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ATP-DEPENDENT SPECTRAL RESPONSE OF OXONOL VI IN AN ATP-P_i EXCHANGE COMPLEX

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(1) Energy transduction in an ATPase complex (complex V) has been studied in two reactions catalyzed by this system, i.e., ATP-dependent spectral shift of oxonol VI, and ATP-P_i exchange activity. (2) Aurovertin alone inhibits 50% of the oxonol shift at 2 μM, and no further inhibition occurs at up to 12 μM. In combination with even weakly effective uncouplers, 4 μM aurovertin fully abolishes the oxonol response. No such effects are observed in the presence of oligomycin and uncouplers. (3) No pH gradient is detectable by quenching of 9-amino-6-chloro-2-methoxyacridine; and nigericin is without effect on the oxonol response. Valinomycin is inhibitory even in the absence of added potassium, due to ammonium ions introduced during the purification steps. Thiocyanate inhibits the dye response by only 10–27%, depending on the preparation. The extent of the oxonol response depends on the ATP/ADP ratio rather than the phosphorylation potential. (4) The dye response in the ATPase complex is 4–7-times less sensitive to bile salts than in submitochondrial particles. The inhibition by cardiolipin can be reversed by the addition of phospholipids. (5) The possibility is discussed that the oxonol response in the ATPase complex reflects, at least in part, a more local, ATP-dependent and energy-related process.

Introduction

Purified preparations of oligomycin- and DCCD-sensitive ATPase complexes have proven to be valuable tools for studying the molecular mechanism of energy transduction and ATP synthesis in mitochondrial, bacterial and chloroplast membranes. These investigations are hampered in some aspects by the fact that important indicators for energy conservation capability in these preparations cannot easily be monitored. Assays for net ATP-P_i exchanges are discontinuous, time-consuming and usually not sensitive enough to reflect small changes in activity. Spectral probes offer additional, continuous and sensitive means for the observation of energy-dependent phenomena in vesicular systems [1–7]. Oxonol VI is a potential-sensitive dye suitable for the study of submitochondrial particles [8], chloroplasts [9] and chromatophores [10]. With this as with other probes, it is not always clear which parameter is actually probed, as calibrations are not always possible [1,9], and other, less relevant phenomena such as surface potentials [11] may play a role.

Abbreviations: AMP-PNP, adenylyl imidodiphosphate; ACMA, 9-amino-6-chloro-2-methoxyacridine; DCCD, *N,N'*-dicyclohexylcarbodiimide; SF 6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile; S-13, 2',5-dichloro-3-tert-butyl-4'-nitrosalicyclanilide; TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; NPA, 2-azido-4-nitrophenol; DNP, 2,4-dinitrophenol; HE-DNP, 2,4-dinitro-6(2-hydroxyethyl)-phenol; NBTU, *N*-tert-butyl-*N'*-*n*-nonylthiourea; NPU, *N*-phenyl-*N'*-*n*-nonylurea; NPTU, *N*-phenyl-N'-*n*-nonylthiourea; NBU, *N*-*tert*-butyl-*N'*-*n*-nonylurea; NBD-Cl, 4-chloro-7-nitrobenzofurazan; NSPM, *N'*-(*N*-*n*-nonyl-4-sulfamoylphenyl)maleimide; PCMB, p-chloromercuribenzoic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DPDS, dipyridyl-(2)-disulfide; diamide, diazenedicarboxylic acid-bisdimethylamide.

In the present work, the ATP-dependent oxonol response of an oligomycin-sensitive ATP-P_i exchange complex (complex V, Ref. 12) was studied in order to establish to what extent this test might be a direct indicator of energy conservation in this preparation, or whether other phenomena such as surface potential changes or direct protein-dye interactions play a role in it. Therefore, the effect of inhibitors, uncouplers and detergents on the spectral response was investigated and compared with ATP-P_i exchange and ATPase activities of this preparation. Data on the oxonol response in submitochondrial particles were used to explore differences and similarities between the ATP-P_i exchange complex (which was not incorporated into liposomes), and submitochondrial particles.

Other preparations of oligomycin-sensitive ATPase complexes have also been reported to sustain an oxonol response [13,14]. A preliminary account of part of the present work has been published [15].

Methods and Materials

Complex V was prepared from phosphorylating submitochondrial particles [16] as described in Ref. 12. Preparations isolated with 0.35 mg cholate per mg protein and an exchange activity of 50–300 nmol $^{33}P_i$ per min/mg complex V (measured without addition of bovine serum albumin or phospholipid) have been used. The ATPase activity of 4–6 μmol ATP per min/mg was 85–90% oligomycin-sensitive. Concentrated as well as diluted solutions of these complexes were clear and the activities were stable to freeze-thawing. Phospholipid contents were determined according to Ref. 17 and found to vary between 7 and 8 μg phosphorus per mg complex protein or approx. 110–130 nmol phospholipid/ nmol complex. Sonicated asolectin has been prepared as in Ref. 18. F_1-ATPase was prepared according to Ref. 19, as modified in Ref. 20.

Protein was determined by the method of Lowry et al. [21]. ATP-$^{33}P_i$-exchange activity was measured at 30 °C according to Pullman [22]. ATPase activity was measured spectrophotometrically at 30 °C according to the method of Pullman et al. [23]. Quenching of the fluorescence emission of ACMA [24] was measured at 480 nm at an excitation wavelength of 410 nm with an Aminco SPF 500 spectrofluorometer. Changes in oxonol VI absorbance were monitored at 630–602 nm with a Shimadzu ultraviolet 300 dual wavelength scanning spectrophotometer. For the determination of the phosphate potential, the reaction mixture was denatured with perchloric acid (6% final concentration). After centrifugation, neutralization with 5 M KOH and removal of $KClO_4$, the concentrations of ADP, ATP and phosphate were determined enzymatically [25] and colorimetrically [26].

Oxonol VI was prepared according to Ref. 13 as follows. Hydroxylamine hydrochloride (15 g, 216 mmol) and ethyl butyrylacetate (22.8 g, 144 mmol) in methanol (92 ml) were refluxed for 90 min. After cooling to room temperature, benzene (150 ml) and water (100 ml) were added and mixed thoroughly in an extraction funnel. The aqeuous phase was discarded, and the benzene layer was washed three times with 50 mol water each, and dried over anhydrous magnesium sulfate. Benzene was removed on a rotation evaporator, and unreacted ethyl butyrylacetate was stripped at 90 ° in a vacuum of about 0.1 mbar. The yield of the orange-colored crude 3-butyl-5-(4H)-isoxazolone was 16.1 g (88%). This product (7.1 g, 56 mmol) was condensed with glutacon aldehyde dianil monohydrochloride (5.3 g, 18.6 mmol) as described for oxonol V [13], except that the reaction mixture was refluxed for 120 min. The yield was 1.74 g (30%). NSPM was prepared essentially according to Ref. 27. NPU and NPTU were prepared analog to NBU and NBTU [28] using phenylisocyanate instead of *t*-butylisocyanate, and phenylisothiocyanate instead of *t*-butylisothiocyanate, respectively. HE-DNP was prepared by nitration of *o*-hydroxyphenetyl alcohol by a procedure similar to that reported in Ref. 29 (Schuermann, M., unpublished data). The products were separated by column chromatography on silica gel with methylene chloride/acetic acid (9 : 1) as eluent, and recrystallized from acetone/water (mp. 109 °).

The sources of the materials used were as follows. Cholid acid, deoxycholic acid and phenylboric acid from EGA-Chemie; crystalline bovine serum albumin, NBD-Cl, phenylglyoxal, and dithioerythritol from Serva; myokinase (rabbit

muscle), nucleotides, oligomycin, PCMB, diamide, *N*-ethylmaleimide and glucose from Sigma (the nucleotides AMP-PNP and UTP were analyzed by Sigma and reported to be free of ATP); hexokinase, lactate-dehydrogenase, pyruvate kinase, phospho*enol*pyruvate, valinomycin, carboxyatractyloside, Ap_5A und ATP from Boehringer Mannheim; pyridoxal phosphate, DTNB and DPDS from Merck; DNP from Eastman; SF 6847 from Sumito Chemical Co.; tetraphenylborate and tetraphenylphosphonium bromide from Ventron, Karlsruhe; asolectin from Associated Concentrates, Inc., and [^{33}P]phosphoric acid from New England Nuclear. NPA [30] and Picryl acetate [31] were synthesized as described.

The following compounds were gifts which we gratefully acknowledge: ACMA from Dr. R. Kraayenhof, Vrije Universiteit Amsterdam, The Netherlands, CCCP from Dr. P.G. Heytler, E.I. Du Pont de Nemours, Wilmington, DE, U.S.A., aurovertin from Dr. R.B. Beechey, Shell Research, Sittingbourne, Kent, U.K., TTFB and nigericin from Dr. E.P. Bakker, University of Osnabrück, F.R.G., phenyldicarbaundecarboranate from Dr. M.F. Hawthrone, University of California, Los Angeles, CA, U.S.A., S-13 from Dr. P. Hamm, St. Louis, Missouri, U.S.A., Monsanto Chemical Co., bonkrekik acid from Prof. M. Klingenberg, University of Munich, F.R.G. All other chemicals were reagent grade quality.

Results

The absorption spectrum of oxonol VI in the presence of complex V undergoes a large red shift upon energization by ATP, similar to the one observed in submitochondrial particles [1]. The oxonol response (Δ absorbance 630–602 nm) increases linearly with the dye concentration until it reaches a plateau at about 20 nmol/mg protein. Assuming M_r values of 500000–600000 for the complex [32,33], saturation is reached at about 10–12 mol oxonol VI per mol of complex V. In submitochondrial particles, maximal response is obtained at 20–25 nmol oxonol VI per mg protein. This corresponds to 36–45 mol per mol membrane-bound F_1 [33], in agreement with Bashford and Smith [1]. All experiments in this communication were done below saturation, i.e., at 10–15 nmol dye per mg protein.

The oxonol response in the ATPase complex is not strictly specific for ATP. Other nucleoside triphosphates elicit two- (ITP, GTP) or four-(UTP) times smaller responses which are concentration-independent in the range tested (1–5 mM). Upon addition of ATP, the response can be increased to the control level, but not beyond. AMP-PNP slows down the development of the ATP-dependent oxonol response and limits it to 50% at a concentration where ATP-P_i exchange is not inhibited *. Surprisingly, ADP elicits a large, but slowly developing oxonol response. This effect is inhibited by AMP or AP_5A, abolished by hexokinase in the presence of glucose, and accelerated by myokinase. Thus, the ADP-effect is most likely due to ATP generated by an adenylate kinase activity present in the complex (cf. Ref. 32).

For a consideration of energy conservation in the complex, the question is relevant whether the phosphorylation potential or the ATP and ADP concentrations determine the extent of the oxonol response. It is seen in Fig. 1, panel A, that the spectral shift is apparently independent of the energy available from ATP hydrolysis when the concentration of phosphate is varied. In contrast, a positive correlation can be seen between the oxonol response and the ATP/ADP ratio (panel B) or, more clearly, the ATP/(ATP + ADP) ratio (panel C). The extent of the ATPase activity at different ATP and ADP concentrations has no direct effect on the spectral response (panel D).

Most inhibitors of ATP synthesis and hydrolysis are also effective inhibitors of the oxonol response (Table I). Notable exceptions are arsenate, 4-chloro-7-nitrobenzfurazan [35], and bathophenanthrolin [36]. Covalent modification by phenylglyoxal [37] and picryl acetate [38] reduce or abolish the spectral response, while pyridoxal phosphate [39] is ineffective. As expected, no adenine nucleotide transport appears to be necessary for the oxonol response, since carboxyatractylate or bongkrekate are without effect. A novel type of inhibition of the dye response is observed with aurovertin. Low concentrations of this inhibitor (2 μM, 1 μg/0.25 mg protein) abolish

* Actually, a 20–30% stimulation is observed under those conditions.

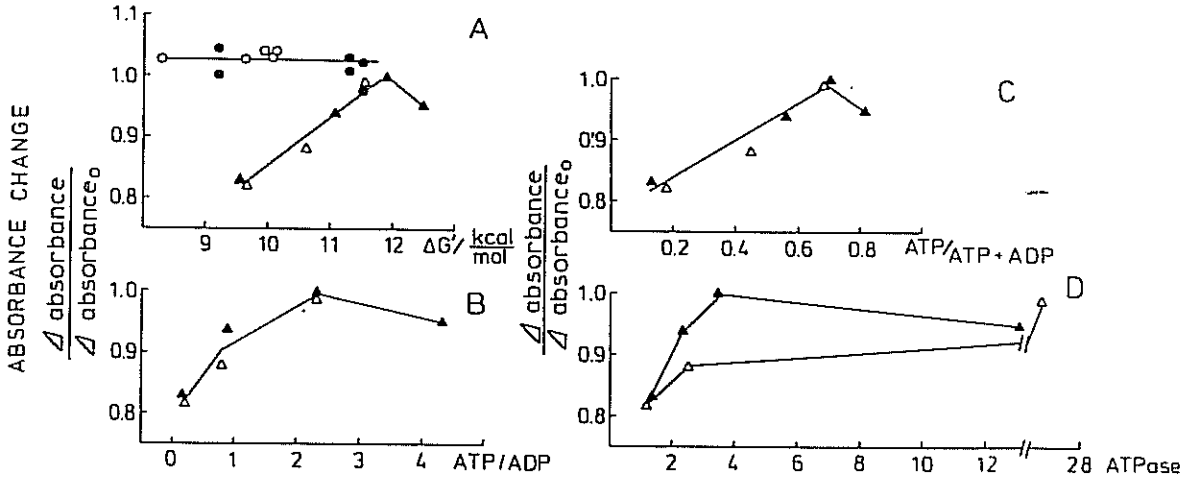

Fig. 1. Effect of phosphorylation potential and nucleotide concentrations on the oxonol response in complex V. The oxonol response as a function of the phosphorylation potential $\Delta G'$ (A), the ATP/ADP ratio (B), the ATP/(ATP+ADP) ratio (C), and the ATPase activity μmol per min/mg (D). The latter was determined from the phosphate concentration differences at the time intervals indicated below. $\Delta G'$ was calculated using $\Delta G'^{\circ} = -40$ kJ/mol ATP hydrolyzed [30]. Closed circles: oxonol response (Δ absorbance/Δ absorbance$_0$) in the presence of phosphate (2.3–102 mM), ADP (1.6–1.8 mM) and ATP (3.5–3.7 mM), 20 s after addition of ATP (5 mM). Open circles: Δ absorbance/Δ absorbance$_0$ in the presence of phosphate (5.2–110 mM), ADP (3.8–4.6 mM) and ATP (1.3–1.9 mM), 1 min after addition of ATP (5 mM), in the presence of phosphoenolpyruvate (2 mM) and pyruvate kinase (3 units). Open triangles: Δ absorbance/Δ absorbance$_0$ in the presence of phosphate, ADP and ATP, 20 s, 3 min and 10 min after addition of ATP (5 mM). Closed triangles: Δ absorbance/Δ absorbance$_0$ in the presence of Ap_5A (0.27 mM), phosphate, ADP and ATP, 20 s, 1 min, 3 min, and 13 min after addition of ATP (5 mM). Oxonol VI (3.8 nmol) and complex V (0.25 mg protein) in 1 ml of assay buffer (0.25 M sucrose, 10 mM $MgSO_4$, 50 mM Tris-sulfate, pH 7.5). Temp., 30 °C.

about 50% of the oxonol response, and no significant increase of inhibition is observed at a 7-fold higher concentration (Fig. 2). In submitochondrial particles, aurovertin exerts the same type of effect, although at a higher level of inhibition (80%, Fig. 2).

The importance of thiol groups in energy transduction [40] and ATP-P_i exchange [41] has been repeatedly demonstrated. In the ATP-dependent oxonol response, however, few of the thiol reagents tested have proven to be inhibitory (Table II). Thus, at the concentrations used, cadmium salts, diamide and *N* – ethylmaleimide are without effect. Somewhat more active are p-chloromercuribenzoate and the disulfides, DTNB and DPDS. NSPM, a lipophilic maleimide, is the only thiol reagent found to be a potent inhibitor.

The effect of ionophores and permeant ions on the oxonol response in complex V and, for comparison, in submitochondrial particles is shown in Fig. 3. In the particles, neither valinomycin nor nigericin, with or without potassium ions, abolish

TABLE I

EFFECTS OF INHIBITORS OF ATP SYNTHESIS, HYDROLYSIS AND TRANSPORT ON THE ATP-LINKED OXONOL VI RESPONSE OF COMPLEX V

Compound	Concn.	Percentage abolishment
Oligomycin	2 nmol/mg	80 [a]
DCCD	16 nmol/mg	100 [a]
Aurovertin D	25-100 nmol/mg	50
Bathophenanthroline + $CoCl_2$	30 + 10 μM	0
NBD-Cl	0.8 mM	21 [a]
Arsenate	10 mM	10
AMP-PNP [b]	50 μM	50
AMP-PNP	1 mM	100
NaN_3	0.4 mM	70 [a]
NH_2OH	20 mM	100
Picrylacetate	40 μM	100 [a]
Phenylglyoxal	35 mM	82
Pyridoxal phosphate	0.8 mM	0
Bongkrekic acid	0.1 mM	0
Carboxyatractyloside	0.35 mM	0

[a] 2 Min after addition.

[b] Added 2 min before energization with ATP.

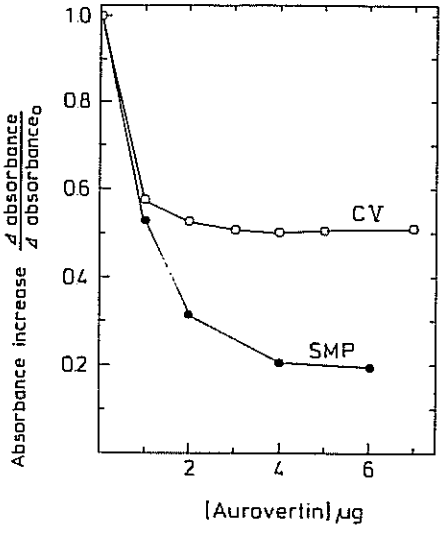

Fig. 2. Effect of aurovertin on the ATP-dependent response of oxonol VI in submitochondrial particles and complex V. Oxonol (3.8 μM), submitochondrial particles (SMP) (0.1 mg protein/ml), complex V (CV) (0.25 mg protein/ml), aurovertin (μg) in 1 ml of assay buffer. Energization with 5 mM ATP, Temp., 30 °C.

the oxonol response to a significant degree, while the combination of all three is fully effective, independent of the order of addition (A,B). Potassium thiocyanate (1–4 mM) abolishes the dye response in submitochondrial particles without further additions, as expected from the inside-out orientation of the vesicles used (A). The situation is in some aspects different in complex V. Thiocyanate has only a partial effect (10–27%) (C) depending on the preparation used, while valinomycin alone, possibly together with the ammonium ions present in the complex V preparation, fully reverses the dye response of the complex (D). The effect of nigericin alone is small (E) and opposite to the one observed in submitochondrial particles in the presence of potassium nitrate [8]. Dialysis of complex V against the assay buffer to remove ammonium ions results in inactivation. However, after applying the centrifuged-column procedure [42] using Sephadex G-50 equilibrated with 0.4 mg cholate per ml assay buffer, the effects of ionophores on complex V become similar to those shown for submitochondrial particles. Thiocyanate remains partially inhibitory (20%) on the oxonol response of the Sephadex-treated preparation. Complex V differs also from submitochondrial particles in the ΔpH-dependent quenching of acridine fluorescence. Independent of the treatment with Sephadex, there is no energy-dependent ACMA response in the presence of complex V, in contrast to submitochondrial particles.

Complex V is prepared in the presence of des-

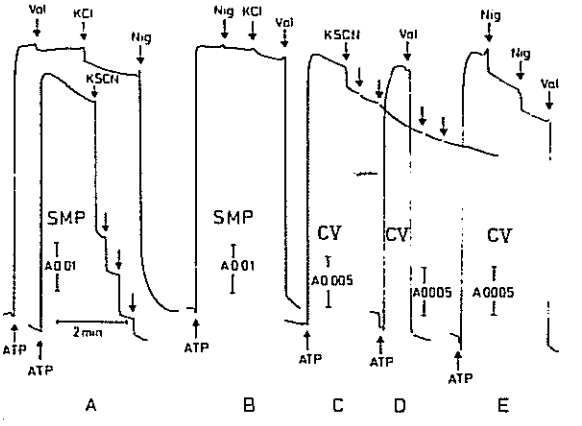

Fig. 3. Effect of ionophores on the energy-dependent absorbance increase of oxonol VI in submitochondrial particles (SMP) and complex V (CV). Additions: 0.2 μg valinomycin/ml (Val), 30 mM KCl, 0.2 μg nigericin/ml (Nig) (except in E, where 0.5 μg was added). 1 mM KSCN. Other conditions as in Fig. 2.

TABLE II

EFFECT OF THIOL REAGENTS ON THE ATP-LINKED OXONOL VI RESPONSE IN COMPLEX V

Compound	Concn. (μM)	Concn. (nmol/mg protein)	Percentage abolishment
N-Ethylmaleimide	200		4
NSPM	10	40	49 [a]
NSPM	20	80	85 [a]
PCMB	50	200	0
PCMB	200	800	47 [a]
DTNB	200		16 (5) [b]
DPDS	200		25 (17) [b]
$CdCl_2$	500		0
+ dithioerythritol	500		0
diamide	500		0

[a] 2 Min after addition.

[b] Sum of rapid and slow abolishment, values in parentheses represent rapid abolishment.

TABLE III

EFFECT OF BILE SALTS AND PHOSPHOLIPIDS ON THE ATP-LINKED OXONOL VI RESPONSE IN COMPLEX V AND SUBMITOCHONDRIAL PARTICLES

Compound	Concn. (mg/mg protein)	Percentage abolishment
Complex V		
Desoxycholate	0.5	50
+ CCCP (20 μM)		100
Cholate	4.0	50
+ CCCP (20 μM)		100
Cardiolipin	0.5	90
+ Asolectin	4	20
Submitochondrial Particles		
Desoxycholate	0.1	50
Cholate	0.55	50

oxycholate (0.3 mg per mg protein) and cholate (0.38 mg per mg protein). The question whether, during the assays for ATP-P_i exchange [12] and oxonol response, complex V is nevertheless present in vesicular form, is relevant for the reaction mechanism in this system. Therefore, the effect of bile salts and phospholipids in the assay mixture was tested *. It is seen in Table III that the concentration of desoxycholate (0.5 mg per mg protein) and cholate (4 mg per mg protein) necessary to inhibit half of the oxonol response are well above those used in the preparation of the complex. For comparison, the corresponding half-inhibitory concentrations in submitochondrial particles are 0.1 mg desoxycholate and 0.55 mg cholate per mg protein, i.e., 5- to 7-fold lower than in complex V. In all cases, the remaining response is fully sensitive to uncouplers. Cardiolipin, a phospholipid with detergent-like properties, has been used to solubilize F_1 from submitochondrial particles [43,44]. In the oxonol response, it is about as inhibiting as desoxycholate. The inhibition can be rapidly reversed by excess soybean phospholipids. It is therefore unlikely that a complete separation of F_1 from the membrane sector occurs.

The effectiveness of uncouplers in decreasing the dye response varies widely. Thus, the strongest uncouplers of oxidative phosphorylation such as

* In the concentration range used, bile salts alone have no effect on the spectrum of oxonol VI.

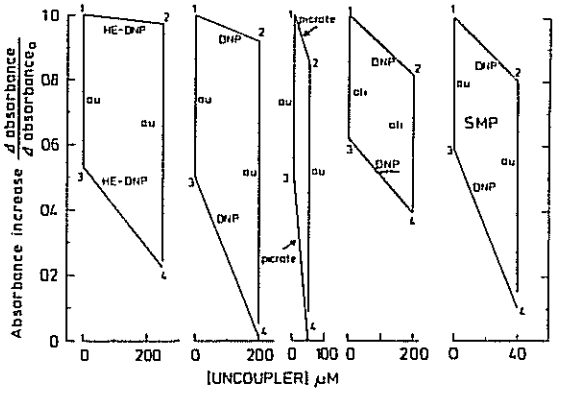

Fig. 4. Combined effect of uncouplers and inhibitors on the ATP-dependent oxonol response in complex V and submitochondrial particles. Changes in oxonol response were determined after addition of uncouplers in the absence (1 → 2) and presence (3 → 4) of inhibitors, and after addition of inhibitors in the absence (1 → 3) and presence (2 → 4) of uncouplers. Conditions as in Fig. 2. Additions: aurovertin (au) (2 μg/ml to complex V, CV, 1 μg/ml to submitochondrial particles, SMP), oligomycin (0.3 μg/ml) (oli), uncouplers (HE-DNP, DNP, picrate) at the indicated concentration, either before or after addition of the inhibitors aurovertin or oligomycin.

SF 6847, S-13 and TTFB fully reverse the oxonol response at low micromolar concentrations, while uncouplers of the nitrophenol type, including

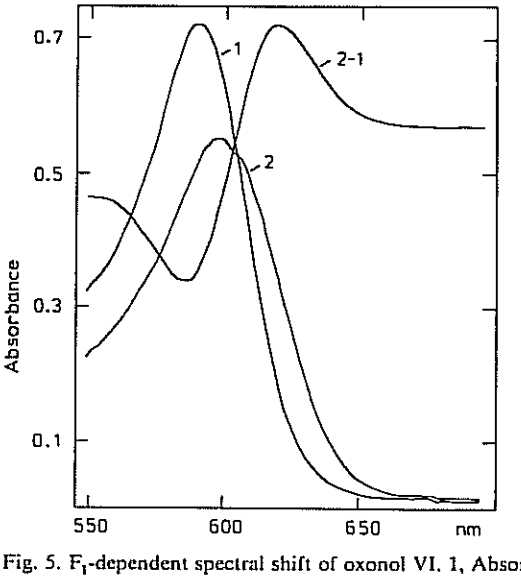

Fig. 5. F_1-dependent spectral shift of oxonol VI. 1, Absorption spectrum of oxonol VI (5 μM) in 1 ml of assay buffer; 2, spectrum after the addition of 0.8 mg protein per ml of F_1-ATPase from bovine heart mitochondria; 2-1, difference spectrum. Temp., 30 °C.

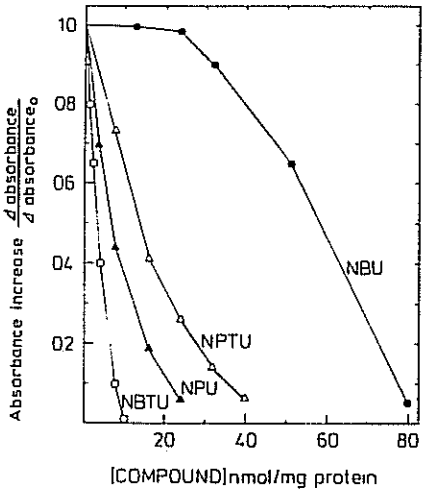

Fig. 6. Effect of lipophilic urea derivatives on the ATP-dependent oxonol response in complex V. Conditions as in Fig. 2.

picrate, are much less effective [15]. HE-DNP, an uncoupler with a half-inhibitory concentration of 40–170 μM in most energy-dependent reactions (Kiehl, R., Schuermann, M. and Hanstein, W.G., unpublished data), shows no effect at concentrations up to 300 μM.

Uncouplers and aurovertin have mutually potentiating effects in complex V and also in submitochondrial particles. It is seen in Fig. 4 that all nitrophenolates tested are much more effective in the presence than in the absence of aurovertin. Conversely, after 10–20% inhibition by uncouplers, aurovertin abolishes nearly all, instead of one half, of the remaining dye response. In contrast, the inhibitions by oligomycin and uncouplers are strictly additive.

For an interpretation of these results, it is important to know whether oxonol, like aurovertin [45,46] and uncouplers [47,48] are capable of direct interaction with F_1. Fig. 5 shows indeed that oxonol VI appears to bind to F_1 and undergo a red shift * similar to the one observed in complex V (Fig. 1) and submitochondrial particles [8].

Lipophilic urea derivatives have been reported to function as uncouplers in mitochondria [49], submitochondrial particles and complex V [5] by an unknown mechanisms. In Fig. 6, the effectiveness of two derivatives each of urea (NBU and NPU) and thiourea (NPTU and NBTU) are compared. All four compounds are capable of fully abolishing the oxonol response at concentration ranging from 2.5 to 20 μM. It is noteworthy that upon substitution of oxygen by sulfur in the nonyl-butyl derivative, the activity is increased by a factor of 9 at 95% inhibition.

Permeant lipophilic ions such as tetraphenyl borate and phenyldicarbaundecaboranate abolish the membrane potential in inside-positive vesicles [51] and decrease the dye response effectively. Phenyl boric acid and tetraphenyl phophonium bromide, which may be regarded as neutral or cationic analogues of tetraphenylborate, are both ineffective as expected.

In quantitative comparison, differences between the oxonol response in the complex V and submitochondrial particles are also apparent. Table IV shows that while some uncouplers and inhibitors, such as NPA, TPB, and DCCD, work equally well inboth system, others show significant differences in effectiveness. Thus, CCCP and oligomycin are about 10-times more inhibitory for the oxonol response of submitochondrial particles, while NBTU is much more effective in complex V.

In Table V, the main ATP-dependent activities of complex V, i.e., ATPase, ATP-P_i exchange and ATP-dependent oxonol response are compared with respect to their inhibition. It is obvious that there is no correlation between ATPase activities and the two energy-linked reactions. An extreme example is the stimulation of ATP-P_i exchange at a concentration of AMP-PNP just enough to inhibit most of the ATPase activity. More importantly, there is also a poor correlation between the effects of modifiers on ATP-P_i exchange and ATP-dependent oxonol response. Thus, at the concentrations used, both valinomycin and PCMB inhibit half of the ATP-P_i exchange activity, while the former abolishes the dye response completely and the latter not at all. The uncouplers DNP (50 μM) and 2-azido-4-nitrophenol (30 μM) decrease the ATP-P_i exchange by 90% while the oxonol response is reduced by only 7 and 25%, respectively. Aurovertin is an effective inhibitor of

* Under similar conditions, no changes in the spectrum of oxonol VI were observed with the following proteins: alcohol dehydrogenase, pyruvate kinase, hexokinase, and trypsin. Bovine serum albumin, however, causes a substantial red shift.

TABLE IV

COMPARISON OF THE CONCENTRATIONS CAUSING 50% ABOLISHMENT OF ATP-LINKED OXONOL VI RESPONSE IN COMPLEX V AND SUBMITOCHONDRIAL PARTICLES BY MODIFIERS OF MITOCHONDRIAL ENERGY LINKED FUNCTIONS

Compound	Concn. Complex V		Concn. submitochondrial particles		Concn. ratio C V/SMP	
	(μM)	(nmol/mg)	(μM)	(nmol/mg)	(μm/μM)	$\left(\frac{\text{nmol}}{\text{mg}} / \frac{\text{nmol}}{\text{mg}}\right)$
CCCP	6	24	0.17	1.7	35.3	14.1
2-azido-4-nitrophenol	70	280	37	370	1.9	0.76
TPB	3.5	14	1.8	18	1.95	0.78
NBTU	0.75	3	2.4	24	0.31	0.125
DCCD		16		30		0.533
Oligomycin		2		0.2		10.0

mitochondrial ATP-P_i exchange and, at high concentrations, also of ATPase activity [52]. At 12 μg per mg protein (6 μM), aurovertin inhibits ATP-P_i exchange fully and ATPase to 90% in complex V. However, as discussed above, even at twice this concentration, the effect of aurovertin on the oxonol response in complex V is limited to 50%. Data obtained with ITP instead of ATP as substrate are also included in this table because they confirm that NTPase, NTP-P_i exchange and NTP-linked oxonol resonse do not correlate.

Discussion

In submitochondrial particles, the spectral response of oxonol VI has been shown to correlate with the phosphate potential during ATP hydrolysis or NADH oxidation [8]. The effects of iono-

TABLE V

EFFECT OF MODIFIERS OF MITOCHONDRIAL ATP-LINKED FUNCTIONS IN COMPLEX V

Compound	Concn.		Percentage ATPase activity	Percentage ATP-P_i exchange activity	Percentage ATP-dependent oxonol VI response
	(mM)	(nmol/mg)			
Nigericin		2.8	100	90	94
Valinomycin		0.9	100	50	0
DCCD		24	100	60	0
NBTU		10	70	85	0
NSPM		80	100	40	15
PCMB	0.05		94	45	100
2-azido-4-nitrophenol	0.03		100	10	75
DNP	0.05		103	10	93
KSCN	5.0		22	8	73
Aurovertin	0.006	25	10	3 [a]	50
AMP-PNP [b]	0.05		10	135	50
ITP [c]	5		150	3	50

[a] Avidin sensitivity is one third of the aurovertin-insensitive activity.

[b] Added 2 min before energization with ATP.

[c] ITPase, ITP-P_i-exchange and ITP-induced oxonol-VI response relative to the ATP-induced value.

phores in this system [8] as well as in chloroplasts [9] confirmed that oxonol VI is a potential sensitive dye [2], responding to an interior positive membrane potential with a red shift in the absorption spectrum. Thus, it appears that in vesicular systems, the oxonol response depends on and reflects energy conservation. It was therefore unexpected that complex V as such, without incorporation into liposomes, should be able to induce a dye response similar to submitochondrial particles [15]. However, since the ATP-P_i exchange activity of this preparation is sensitive to valinomycin [12], some kind of spontaneous vesicle formation may be presumed to take place under assay conditions.

In the present work, the oxonol response of complex V has been studied using different substrates, uncouplers, inhibitors, permeant ions and ionophores. In the following, the question will be discussed whether this response directly reflects energy transduction, i.e., the membrane potential component of the proton electrochemical gradient, or whether more localized phenomena have to be taken into account as well. Therefore, comparisons will be made (a) in complex V between the oxonol response and an energy-dependent reaction, i.e. ATP-P_i exchange, and (b) between the spectral response in submitochondrial vesicles and complex V whose structural organization is unknown.

The extent of the oxonol response induced by different nucleoside triphosphates (NTP) decreases in the order ATP > ITP = GTP > UTP, similar to NTP-P_i exchange [12] and other activities such as oligomycin-sensitive NTPase [53,54], and the NTP-induced inhibition of ATPase by the mitochondrial inhibitor protein [53]. All energy-transfer inhibitors and most uncouplers tested either decrease or abolish the dye response as well as the ATP-P_i exchange in complex V.

There are, nevertheless, significant differences in the degree to which these reactions can be inhibited (Table V). Thus, uncouplers of the nitrophenolate type inhibit ATP-P_i exchange strongly at concentration where little effect on the oxonol response is seen. AMP-PNP and aurovertin, two inhibitors known to interact with F_1-ATPase, have opposite effects on ATP-P_i exchange. Under conditions where half of the oxonol response is abolished, AMP-PNP stimulates the ATP-P_i exchange while aurovertin is nearly completely inhibitory. These data suggest that ATP-P_i exchange and oxonol response in complex V are two qualitatively similar reactions with mechanisms that may be different in a number of steps. This is also suggested by the experience that there is no strong correlation between the exchange activity and the extent of the oxonol response in different preparations of complex V. In a comparison of submitochondrial particles and complex V (Table IV), significant quantitiative differences between the effects of uncouplers and energy-transfer inhibitors become apparent. The addition of detergents, and the removal of neighboring proteins during the purification of the complex may be responsible for this.

More important are differences in the effects of ionophores and permeant ions. Thus, without potassium, valinomycin has an insignificant effect on the oxonol response in submitochondrial particles, while in complex V it is fast and fully abolished under the same conditions (Fig. 3). The reason is that from the method of preparation, complex V solutions always contain ammonium sulfate. In vesicles containing ammonium ions, valinomycin short-circuits membrane potential and pH-gradient in a mechanisms established for submitochondrial particles [55]. The absence of an ATP-dependent ACMA response in complex V, however, may require a different explanation, because removal of ammonium sulfate does not restore the response.

Submitochondrial particles and complex V differ in the sensitivity of their oxonol response toward thiocyanate, a permeant ion used in the determination of inside-positive transmembrane potentials [56]. At millimolar concentrations, thiocyanate inhibits ATP-P_i exchange in inverted vesicles from Escherichia coli [57,58] and the oxonol response in submitochondrial particles (Fig. 3). In complex V, however, 1 mM thiocyanate produces only a small effect which cannot by increased by up to 5-fold higher concentrations (Fig. 3). A high permeability barrier for this anion may be responsible for this finding [57].

It is also possible that complex V in solution is not present in a classical vesicular form. Data on the effect of detergents (Table III) support such a contention. Thus, in the presence of 4 mg cholate or 0.5 mg desoxycholate per mg of protein, half of

the oxonol response is still present and fully uncoupler-sensitive. To our knowledge, there is no precedent for phospholipid vesicles being intact under these conditions. In submitochondrial particles, much lower concentrations (0.55 mg cholate or 0.1 mg deoxycholate per mg of protein) are necessary for abolishing 50% of the dye response. It is, of course, not impossible that complex V vesicles are more stable towards detergents because they contain less phospholipids. It is also possible that in the enzyme, there is a volume with solvent character similar to the one proposed by Wagner et al. [60] for CF_1. Such a speculative molecular vesicle formed by proteins of the complex would have to be impermeable to thiocyanate, resistant to high-detergent concentrations, yet permeable to valinomycin and oxonol VI.

If complex V should not possess a vesicular structure similar to submitochondrial particles, then other, more localized phenomena capable of eliciting an oxonol response have to be considered instead of a transmembrane potential. Changes in surface potential need to be discussed here. Protons and other cations decrease the magnitude of negative surface potentials and may therefore enhance the binding of anionic probes [59]. Specifically, calcium ions increase the binding of oxonol VI to sarcoplasmic reticulum vesicles and produce a spectral red-shift in the absence of a transmembrane potential [11]. For the oxonol response in complex V, changes in surface potential are probably least important because all spectral experiments were done in the presence of 10 mM magnesium sulfate. A (negative) potential at the outside surface is therefore probably already quite small [59].

Several aspects of the results point toward a mechanism where oxonol is bound at the F_1 part of the complex, undergoing a spectral red-shift in response to changes brought about by ATP as a ligand. These changes may include redistribution of charges or alterations in the hydrophobicity of the binding environment or both. Consistent with this hypothesis is the finding that the ATP/ADP ratio rather than the phosphorylation potential determines the extent of the oxonol response, and the data showing direct binding of oxonol to F_1-ATPase (Fig. 5). The cooperative effects of uncouplers and aurovertin (and the absence of such effects with oligomycin) can also be rationalized on the basis of interdependent molecular interactions of oxonol, ATP, aurovertin and uncouplers.

Independent of the detailed mechanism, the oxonol response in complex V appears to be a useful and flexible tool for studying energy-dependent phenomena in this system. Precisely which aspect of the proton translocating mechanism can be probed by oxonol is not clear yet. From the dependence on the ATP/ADP ratio rather than the phosphorylation potential it would appear that mainly the forward reaction, i.e., ATP-dependent proton pumping, is monitored, rather than both forward and back reactions which are involved in ATP-P_i exchange.

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