

Studies of Energy-Linked Reactions

LOCALIZATION OF THE SITE OF ACTION OF TRIALKYL TIN IN YEAST MITOCHONDRIA

By KELVIN CAIN and DAVID E. GRIFFITHS

Department of Molecular Sciences, University of Warwick, Coventry CV4 7AL, U.K.

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Ligand-binding studies with labelled triethyltin on yeast mitochondrial membranes showed the presence of high-affinity sites ($K_D = 0.6 \mu\text{M}$; $1.2 \pm 0.3 \text{ nmol/mg}$ of protein) and low-affinity sites ($K_D > 45 \mu\text{M}$; $70 \pm 20 \text{ nmol/mg}$ of protein). The dissociation constant of the high-affinity site is in good agreement with the concentration of triethyltin required for inhibition of mitochondrial ATPase (adenosine triphosphatase) and oxidative phosphorylation. The high-affinity site is not competed for by oligomycin or venturicidin, indicating that triethyltin reacts at a different site from these inhibitors of oxidative phosphorylation. Fractionation of the mitochondrial membrane shows a specific association of the high-affinity sites with the ATP synthase complex. During purification of ATP synthase (oligomycin-sensitive ATPase) there is a 5–6-fold purification of oligomycin- and triethyltin-sensitive ATPase activity concomitant with a 7–9-fold increase in high-affinity triethyltin-binding sites. The purified yeast oligomycin-sensitive ATPase complex contains approximately six binding sites for triethyltin/mol of enzyme complex. It is concluded that specific triethyltin-binding sites are components of the ATP synthase complex, which accounts for the specific inhibition of ATPase and oxidative phosphorylation by triethyltin.

Trialkyltin compounds are potent inhibitors of oxidative phosphorylation (Aldridge & Street, 1964; Sone & Hagihara, 1964; Rose & Aldridge, 1972) and inhibit dinitrophenol-stimulated ATPase activity as well as P_i -ATP-exchange activity, indicating a site of action in the ATP synthase complex similar to that of oligomycin and dicyclohexylcarbodi-imide. In addition, trialkyltin compounds catalyse a Cl^-/OH^- -exchange reaction in mitochondria and chloroplasts (Selwyn *et al.*, 1970; Stockdale *et al.*, 1970; Watling & Selwyn, 1970; Dawson & Selwyn, 1974), and facilitate swelling of mitochondria in Cl^- -containing media. It is not known if the Cl^-/OH^- -exchange (antiport) system in mitochondria represents a specific transport property of trialkyltin compounds, or whether it is the result of reactions with a specific site in the mitochondrial inner membrane which may be a component of the ATP synthase complex.

Interaction with the ATP synthase complex is indicated by the inhibition by trialkyltins of the oligomycin-sensitive ATPase from ox heart (Kagawa & Racker, 1966; Tzagoloff *et al.*, 1968; Byington, 1971) of F_1 -ATPase. The demonstration that tributyltin (1 nmol/mg of protein) induces respiratory control and lessens the proton permeability of

coupling-factor-deficient submitochondrial particles (Dawson & Selwyn, 1975) indicates that trialkyltin compounds have a similar mode of action to oligomycin and dicyclohexylcarbodi-imide on hydrophobic protein subunits of the ATP synthase complex.

Previous studies on the binding of trialkyltin compounds to rat liver mitochondrial membranes (Aldridge & Street, 1970) indicate the presence of high-affinity and low-affinity binding sites, and the dissociation constant for the high-affinity binding site is similar to the concentrations required to inhibit oxidative phosphorylation. However, the high-affinity site has not been specifically localized on any component of the oxidative-phosphorylation system. Biochemical-genetic studies (Lancashire & Griffiths, 1975a) support the localization of the trialkyltin-interaction site in the oligomycin-sensitive ATPase, and genetic studies of cytoplasmic (mitochondrial) trialkyltin-resistant mutants indicate that the trialkyltin-interaction site involves a different protein component from that involved in the oligomycin-interaction site (Griffiths *et al.*, 1975).

The present paper describes studies with triethyl- $[^{113}\text{Sn}]$ tin which localize most of the high-affinity triethyltin-binding sites in yeast mitochondria in the oligomycin-sensitive ATPase complex described by Tzagoloff *et al.* (1973).

* Abbreviations: ATPase, adenosine triphosphatase; F_1 -ATPase, mitochondrial ATPase (Pullman *et al.*, 1960).

Materials

The source of many of the materials and reagents used has been described previously (Griffiths & Houghton, 1974).

Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] and Triton X-100 were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Triethyl[¹¹³Sn]tin chloride (8.6 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Trialkyltin chlorides and triethyltin sulphate were obtained as previously described (Lancashire & Griffiths, 1975a) and used as ethanolic solutions. Spectrophore dialysis membrane (mol.wt. cut-off 6000–7000) was from Raven Scientific, Haverhill, Suffolk, U.K., and washed for several hours in water before use. All other reagents were of A.R. grade.

Methods

Growth and isolation of yeast, isolation of yeast mitochondria, preparation and isolation of yeast submitochondrial particles, the assay of protein, ATPase activity and inhibitor sensitivity (I_{50} values) are essentially as described by Griffiths & Houghton (1974).

The partially purified oligomycin-sensitive ATPase was prepared by extraction of yeast submitochondrial particles with 0.5% (w/v) Triton X-100 essentially as described by Tzagoloff & Meagher (1971). This preparation is referred to as the Triton X-100 extract, and the residue after extraction is termed the Triton-extracted particles. Purified oligomycin-sensitive ATPase was prepared from the Triton X-100 extract by density-gradient centrifugation as described by Tzagoloff & Meagher (1971). Yeast submitochondrial particles depleted of F_1 -ATPase and oligomycin-sensitivity-conferring protein were prepared by extraction with NaBr and NH_3 as described by Tzagoloff (1971) and are referred to as depleted particles in the text.

Triethyl[¹¹³Sn]tin was assayed by liquid-scintillation counting in a Packard 4325 scintillation counter. Samples were contained in 1 ml of aqueous solution and added to 9 ml of a Triton X-100-based scintillant solution [1 litre of toluene, 0.5 litre of Triton X-100, 7 g of 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole]. Standards were counted for radioactivity with each batch of samples. Under these conditions the efficiency of counting was 60%.

Binding studies with triethyl[¹¹³Sn]tin

Binding of triethyltin to mitochondria and submitochondrial particles was carried out by incubating triethyl[¹¹³Sn]tin with 1–2 mg of mitochondrial protein in 5.0 ml of buffer (0.5 M-sorbitol/10 mM-Hepes/KOH, pH 7.5) in cellulose nitrate centrifuge

tubes at 5°C for 30 min. Mitochondria and submitochondrial particles were separated by centrifugation at 100000g in the 40.3 rotor of the Beckman ultracentrifuge for 30 min at 5°C. Samples of the supernatant were taken for scintillation counting, and the remaining supernatant was discarded and the precipitate solubilized by leaving overnight in 1.0 ml of 2% (w/v) Triton X-100. The supernatant and precipitate were assayed for radioactivity by scintillation counting to estimate the concentration of free and bound ligand. There was no significant binding of triethyl[¹¹³Sn]tin to the cellulose nitrate tubes.

Equilibrium-dialysis studies with detergent-dispersed oligomycin-sensitive ATPase were carried out in a multicavity micro-dialysis cell (cat. no. 6779-09; Cole-Parmer, Chicago, IL, U.S.A.) rotated at 20 rev./min. The dialysis cell contained approx. 150 µg of oligomycin-sensitive ATPase in 0.5 ml of sucrose buffer [0.36 M-sucrose/10 mM-Tris/ H_2SO_4 , pH 7.5, containing 0.1% (w/v) Triton X-100]. The other chamber contained 0.5 ml of the same buffer containing 0.4 µM–0.4 mM-triethyl[¹¹³Sn]tin. Under these conditions there was no significant binding of triethyltin to the dialysis membrane or incubation chamber. Samples of both chambers of the dialysis cell were removed for scintillation counting after dialysis for 24 h at 5°C. Control experiments showed complete equilibrium and recovery, also no decrease in the ATPase activity or modification of sensitivity to inhibitors over this period. The results from binding experiments were plotted by the procedure of Scatchard (1949) and analysed by trial and error.

Results

The yeast mitochondrial oligomycin-sensitive ATPase is readily solubilized from the mitochondrial inner membrane by 0.5% (w/v) Triton X-100, in contrast with the comparatively drastic conditions required to solubilize the oligomycin-sensitive ATPase complex of heart and liver mitochondria, and retains sensitivity to oligomycin, venturicidin and triethyltin which approximates to that of the membrane-bound enzyme (Griffiths & Houghton, 1974). This provides a suitable method for determining the association of high-affinity binding sites for triethyltin with components of the yeast mitochondrial membrane, particularly the oligomycin-sensitive ATPase.

Fig. 1 shows that a similar degree of sensitivity to triethyltin is observed in the oligomycin-sensitive ATPase in submitochondrial particles and the Triton X-100 extract, and a slightly modified sensitivity in purified oligomycin-sensitivity ATPase. Purified F_1 -ATPase exhibited less than 10% inhibition by triethyltin, even at 500 nmol/mg of protein. Triethyltin is not bound covalently, as the amount of inhibitor bound can be totally removed by three

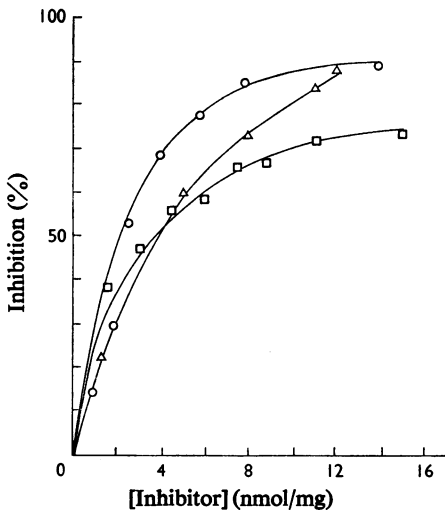


Fig. 1. Inhibition of detergent-solubilized oligomycin-sensitive ATPase and membrane bound oligomycin-sensitive ATPase by triethyltin

○, Submitochondrial particles (specific activity, $5 \mu\text{mol}/\text{min}$ per mg of protein); △, Triton X-100 extract (specific activity, $9 \mu\text{mol}/\text{min}$ per mg of protein); □, purified oligomycin-sensitive ATPase (specific activity, $27 \mu\text{mol}/\text{min}$ per mg of protein). ATPase assays were carried out at pH 9.5 at 30°C and P_i release was measured as described by Griffiths & Houghton (1974). The enzyme preparations used are described in the Methods section.

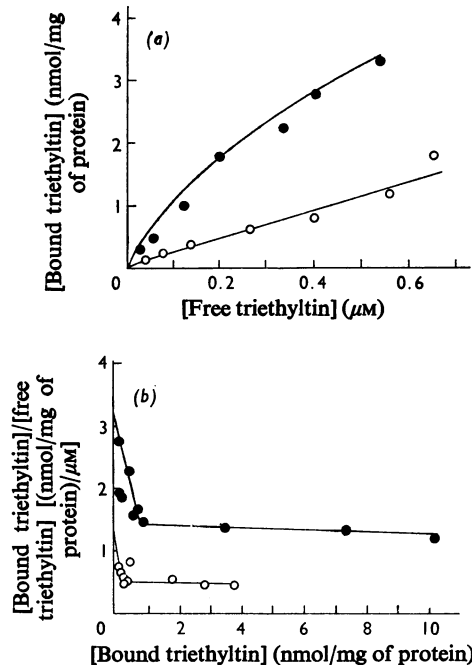


Fig. 2. Binding of triethyltin to particles and Triton X-100-extracted particles

(a) Amount bound plotted against free ligand concentration; (b) data analysed by the method of Scatchard (1949), with the bound/free ratio plotted against the amount bound. ●, Particles (no. of high-affinity sites = $0.9 \text{ nmol}/\text{mg}$, $K_D = 0.43 \mu\text{M}$; no. of low-affinity sites = $60 \text{ nmol}/\text{mg}$, $K_D = 40 \mu\text{M}$). ○, Triton X-100-extracted particles (no. of high-affinity sites = $0.2 \text{ nmol}/\text{mg}$, $K_D = 0.25 \mu\text{M}$; no. of low-affinity sites = $50 \text{ nmol}/\text{mg}$, $K_D = 80 \mu\text{M}$).

washes with binding medium, with restoration of ATPase activity.

Ligand-binding studies with triethyltin and yeast mitochondrial particles show that there are two classes of binding site (Fig. 2), as shown by Aldridge & Street (1970) for liver mitochondria. Analysis of the binding data indicates a class of high-affinity sites ($K_D = 0.6 \pm 0.2 \mu\text{M}$; $1.2 \pm 0.3 \text{ nmol}/\text{mg}$ of protein) present in low concentration and a class of low-affinity sites ($K_D > 45 \mu\text{M}$; $70 \pm 20 \text{ nmol}/\text{mg}$ of protein) present in high concentration. The dissociation constant and concentration of high-affinity sites is in good agreement with the concentrations of triethyltin required for inhibition of mitochondrial ATPase, whereas the low-affinity sites probably represent non-specific binding to membrane proteins and phospholipid.

Extraction of yeast submitochondrial particles with 0.5% (w/v) Triton X-100 removes oligomycin-sensitive ATPase (triethyltin-sensitive ATPase), with a corresponding decrease in the number of high-affinity binding sites for triethyltin present in the Triton X-100-extracted particles (Fig. 2 and Table 1). The concentration of low-affinity binding sites in the

Triton X-100-extracted particles is not markedly affected, and it appears that most of the high-affinity sites are extracted with the oligomycin-sensitive ATPase into the Triton X-100 extract. However, up to 30% of the high-affinity sites are retained in Triton-extracted particles, which suggests that all of the high-affinity sites are not in tight association with the oligomycin-sensitive ATPase complex.

Association of the high-affinity binding sites with oligomycin-sensitive ATPase is seen on examination of the ligand-binding properties of the purified complex (Fig. 3). Only one class of binding site is found, of relatively high affinity ($K_D = 6\text{--}7 \mu\text{M}$; $11\text{--}12 \text{ nmol}/\text{mg}$ of protein), and there is no evidence for the low-affinity binding sites ($K_D > 45 \mu\text{M}$) found in mitochondrial particles. There is also an increase in the concentration of high-affinity sites, which is consistent with the degree of purification of the oligomycin-sensitive ATPase from sub-mitochondrial particles.

Table 1. *Binding of triethyltin to mitochondrial oligomycin-sensitive ATPase*

Triethyltin binding to various mitochondrial preparations was determined as described in the Methods section. The results are expressed where appropriate as means \pm S.D.

Preparation	No. of observations	High-affinity sites		Low-affinity sites		Specific activity of triethyltin-sensitive ATPase ($\mu\text{mol}/\text{min}$ per mg)
		K_D (μM)	No. of sites (nmol/mg)	K_D (μM)	No. of sites (nmol/mg)	
Particles	4	0.6 ± 0.2	1.2 ± 0.3	45 ± 9	70 ± 20	5.7 ± 0.5
Triton X-100 extracted particles*	4	0.3 ± 0.1	0.35 ± 0.2	76 ± 15	55 ± 9	1.2 ± 0.2
Oligomycin-sensitive ATPase	2	6.9	11.9	—	—	27
Depleted particles	1	1.2	4.5	41	102	0

* In this case the residual ATPase was insensitive to triethyltin.

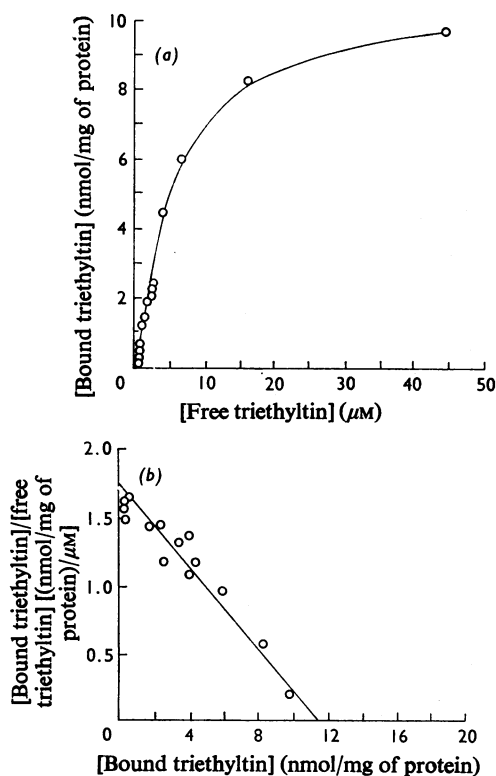


Fig. 3. *Binding of triethyl[^{113}Sn]tin to purified oligomycin-sensitive ATPase*

(a) Amount bound plotted against free ligand concentration; (b) data analysed by the method of Scatchard (1949) with bound/free ratio plotted against the amount bound. $K_D = 6.9 \mu\text{M}$, no. of sites = $11.6 \text{ nmol}/\text{mg}$.

Possible reasons for the tenfold decrease in affinity for triethyltin are discussed below, but the results clearly establish an association of high-affinity sites for triethyltin with the oligomycin-sensitive ATPase.

That the high-affinity binding sites for triethyltin are associated with the hydrophobic subunits of the oligomycin-sensitive ATPase is shown in experiments where the F_1 -ATPase and oligomycin-sensitivity-conferring protein are preferentially removed from the membrane by NaBr/NH_3 extraction (Griffiths & Houghton, 1974), leaving most of the hydrophobic subunits of the oligomycin-sensitive ATPase in the depleted particles. NaBr/NH_3 extraction does not remove the high-affinity binding sites (Fig. 4 and Table 1). In fact, there is an increase in the concentration of high-affinity sites, reflecting the increased concentration of hydrophobic subunits of the oligomycin-sensitive ATPase complex present in the depleted particles.

The effect of pH on binding of triethyltin to particles was investigated, as this may give some indication of the pK_a values of functional groups involved in binding. Aldridge & Rose (1969) have proposed that two histidine residues per binding site are involved as a result of studies of the effect of pH on ADP-stimulated respiration, which is supported by studies of triethyltin-binding sites on a model system. Table 2 shows that there is little change in binding of triethyltin in the pH range 6.0–7.5 and a decrease in binding above pH 7.5. Similar results have been reported by Dawson & Selwyn (1974), and the involvement of histidine residues of the triethyltin-binding site remains to be established.

Oligomycin and venturicidin have a similar mode of action to triethyltin (Sone & Hagihara, 1964; Stockdale *et al.*, 1970) and it is assumed that they have a similar site of action. However, significant differences in the mode of action of triethyltin have been noted by Aldridge & Street (1971), and biochemical-genetic studies in this laboratory (Lancashire & Griffiths 1975a,b) indicate that the resistance determinants that specify triethyltin resistance, and hence the components of the binding site, are different from those which specify oligomycin- and venturicidin-resistance.

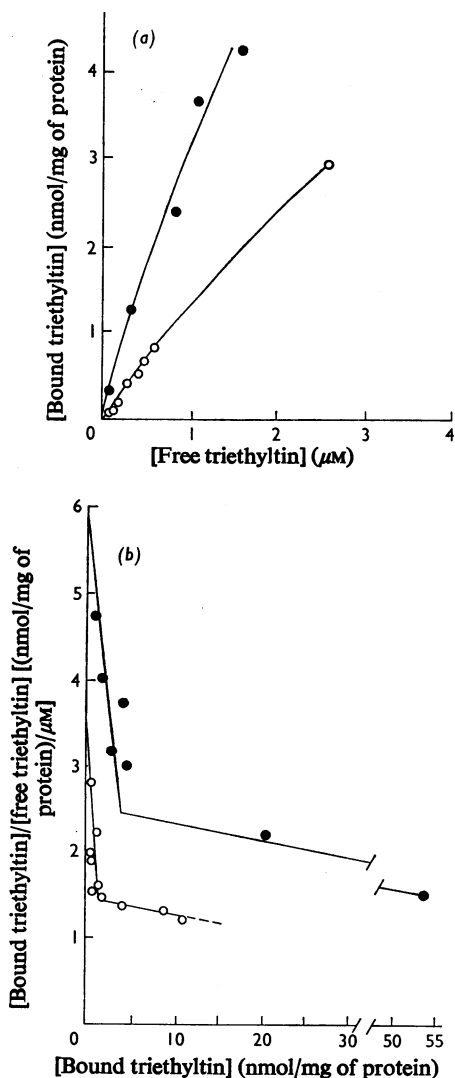


Fig. 4. Binding of triethyl[¹¹³Sn]tin to depleted particles (a) Amount bound plotted against free ligand concentration; (b) data analysed by the method of Scatchard (1949) with the bound/free ratio plotted against the amount bound. ●, Depleted particles (no. of high-affinity sites = 4.5 nmol/mg, $K_D = 1.2 \mu\text{M}$). ○, Control, i.e. binding to particles (no. of high-affinity sites = 0.9 nmol/mg, $K_D = 0.43 \mu\text{M}$; no. of low-affinity sites = 60 nmol/mg, $K_D = 40 \mu\text{M}$).

Fig. 5 shows that oligomycin and venturicidin have no effect on the binding of triethyltin to mitochondrial particles even at concentrations 10 times higher than that at which they cause 50% inhibition of ATPase activity (I_{50} value), and support the conclusion that triethyltin binds at a separate and distinct binding site.

Table 2. Binding of triethyltin to high- and low-affinity sites. Binding of particles was measured as described in the Methods section. In (a) particles were incubated with 0.42 nmol of triethyltin/mg of protein; this titrates predominantly high-affinity sites. In (b) particles were incubated with 42 nmol of triethyltin/mg of protein, thus titrating the low-affinity sites. The results are expressed in terms of the bound/free ratio described by Scatchard (1949).

pH	Bound/free ratio	
	(a)	(b)
6.0	2.7	1.7
6.8	2.7	1.4
7.0	2.6	1.3
7.5	2.4	1.25
8.0	2.1	1.1
8.5	1.4	0.8

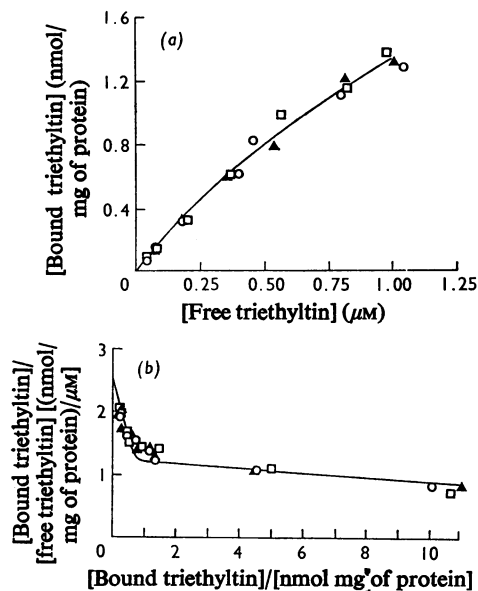


Fig. 5. Effect of oligomycin and venturicidin on triethyl[¹¹³Sn]tin binding to particles

Particles were preincubated for 30 min with inhibitors before carrying out triethyl[¹¹³Sn]tin binding. Analysis of binding data was as described for Fig. 2. ○, Control, i.e. no inhibitor; ▲, 2 μg of venturicidin/mg; □, 10 μg of oligomycin/mg. No. of high-affinity binding sites = 1.0 nmol/mg, $K_D = 0.5 \mu\text{M}$; no. of low-affinity binding sites = 61 nmol/mg, $K_D = 50 \mu\text{M}$.

Discussion

The yeast mitochondrial inner membrane has been shown to have high-affinity binding sites and low-affinity binding sites for triethyltin compounds, as shown by Aldridge & Street (1970) for liver mito-

chondria. The dissociation constant (K_D) for the high-affinity binding site is of the required value to explain inhibition of ATPase and oxidative phosphorylation by triethyltin (Table 1). Extraction of the high-affinity sites concomitant with extraction of the oligomycin-sensitive ATPase complex from the mitochondrial membrane indicates a specific association with the oligomycin-sensitive ATPase complex and thus locates the site of inhibition of oxidative phosphorylation on a component of the complex.

Table 1 shows that there is a 5–6-fold degree of purification with respect to oligomycin- and triethyltin-sensitive ATPase activity concomitant with a 7–9-fold increase in high-affinity triethyltin-binding sites. This indicates that a specific stoichiometry is maintained during the purification procedure. Assuming that the F_1 -ATPase represents 10% of the membrane protein and thus a content of approx. 0.2–0.3 nmol of F_1 -ATPase/mg of membrane protein, a value of approx. 5–6 binding sites per oligomycin-sensitive ATPase complex can be calculated. A similar stoichiometry is obtained in the purified oligomycin-sensitive ATPase complex on the basis of a mol.wt. of 486 000 (Tzagoloff & Meagher, 1971). The high-affinity site in the purified oligomycin-sensitive ATPase has a higher K_D value (6.9 μ M) than that of the membrane-bound enzyme ($K_D = 0.6 \mu$ M), and this is also reflected in an increase in the measured I_{50} value for inhibition by triethyltin (Fig. 1). This is probably due to a conformational change in the lipid-depleted enzyme caused by its removal from the highly ordered hydrophobic lipid-enriched membrane environment. The 25–30% of high-affinity binding sites which remain in the membrane with unextracted ATPase (Table 1) probably represent tightly bound oligomycin-sensitive ATPase or oligomycin-sensitive ATPase that has been modified during the extraction procedure.

Trialkyltins do not inhibit F_1 -ATPase, and extraction of F_1 -ATPase, and the oligomycin-sensitivity-conferring protein (Fig. 4) does not remove the high-affinity binding sites from the membrane. These results indicate that the high-affinity binding site is not associated with components of F_1 -ATPase, but indicate a specific association with hydrophobic components of the membrane. These components are also specific components of the intact functional oligomycin-sensitive ATPase complex, which in yeast mitochondria is readily extractable by Triton X-100 (Tzagoloff & Meagher, 1971). It has been demonstrated previously that the yeast oligomycin-sensitive ATPase complex contains the sensitivity sites for oligomycin, venturicidin, triethyltin and dicyclohexylcarbodi-imide (Griffiths & Houghton, 1974), and biochemical-genetic studies indicate that at

least three polypeptide species are involved (Griffiths, 1975). The retention of inhibitor-sensitivity in a detergent-dispersed oligomycin-sensitive ATPase complex of defined subunit composition would indicate that this complex represents a functional entity which is integrated into the oxidative-phosphorylation complex of the mitochondrial inner membrane.

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