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LIPID PROTEIN INTERACTIONS IN MITOCHONDRIA

VII. A COMPARISON OF THE EFFECTS OF LIPID REMOVAL AND LIPID PERTURBATION ON THE KINETIC PROPERTIES OF MITOCHONDRIAL ATPase

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Summary

We investigated the kinetics of mitochondrial ATPase in bovine heart mitochondria and submitochondrial particles upon treatment with phospholipase A_2, or upon addition of *n*-butanol to perturb the lipid protein interactions. The changes observed are the following:

(1) Lipid removal or perturbation with butanol is accompanied by loss of ATPase activity with decrease of both V and of the K_M for ATP.

(2) There are changes of activation energy of ATPase activity at temperatures above the discontinuity normally observed for membrane-bound enzymes in mitochondria. In particular, butanol abolishes the discontinuity, and induces a constant activation energy of about 32 kcal/mol in the range 8—37°C.

(3) Butanol modifies the pH dependence of ATPase shifting the pH optimum from around 10 to less alkaline values. The optimum for Mg^{2+} concentration is increased by the solvent.

(4) Treatment with phospholipase A_2 results in a removal of oligomycin-sensitive ATPase, whereas butanol addition prevents oligomycin inhibition of ATPase.

(5) In beef heart mitochondria, a spin-labelled analog of the inhibitor, dicyclohexyl carbodiimide, did not show any change in environment upon butanol addition, unlike that found in mitochondria from *Saccharomyces cerevisiae*.

Abbreviation: NCCD, *N*-2,2,6,6-tetramethylpiperidyl-1-oxyl-*N'*-cyclohexyl carbodiimide.

Introduction

In a previous report we showed that organic solvents affect the activity of mitochondrial ATPase by exerting changes in the physical state of the membrane phospholipids [1]. In a later study, we showed that physical state changes parallel the enzymic alterations [2] and we suggested that lipid perturbation induced conformational changes in the membrane proteins.

The conformational role of lipids was studied either by lipid removal or by lipid perturbation [3].

We have now studied the catalytic activity of the mitochondrial ATPase, which is a very complex enzyme system [4–6] consisting of an extrinsic portion (F_1) [7] containing the catalytic site for ATP hydrolysis, and an intrinsic portion, or membrane factor [8]. The enzyme is characterized by a property described by Racker [9] as allotropy: the isolated F_1, which is water-soluble and does not bind lipids, is active in absence of lipids, is cold-labile and insensitive to the energy transfer inhibitors, oligomycin and dicyclohexyl carbodiimide; the membrane-bound enzyme, on the other hand, requires phospholipids for activity, is cold-stable, and is inhibited by oligomycin and dicyclohexyl carbodiimide. The above inhibitors do not bind to F_1 but to the membrane, apparently to intrinsic polypeptide chains in the ATPase complex [8,10]. The conformation and environment of the ATPase in the intact membrane may therefore be different from the conformation in isolated F_1.

We have compared the action of organic solvents on the properties of mitochondrial ATPase with the effects of lipid removal by means of phospholipase A_2.

Materials and Methods

Bovine heart mitochondria [11] and sonicated submitochondrial particles [12] were prepared as discussed previously [1,2]. F_1 was extracted from submitochondrial particles by incubation with cardiolipin [13] or from an oligomycin-sensitive ATPase by heating at 65°C [14].

ATPase activity was assayed in two ways: One procedure involved the colorimetric assay described earlier [15]. For the determination of K_M, where very low activities are measured at low substrate concentrations, we used a titrimetric procedure by titration of H^+ produced at pH 7.2 using a pH-Stat radiometer.

The activation energy of ATPase was calculated from Arrhenius plots.

Phospholipase A_2 was used to extract the lipids [16]; free fatty acids and lysophospholipids were removed by repeated washings with a buffer containing fatty acid-poor albumin.

Lipid phosphorus was assayed according to Marinetti [17] and protein with either a biuret method [18] or according to Lowry et al. [19]. 50 μM NCCD (*N*-2,2,6,6-tetramethylpiperidyl-1-oxyl-*N'*-cyclohexyl-carbodiimide), a site-directed, spin-label analog of dicyclohexyl carbodiimide [20] was added to 30 mg/ml mitochondrial membranes in 0.25 M sucrose, 5 mM Tris-HCl (pH 7.5), containing 1 mM $K_3Fe(CN)_6$ to prevent reduction of the nitroxide radical. Excess NCCD was removed by dialysis. The spectra were recorded with a Decca

radar XI EPR spectrometer [21], at 20°C (microwave frequency 9.52 GHz; amplifier gain, $5 \cdot 10^2$; modulation amplitude, 2 gauss; time constant, 0.3 s).

Results

ATPase activity

Treatment of submitochondrial particles with phospholipase A_2 resulted in progressive loss of ATPase activity, which paralleled the loss in lipid phosphorus, as found by others [16,22]. A similar loss of activity was found upon perturbation of the membrane with *n*-butanol [1]; butanol inhibited ATPase in both mitochondria and submitochondrial particles, but was more effective in the latter, where the inhibition curve was hyperbolic and inhibition already evident at low butanol concentration (apparent K_i, 120 mM). In mitochondria, a sigmoidal inhibition curve was found with an apparent K_i = 400 mM.

Dependence on substrate concentration

Double-reciprocal plots of ATPase activity versus ATP concentration show that progressive delipidation by means of phospholipase A_2 lowers both the V and K_M of the enzyme (Fig. 1).

This type of inhibition also resulted from perturbation of submitochondrial particles by addition of *n*-butanol; 0.1 M butanol induced a K_M decrease from 0.20 to 0.07 mM. Lineweaver-Burk plots, as well as plots $[S]/v$ vs. $[S]$, of v vs. $v/[S]$, and of $[I](1-i)/i$ vs. $[S]$ (where i stands for fractional inhibition), suggest an 'uncompetitive' or 'coupling' inhibition [23]. The decrease of both V and K_M, however, besides binding of the inhibitor only to the enzyme substrate complex, may be ascribed to stabilization of an enzyme-substrate inter-

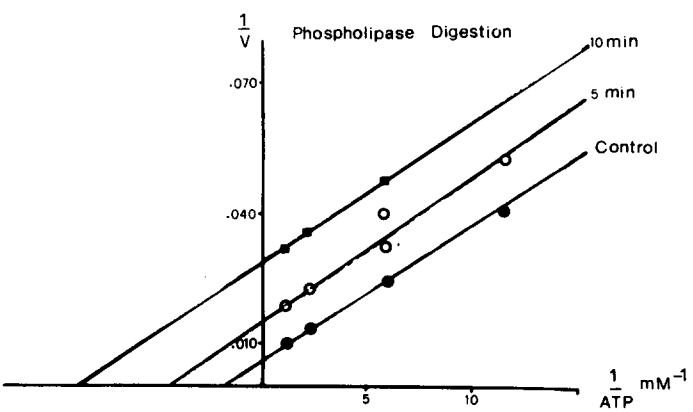

Fig. 1. Effect of phospholipase A_2 hydrolysis on double reciprocal plots of ATPase activity against ATP concentration in submitochondrial particles. The phospholipase digestion was accomplished in the medium described by Fleischer and Fleischer [16]. ●———●, control incubated without phospholipase (phosphorus content 16.5 μg/mg protein); ○———○, 5 min digestion (P content 8.6 μg/mg protein); ■———■, 10 min digestion (P content 6.1 μg/mg protein). ATPase activity was expressed in nequiv. NaOH per min per mg protein in a pH-Stat experiment.

mediate [24]:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} E + P$$

If lipid removal or perturbation lower both the k_{-1} and k_2, the results would be an apparent decrease of both V (k_2) and $K_M(k_{-1} + k_2)/k_1$.

Temperature dependence

Changes of slope in Arrhenius plots of membrane-bound enzymes are probably the result of conformational changes of the enzyme proteins above and below the critical temperature [25]; the origin of the conformational change is due to a phase change of the lipids surrounding the enzyme [26].

In mitochondrial ATPase, a slope change in the Arrhenius plot was found at 17—20°C, with an increase in activation energy below that temperature from about 10 kcal/mol to more than 30 kcal/mol [27,28]. The effect of lipid removal by means of phospholipase A_2 is shown in Fig. 2. There is no large change in the temperature of the slope change, but a slight increase in activation energy is apparent above the discontinuity; we did not find high increases of slope, but it should be kept in mind that phospholipase A_2 does not remove all the lipids from the membrane (8.6 μg P/mg protein). The effect of butanol is also shown in Fig. 2: a complete loss of the discontinuity is observed, due to an increase in activation energy at the higher temperatures. The same effect has been shown by addition of Triton X-100 [28].

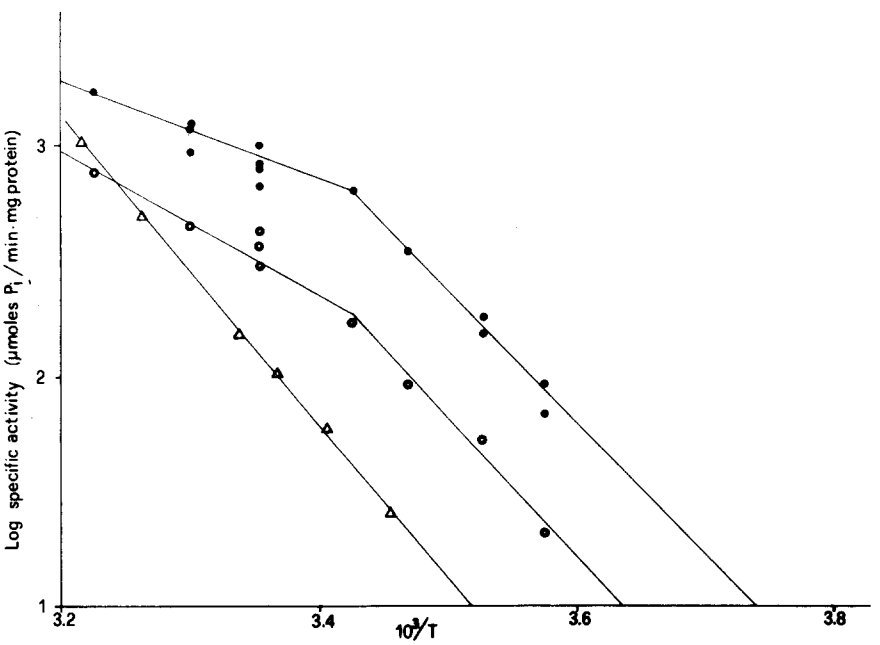

Fig. 2. Effect of phospholipase A_2 hydrolysis and of butanol treatment on the Arrhenius plot of ATPase in mitochondrial membranes. ●———●, submitochondrial particles, no treatment; ○———○, submitochondrial particles, phospholipase A_2 hydrolysis (5 min incubation); △———△, mitochondria, *n*-butanol (0.35 M).

Mg^{2+} activation

0.15 M *n*-butanol increased the apparent K_M for Mg^{2+} activation from 0.2 to 1.0 mM.

pH dependence

0.3 M butanol decreased the pH optimum from pH 10.3 to 8.6.

Oligomycin sensitivity

Treatment of submitochondrial particles with phospholipase A_2 results in a removal of oligomycin-sensitive ATPase, whereas butanol addition prevents oligomycin inhibition of ATPase (Fig. 3). We have previously shown [1] that oligomycin is still capable of inhibiting butanol-treated mitochondria, when the inhibitor is added in very large amounts. The increase in apparent K_i of oligomycin inhibition did not appear to be the result of displacement of oligomycin by butanol for the membrane site of action of the inhibitor, since the effect was shared by dicyclohexyl carbodiimide, which is covalently bound to a subunit of the ATPase complex. Lipid removal or solvent treatment do not result in detachment of F_1 from the membrane [29].

Fluoride inhibition

Fluoride inhibits mitochondrial ATPase activity at low pH (Goffeau, A., personal communication); the inhibition curve is sigmoidal and is not significantly affected by butanol. Hill plots show a slight change of *n*, induced by butanol, from −1.36 to −1.08.

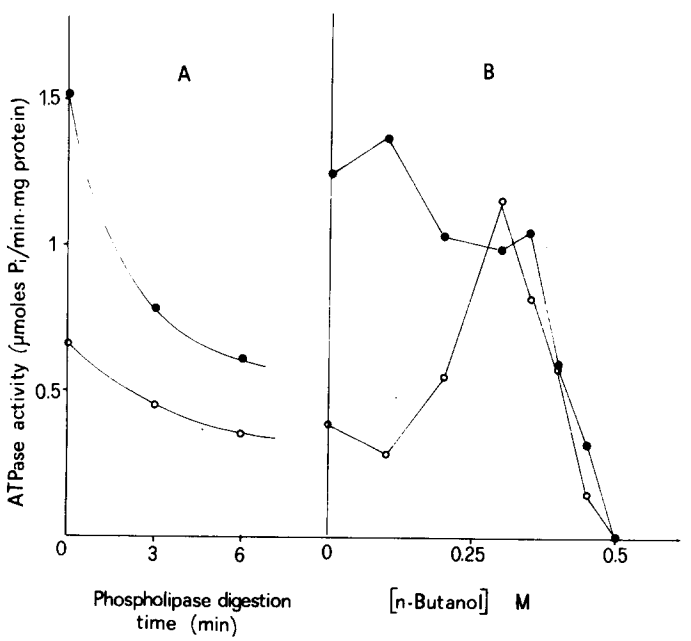

Fig. 3. Effect of phospholipase A_2 and of *n*-butanol on oligomycin sensitivity of ATPase. A. Phospholipase A_2 (in submitochondrial particles). ●———●, control; ○———○, oligomycin, 1 μg/ml. B. Butanol (in mitochondria). ●———●, control; ○———○, oligomycin, 0.07 μg/ml.

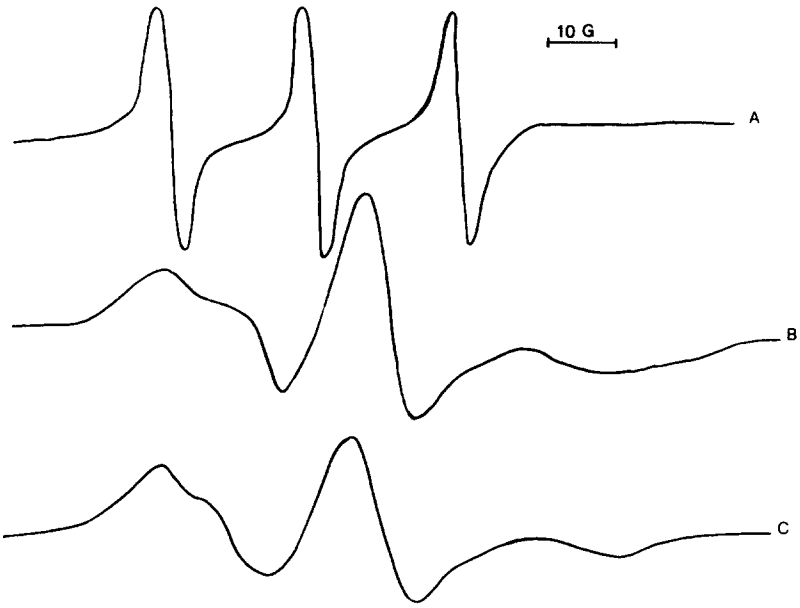

Fig. 4. Effect of *n*-butanol on the ESR spectrum of NCCD bound to submitochondrial particles. A. Spectrum of the spin label in water. B. Spectrum of the spin label bound to submitochondrial particles. C. Spectrum of the spin label bound to submitochondrial particles in presence of 100 mM *n*-butanol.

Studies with NCCD

We have studied the microenvironment of the subunit bearing the dicyclohexyl carbodiimide-binding site, which is an intrinsic protein in the mitochondrial membrane [10] by using the spin labeled dicyclohexyl carbodiimide analog, NCCD [20]. Changes in NCCD environment, either in the lipid or in the protein, may be sensed by the paramagnetic spectrum of the probe [30]; by this means, a conformational change induced by lipid perturbation on the dicyclohexyl carbodiimide-binding protein may be detected.

The effect of butanol on the NCCD spectrum in submitochondrial particles is shown in Fig. 4. The immobilized spectrum of protein-bound NCCD is not modified by 100 mM butanol. This is in contrast with yeast mitochondria, where the spectrum is changed to that of a more mobile nitroxide [31]. The difference suggests displacement of NCCD in yeast mitochondrial particles from the binding site to the phospholipid bilayer; this is not possible in bovine particles, where NCCD is covalently bound. The spectral change is, therefore, a change in environment of the lipids, whereas no conformational change in the protein is detected by this technique.

Discussion

The modifications of the kinetic properties of mitochondrial ATPase, arising from lipid removal using phospholipase A_2 or lipid perturbation with *n*-butanol, concern a decrease of both V and K_M for ATP, a decrease of pH optimum, an increase in activation energy and changes in oligomycin inhibition. The fact

that similar changes are observed by lipid removal and solvent treatment of the membrane, points out to an indirect effect, mediated by the lipids, also in the case of solvent addition.

Modification of kinetic parameters of membrane-bound enzymes upon lipid removal or addition of alcohols or other solvents has been reported in the literature. Removal of the lipids results in lower activity of several membrane-bound enzymes; in general a decrease of both V and K_M is apparent (cf. Ref. 3). A possible interpretation of a K_M decrease by lipid removal may be in an increased accessibility of the substrates to the active sites after membrane disruption by means of delipidation or of organic solvents addition, at the same time that the enzyme activity is decreased (lower V); an alternative explanation is an increased stability of a substrate-enzyme complex due to a conformational change of the active site, accompanied by a decreased rate of formation of the products.

Studies suggesting a stabilization of an enzyme-substrate complex by organic solvents are available in the literature [32—34]. We have found that butanol addition to isolated F_1 also inhibits ATPase activity with decrease of both V and K_M suggesting that the solvent may affect hydrophobic regions of the enzyme also when in the lipid-free state, leading to a change in its structure. The fact that a similar effect on ATPase is shown by phospholipase digestion is in favor of a dual effect, both on the lipid and on the protein, of solvent addition. The alternative explanation that the hydrolysis products of phospholipase A_2 (lysophospholipids and free fatty acids) may act as detergents on F_1 and induce similar changes is unlikely, because thorough washings with albumin have been accomplished after phospholipase digestion.

The correlation between lipid phase transitions and discontinuities in Arrhenius plots of mitochondrial ATPase has been taken as a conformational probe of the enzyme [25]. Upon addition of butanol to the membrane, no discontinuity is apparent any more and the activation energy is increased at all temperatures to values in the range of those below the break in unperturbed ATPase, suggesting disorganization of lipid protein interactions, in agreement with the spin label studies [2]. This interpretation is strengthened by the fact that Triton X-100, which breaks lipid protein interactions but does not alter protein structure directly [35] abolishes the break in the Arrhenius plot of ATPase [27].

Butanol addition abolishes oligomycin and dicyclohexyl carbodiimide inhibition of ATPase. The concentration of butanol required for this effect is in the range of concentration which disrupts lipid protein interactions in mitochondrial membranes as probed by spin labels [1,2,21]. We have previously shown that butanol does not detach ATPase from the membrane [29]. Azzi et al. [20,30] have shown by means of ESR techniques that the paramagnetic dicyclohexyl carbodiimide analog, NCCD, and Mn-ATP are within a distance of about 20 Å in normal submitochondrial particles, but ether treatment [36] increases this distance to above 35 Å. It is tempting to suggest that lipid removal or perturbation with butanol induce a dislocation of ATPase subunits. This interpretation is also in accordance with that of Kozlov and Chernyak [37] on the increased sensitivity of ATPase to *N*-cyclohexyl-*N'*-β-(4-methylmorpholine)ethyl carbodiimide after butanol treatment of mitochondria.

Contrary to what has been found for yeast mitochondria [31] it has not been possible to detect conformational changes of the dicyclohexyl carbodiimide-binding subunit by using the dicyclohexyl carbodiimide paramagnetic analog NCCD. The lack of a change detectable with this method does not mean, however, that no conformational change has occurred.

The origin of the kinetic changes reported in this investigation is not shown by the experiments, but it is tempting to suggest that they are the result of conformational changes in the enzymic protein. Direct studies with physical means, such as circular dichroism, have suggested that changes of secondary structure are induced by organic solvents [3,38] and by lipid depletion [39].

As a final consideration, we point out that this investigation is strongly relevant to the mechanism of action of general anesthetics at the membrane level. It is described elsewhere [3,40] that other organic solvents, which are used in clinics as general anesthetics, have effect on ATPase comparable with those of *n*-butanol. The working hypothesis has been advanced that anesthetics via inducing changes in lipid protein interactions, affect the conformation and activity of membrane-bound catalytic proteins.

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