, but no strictly quantitative correlation between extent of oxonol response and ATP-P_i exchange activity.

The cumulative effect of aurovertin and uncouplers (Fig. 6) is reminiscent of the interaction of HCO_3^- and aurovertin on F_1 [11]. Literature data suggest that under the conditions used, one aurovertin binding site is fully saturated[12], ATPase is nearly (Table I) and ATP-P_i exchange is completely inhibited[13]. At the same time, the oxonol response is only decreased to 50 ($\pm$ 5) % (Figs. 4 and 6), and is now much more uncoupler sensitive. These data suggest that at least a part of the oxonol response is not dependent on rapid turn-over of the ATP-P_i exchange complex. The effect of uncouplers in the presence of aurovertin may then be due to direct interaction with complex V. Furthermore, it is possible that oxonol under the conditions used ($\sim$2-fold molar excess over complex V) may, at least in part, report charge separation within complex V at the molecular level. The differences in uncoupler effectiveness in complex V and submitochondrial particles (Table II) may reflect energization differences. As stated above, a ΔpH is probably largely absent in complex V. Thus, in going from CCCP to PCB^- (phenyl dicarbaundecaboranate), the order of uncouplers may reflect increasing ability to abolish charge separation.

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REFERENCES

1. Stiggall, D.L., Galante, Y.M. and Hatefi, Y. (1978) J. Biol. Chem. 253, 956-964.
2. Bashford, C.L. and Thayer, W.S. (1977) J. Biol. Chem. 252, 8459-8463.
3. Toson, G., Contessa, A.R. and Bruni, A. (1972) Biochem. Biophys. Res. Comm. 48, 341-347.
4. Lee, C.-P., Huang, C.H. and Cierkosz, B.I.T. (1974) Membrane Proteins in Transport and Phosphorylation, North Holland, Amsterdam, pp. 161-170.
5. Linnet, P.E. and Beechey, R.B. (1979) Meth. Enz. 55, 472-518.
6. Kiehl, R., Schuermann, M. and Hanstein, W.G., in preparation.
7. Grinius, L.L., Jasaitis, A.A., Kadziauskas, Yu.P., Liberman, E.A., Skulachev, V.P., Topali, V.P., Tsofina, L.M. and Vladimirova, M.A. (1970) Biochim. Biophys. Acta 216, 1-12.
8. Schuurmans, J.J., Casey, R.P. and Kraayenhof, R. (1978) FEBS Letters 94, 405-409.
9. Smith, J.C., Russ, P., Cooperman, B.S. and Chance, B. (1976) Biochemistry 15, 5094-5105.
10. Montal, M., Chance, B. and Lee, D.-P. (1970) J. Membr. Biol. 2, 201-234.
11. Ebel, R.E. and Lardy, H.A. (1975) J. Biol. Chem. 250, 4992-4995.
12. Chang, R.-M. and Penefsky, H.S. (1974) J. Biol. Chem. 249, 1090-1098.
13. Lee, C.-P. and Ernster, L. (1968) Eur. J. Biochem. 3, 391-400.