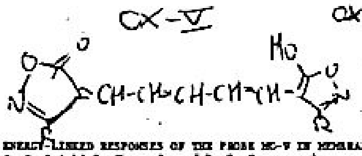
OX-V   OX-V R = ⌬



OX-VI R = CH2CH2CH3
OX-VII, R = CH3

10. International Congress of Biochem, Hamburg, July 25. to 31 (1976)

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06-6-179 ENERGY-LINKED RESPONSES OF THE PROBE MC-V IN MEMBRANES

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The probe MC-V has recently been identified as bis-[3-phenyl-5-isoxazolone-(4)]-pentamethine oxonol. The anionic form is the fluorescent species, the neutral form being insoluble in aqueous media (1). MC-V has been studied in asolecithin vesicles, reconstituted ATPase vesicles, submitochondrial particles (SMP) and mitochondria. In these preparations and in R. rubrum chromatophores energized with light (2), a fluorescence decrease and a red shift of the 620-650 nm absorption spectrum is caused by substrate addition; in pigeon heart mitochondria (PHM), however, the small absorption change is in the opposite sense as in SMP and increases linearly with the dye to PHM protein ratio. The uncoupler-sensitive, energy-linked MC-V spectral changes are reversed by reagents known to affect the ΔΨ component of the protonmotive force. In reconstituted ATPase vesicles or SMP, addition of NH_4Cl causes an enhancement of the energy-linked MC-V spectral changes; subsequent addition of valinomycin or thiocyanate reverses the MC-V responses. Preliminary results of binding studies suggest that energization of EDTA SMP causes an increase in the bound fraction of the dye leading to quenching of the fluorescence of this fraction. In PHM, energization may cause an ejection of MC-V from the membrane causing a decrease in fluorescence and an apparent blue shift of the absorption spectrum. In, SMP, additional fluorescence changes are associated with the bound portion of MC-V; these spectral effects may be due to quantum yield, electrochromic, or other changes. In vesicles, the absorption and fluorescence changes caused by valinomycin-induced K^+ efflux and influx are of the opposite sense. The absorption changes are consistent with those observed in SMP and PHM but the fluorescence changes are not, possibly because of weaker binding of MC-V to asolecithin. (1) with P. Russ, submitted to Biochemistry, (2) B. Chance and M. Baltscheffsky, in Biomembranes Vol. 7, P. 33, 1975. This research was supported by National Instututes of Health grants GM-05226-01 and GM-12202.

06-6-180 LIPOPHILIC THIOUREA —— —— INHIBITORS OF OXIDATIVE PHOSPHORYLATION

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N-Mono-nonyl-thiourea (MNT) and 6-nonyl-2-thiouracil (NTU) are very rapid reagents for protein sulfenyl groups (RS^+), as found with β-Lactoglobulin sulfenyl iodide. To test our working hypothesis that an oxidized sulfur function, such as sulfenyl groups, may be involved in the respiratory-chain ATP synthesis, lipophilic thioureas and thiouracils has been studied. Both substances (140 nmole MNT or 230 nmg NTU/mg protein) inhibit state 3 respiration in ox heart mitochondria with the substrates glutamate + malate [1]. The stimulate state 4 respiration with succinate to 70%, and with ascorbate + TMPD to the maximum of state 3 respiration. A merely lipophilic interaction can be excluded, for equimolar amount per mg protein of the oxygen analogues of MNT and NTU are inactive. For thiourea in strongly acidic solution split cystine into the mixed disulfide and cysteine,

$$[R-\overset{H}{\underset{+}{S}}-S-R-]X \longrightarrow RSX + RSH$$

the inhibitory effect of MNT or NTU can be related to such a protonized disulfide, an activated thioester of the sulfenic acid. This may be a mechanistic linkage to a proton-driven ATP synthesis, X ~ I being the protonized disulfide and the protonation the coupling step. With the formation of a proton-induced sulfenyl group a thiol group is liberated (eq. 1), which is assumed to react in mitochondria with low concentrations of lipophilic thiol reagents.

[1] E. Bäuerlein and R. Kiehl (1976) FEBS Letters 61, 68-71.

06-6-181 ENDOGENOUS FREE FATTY ACIDS AS POSSIBLE REGULATORS OF OXIDATIVE PHOSPHORYLATION

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Incubation of mouse-liver mitochondria at 23° results in a progressive increase in the endogenous level of free fatty acids. A decline in respiratory control, ADP/O ratio, uncoupler-stimulated ATP'ase, and uncoupler-, calcium- and valinomycin-stimulated respiration parallels this rise in free fatty acids. These fatty acids are available for oxidation in the presence of acetylcarnitine or ATP, CoA and carnitine. Bovine serum albumin reduces this oxidation and concomitantly restores respiratory control, ADP/O ratio, uncoupler-stimulated ATP'ase and the uncoupler-, calcium- and valinomycin-stimulated respiration. Addition of ATP, CoA, carnitine and magnesium to these loosely-coupled mitochondria also reduces their free fatty acid content and restores the above mitochondrial functions to near-normal levels. Finally, pretreatment of mitochondria with a variety of respiratory substrates counteracts the loss of some of these functions by substantially reducing the accumulation of free fatty acids. However this protective effect of substrate is nullified by KCN, antimycin, rotenone and DNP; all of which allow for increased accumulation of free fatty acids.

These data show a direct correlation between loose-coupling and high levels of free fatty acids suggesting that endogenous free fatty acids may function as regulators of oxidative phosphorylation. Furthermore, the endogenous level of free fatty acids in mitochondria could be controlled by ATP or high-energy intermediates generated by oxidative processes.

06-6-182 METABOLIC EFFECT OF SOME UNCOUPLERS OF OXIDATIVE PHOSPHORYLATION

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Adenosine-triphosphate-phosphoribosyl transferase from Escherichia coli is inhibited by dicoumarol, dinitrophenol and pentachlorophenol in competition with ATP. K_i was approximately 60 μM for dicoumarol and 50μM for pentachlorophenol. Carbonyl-cyanide meta-chlorophenyhydrazone did not seem to have any kinetic effect. Dicoumarol is bound to the extent of 6 sites per enzyme hexamer with a K_{diss} of 50 μM. Dicoumarol and pentachlorophenol partly prevents the binding of ATP and AMP to the transferase. While AMP slightly prevented the binding of dicoumarol to the transferase, ATP had no such effect. The reversed reaction is inhibited by dicoumarol and pentachlorophenol without changes in ($S_{0.5}$) for phosphoribosyl-adenosine triphosphate. Dicoumarol or pentachlorophenol had little effect upon the binding of phosphoribosyl-adenosine triphosphate. Dicoumarol, dinitrophenol and pentachlorophenol diminish the yield of phosphoribosyl-adenosine triphosphate in the transferase reaction apparently by acting as parasite substrates; carbonyl-cyanide meta-chlorophenylhydrazone had no effect.

06-6-183 ON THE STRUCTURE OF THE MITOCHONDRIAL UNCOUPLER BINDING SITE.

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Mitochondria and submitochondrial particles bind uncouplers such as 2-azido-4-nitrophenol (NPA) in two ways: 1) by a partition equilibrium, and 2) at a specific, saturable binding site characterized by dissociation constants K_D and other parameters. The values of K_D and $K_{D,i}$ (from competition studies) of uncouplers such as azide, dinitrophenol, NPA, pentachlorophenol, trinitrophenol and S-13, and the concentrations necessary for 50% uncoupling ($\phi_{1/2}$) are directly proportional ($\phi_{1/2} \approx K_D/3$). A binding site able to accomodate very small (azide) as well as very large (S-13) uncoupling molecules is likely to be limiting in one or two dimensions only. One is apparently the distance between the negative charge and the para-substituent at the adjacent aromatic system (4 - 8 Å), another the thickness of the aromatic system. It is conceivable therefore that the uncoupler binding site has the shape of a cleft, either in a single protein, or as part of the contact interface between two adjacent proteins.

Photo-affinity labeling studies utilizing the NPA anion have shown that two proteins are labeled to a major degree: 1) a protein of a MW of 31,000, which is not identical with either subunit 3 of F_1-ATPase, the NEM-binding protein associated with the ADP/ATP carrier, the carboxy-atractylate binding protein, nor with factor B. This protein, the "uncoupler binding protein", is necessary for uncoupler equilibrium binding. 2) A protein of a MW of 56,000, which has been identified with subunit 1 of F_1-ATPase. These data together with the above considerations suggest the possibility that the uncoupler binding site is composed of both the uncoupler binding protein and subunit 1 of F_1-ATPase. The implications for the mechanism of uncoupling and of oxidative phosphorylation will be discussed. (Supp. by GM 19734).

Melittin

06-6-184 A LINEAR PEPTIDE AS A POTENT UNCOUPLER OF OXIDATIVE PHOSPHORYLATION

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Melittin, an amphiphilic peptide from bee venom has been recognised as a membrane lytic (mainly hemolytic) agent. However, marked influences on membrane enzyme systems possibly unrelated to membrane destruction occur already at sublytic concentrations. An example for such an effect may be the uncoupling ability of the peptide. This property has been investigated in rat liver mitochondria by reactions characteristic for uncouplers. A 50% decrease in respiratory control and of the ADP/O quotient occured at 2-4 nmoles melittin/mg protein, concomitantly with a maximal (over 10-fold) activation of the ATPase. Dinitrophenol requires considerably higher concentrations (10-20 nmoles/mg) for 50% uncoupling. The atractyloside sensitivity of the melittin-induced ATPase, the lack of oxidation of exogenous NADH and the absence of passive swelling under the conditions mentioned point to the intactness of the mitochondrial membranes. Comparative studies with various detergents show the occurence of similar but less clear effects only at much higher concentrations. An ionophoric action as described for certain cyclic peptide antibiotics or nonionic detergents does not seem likely for melittin on ground of its structure. Thus, melittin seems to be an uncoupler of unusual structure: it is a basic peptide of the comparatively high molecular weight of 2840, whereas the routinely used synthetic uncouplers are mostly weak aromatic acids of much lower molecular weights.

Hamburg, FRG, Aug. 1-2, 1976

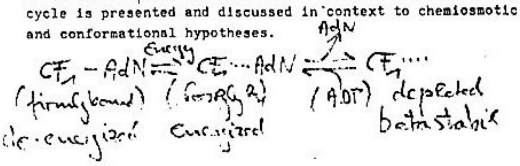
H. Strotmann: Energy-dependent exchange of CF_1-bound adenine nucleotides in chloroplasts

Chloroplast coupling factor (CF_1) contains firmly bound adenine nucleotides, which are not removed by washing and exhibit only a marginal exchange in the de-energized state of the chloroplasts. Evidence is presented for the substrate role of bound adenylates in the process of photophosphorylation. By energization of the thylakoids, a rapid exchange of bound adenine nucleotides for free ADP or ATP is induced. The significance of adenylate exchange in photophosphorylation is demonstrated.

Exchange can temporally be separated into two partial reactions, energy-dependent release of the bound substrate molecules and energy-independent re-binding of free nucleotide. The former reaction is driven by a pH gradient across the thylakoid membrane. The adenylate-depleted conformation is ready for incorporation of free adenine nucleotides over several minutes. The adenylate binding condition, once established, does not require an energized state of the thylakoids for its maintainance and persists even after solubilization of the enzyme. However, as soon as a new adenine nucleotide is incorporated, CF_1 is re-transferred to the non-exchangeable form.

In contrast to adenine nucleotide binding, the ability of $^{32}P_i$ incorporation into CF_1-bound ATP rapidly decreases, when the energy source is taken off.

A model of CF_1-linked reactions of the photophosphorylation cycle is presented and discussed in context to chemiosmotic and conformational hypotheses.



The Action of Lipophilic Trapping Agents for Sulfenyl (RS^+) and Thiol Groups in Mitochondrial Energy Transduction.

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N-Mono-nonyl-thiourea (MNT) and 6-nonyl-2-thiouracil (NTU) are very rapid reagents for protein sulfenyl groups (RS^+), as found with β-lactoglobulin sulfenyl iodide [1]. Therefore we studied the action of such lipophilic thioureas and thiouracils of increasing chain length in the mitochondrial energy transduction to test our working hypothesis, that an oxidized thiol group, a sulfenyl group (RS^+), may be involved in the respiratory chain dependent ATP synthesis. Sulfenyl groups can activate inorganic phosphate by the formation of an sulfenic-phosphoric acid anhydride intermediate ($R-S-O-PO_3H_2$), as found by chemical model reactions [2].

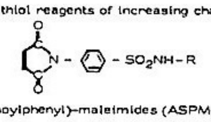
Thiourea (MNT) as well as thiouracil (NTU) with nonyl carbon chains inhibit best state 3 respiration in ox heart mitochondria with the substrates glutamate + malate [3]. They stimulate state 4 respiration with succinate to 70 %, and with ascorbate + TMPD to the maximum of state 3 respiration. A pure lipophilic interaction can be excluded, as equimolar amounts per mg protein of the oxygen analogues of MNT and NTU, the n-nonyl-urea and 6-nonyl-uracil resp. are inactive.

Since thiourea cleave cystine in strongly acidic solution into the mixed disulfide and cystein [4],

$$[Cys-\overset{H}{\underset{+}{S}}-S-Cys]\,X^{\ominus} + S{=}C(NH_2)_2 \rightarrow Cys-SH + [Cys-S-S-C(\overset{+}{N}H_2)(NH_2)]\,X^-$$

the inhibitory effect of MNT and NTU could be attributed to the reaction of a protonized disulfide within the mitochondria, i.e., an activated thioester of the sulfenic acid, with these reagents. This may be a mechanistic linkage to a proton-driven ATP synthesis. With the formation of one proton-induced sulfenyl group one thiol group has to be liberated.

In a second approach to the proposed mechanism the action of lipophilic thiol reagents of increasing chain length, e.g. N-(N-Alkyl-4-



sulfamoylphenyl)-maleimides (ASPM-analogues [5]) in the mitochondrial energy transduction were studied. Once more the n-nonyl-derivative (NSPM) is the most active. NSPM (16 nmoles/mg protein) inhibits state 3 respiration in ox heart mitochondria with the substrates glutamate + malate. NSPM (20 nmoles/mg protein) stimulate state 4 respiration with succinate to 90 % of state 3 respiration. Again a pure lipophilic interaction can be excluded, for equimolar amounts per mg protein of the saturated compound, the N-(N-n-nonylsulfamoyl-phenyl)-succinimide is inactive.

The very similar features in reactivity of both, sulfenyl and thiol reagents, on mitochondria support the hypothesis of a protonized disulfide as an intermediate in proton-driven ATP synthesis.

[1] E. Bäuerlein and R. Kiehl, in preparation.

[2] E. Bäuerlein (1974) in: Glutathione (Flohe, L. et al., eds). pp. 44-55, Georg Thieme Publishers, Stuttgart.

[3] E. Bäuerlein and R. Kiehl (1976), FEBS Letters 61, 68-71.

[4] G. Toennies (1937), J. Biol. Chem. 120, 297-313.

[5] R. Kiehl and E. Bäuerlein, in preparation.

We interprete these observations as follows: OPDM modifies CF1 in a way that it is rapidly opened for proton conduction by either of two activators, some unknown factor of the electron transport chain, or the electric potential difference. The selectivity for one and only one proton per electron transport chain might reflect the stoichiometrical abundance of CF1, which according to the pertinent literature is about one per chain. If so, it has to be postulated that the activated CF1 closes again after the passage of one proton.

1 Weiss, M. A. & McCarty, R. I., *Biochem. J.*, in press.
2 Junge, W. (1977) *Annu. Rev. Plant. Physiol.* 28, 503 - 536.
3 Wagner, R. & Junge, W. (1977) *Biochim. Biophys. Acta* 462, 259 - 272.

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E. Bäuerlein, R. Kiehl and C. Solis

The Action of Lipophilic Thiol and Sulfenyl Group Reagents on Mitochondrial Energy Transduction

Following the working hypothesis that protonated disulfides, as activated thioesters of the sulfenic acid (RSOH), may be a part of the energy transduction in the electron transport phosphorylation, lipophilic trapping agents for the thiol as well as the sulfenyl ($RS^{\oplus}$) group are introduced.

1) Effects on coupled respiration of beef heart mitochondria

The lipophilic maleimide NSPM[1] as well as the corresponding thiouracile NTU[2] inhibit coupled respiration with glutamate + malate or β-hydroxybutyrate, and not with succinate as substrate. In contrast to the sulfenyl group reagent NTU higher concentrations of NSPM as well as of the correlated succinimide NSPS[1] inhibit the electron transport only, if glutamate + malate are the substrates. Thus two modes of reactions of NSPM are detected, a chemical and a purely lipophilic one.

2) Effects on submitochondrial particles

Respiration as well as the reduction of ubiquinone is inhibited by NSPM, NSPS and NTU with NADH, and not with succinate as substrate. No effect is found on the ATP- or energy driven transhydrogenase. This inhibition appears to be a competition with ubiquinone in the interaction with complex I and can be ascribed to the above mentioned "purely lipophilic" mode of reaction.

3) Identification and isolation of the [^{14}C]NSPM-binding protein

After the incubation of well-coupled mitochondria with 6.9 nmol [^{14}C]NSPM/mg protein for 5 min, causing nearly complete inhibition of state 4 $\rightarrow$ state 3 transition, a low molecular weight NSPM-binding protein (< 13000) can be extracted by ethanol from the mitochondrial suspension. After separation of the phospholipids by ether and $CHCl_3/CH_3OH$, a water soluble protein is isolated by column chromatography with Sephadex LH 20.

Abbreviations:
NSPM, *N*-(*N*-n-nonyl-4-sulfamoylphenyl)maleimide; NSPS, *N*-(*N*-n-nonyl-4-sulfamoylphenyl)succinimide; NTU, 6-n-nonyl-2-thiouracil.

1 Kiehl, R. & Bäuerlein, E. (1976) *FEBS Lett.* 72, 24 - 28.
2 Bäuerlein, E. & Kiehl, R. (1976) *FEBS Lett.* 61, 68 - 71.

Priv. Doz. Dr. E. Bäuerlein, Max-Planck-Institut für Medizinische Forschung, Abteilung Naturstoff-Chemie, Jahnstr. 29, D-6900 Heidelberg.

D. Kuschmitz and B. Hess

Über die Kopplung von Proton- und Lichtzyklus in Bacteriorhodopsin

Die Untersuchung der „Stöchiometrie" von $H^{\oplus}$/Bacteriorhodopsin (BR) während des photochemischen Zyklus mit spektrophotometrischen und fluorometrischen Methoden unter Benutzung von Methylumbelliferon als pH-Indikator ergab die folgenden Verhältnisse:

Unter stationären Belichtungsbedingungen wird ein Verhältnis von 0.5 ($H^{\oplus}$/BR) in wäßrigen Suspensionen von Purpurmembran gefunden, das mit zunehmender Salzkonzentration (KCl oder NaCl) auf Werte um 1.8 anwächst in Übereinstimmung mit maximalen Verhältnissen, die früher nach Laserblitzaktivierung erhalten wurden. Wenn von stationärer Belichtung zu Pulslichtaktivierung übergegangen wird, fällt das $H^{\oplus}$/BR-Verhältnis in Gegenwart von Salz (nicht aber in wäßriger Suspension) auf Werte um 1 ab und die Zerfallskinetik von BR-412 wird zweiphasig. Mit abnehmenden Lichtintensitäten unter stationärer Belichtung wurde dagegen, wiederum in Gegenwart von Salz, ein Anwachsen des $H^{\oplus}$/BR-Verhältnisses gefunden. – Die Ergebnisse zeigen, daß das Verhältnis von freigesetzten mol $H^{\oplus}$ zu mol Rhodopsin keine stöchiometrische Größe darstellt, sondern unter anderem durch das Oberflächenpotential

11. FEBS Meeting Copenhagen, Aug. 1977 Abstracts

A 4-13 705 5/6/7

GENERATION OF PHOTOPOTENTIALS BY BACTERIORHODOPSIN, ASSOCIATED WITH LIPID-IMPREGNATED MILLIPORE FILTERS

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Millipore filters impregnated with hexadecane and phospholipids were incubated on one side with bacteriorhodopsin-containing phospholipid vesicles and Ca^{++}-ions. Upon illumination a photopotential was generated across these filters. In comparison with the planar membrane system of Drachev et al. (Nature 249 (1974) 321-324), this system is much more stable. Moreover, the membrane area has been drastically increased. As a measure for the association of the vesicles with the filter we used the photopotential that is generated during short illumination periods. The experiments show that the association is a time-dependent process, which is a function of both the vesicle- and Ca^{++}-concentration. Furthermore, the photopotential depends on the ratio of bacteriorhodopsin to phospholipid in the vesicles. Values up to 200 mV have been obtained. Uncouplers like FCCP cause a decrease of the photopotential. Relatively low concentrations of nigericin produce an increase of the photopotential, which can be reversed by valinomycin if the vesicles contain K^+-ions. Results of a systematic study of the influence of nigericin and valinomycin on the photoeffect as a function of the ionic composition of the internal compartment of the vesicles and of the medium on both sides of the filter will be presented. The experiments indicate that at least part of the vesicles is associated with the filter in such a way that the vesicles retain their original enclosed medium.

A 4-13 706 5/6/7 LR

Arylazido nucleotide analogue as photoaffinity labels for the mitochondrial adenosine triphosphatase

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Arylazido-aminobutyryl-adenosine diphosphate analogue (NAP-GABA-ADP) has been used as photoaffinity label of the mitochondrial F_1-ATPase. Upon irradiation by light, (^{3}H)NAP-GABA-ADP covalently binds to F_1-ATPase. The bound radioactivity has been measured in slices of the SDS-polyacrylamide gel and found to be localized on the α and β subunits. When F_1-ATPase is preincubated in the dark with either ADP, ATP or AMP-PNP together with (^{3}H)NAP-GABA-ADP, the covalent labeling of α and β subunits is strongly decreased. (^{3}H)NAP-GABA incubated in the light with F_1-ATPase is incorporated into F_1-ATPase to a much smaller degree than (^{3}H)NAP-GABA-ADP and its incorporation is strongly prevented by NAP-GABA-ADP. The uncoupler pentachlorophenol (PCP) competes efficiently for binding with (^{3}H)NAP-GABA but not with (^{3}H)NAP-GABA-ADP.
In parallel kinetic experiments, it was shown that NAP-GABA-ADP inhibits the hydrolysis of ATP by F_1-ATPase both in the dark or after light irradiation. PCP alone stimulates the ATPase activity both in the dark and after photoirradiation. NAP-GABA stimulates F_1-ATPase in the dark, and inhibits F_1-ATPase upon photoirradiation. The inhibition of F_1-ATPase by NAP-GABA can be prevented by addition of PCP before photoirradiation. NAP-GABA does not uncouple mitochondrial respiration. These results are discussed in the light of specific binding of NAP-GABA-ADP to ADP/ATP binding sites on the α and β subunits of F_1-ATPase.

A 4-13 707 5/6/7

Inhibition of oxidative phosphorylation by the lipophilic maleimide NSPM and isolation of the NSPM-binding low molecular weight protein

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Oxidative phosphorylation as well as uncoupling by DNP or FCCP is inhibited by 12 - 16 nmoles NSPM/mg protein with glutamate + malate as substrates, whereas with succinate state 4 respiration is stimulated by the same concentration of NSPM to 90 % of state 3 respiration [1]. In submitochondrial particles electron transport is inhibited by 30 - 40 nmoles NSPM/mg protein with NADH, and not with succinate, which parallels the reaction of 90 - 100 nmoles NSPM/mg protein in intact mitochondria. For equal amounts of the corresponding succinimide NSPS inhibits electron transport, this inhibition can be explained by pure lipophilic interaction. To elucidate the action of the low concentrations of NSPM, well-coupled mitochondria were incubated with 6.9 nmoles ^{14}C-NSPM/mg protein (90 % inhibition). Three radioactive bands are detected by SPAGE; the main band can be extracted by ethanol, liberated from phospholipids by ether and now separated by LH20-column chromatography in water. This protein (MG ~ 6-8000) is very probably responsible for the specific inhibition, and its amino acid analysis is different from subunit 9 and the ATPase inhibitor.

NSPM = N'-(N'-n-nonyl-4-sulfamoylphenyl)-maleimide

[1] Kiehl, R., and Bäuerlein, E. (1976) FEBS Letters 72, 24.

A 4-13 708 5/6/7

Control of the Ratio of the Proton- and Photochemical Cycle in Bacteriorhodopsin

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The "stoichiometry" of H^+/Bacteriorhodopsin (BR) during a photochemical cycle was analyzed with spectrophotometric and fluorometric techniques using methylumbelliferon as pH-indicator. - Whereas earlier experiments (1) gave a H^+/BR ratio of maximum 1.8 upon laser flash activation of the purple membrane, the control of this ratio was found to be dependent on the following experimental conditions: under photo-steady-state conditions a ratio of 0.5 is found in aqueous suspension of BR which increased with rising salt concentrations (KCl or NaCl) up to 1.8. During phototransients the ratio decreases in the presence of salt to a value near unity concomitantly with the appearance of a salt-dependent biphasicity in the BR-412 decaykinetic. With decreasing light intensities under photo-steady-state conditions an apparent increase in the H^+/BR ratio is observed again facilitated by the presence of salt. - The results indicate that the ratio of the photochemical and proton cycle is controlled by the surface potential of the purple membrane and cannot be defined as a true stoichiometry. It is concluded that a second proton pool is induced on lowering the apparent surface potential with increasing salt concentration distinguishable from another proton pool existing at apparent high surface potential.

(1) Hess, B., Kuschmitz, D., and Oesterhelt, D. (1976) IUB 10th Internat. Cong. Biochem. Hamburg, Abstr. 06-2-205

A 4-13 709 5/6/7

Subunit Structure of H^+-Translocating ATPase. Image Reconstruction and Active Subunits

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H^+-translocating ATPase (TF_0·F_1) of thermophilic bacterium PS3 was composed of catalytic moiety (TF_1) and H^+-channel moiety (TF_0).[1,2] TF_1 was crystallized[3] and its molecular structure was observed by computerized image reconstruction (Fig. 1) from electron micrograph. TF_1 was dissociated into 5 kinds of subunits (α, 56,000, β, 53,000, γ, 32,000, δ, 15,500, ε, 11,000 daltons) and ATPase activity was reconstructed from β and γ subunits.[4] TF_0 was composed of DCCD-binding protein (5,400) and two other subunits (19,000 and 13,500). Passive H^+ translocation through liposomes inlaid with TF_0 was completely inhibited by either DCCD, anti-TF_0 or complex containing γ+δ+c. The γ+δ+c complex was shown to be a gate of H^+ as well as connector of α+β complex.[2] The liposomes containing TF_0-F_1 and saturated branched phospholipids generated $\Delta\mu H^+$ of 300mV on addition of ATP-Mg, and synthesized ATP (53 nmoles/mg protein) when 205 mV of $\Delta\mu H^+$ was imposed by acid incubation followed by the addition of alkali.[5,6,7]

1. J. Biol. Chem. 250, 7910, 7917 (1975).
2. Proc. Natl. Acad. Sci. 74, (3) in press.
3. J. Biochem. 80, 141 (1976).
4. J. Biol. Chem. 252, in press (1977).
5. J. Membrane Biol. 30, 121 (1976).
6. J. Biol. Chem. 252, in press (1977).
7. Federation Proc. 36, (6) (1977).

Fig. 1. Reconstituted Image of TF_1 Molecule. Digital and analogue output are overlapped. —— 25 Å

A 4-13 710 5/6/7

ATPase Proteolipid Coded by a Mitochondrial Gene Determining Oligomycin Resistance in Aspergillus nidulans

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The major protein extractable with neutral chloroform: methanol (2:1) from whole mitochondria of an extranuclear oligomycin-resistant mutant of A. nidulans and its parental strain has been purified to electrophoretical homogeneity and identified as the smallest subunit of the mitochondrial ATPase complex. The proteins of both strains are synthesized on cycloheximide-resistant mitochondrial ribosomes, co-migrate in urea-SDS-gels with an apparent m.w. of 6000 and are similar in their amino acid composition (minimal m.w. around 7000, polarity 38 %). Both proteins have retained the initiating amino acid N-formylmethionine specific for bacterial and mitochondrial protein synthesis, and both contain valine as C-terminus. However, a second internal methionine residue is missing in the mutant protein, together with some other amino acids. The mutational alteration of this mitochondrially coded integral membrane protein not only confers oligomycin resistance to the solubilized ATPase complex but also causes a structural alteration of the inner mitochondrial membrane, resulting in binding of excess cytochrome c and in impaired cellular growth rate.

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Energy Conservation in Biological Membranes

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Abstracts

H. Baltscheffsky

Evolutionary Aspects of Energy Metabolism

Currently, an increasingly detailed picture of the molecular evolution of biological energy metabolism is developing. Recent progress in the determination of protein primary, secondary and tertiary structures has initiated consideration of how energy conservation processes such as fermentation, photophosphorylation and oxidative phosphorylation originated and evolved. The tentative evolutionary schemes are based on the strongly supported assumptions that gradually divergent trends in protein structure are evidence for a single common ancestor and that a protein with a certain structure and function may give rise to a protein with different structure and either retained or different function through gene duplication, mutation, etc.

Numerous observations indicate that in protein molecular evolution over billions of years three-dimensional structural features are more conserved than amino acid sequences. The β-pleated sheet structure appears to be of particular evolutionary significance in general[1], as well as in proteins involved in redox reactions[2,3] and in phosphate transfer[4] in the various cellular energy conversion systems. Evidence that β-structures may have already been important when life on earth began[5,6] points to the possibility of a continuity in protein β-structure evolution since that time.

For both electron transport chains[2,3] and fermentation reaction sequences[4] it may be assumed that stepwise evolution from protein to neighbour protein has occurred. The recent solubilization[7] of membrane-bound inorganic pyrophosphatase, which catalyzes the light-induced formation of energy-rich pyrophosphate from orthophosphate[8] from *Rhodospirillum rubrum* chromatophores, may open the possibility of investigating whether stepwise protein molecular evolution has also occurred along the pathway of electron transport-coupled phosphorylation.

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Y. Hatefi, Y. M. Galante and R. Kiehl

Organization of the Mitochondrial Respiratory Chain

In mitochondria, the machinery for oxidative phosphorylation is located in the inner membrane in the

form of five enzyme complexes. Complexes I, II, III and IV (plus cytochrome *c* and ubiquinone) make up the respiratory chain, while Complex V is responsible for ATP synthesis and hydrolysis. Complex I catalyzes electron transfer from NAD(P)H to ubiquinone. It is composed of 16 - 18 polypeptides, FMN, and 5 iron-sulfur centers. A soluble iron-sulfur flavoprotein with M_r = 75000 ± 6% (three subunits) is the primary NAD(P)H dehydrogenase. Complex II catalyzes electron transfer from succinate to ubiquinone. It is composed of 4 polypeptides. Two polypeptides belong to succinate dehydrogenase, which is an iron-sulfur flavoprotein with M_r = 97000 ± 4%. In addition to succinate dehydrogenase, Complex II contains a low potential cytochrome *b* whose reduced form at 77 K exhibits absorption maxima at 557.5, 550, 531, 523 and 422 nm. Complex III catalyzes electron transfer from reduced ubiquinone to cytochrome *c*. It is composed of 7 - 8 polypeptides. The identified electron carriers of Complex III are two *b*-type cytochromes, cytochrome c_1 an iron-sulfur protein, and a component with cytochrome *b*-like electron transfer properties and an absorption peak when reduced at 77 K at 558 nm. Complex IV catalyzes electron transfer from ferrocytochrome *c* to molecular oxygen. It is composed of 7 polypeptides, and contains hemes a, a_3 and two atoms of copper per mole. In addition to the above, each enzyme complex also contains 20 - 30% phospholipids by dry weight, and Complexes I and III contain bound ubiquinone. Complexes I, II, III and IV have been physically and functionally recombined in the presence of cytochrome *c* (and added ubiquinone where necessary) to reconstitute the entire electron transport system ($\begin{matrix}\mathrm{I}\\\mathrm{II}\end{matrix}$ > III-*c*-IV) or segments thereof (I-III, II-III, $\begin{matrix}\mathrm{I}\\\mathrm{II}\end{matrix}$ > III, I-III-*c*-IV, II-III-*c*-IV) with the expected overall activities of the participating complexes. In mitochondria, Complexes I, III and IV contain the energy coupling sites 1, 2 and 3, respectively. The oxidation energy captured by these complexes is transferred to Complex V for ATP synthesis. Recently, the latter complex has been obtained in a highly purified form in our laboratory. It is composed of 11 polypeptides, of which 10 have been identified as follows: the five F_1 subunits, the oligomycin-sensitivity-conferring protein, the dicyclohexylcarbodiimide-binding protein, the uncoupler-binding protein, coupling factor F_6, and provisionally coupling factor B. Complexes I to V are present in the mitochondrial inner membrane in the approximate ratio of I 1 : II 2 : III 3 : IV 7 : V 3, and together they make up about 50% of the inner membrane protein.

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Mitochondrial Cytochrome b

Mitochondrial cytochrome *b* is an approximately 28000 M_r polypeptide chain containing a single non-convalently bound ferroprotoporphyrin IX prosthetic group. As a dimer (or perhaps as a tetramer) it forms part of the ubiquinone: cytochrome *c* oxidoreductase. This is a 250000 M_r (or perhaps 500000 M_r) membraneous multiprotein complex which contains additionally cytochrome c_1 (M_r 31000), an iron-sulfur protein (M_r 25000) and five subunits without known prosthetic groups (M_r 8000, 12000, 14000, 45000, 50000). The complex catalyzes electron transport from reduced ubiquinone to ferricytochrome *c* and is a site for the transformation of electrogenic energy into a form suitable for ATP synthesis. Apparently, cytochrome *b* is the membranous section of the multiprotein complex which spans the inner mitochondrial membrane, whereas cytochrome c_1 is located at the cytoplasmic surface of the membrane and the 45000 and 50000 M_r subunits at the matrix surface.

Cytochrome *b* is the only polypeptide of the multiprotein complex which is a product of the mitochondrial genetic system. It is coded for by a gene on the mitochondrial DNA and translated on mitochondrial ribosomes. The other subunits of the multiprotein complex are synthesized in the cytoplasm.

A synopsis of our knowledge on the genetics, biogenesis, structure and function of cytochrome *b* will be given.

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Beef Heart Cytochrome bc_1 Complex (Complex III): Isolation and Characterization of the Polypeptide Subunits Carrying Heme b

When the bc_1 complex is isolated from antimycin-loaded beef heart mitochondria in Triton X-100 by hydroxyapatite chromatography[1], it consists of six polypeptide subunits with molecular weights of 12000, 29000 (cytochrome c_1), 2 × 30000 (2 × cytochrome *b*) and finally

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A FRAGMENT OF SUBUNIT γ REMAINS TIGHTLY BOUND TO THE E. COLI F_1-ATPase AFTER TRYPSINIZATION. Jeffrey B. Smith* and Christopher Wilkowski* (SPON: H.H. Williams) Sec. Biochem., Molec. & Cell Biol., Cornell Univ., Ithaca, N.Y. 14853

Antiserum specific for the γ subunit of the F_1-ATPase of E. coli (ECF_1) strongly inhibits both the membrane-bound and purified ECF_1. Surprisingly, the digestion of ECF_1 with trypsin, which was reported by Nelson et al (PNAS 71, 2720, 1974) to remove the γ, δ and ε subunits without decreasing ATPase activity, did not alter the sensitivity to inhibition by the antiserum. Even a more exhaustive digestion with trypsin failed to decrease the inhibition by the anti-γ serum. On SDS gels, trypsinized ECF_1 consists mainly of α and β and two small polypeptides (MW~10,000) that migrated near the dye front. Both were eluted from SDS gels and found to form an immunoprecipitate with anti-γ serum but not with antisera specific for α,β,γ or ε. Since the γ fragment(s) was not removed from ECF_1 by repeated molecular sieve chromatography, it is firmly bound to the α and/or β subunits. After inactivating four (α,β,γ,ε) subunit ECF_1 by freezing in high salt, the reconstitution of ATPase activity was blocked by anti-β and anti-γ sera but unaffected by anti-ε serum. These results suggest that a portion of γ is essential for ATPase activity. The γ chain may form part of the catalytic site or the interaction of γ with α and/or β protects the catalytic site. (Supported by NSF Grant PCH 75-20287 and NIH Fellowship GM 02419).

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ELECTRON TRANSPORT PROTEINS ASSOCIATED WITH PROLINE FERMENTATION IN CLOSTRIDIUM STICKLANDII. Belinda Sero* (SPON: T. C. Stadtman). NIH Bethesda, MD 20014

The reductive ring cleavage of D-proline to 5-aminovalerate is catalyzed by a membrane-bound multienzyme complex in C. sticklandii. Under physiological conditions, NADH is the electron donor, and D-proline is the terminal electron acceptor. The reducing equivalents generated from the oxidation of NADH are transferred sequentially through a flavoprotein, a metalloprotein, proline reductase and ultimately to D-proline. The flavoprotein, which functioned as a NADH dehydrogenase, was found to have a molecular weight of 38,000. It is associated specifically with the proline reductase complex. It cannot be replaced by several other flavoproteins isolated from C. sticklandii for electron transport between NADH and proline reductase. Absorption and fluorescence spectroscopy demonstrated the presence of FAD. An additional protein, also isolated from the reductase complex, is required to reconstitute the active NADH-linked proline reduction. Inhibition of the electron transport by EDTA and diethyldithiocarbamate suggests that this may be a metalloenzyme. EDTA did not inhibit the terminal proline reductase activity when dithiothreitol was used as an artificial electron donor. These results suggest that the metalloprotein functions as an intermediate electron carrier between the flavoprotein and proline reductase. Additionally, electron transport was inhibited by antimycin A, rotenone and 2-n-nonyl-4-hydroxyquinoline-N-oxide.

1387

COMPOSITION OF THE MITOCHONDRIAL UNCOUPLER- AND OLIGOMYCIN-SENSITIVE ATP-Pi EXCHANGE COMPLEX (COMPLEX V). Y. H. Galante,* R. Eichl,* and S. Y. Wong* (SPON: J. Spizizen). Dept. of Biochem., Scripps Clinic and Research Foundation, La Jolla, CA 92037.

Removal of minor Complex V contaminants by chromatography on Agarose A5M in the presence of cholate has yielded a highly purified preparation with 11 polypeptides and ATPase and ATP-Pi exchange activities of 8-10 x 10^3 and 110-120 (or 210-230 when corrected for ATP hydrolysis during exchange) nanomol/min/mg protein, respectively. As isolated, the preparation contains only about 0.1 μmol phospholipids per mg protein, and requires added phospholipids for activity. Thus, its ATPase activity is stimulated 15-20 fold upon addition of sonicated phospholipids (~20 μg phospholipid phosphorus per mg protein) directly to a reaction mixture containing enzyme and substrate. Purification of Complex V involves also the loss of some F_1-ATPase, which should be added back to attain maximal ATP-Pi exchange activity. Ten polypeptides of Complex V have been identified by comparison of M_r values and coelectrophoresis with pure, authentic preparations, as well as by affinity labeling with appropriate radioactive reagents. They are: the 5 subunits of F_1, the oligomycin-sensitivity-conferring protein, the dicyclohexylcarbodiimide-binding protein, the uncoupler-binding protein, coupling factor F_6, and provisionally coupling factor B (M_r 11-12 x 10^3). In collaboration with Y. Hatefi (This work was supported by USPHS grant AM08126 and NSF grant PCH 76-01378 to Y. H.)

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INTERACTION OF UNCOUPLERS WITH THE MITOCHONDRIAL MEMBRANE Nandini V. Katre* (SPON: Y. Suyama) Dept. of Biochem. & Biophys., Univ. of Penna., Phila. Pa. 19104.

A potent uncoupler, ($u_{\frac{1}{2}}$ = 0.2μM) of oxidative phosphorylation, 2-NO_2,4-N_3 carbonylcyanidephenylhydrazone (N_3CCP), with 50 mCi ^3H/m mole, was synthesized. Equilibrium binding studies showed a high affinity binding site (Kd = 0.6 ± 0.3 μM) in the mitochondrial membrane at a concentration of 1.4 ± 0.2 sites/cyt. a. All other tested uncouplers compete for this binding site consistent with their uncoupling activity. Removal of about 80% of mitochondrial phospholipids did not significantly alter high affinity binding, suggesting involvement of protein(s). Following photolysis of the $-N_3$ group 50-60% of the total ^3H was covalently bound to the membrane fraction and 80-85% of this to a single peptide of 12-15,000 daltons on SDS - polyacrylamide gel electrophoresis. Radioactive incorporation into the peptide could be prevented by adding saturating amounts of other uncouplers before photolysis. The labeled peptide could be extracted from mitochondria or oligomycin - sensitive ATPase with $CHCl_3$: CH_3OH (2:1), suggesting involvement of a proteolipid from the membrane component(s) of the latter. Uncouplers of mitochondrial oxidative phosphorylation appear to act by binding to a specific site, where they interact with a proteolipid component of the membrane. (Collaboration with D.F. Wilson, Supported by GM 12202)

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06-6-R57

ELECTRON DONORS AND ACCEPTORS OF MITOCHONDRIAL ATPase IN THE PROCESS OF ENERGY TRANSDUCTION.

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The hydrolytic activity of rat liver mitochondrial ATPase was affected by a number of electron carriers. Their oxidized or reduced form stimulated in a different degree each of the three postulated catalytic sites of the enzyme. F_1-ATPase was activated and became insensitive to bicarbonate or dinitrophenol stimulation in the presence of NADH, FADH, CoQH₂ and red cyt c; at the same time sensitivity to oligomycin appeared. F_1-ATPase was activated and could still be further stimulated by dinitrophenol or bicarbonate in the presence of FAD, CoQ and cyt c; ATPase remained oligomycin insensitive. The different affinity of ATPase for the electron carriers was modified by ATP of ADP. These results support the idea of a model for oxidative phosphorylation (Santiago, E. and López-Moratalla, N. (1978) Rev. esp. Fisiol., 34, 481-490) which proposes that energy transduction takes place coupling the phosphorylation reactions with the electron flow through each of the three catalytic sites of ATPase constituted by a pair of Fe atoms with ligands ATP, ADP and Pi.

06-6-R58

COUPLING FACTOR B IS A COMPONENT OF THE ENERGY TRANSDUCING ATPase COMPLEX OF MITOCHONDRIA.

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Three types of experiments support the above conclusion:

1. An ATPase complex (AEC) has been prepared from Factor B (B)-deficient AE-particles. It shows 5-fold stimulation of P_i-ATP exchange (EX) activity. A membrane protein (AE-MP) fraction derived from above AEC by extraction with NaBr has low EX even after F_1 addition. EX is stimulated over 10-fold on the addition of B. AE-MP pretreated with N-ethylmaleimide regains oligomycin sensitive ATPase activity on addition of F_1 alone but EX is zero. B restores EX substantially to AE-MP + F_1. B cannot be substituted by BSA, ATPase inhibitor, OSCP, F_6 or any combination of these. Besides showing absolute requirement of B for EX, the data indicate that F_1 binds to MP and is active in the absence of B. Experiments involving preincubation followed by centrifugation show that B and F_1 bind MP independently of each other.
2. The B-deficient AEC shows no Ouchterlony precipitin line with B-antibody but intact AC shows a reaction. NaBr extracts of intact ATPase complex (AC) contain B.
3. The content of F_1 and B in AC has been determined by labeling with N-^{3}H-ethylmaleimide followed by SDS-PAGE and identification of B region by coelectrophoresis with Flourescamine labeled B. Their stoichiometry is close to one.

06-6-R59

INTERACTION OF [^{14}C]DICYCLOHEXYLCARBODIIMIDE WITH COMPLEX V

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Complex V is the isolated ATP synthetase enzyme complex of beef heart mitochondria. It catalyzes oligomyin- and dicyclohexylcarbodiimide (DCCD)-sensitive ATP hydrolysis and ATP-^{32}Pi exchange. The exchange is also sensitive to uncouplers. At 0°C and pH 7.5, [^{14}C]DCCD labels Complex V in 3 regions, when labelled Complex V is examined by dodecylsulfate-acrylamide gel electrophoresis: (a) A wide band centered at $M_r \sim 22{,}000$ with a capacity of 12 nmol DCCD/mg protein, (b) a narrow band at $M_r \sim 13{,}000$ (extractable with 2:1 chloroform-methanol) with a capacity of 6-6.5 nmol/mg protein, and (c) a third region beyond the dye front with a capacity of 1 nmol/mg Complex V protein. [^{14}C]DCCD incorporation into Complex V is biphasic. The initial, rapid phase correlates with inhibition of ATP-Pi exchange, which is more rapid than ATPase activity inhibition. DCCD incorporation into band (b) also correlates with ATPase activity inhibition up to 80%. Extrapolation to 100% gives 3-3.5 nmol DCCD in this band per mg Complex V. Rutamycin (50 nmol/mg) and venturicidin (100 nmol/mg) inhibited DCCD (24 nmol/mg added to the reaction mixture) incorporation into band (b) by >50%. Dibutylchloromethyltin chloride abolished the labelling of (b) and increased the labelling of (a). Aging of labelled Complex V had a similar effect. The water-soluble carbodiimide, 1-ethyl-3 (3-dimethyl-aminopropyl)-carbodiimide did not inhibit ATPase activity and did not affect DCCD binding.

06-6-R60

PURIFICATION, COMPOSITION, AND RESOLUTION-RECONSTITUTION OF MITOCHONDRIAL COMPLEX V.

Yves M. Galante, Siu-Yin Wong, and Youssef Hatefi, Dept. of Biochemistry, Scripps Clinic and Research Fndn., La Jolla, CA 92037, U.S.A.

Complex V has been purified from beef-heart mitochondria, and freed from minor contamination by known components of electron transfer complexes. The purified preparation contains 14 polypeptides when examined by two-dimensional gel electrophoresis in presence of dodecylsulfate, and 3-4 µg phospholipid phosphorus per mg protein. It catalyzes oligomycin- and dicyclohexylcarbodiimide (DCCD)-sensitive ATP hydrolysis, and uncoupler-sensitive ATP-Pi exchange. A number of Complex V polypeptides have been identified as follows: F_1-ATPase 5 subunits (coelectrophoresis and M_r comparison with pure F_1), oligomycin-sensitivity-conferring protein (same criteria using pure OSCP), uncoupler-binding protein (photoaffinity labelling with [^{3}H]2-azido-4-nitrophenol), DCCD-binding protein (labelling with [^{14}C]DCCD and correlation of labelling with activity inhibition), coupling factor F_6 (isolation from Complex V). In addition, polypeptides with M_r values corresponding to ATPase inhibitor (minor amounts) and purified factor B [M_r 11-12 x 10^3: You and Hatefi, Biochim. Biophys. Acta (1976) 423, 398-412] are present. Complex V has been resolved with respect to F_1 and OSCP by treatment with chaotropes, and reconstituted by addition of pure F_1 and OSCP with >90% oligomycin sensitivity of ATPase activity. Results regarding the inhibitors of Complex V, especially ferrous bathophenanthroline and phenylglyoxal and their modification of structure of F_1 and Complex V, will be presented.

06-6-R61

THE COUPLING FACTOR FROM PHOTOSYNTHETIC MEMBRANES OF A THERMOPHILIC BLUE-GREEN ALGA.

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Thylakoid membranes of the thermophilic blue-green alga Mastigocladus laminosus can be prepared by digesting the cells with lysozyme. The membranes are washed several times in a buffer, containing Mg^{2+} and phosphate. The membranes are active in photosystem-II sensitized and photosystem-I sensitized electron transport reactions and in photophosphorylation. The optimal temperature of these membranes lies at 50°C. They are stable at 4°C for several days and can be stored at -25°C without loss in activity. From these membranes a coupling factor was extracted with chloroform and purified on a sucrose gradient. The factor shows a Ca^{2+}-dependent ATPase activity with an optimum at 50°C. For optimal activity 20 mM Ca^{2+} are needed. The activity is latent and has to be activated with higher concentrations of trypsin than with the coupling factor of spinach chloroplasts. The thermophilic algal factor cannot be heat activated as the spinach factor. In SDS-gelelectrophoresis the algal factor shows 4 subunits, corresponding approximately to the α, β, γ and ε subunit of the spinach factor.

06-6-R62

PHOTOPHOSPHORYLATION AND ATPASE ACTIVITIES AFTER SINGLE [illegible] LIGHT FLASHES IN CHROMATOPHORES FROM [illegible] rubrum. Margareta Baltscheffsky and [illegible], Dept. of Biochemistry, Arrhenius Laboratory, [illegible] of Stockholm.

Employing a new, purified luciferase [illegible] luminescence in the presence of ATP [illegible] and Thore [illegible] Anal. Biochem. [illegible] we have [illegible] measured photophosphorylation and ATPase activities in [illegible] from [illegible] rubrum. We have found that [illegible] single short light flash, eliciting only one turnover [illegible] electron transport system, gives rise to ATP formation [illegible] yield of ATP formed after the first turnover is only [illegible] of that formed after subsequent turnovers [illegible] [illegible]

[illegible]