superaggregates to an active tetramer of 360000 daltons and a slow phase of half-time 15 s representing the subsequent dissociation of tetramer to an inactive dimer. When enzyme at the same concentration is mixed with 5 mm-ATP, only the rapid phase of tetramer production is observed. Although it is well known that phosphofructokinase is strongly inhibited by ATP at pH 6.8 and that the effect of citrate is apparently synergistic, these light-scattering studies show that the mechanism for inhibition by citrate is different from that by ATP. The dissociation of tetramer by citrate may be reversed by ATP, fructose 6-phosphate and fructose 1,6-bisphosphate.

Studies at low enzyme concentration reveal that the conformational change induced by ATP before dissociation is not a simple one-step mechanism.

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**Organization of Oligomycin-Sensitive Adenosine Triphosphatase**

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Structural information pertaining to the sites of ATP synthesis and hydrolysis in mitochondrial membranes has been obtained by the use of extrinsic probes which act at the catalytic site of the ATPase* complex (Azzi et al., 1969; Nordenbrand & Ernster, 1971). A site-directed spin label, N-(2,2,6,6-tetramethylpiperidyl-1-oxyl-N'-cyclohexyl)-carbodi-imide, has been used successfully in ox heart submitochondrial particles (Azzi et al., 1973). The compound is an analogue of dicyclohexylcarbodi-imide, an inhibitor of mitochondrial ATPase (Robertson et al., 1968), and conserves its inhibitory capacity almost intact (Azzi et al., 1973). Although both compounds have been shown to bind covalently in ox heart mitochondria to extents comparable with those giving maximal inhibition of ATPase, mitochondria from aerobically grown yeast do not bind either of the compounds strongly. We have found that dicyclohexylcarbodi-imide totally inhibits the oligomycin-sensitive ATPase in mitochondria from aerobically grown yeast at 5 μg/mg of protein (I50 = 0.9 μg/mg). Purified preparations of the oligomycin-sensitive ATPase (Tzagoloff & Meagher, 1971) have also been shown to be inhibited by the compound (I50 = 1.3 μg/mg of protein). We have incubated inhibitory amounts of 14C-labelled dicyclohexylcarbodi-imide with mitochondria, sonic particles and purified oligomycin-sensitive ATPase for 18 h at 0°C and found only 3–5% of the applied radiolabel bound, unlike ox heart mitochondria, which bind 80% of the inhibitor. A reaction involving dicyclohexylcarbodi-imide is taking place, since t.l.c. of the free inhibitor shows a predominance of the hydration product dicyclohexylurea.

In promitochondrial particles from anaerobically grown yeast, the binding of 14C-labelled dicyclohexylcarbodi-imide is greatly increased, and this is due in part to the presence of a high-affinity site, KD = 1.7 × 10^{-6} M, which is not detectable in mitochondria from aerobically grown yeast. There are approximately 1.5 nmol of high-affinity site/mg of protein. The high-affinity site is also present in mitochondria from catabolite-repressed yeast and in mitochondria from aerobically grown yeast depleted of oligomycin-insensitive ATPase, as shown in Fig. 1.

The presence of the compound binding dicyclohexylcarbodi-imide in oligomycin-sensitive ATPase from promitochondrial particles and mitochondrial particles (depleted of oligomycin-insensitive ATPase) is shown by precipitation of bound inhibitor from Triton X-100 extracts by antisera to oligomycin-sensitive ATPase. Negligible precipitation of radiolabel is found with pre-immune sera. Chloroform/methanol extraction of promitochondria labelled with 14C-labelled dicyclohexylcarbodi-imide removes 95% of the bound radiolabel. T.l.c. of the chloroform/methanol extract shows that the radiolabel is associated with a slow-moving component which co-migrates with

* Abbreviation: ATPase, adenosine triphosphatase.
Fig. 1. Binding of $^{14}$C-labelled dicyclohexylcarbodi-imide to yeast submitochondrial particles

Submitochondrial particles (2mg) were incubated with various amounts of $^{14}$C-labelled dicyclohexylcarbodi-imide at 4°C for 18 h in 5ml containing sucrose (0.25M) and Tris/HCl (10mm), pH7.5. The suspension was centrifuged at 100000g for 30min and the pellet solubilized in 2% (w/v) Triton X-100. Radioactivity in both pellet and supernatant was determined by liquid-scintillation counting. △, NaBr/NH$_3$-extracted submitochondrial particles from aerobic yeast; ○, promitochondrial particles from anaerobic yeast; □, submitochondrial particles from aerobic yeast.

Subunit 9 of the oligomycin-sensitive ATPase and not with either dicyclohexylcarbodi-imide or dicyclohexylurea.

We have studied the microenvironment associated with subunit 9 of the oligomycin-sensitive ATPase in yeast and beef heart mitochondria by using the spin label ($N$-2,2,6,6-tetramethylpiperidyl-1-oxyl)-$N'$-(cyclohexyl)carbodi-imide. Mitochondrial particles (30mg/ml) were incubated at 4°C in the presence of the spin label (50μM) in a medium of 0.25M-sucrose, 5mM-Tris/HCl, pH7.5, containing 1mM-potassium ferri-cyanide to prevent reduction of the nitroxide radical. Excess of label was removed by dialysis. The spectrum of the spin label bound to mitochondrial particles is shown in Fig. 2. No e.p.r. signal can be detected in spin-label-treated mitochondria from aerobically grown yeast, which is consistent with low $^{14}$C-labelled dicyclohexylcarbodi-imide binding to these membranes. Although an immobilized spectrum is found in spin-label-treated promitochondria, a stronger signal is found in sonicated promitochondrial membranes. This suggests a greater accessibility of the carbodi-imide-binding site on the inner face of the inner mitochondrial membrane, i.e. the M-side of the inner membrane, the location of $F_1$ ATPase. The spectrum in Fig. 2(b) is not due to free spin-label or its hydration product $N$-(2,2,6,6-tetramethylpiperidyl-1-oxyl)-$N'$-(cyclohexyl)-urea, as shown by extraction and chromatography of the bound inhibitor. The signal is presumed to be associated with a spin-label–protein adduct as found for $^{14}$C-labelled dicyclohexylcarbodi-imide.

The dicyclohexylcarbodi-imide-binding site in ox heart and yeast mitochondria appears to be quite different. The compound inhibits yeast mitochondrial ATPase, but there is no covalent binding as is found in ox heart, where the compound can be shown
Mitochondrial particles (30mg/ml) were incubated at 4°C in the presence of the spin label (50μM) in 1.0ml containing sucrose (0.25M) and Tris/HCl (5mM), pH 7.5. Potassium ferricyanide (1mM) was included to prevent reduction of the nitroxide radical. Excess of label was removed by dialysis. (a) Spectrum of the spin label in water; (b) spectrum of the spin label bound to yeast promitochondria; (c) as (b), treated with butan-1-ol (100mM); (d) spectrum of spin label bound to ox heart submitochondrial particles and treated with butan-1-ol (100mM).

to be bound to a chloroform/methanol-soluble proteolipid. Moreover, addition of butan-1-ol causes an increase in the mobility of spin label bound to yeast mitochondrial membranes but not of spin label bound to ox heart mitochondria.

The major features of the interaction of dicyclohexylcarbodi-imide with yeast mitochondria are as follows. (a) The dicyclohexylcarbodi-imide- (or the spin-label-analogue-) binding site is only apparent in promitochondrial particles or oligomycin-insensitive--ATPase-depleted mitochondrial particles. (b) We have evidence that dicyclohexylcarbodi-imide or the spin-label analogue is bound to subunit 9 of the yeast oligomycin-sensitive ATPase complex as shown by the chloroform/methanol solubility of the dicyclohexylcarbodi-imide-binding component, the precipitation of the component with antisera to oligomycin-sensitive ATPase and co-migration with purified subunit 9 in t.l.c. (c) The importance of protein–lipid interactions in the binding of dicyclohexylcarbodi-imide or the spin label to yeast mitochondria as opposed to ox heart mitochondria is shown by the change in fluidity of the environment of subunit 9 on addition of butan-1-ol. It has been shown that hydrophobic alcohols such as butan-1-ol have the ability to disorganize the lipid–protein interactions in the membrane (Lenaz et al., 1975). (d) The data suggest that the conformation of the oligomycin-sensitive ATPase, which depends on lipid–protein (perturbed by butan-1-ol) or protein–protein (modified by depletion of F1 or oligomycin-sensitive ATPase complex) interactions, is the determining factor in dicyclohexylcarbodi-imide binding in yeast mitochondria. The binding of dicyclohexylcarbodi-imide or the spin label to promitochondria may prove a useful way of studying the biogenesis of the oligomycin-sensitive ATPase complex.

Preliminary investigations show that the 14C-labelled dicyclohexylcarbodi-imide-binding site in oligomycin-sensitive ATPase from promitochondria and the F0
membrane component in membranes from aerobic mitochondria depleted of F has a molecular weight of 45000, and that this is converted to a low-molecular-weight component by treatment with organic solvents, as found in biogenesis experiments establishing the mitochondrial synthesis of subunit 9 (Tzagoloff & Akai, 1972). The number of components in the 45000-molecular-weight component and in the 8000-molecular-weight subunits derived from it has not been determined.


The Protonmotive Force, the Phosphate Potential and Respiratory Control: the p-Zone Interpretation

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The protonmotive force in state 4, determined according to the classical formulation of the chemiosmotic hypothesis, has invariably proved less than the value required by estimates of the phosphate potential (Mitchell & Moyle, 1969; Padan & Rottenberg, 1973; Nicholls, 1974). The deficit has never been satisfactorily explained. The p-zone interpretation of the hypothesis (Archbold et al., 1974, 1975a,b,c) recognizes the importance of the fixed charges on the energized inner membrane in developing a protonmotive force and can be shown to satisfy within strictly physiological conditions the existence of the required state-4 phosphate potential of 300mV.

The force across the membrane depletion layer can be derived from the expression giving the maximum electrostatic potential within a fixed charge lattice (Mauro, 1962; Coster, 1965):

\[ \Delta \psi = \frac{kT}{q} \ln \frac{N^-N^+}{p_0n_1} + V_m \]

where \( N^- \) and \( N^+ \) are the membrane fixed charges; \( p_0 \) and \( n_1 \) the positive and negative counter-ion concentrations in the bulk phase; \( k \) is the Boltzmann constant, \( T \) the temperature on the Kelvin scale and \( q \) the charge on each particle. \( V_m \) is the membrane potential measured in the bulk phase; in the energized membrane it is likely to be very small compared with the first term. The total potential across the depletion layer is therefore given by

\[ \Delta \psi \approx \frac{kT}{q} \ln \frac{N^-N^+}{p_0n_1} \]

We assume that \( N^- \) and \( N^+ \) are equal and that they are correctly assessed as volume, rather than surface, densities of charge; \( p_0 \) and \( n_1 \) are also regarded as being equal, but a tenfold disparity in these values would involve only a 20% change in the calculated values of \( \Delta \psi \) at 300mV.

Fig. 1 gives evaluations of \( \Delta \psi \) for a series of relevant \( p_0, n_1, N^- \) and \( N^+ \) estimates. The hatched area gives the solutions for \( \Delta \psi = 300 \pm 10 \text{mV} \). The evidence already