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Dibutyltin-3-hydroxyflavone Titrates a Dissociable Component (Cofactor) of Mitochondrial ATP Synthase: An Energy-transfer Component Linked to the Ubiquinone Pool

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Dibutyltin-3-hydroxyflavone, $Bu_2Sn(of)$, is a new fluorescence probe inhibitor of F_1F_0-ATPase and oxidative phosphorylation which inhibits by titration of an unidentified component of F_0. Its site of action is closely related to that of the trialkyltins and of venturicidin. This F_0 component is part of a pool of this component which is present in the heart mitochondrial inner membrane at levels of 5–7 nmol (mg protein)$^{-1}$ [18 ± 3 $Bu_2Sn(of)$ sites per mol F_1F_0-ATPase]. However, ATPase activity in submitochondrial particles is near maximally inhibited by titration of approx. three $Bu_2Sn(of)$ sites per mol F_1F_0-ATPase.

Over 60% (60–80%) of the $Bu_2Sn(of)$ interaction sites can be lost during the purification of F_1F_0-ATPase from submitochondrial particles. The number of $Bu_2Sn(of)$ interaction sites in various F_1F_0-ATPase preparations is variable. The high numbers of $Bu_2Sn(of)$ sites per mol F_1F_0-ATPase for heart mitochondria (18–21) and submitochondrial particles (15–19.5) decline in ATP synthase (11–15) to the low values obtained in Complex V (7–10.5) and the minimal values observed in highly purified F_1F_0-ATPase (3.5–5.6), thus indicating a variable dissociable component or cofactor of ATP synthase.

The $Bu_2Sn(of)$ interaction site, a component of ATP synthase, is responsive to the redox status of the respiratory chain and the interaction with $Bu_2Sn(of)$ is with the reduced form of this component. Fluorescence titration studies show that this component is in redox equilibrium with the ubiquinone pool of the respiratory chain. It is proposed that this redox component serves as an inhibitor titratable cofactor pool which cycles through an F_0 interaction site (or sites) via a system which serves as an energy-transfer link between the respiratory chain and ATP synthase.

Keywords: Dibutyltin-3-hydroxyflavone, mitochondria, ATP, oxidative phosphorylation, energy transfer, ubiquinone

INTRODUCTION

The extensive evidence for the binding change mechanism for ATP synthase has been reviewed recently.[1,2] There appears to be general acceptance that conformational changes linked to proton translocation can change the properties of catalytic sites in F_1. However, the mechanism of F_0 proton translocation has not been established and the limitations of the current evidence have been discussed.[1]

Discussions of localized movement of protons from electron-transport components to the ATP synthase,[3,4] as opposed to scalar proton transfers, and of conformational interactions during intramembrane collision processes,[5,6] all require evidence of the interaction of ATP synthase and a redox component but no definitive evidence has been presented. Such interactions could be mediated by a pool of mobile lipophilic component(s) of the inner mitochondrial membrane which interacts with ATP synthase and which is in redox equilibrium with a component or components of the respiratory chain. Possible candidates are (a) the ubiquinone pool which is present in the inner membrane at 5–8 nmol (mg protein)$^{-1}$; (b) the mitochondrial thiol pool (thiol, dithiol, thioester) which is present in the inner membrane at levels at least three times the ubiquinone pool. However, the majority of the thiol pool is made up of protein-bound thiols and evidence for a relevant mobile thiol pool is limited.

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The extensive studies by Aldridge and co-workers on the mode of action of trialkyltin energy-transfer inhibitors led to the formulation of a mechanism of oxidative phosphorylation involving an array of histidine residues as proton acceptors.[7] It was shown that the mitochondrial inner membrane contained triethyltin binding sites at a level of ~6 nmol (mg protein)$^{-1}$, i.e. a pool of binding sites. Inhibition of oxidative phosphorylation with pyruvate and succinate as substrates and of DNP-stimulated ATPase was achieved after binding (titration) of only 10–15% of these sites, i.e. titration of only one site per 7–10 sites. However, studies of phosphorylation linked to ascorbate oxidation indicated that the total pool of sites was involved.[7]

Previous studies in this laboratory using dibutyl(chloromethyl)tin chloride (DBCT), an affinity probe inhibitor of oxidative phosphorylation and F_1F_0-ATPase, showed that [^{3}H]DBCT labelled a component of the yeast mitochondrial inner membrane which was present at levels of 6–8 nmol (mg protein)$^{-1}$, i.e. at levels similar to that of the ubiquinone pool.[8,9] Approximately one-third of the DBCT interaction sites remained in the yeast mitochondrial inner membrane after detergent extraction of F_1F_0-ATPase, thus indicating the presence of a pool of interaction sites in addition to those associated with the ATPase complex.[9] In addition, studies of the inhibition by DBCT of the ATP-driven NAD^+ reduction by succinate reaction show a lag phase which is dependent on DBCT concentration, followed by a reaction phase of decreasing activity.[9,10] These results are consistent with the titration of a component of ATP synthase leading to inhibition of activity and its slow replacement by an active component from the pool leading to reactivation of activity, i.e. cycling of this component through an F_0 reaction site. Complete inhibition is achieved by total titration of the pool. Similar results are obtained with dibutyltin dichloride and trialkyltins.[10,11] These results with organotins are in contrast to the mode of action of oligomycin, which inhibits immediately at concentrations similar to the concentration of F_1F_0-ATPase.

The interaction site of DBCT was not identified definitively although the [^{3}H]DBCT derivative of lipoic acid and a ^{3}H-labelled derivative of a low-molecular-mass (~4 kDa) peptide were found as products of [^{3}H]DBCT labelling studies.[12] A role for lipoic acid or a similar dithiol was proposed and supporting evidence was presented in experiments showing reversal of DBCT inhibition of ATPase and oxidative phosphorylation by dihydrolipoate[13] and inhibition of ATPase and oxidative phosphorylation by reduced 8-methyl-lipoate,[14] a lipoic acid analogue known to cause growth inhibition.[15] However, no definitive experiment for a role for lipoic acid or a related thiol component involving a linkage to the respiratory chain was established.

This paper describes experiments with dibutyltin-3-hydroxyflavone bromide, Bu_2Sn(of), a new fluorescence probe inhibitor of F_1F_0-ATPase and oxidative phosphorylation which titrates an unidentified component of F_0.[16,17] Fluorescence titration experiments show that the number of Bu_2Sn(of) interaction sites in mitochondria and submitochondria particles (SMP) are in excess of the 9–12 subunit *c* content of F_1F_0-ATPase and that there is a pool of Bu_2Sn(of) interaction sites which is present in the mitochondrial inner membrane at levels similar to that of the ubiquinone pool. Evidence is also presented that interaction with Bu_2Sn(of) is dependent on the reduction status of a component of the inner membrane and that it is in redox equilibrium with the ubiquinone pool of the respiratory chain. This component thus has the properties of an energy-transfer component which provides a link between ATP synthase and the respiratory chain.

The implications of these findings for current studies of the mechanism of oxidative phosphorylation are discussed and a minimal hypothesis for the mechanism of action of ATP synthase is presented. A preliminary report of part of this work has been presented previously.[18]

MATERIALS AND METHODS

The preparation of beef heart mitochondria (BHM) and heart submitochondrial particles (SMP), protein assay, ATPase assay, sources of chemicals and inhibitors have been described in previous papers.[12,16,17] Ubiquinone-1 (UQ_1) was a gift from Dr P. Jewess, Shell Research Ltd, Sittingbourne, UK

Enzymes

The following F_1F_0-ATPase or ATP synthase preparations were used in these studies.

ATP synthase

This was prepared by lysolecithin extraction of SMP.[19] The partially purified preparation

obtained prior to the final density gradient purification step was used; it was estimated to be 65–70% pure on the basis of its examination by polyacrylamide gel electrophoresis and the content of α and β subunits of F_1-ATPase. The F_1F_0-ATPase content was estimated to be ~1.0–1.2 nmol (mg protein)$^{-1}$.

Complex V

Complex V was prepared as described by Stiggall *et al.*[20] and was a gift from Dr Y. Hatefi. The preparation was estimated to be 70–75% pure (Y. Hatefi, personal communication) and was estimated to have a content of 1.1–1.3 nmol F_1F_0ATPase (mg protein)$^{-1}$.

F_1F_0ATPase

The highly purified preparation was a gift from Dr J. E. Walker and was estimated to have a content of 1.71–1.88 nmol F_1F_0-ATPase (mg protein)$^{-1}$ based on molecular masses ranging from 580 to 530 kDa.

Dibutyltin-3-hydroxy flavone, $Bu_2Sn(of)$

$Bu_2Sn(of)$ was prepared as described previously,[16,17] with the following minor variations. Equal volumes of a 10 mM ethanolic solution of dibutyltin bromide and a 10 mM ethanolic solution of 3-hydroxyflavone were mixed in a graduated flask and heated in the dark at 60 °C for 30 min. The mixture was allowed to stand in the dark overnight at room temperature and then made up to volume with ethanol. The resultant 5 mM solution of dibutyltin-3-hydroxyflavone bromide, $Bu_2Sn(of)$, was stable for at least two months when stored in the dark.

Alternatively, equimolar amounts of dibutyltin dibromide were added to hot (>60 °C) ethanolic solutions of 3-hydroxyflavone using an autopipette and small volumes of ethanol washings. After 10 min at 60 °C the mixture was allowed to stand in the dark at room temperature overnight and then made up to the appropriate volume with ethanol.

Using this method, 50 mM $Bu_2Sn(of)$ solutions can be prepared from which crystalline $Bu_2Sn(of)$ is readily obtained by slow evaporation on standing in the dark for several days. Rapid evaporation in a rotary evaporator and consequent formation of oils should be avoided as inactive dismutation products are formed. Dibutyltin-3-hydroxyflavone chloride can be made in similar fashion to the procedures outlined above using dibutyltin dichloride. The use of dibutyltin dichloride has significant cost advantages and no significant differences in enzymic or fluorescence titration properties have been noted in the use of the chloride or bromide derivatives of $Bu_2Sn(of)$. All preparative and handling procedures using organotins should be carried out in a fume cupboard with adequate glove and face-mask protection.

Fluorescence assays

Fluorescence assays were carried out at room temperature (18–20 °C) in a Perkin–Elmer LS-5 spectrofluorimeter: excitation, 400 nm; emission, 450 nm; 5 nm and 10 nm slit widths, respectively.[16,17] The standard incubation mixture contains 50 mM Hepes buffer, pH 7.4, 0.25 M sucrose, 0.5 mM EGTA (HSE buffer) plus variable amounts of mitochondrial protein and 0.5–1 μM $Bu_2Sn(of)$ in a final volume of 2.0 cm^3. $Bu_2Sn(of)$, UQ_1 and various inhibitors were added as small volumes of ethanolic solutions and the final ethanol concentration did not exceed 0.5% w/v. NADH, succinate, malonate and ascorbate–tetramethylphenylenediamine (ascorbate–TMPD) were added as small volumes of aqueous solutions.

Estimation of $Bu_2Sn(of)$ sites per mg protein

Scatchard analysis: fixed protein versus variable [$Bu_2Sn(of)$]

These experiments were carried out essentially as described by Chang and Penefsky for studies of aurovertin.[21,22] ΔF values (corrected for blank values) were determined by addition of variable amounts of $Bu_2Sn(of)$ (0–2 μM) to fixed amounts of mitochondrial protein (BHM, SMP, F_1F_0-ATPases). This was done in individual experiments and not by successive additions of $Bu_2Sn(of)$ to the same incubation mixture. The relationship between ΔF and [bound ligand] was determined from $1/\Delta F$ versus 1/[protein] plots as described.[21,22] The [free ligand] was determined by difference. Bound/free (B/F) versus Free (F) plots were utilized to determine the number of $Bu_2Sn(of)$ sites per mg protein (Fig. 1).

Direct plot: fixed [$Bu_2Sn(of)$] versus variable protein

ΔF values (corrected for blank values) were determined at fixed concentrations of $Bu_2Sn(of)$ and variable amounts of protein. The intersection points of ΔF values at limiting protein levels and

ΔF values at excess protein levels give values which approximate to the number of $Bu_2Sn(of)$ sites per mg protein (Fig. 1). These values are 10–15% higher than those obtained by Scatchard analysis. However, it is a simple reliable method which is internally consistent and does not rely on a separate determination of a fluorescence enhancement factor which may be a source of error.

Determination of FEΔF per mg protein

This method is a determination of the ΔF value at a fixed protein concentration in the presence of excess $Bu_2Sn(of)$; 2–3 μM. It gives a rapid assay of the number of $Bu_2Sn(of)$ binding sites expressed in ΔF units and it can be used to compare one membrane preparation with another, particularly in cell fractionation and membrane fractionation studies.[17] It should be used with caution when dealing with detergent-solubilized preparations where significant changes in the fluorescence enhancement factor can occur.

RESULTS

Previous fluorescence titration experiments[17,18] showed that the number of $Bu_2Sn(of)$ interaction sites per protein unit did not increase relative to the degree of purification of F_1F_0-ATPase from SMP. This indicates that there is a loss of $Bu_2Sn(of)$ interaction sites during the purification of F_1F_0-ATPase from SMP and that the number of $Bu_2Sn(of)$ interaction sites on F_1F_0-ATPase is variable. The number of $Bu_2Sn(of)$ interaction sites in membrane-bound and solubilized F_1F_0-ATPase has been further investigated.

Fluorescence titration studies on membrane-bound and solubilized F_1F_0-ATPases

Figure 1 shows titration studies (A, direct plot—fixed fluorophore versus variable protein; B, Scatchard plot—fixed protein versus variable fluorophore) with SMP and a highly purified F_1F_0-ATPase from which values of $Bu_2Sn(of)$ sites per mg protein and $Bu_2Sn(of)$ sites per mol F_1F_0-ATPase are determined. It should be noted that estimations of the number of $Bu_2Sn(of)$ sites per mol F_1F_0-ATPase require values for the content of F_1F_0-ATPase per mg protein which have not been directly determined; for example, values of 0.34–0.4 nmol F_1F_0-ATPase (mg protein)$^{-1}$ have been used for SMP based on studies by other

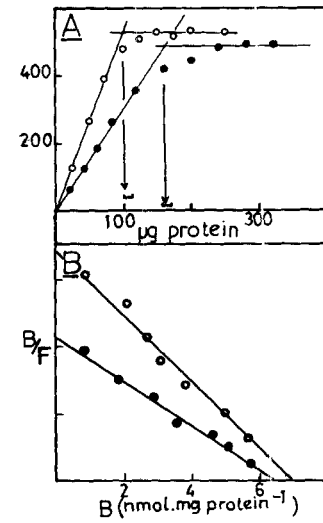

Figure 1 Fluorescence titration of SMP and F_1F_0-ATPase with $Bu_2Sn(of)$. Incubation conditions and fluorimetry are described in the Materials and Methods section.

(A) Direct plot (fixed fluorophore versus variable protein). To cuvettes containing variable amounts of protein in HSE buffer, pH 7.4, 1 nmol $Bu_2Sn(of)$ was added with efficient mixing. Total volume 2.0 cm^3. ΔF values were estimated after 10 min incubation after subtraction of suitable control blank values. Usually two $Bu_2Sn(of)$ concentrations were used, 0.5 μM and 0.25 μM. ○, F_1F_0-ATPase (purified); titration gives a value of 9.5 ± 0.2 nmol $Bu_2Sn(of)$ (mg protein)$^{-1}$, i.e. 5–5.5 mol $Bu_2Sn(of)$ (mol F_1F_0-ATPase)$^{-1}$ based on molecular masses of $(5.85–5.3) \times 10^5$ [1.71–1.88 nmol F_1F_0-ATPase (mg protein)$^{-1}$]. ●, SMP, titration gives a value of 6.25 ± 0.2 nmol $Bu_2Sn(of)$ (mg protein)$^{-1}$, i.e. approximately 15–19 mol (mol F_1F_0-ATPase)$^{-1}$ based on values of 0.34–0.4 nmol F_1F_0-ATPase (mg protein)$^{-1}$.[23]

(B) Scatchard plot (fixed protein versus variable fluorophore). The general methods used were based on the method of Chang and Penefsky.[21,22] Fluorescence enhancement values were determined from $1/\Delta F$ versus 1/protein plots[21,22] for the determination of bound fluorophore, and free fluorophore was determined by difference. Each experimental point was determined in separate incubation mixtures and not by successive additions of $Bu_2Sn(of)$ to the same cuvette. Fixed amounts of F_1F_0-ATPase (50 μg or 100 μg) were used for each range of $Bu_2Sn(of)$ concentrations. Ordinate units, $B/F \times 1/\mu M$. K_D values, 0.1–0.2 μM. ○, F_1F_0-ATPase purified; titration gives a value of 7 nmol $Bu_2Sn(of)$ (mg protein)$^{-1}$, i.e. approximately 3.7–4.1 mol $Bu_2Sn(of)$ (mol F_1F_0-ATPase)$^{-1}$ based on values of 1.71–1.88 nmol F_1F_0-ATPase (mg protein)$^{-1}$. ●, SMP; titration gives a value of ~6.5 nmol $Bu_2Sn(of)$ (mg protein)$^{-1}$, i.e. approximately 16–19 mol $Bu_2Sn(of)$ (mol F_1F_0-ATPase)$^{-1}$ based on values of 0.34–0.4 nmol F_1F_0-ATPase (mg protein)$^{-1}$.

Table 1 $Bu_2Sn(of)$ content of ATPase preparations

ATPase	$Bu_2Sn(of)$ sites content[a] [sites (mol F_1F_0-ATPase)$^{-1}$] Direct plot method (A)[b]	Scatchard plot method (B)[b]	F_1F_0-ATPase content[c] [nmol F_1F_0-ATPase (mg protein)$^{-1}$] (C)	$Bu_2Sn(of)$ sites content [FEΔF (mg protein)$^{-1}$] (D)
BHM	19–22 (3)[d]	18–20 (3)	0.2–0.22	1020 ± 80
SMP	15.5–18.5 (3)	16–19.5 (3)	0.34–0.4	2100 ± 100
ATP synthase	12.3–15 (1)	11–13 (1)	1.0–1.2	8600
Complex V	7.5–9.0 (2)	6–8 (2)	1.1–1.3	4860
F_1F_0-ATPase	5–5.6 (3)	3.5–4.4 (3)	1.71–1.88	5950

[a] Presented as a range of values due to the uncertainties in the values listed in column C. [b] Methods described in Fig. 1. [c] Not determined directly: accepted literature values, or values calculated as described in the Materials and Methods section. [d] Numbers in parentheses are the number of assays.

workers.[23] Similarly, values of 1.71–1.88 nmol F_1F_0-ATPase (mg protein)$^{-1}$ are utilized based on estimates of the molecular mass of the complex ranging from 580 kDa to 530 kDa. A range of calculated values with these limits is presented in Fig. 1, and from similar studies in Table 1.

Despite these uncertainties in the estimation of the content of F_1F_0-ATPase per mg protein, it is clearly demonstrated that the content of $Bu_2Sn(of)$ sites for SMP [$\sim 17.5 \pm 2$ sites (mol F_1F_0-ATPase)$^{-1}$] exceeds the 9–12 subunit *c* content of F_1F_0-ATPase[24] and that the value for purified F_1F_0-ATPase has declined to $\sim 4.5 \pm 1$ $Bu_2Sn(of)$ sites (mol F_1F_0-ATPase)$^{-1}$. Thus, in SMP, the average number of $Bu_2Sn(of)$ sites per mol F_1F_0-ATPase is over 45% (45–95%) greater than the 9–12 subunit *c* present per mol F_1F_0-ATPase. In contrast, in purified F_1F_0-ATPase the number of $Bu_2Sn(of)$ sites is less than 50% of the 9–12 subunit *c* present per mol F_1F_0-ATPase, assuming that there is no loss of subunit *c* content during the purification procedure. Thus over 60% (60–80%) of the $Bu_2Sn(of)$ interaction sites can be lost during the isolation of F_1F_0-ATPase from SMP.

Similar studies with heart mitochondria (BHM), ATP synthase[19] and Complex V[20] are summarized in Table 1 together with data for SMP and purified F_1F_0-ATPase for comparison. Fluorescence enhancement at maximal fluorophore concentration, FEΔF (mg protein)$^{-1}$,[17] increases as expected with SMP, as compared with BHM.[17] However, solubilized purified F_1F_0-ATPase preparations which are 5–6-fold purified as compared with SMP, and thus should have FEΔF (mg protein)$^{-1}$ values of 10 000–12 000, exhibit variable values which are all lower than expected. ATP synthase, the lysolecithin-extracted preparation described by Sanadi and co-workers,[19] is the only solubilized preparation which has FEΔF (mg protein)$^{-1}$ values which approach the expected values but the values for Complex V and purified F_1F_0-ATPase are markedly lower than expected.

Similar results are obtained on analysis of the values of $Bu_2Sn(of)$ sites per mol F_1F_0ATPase of different membrane-bound and solubilized F_1F_0-ATPase preparations (Table 1). The high values obtained for BHM (18–21) and SMP (15–19.5) decline in ATP synthase (11–15) to the low values obtained in Complex V (6–9) and the minimal values observed in purified F_1F_0-ATPase (3.5–5.6).

$Bu_2Sn(of)$ thus titrates a component of F_0 which is necessary for oxidative phosphorylation, F_1F_0-ATPase activity and associated proton translocation[17] and which is present in the mitochondrial inner membrane at 6–7 nmol (mg protein)$^{-1}$ (18 ± 3 times the content of F_1F_0-ATPase). However, near-maximal inhibition of ATPase activity in SMP is achieved by titration of approximately three $Bu_2Sn(of)$ sites per mol F_1F_0-ATPase (Fig. 2). It is proposed that the pool of $Bu_2Sn(of)$ sites serves as a cofactor pool which cycles through an F_0 interaction site (or sites) in a system which serves as an energy-transfer link between ATP synthase and redox components of the respiratory chain. The following experiments support the proposition that the reduction state of the mitochondrial inner membrane determines the interaction of $Bu_2Sn(of)$ with its interaction site and that this component is a redox component which is in redox equilibrium with the ubiquinone pool.

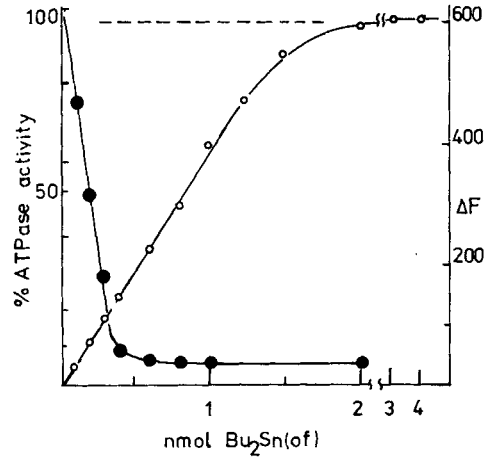

Figure 2 Titration of mitochondrial F_1F_0-ATPase by $Bu_2Sn(of)$. Experimental conditions are described in the Materials and Methods section. Aliquots (0.2–2 µl) of ethanolic solutions of 0.5 mM OR 1 mM $Bu_2Sn(of)$ were added to suspensions (0.33 mg SMP protein in 2.0 cm^3 HSE buffer). The ΔF values were recorded and blanks corrected for light scattering. Samples of each $Bu_2Sn(of)$-treated suspension were taken for ATPase assay as described in Refs 16, 17. The I_{50} value was 0.6–0.7 nmol (mg protein)$^{-1}$. 90% inhibition was 1.1–1.3 nmol (mg protein)$^{-1}$, i.e. 16–20% of the maximal fluorescence titration and approximately 2.8–3.6 $Bu_2Sn(of)$ sites per mol F_1F_0-ATPase. ○, ΔF (corrected values); ●, % ATPase activity [100% ATPase activity = 1.45 µmol min^{-1} (mg protein)$^{-1}$].

Interactions with F_0 inhibitors and redox interactions

The fluorescence enhancement observed on interaction of $Bu_2Sn(of)$ with F_0 indicates a specific interaction at a defined but unidentified component. Back-titration studies with other F_0 inhibitors[17,25] show that trialkyltins such as Bu_3SnCl back-titrate and compete for *all* the $Bu_2Sn(of)$ interaction sites with high efficiency. The macrolide inhibitor, venturicidin, is also a particularly effective competitor for the $Bu_2Sn(of)$ interaction site but titrates only about 65% of the total sites. In contrast, the macrolide inhibitor oligomycin is a poor competitor for the $Bu_2Sn(of)$ interaction site and normally titrates only 15–25% of $Bu_2Sn(of)$ sites, although maximal back-titration of up to 35% of the $Bu_2Sn(of)$ sites has been observed.[25]

These studies show that venturicidin and trialkyltins act a closely related site to the $Bu_2Sn(of)$ interaction site, whereas oligomycin probably acts at a different locus.[25] However, the oligomycin interaction site is sufficiently close to interact or modify the $Bu_2Sn(of)$ interaction detected by

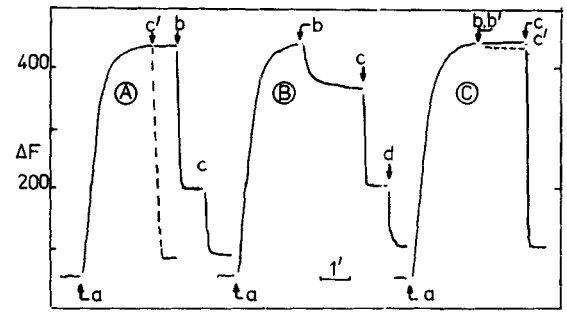

Figure 3 Effects of venturicidin, tributyltin (TBT), oligomycin and 8-methyl-lipoate (8-MLA) on $Bu_2Sn(of)$ interaction with SMP. Incubation conditions and assay components are described in the Materials and Methods section. Total volume, 2.0 cm^3; SMP, 0.2 mg protein. The following additions were made:

A a, 1 nmol $Bu_2Sn(of)$; b, 4 nmol venturicidin; c, 5 nmol TBT; c', 5 nmol TBT.

B a, 1 nmol $Bu_2Sn(of)$; b, 2 nmol oligomycin; c, 2 nmol venturicidin; d, 5 nmol TBT.

C a, 1 nmol $Bu_2Sn(of)$; b, 10 nmol 8-MLA; b', 10 nmol reduced 8-MLA; c,c', 5 nmol TBT.

fluorescence enhancement. In contrast, the F_0 inhibitor reduced 8-methyl-lipoate shows no evidence of interaction with the $Bu_2Sn(of)$ interaction site (Fig. 3). Reduced 8-methyl-lipoate may thus interact with a component of F_0 other than the well-established macrolide interaction sites on subunit *c* and subunit *b*.

Similar studies have been utilized to demonstrate that the $Bu_2Sn(of)$ interaction site responds to the redox status of the mitochondrial inner membrane. Previous studies[17] with freshly prepared heart and liver mitochondria and SMP failed to demonstrate any effects of substrate or respiratory chain inhibitors on fluorescence enhancement on binding of $Bu_2Sn(of)$. However after storage at −30 °C, studies of frozen–thawed BHM and SMP showed that the fluorescence yield was 20–30% lower than that obtained with the original preparations. It was found that storage at −30 °C of mitochondria and SMP under conditions that have been established for maintenance of oxidative phosphorylation (addition of succinate; succinate plus ATP) all protect mitochondria and SMP from the decline in fluorescence yield (Fig. 4). This indicates that maintenance of mitochondria in a *reduced* condition is a requirement for maximal fluorescence yield and that interaction with $Bu_2Sn(of)$ requires reduction of a membrane component.

Figure 4 shows that addition of succinate or NADH to frozen–thawed mitochondria leads to

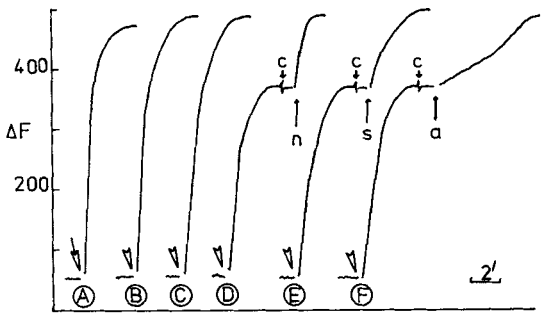

Figure 4 Effects of substrates on $Bu_2Sn(of)$ interaction. Experimental conditions are as described in the Materials and Methods section. Mitochondrial protein, 0.5 mg. The initial addition was 1 μl of 1 mM $Bu_2Sn(of)$.

A Fresh mitochondria.
B Fresh mitochondria plus 1 mM succinate.
C Frozen–thawed mitochondria (stored at −30 °C) plus 1 mM succinate.
D Frozen–thawed mitochondria (stored at −30 °C). Additions: c, 1 mM NaCN; n, 20 μM NADH.
E Frozen–thawed mitochondria. Additions: c, 1 mM NaCN; s, 1 mM succinate.
F Additions: c, 1 mM NaCN; a, 0.5 mM ascorbate–TMPD.

restoration of maximal fluorescence enhancement, particularly in the presence of antimycin or cyanide, maximal effects being obtained in the presence of cyanide. Figure 5 shows that similar effects are observed with SMP which had been stored at −30 °C and frozen and thawed at least three times. Restoration of maximal fluorescence

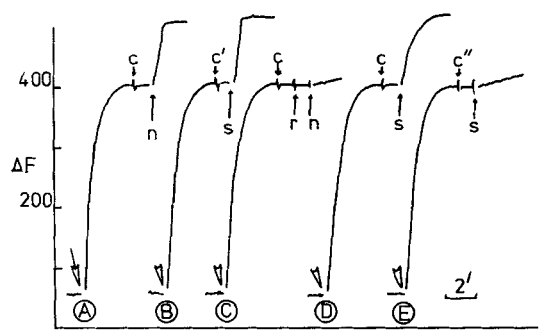

Figure 5 $Bu_2Sn(of)$ interactions: effects of substrates and inhibitors on frozen–thawed SMP. Experimental conditions were as described previously. Frozen–thawed SMP, 0.25 mg protein. The initial addition was 1 μl of 1 mM $Bu_2Sn(of)$. The following additions were then made:

A c, 1 mM NaCN; n, 20 μM NADH.
B c, 1 mM NaCN plus 1 μM rotenone or 1 μM piericidin; s, 1 mM succinate.
C c, 1 mM NaCN; r, 1 μM rotenone; n, 20 μM NADH.
D c, 1 mM NaCN; s, 100 μM succinate.
E c″, 1 mM NaCN plus 50 μM thenoyltrifluoracetone; s, 100 μM succinate.

by NADH was blocked by rotenone and by piericidin, inhibitors of NADH–UQ reductase activity. However, the restoration of maximal fluorescence by succinate was not blocked by these inhibitors but was markedly inhibited by 50 μM thenoyltrifluoroacetone. These experiments indicate that reduction of the ubiquinone pool in the inner membrane may be involved in restoration of maximal fluorescence enhancement.

These experiments permit the following conclusions:

(a) that the $Bu_2Sn(of)$ interaction site, a component of ATP synthase, is responsive to the redox status of the respiratory chain;
(b) that interaction with $Bu_2Sn(of)$ requires *reduction* of a component of the interaction site;
(c) that this component is in redox equilibrium with the ubiquinone pool of the respiratory chain as NADH–UQ reductase and succinate–UQ reductase activity restores maximal fluorescence enhancement.

Further evidence for an interaction with the ubiquinone pool is presented in experiments with added UQ_1, a short-chain analogue of UQ_{10} which has been shown to equilibrate rapidly with the inner membrane UQ_{10} pool.[26] Figure 6 shows that addition of UQ_1 to BHM or SHP blocked with cyanide or antimycin A back-titrates the $Bu_2Sn(of)$ fluorescence enhancement, presumably due to oxidation of the component which reacts with $Bu_2Sn(of)$. Similarly, preincubation of cyanide-blocked BHM or SMP with UQ_1 leads to a depressed fluorescence enhancement yield on addition of $Bu_2Sn(of)$. In both cases the maximum fluorescence yield is attained on addition of NADH in a rotenone- or piericidin-sensitive reaction or an addition of succinate in the presence or absence of these inhibitors (Fig. 6).

Equilibration of the $Bu_2Sn(of)$ interaction site with the ubiquinone pool is shown in Fig. 7, where UQ_1 additions are made to cyanide-blocked mitochondrial suspensions titrated with $Bu_2Sn(of)$ in the presence of either excess NADH or excess succinate. Under these conditions the ubiquinone pool is maintained continuously in a maximally reduced condition. Successive additions of UQ_1 lead to a rapid drop in fluorescence followed by a slow recovery phase in the presence of either NADH or succinate. In the presence of NADH plus rotenone, the rapid fluorescence drop (oxidation) on addition of UQ_1 is more extensive because of the total inhibition of the

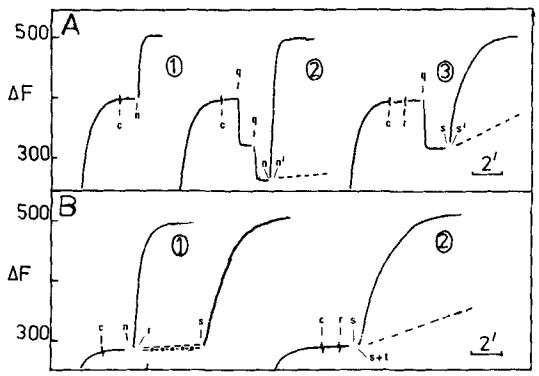

Figure 6 $Bu_2Sn(of)$ interactions: effects of UQ_1 and oxidation and reduction of the binding component. Experimental conditions are as described in Fig. 5. 0.25 mg Frozen–thawed SMP protein, 0.25 mg protein.

Experiment A: The traces and general procedure were similar to Fig. 4 but the lower 50% of the trace is not shown nor is the initial addition of 1 nmol $Bu_2Sn(of)$. UQ_1 was added as 1 μl of a 5 mM UQ_1 solution. The following additions were made:

1 c, 1 mM NaCN; n, 20 μM NADH.
2 c, 1 mM NaCN; q, 5 nmol UQ_1; n, 20 μM NADH; n', 1 μM rotenone plus 20 μM NADH (broken trace).
3 c, 1 mM NaCN; r, 1 μM rotenone; q, 5 nmol UQ_1; s, 0.5 mM succinate; s', 50 μM thenoyltrifluoroacetone plus 0.5 mM succinate (broken trace).

Experiment B: Here the UQ_1 (10 nmol) was preincubated in the incubation mixture for 1 min prior to the addition of 1 μl of 1 mM $Bu_2Sn(of)$. The lower 50% of the trace is not shown, nor are the initial additions of UQ_1 and $Bu_2Sn(of)$. Preincubation with UQ_1 led to a lower initial ΔF than in experiment A, due to oxidation of the binding component. The following additions were made:

1 c, 1 mM NaCN; n, 40 μM NADH; r, 1 μM rotenone (broken trace) *or* 1 μM piericidin A (dotted trace); s, 1 mM succinate.
2 c, 1 mM NaCN; r, 1 μM rotenone; s, 1 mM succinate; t, 50 μM thenoyltrifluoroacetone (broken trace).

reductive recovery phase (Fig. 7, trace 2). No effect of rotenone is seen in the presence of succinate (Fig. 7, trace 3), but thenoyltrifluoroacetone (50 μM) causes marked inhibition of the recovery phase in the presence of succinate. These experiments show that UQ_1 addition causes the rapid oxidation of a component monitored by $Bu_2Sn(of)$ followed by a slow reductive phase involving reduced ubiquinone generated by NADH–UQ reductase (Complex 1) or succinate–UQ reductase (Complex II). More surprising is the finding that the ascorbate–cytochrome *c* and ascorbate–TMPD redox couples also equilibrate with the $Bu_2Sn(of)$ interaction site. Figure 7, trace 5, shows the rapid oxidation by Q_1 and the

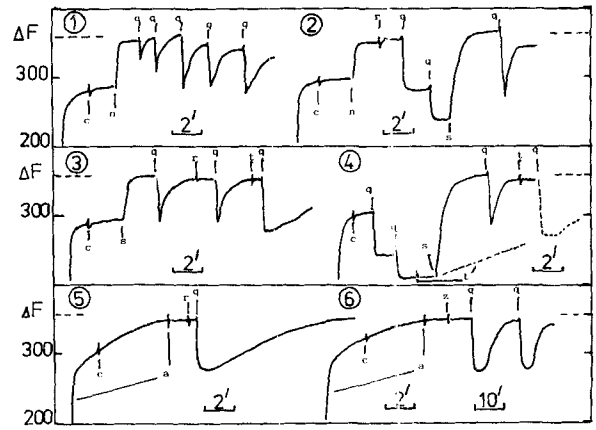

Figure 7 $Bu_2Sn(of)$ interactions: evidence for a redox cycle involving UQ_1 and the binding component (T-pool). The experimental system was as previously described in Fig. 6 but here the UQ_1 additions were made to a cyanide-blocked system *after* addition of substrate. Under these conditions there was continuous reduction of the Q-pool by NADH or succionate and continuous re-reduction of the $Bu_2Sn(of)$ binding component. SMP, 0.2 mg protein. The following additions were made:

1 c, 1 mM NaCN; n, 20 μM NADH; q, 10 nmol UQ_1.
2 r, 1 μM rotenone or 1 μM piericidin A; s, 1 mM succinate.
3 c, 1 mM NaCN; s, 1 mM succinate; q, 10 nmol UQ_1. r, 1 μM rotenone or 1 μM piericidin A; t, 50 μM thenoyltrifluoroacetone.
4 t, 50 μM thenoyltrifluoroacetone (broken trace only).
5 c, 1 mM NaCN; a, 1 mM ascorbate, 0.1 mM TMPB; r, rotenone; q, 10 nmol UQ_1.
6 z, 2 μg antimycin A.

very slow re-reduction by ascorbate or ascorbate–TMPD. This reaction is not sensitive to antimycin or uncoupling agents but its rate is only 15–20% of the rate observed in the presence of NADH or succinate.

DISCUSSION

This paper presents studies of a component of ATP synthase (F_1F_0-ATPase) which is monitored by a fluorescent probe inhibitor, $Bu_2Sn(of)$, and which is shown to be part of a pool of this component which is present in the heart mitochondrial inner membrane at levels similar to that of the ubiquinone pool. As these findings have implications for studies of the mechanism of oxidative phosphorylation it is appropriate at this stage to summarize the major fentures of the

evidence presented here and in previous studies.[17,18]

$Bu_2Sn(of)$ is an energy-transfer inhibitor which inhibits mitochondrial oxidative phosphorylation and F_1F_0-ATPase,[17,18] and the mitochondrial inner membrane component titrated by $Bu_2Sn(of)$ exhibits the following properties.

(a) It is present in the mitochondrial inner membrane at pool levels of 6–7 nmol (mg protein)$^{-1}$ [$\sim 18 \pm 3$ $Bu_2Sn(of)$ sites (mol F_1F_0-ATPase)$^{-1}$, but inhibition of F_1F_0-ATPase requires titration of only ~ 3 $Bu_2Sn(of)$ sites (mol F_1F_0-ATPase)$^{-1}$.[18] It is present in purified F_1F_0-ATPase preparations and in purified F_0.[17,18] One class of binding sites is observed (K_D, 0.1–0.2 μM), in contrast with previous studies with trialkyltins, discussed in Refs 9, 10.

(b) The majority of this component pool appears to be associated with the ATP synthase complex but up to 60–80% of this component is dissociable during the purification of ATP synthase.[18]

(c) The location or interaction site of this component on ATP synthase is closely related to the interaction site of venturicidin and the trialkyltins. Oligomycin appears to act at a different site, as does the other F_0 inhibitor, dihydro-8-methyl-lipoate.[14]

(d) The interaction site of $Bu_2Sn(of)$ is affected by the redox status of mitochondria and SMP. Interaction with $Bu_2Sn(of)$ occurs maximally under conditions where the redox components of the respiratory chain, including the ubiquinone pool, are maximally reduced and thus appears to involve the *reduced* form of the interaction site.

(e) The $Bu_2Sn(of)$ interaction site is in redox equilibrium with the ubiquinone pool as it can be rapidly oxidized by UQ_1 and then reduced by the action of NADH–UQ reductase and/or succinate–UQ reductase.

Thus this component has the properties of an energy-transfer component which can serve as a link between the ATP synthase complex and the respiratory chain and is an active component of ATP synthase. Its presence in the mitochondrial inner membrane at levels ~15–21-fold greater than that of F_1F_0-ATPase and at levels at least 50% greater than the levels of subunit *c* present in F_0 indicates a pool function for this component. The demonstration (Table 1) that the high numbers of $Bu_2Sn(of)$ sites per mol F_1F_0-ATPase observed in mitochondria and SMP decline in various F_1F_0-ATPase preparations to the minimal values observed in highly purified F_1F_0-ATPase indicates a variable dissociable component or cofactor of ATP synthase.

The minimum number of $Bu_2Sn(of)$ interaction sites required for an active ATP synthase complex is not known. The minimal values of 3.5–5.6 $Bu_2Sn(of)$ sites (mol F_1F_0ATPase)$^{-1}$ observed in highly purified F_1F_0ATPase (Fig. 1; Table 1) and the maximal inhibition of ATPase activity observed on titration of ~ 3 $Bu_2Sn(of)$ sites (mol F_1F_0-ATPase)$^{-1}$ (Ref. 18 and Fig. 2) point to values of 3–6 sites per mol F_1F_0-ATPase. However, back-titration studies with mitochondria and SMP show that ~65% of the $Bu_2Sn(of)$ interaction sites are back-titratable by venturicidin.[25] If interaction with venturicidin, a specific F_0 inhibitor, is taken as an index of specific interaction with ATP synthase, then an active functional ATP synthase may contain $\sim 12 \pm 2$ $Bu_2Sn(of)$ interaction sites.

In this context, it should be noted that these levels of $Bu_2Sn(of)$ sites are approached (Table 1) in the lysolecithin-extracted ATP synthase of Sanadi and co-workers[19] which exhibits high P_i–ATP exchange activity. These workers[27] attribute the differences in P_i–ATP exchange activity in ATP synthase (high), Complex V (low), and F_1F_0-ATPase (zero) to differences in the content of Factor B (F_B). It may not be fortuitous that the decline in P_i–ATP exchange activity and F_B content observed in these preparations[27] is paralleled by a decrease in $Bu_2Sn(of)$ interaction sites (Table 1). Also, as F_B is a dithiol protein[28] and thus a target site for organotins, there may be a causal relationship between the $Bu_2Sn(of)$ interaction sites and an enzymatic function of F_B.

These considerations may be of general applicability in studies of isolated ATP synthase and/or F_1F_0-ATPase. Many solubilized F_1F_0-ATPase preparations which are used for reconstitution studies in liposomes for H^+-gradient-driven ATP synthase may be deficient in important components such as F_B and the $Bu_2Sn(of)$-titratable component. In addition, the sensitivity to trialkyltins and venturicidin, a major index of the activity of this component, has not been tested in many of the experimental systems used for reconstitution of ATP synthase, but modified sensitivity to trialkyltins has been reported. Reconstituted ATP synthase preparations which do not exhibit the original sensitivity to trialkyltins, $Bu_2Sn(of)$ and venturicidin may thus exhibit activity indicative of

an incomplete or partial reaction system which is not wholly representative of the original membrane-bound enzyme.

These considerations also apply in studies involving bacterial mutants where DCCD-sensitive ATP-driven proton translocation is used as the definitive assay which is equated with ATP synthase. The assumption that proton-translocating ATPase is identical to ATP synthase may be incorrect.

Redox interactions of the $Bu_2Sn(of)$ interaction site

Figure 4 shows that the $Bu_2Sn(of)$ interaction site is responsive to the redox status of the inner membrane and that this assay (no added substrate, no cyanide block) can be utilized to evaluate the redox status of the $Bu_2Sn(of)$-titratable component. In freshly prepared mitochondria and SMP this component is over 95% reduced, presumably due to the presence of endogenous substrates. In frozen–thawed and −30 °C stored preparations this component becomes 20–25% oxidized but can be fully reduced by NADH in a rotenone- or piericidin-sensitive reaction or by succinate in a thenoyltrifluoroacetone-sensitive reaction. These experiments indicate the involvement of the ubiquinone pool and a reduction product of NADH–UQ reductase and/or succinate–UQ reductase (ubisemiquinone or ubiquinol) in the reductive regeneration reaction.

These conclusions are supported by the experiments with added UQ_1 (Fig. 6) which show rapid oxidation of the $Bu_2Sn(of)$-titratable component on addition of UQ_1 and its subsquent reduction by NADH and/or succinate. In similar experiments, ubiquinol (UQ_1H_2) is not active in restoring reduction of the component in mitochondria and SMP and in UQ-treated preparations (data not presented). This lack of activity of UQ_1H_2 is surprising but an enzymically generated ubisemiquinone species or a linked iron–sulphur complex may be the active reductant rather than ubiquinol.

The pathways of electron transport and proton transport between the $Bu_2Sn(of)$-titratable component and UQ in the oxido-reduction reactions described in Fig. 6 remain to be established. Nevertheless, it is clear that the oxidation of this component by UQ_1 is not affected by known respiratory-chain inhibitors (rotenone, piericidin, thenoyltrifluoroacetone, antimycin A). It remains to be established whether the observed inhibitions of re-reduction of the component by rotenone, piericidin and thenoyltrifluoroacetone reflect either the inhibition of the reduction of the Q-pool component which is involved in re-reduction of the $Bu_2Sn(of)$-titratable component *or* an as-yet undefined intrinsic property of NADH–UQ reductase and succinic–UQ reductase.

It is concluded that the $Bu_2Sn(of)$-titratable component, a component of ATP synthase, is a redox species which is in redox equilibrium with the ubiquinone pool (Q-pool) and that a reversible oxido-reduction cycle (Fig. 8) operates between the Q-pool and the pool of the $Bu_2Sn(of)$-titratable component (T-pool). This cycle does not appear to involve the known redox components of the respiratory chain except insofar as a reduced component of the Q-pool (ubiquinol, ubisemiquinone, associated iron–sulphur complexes) is required. The generation of this reduced Q-pool component represents the input point from the respiratory chain and accounts for the sensitivity to the NADH–UQ reductase inhibitors, rotenone and piericidin, and the succinic–UQ reductase inhibitor, thenoyltrifluoroacetone. The input point from ascorbate–TMPD in the presence of antimycin and the slow rate of re-reduction are less clear and require further investigation as to the possible role of the Q-pool. The experiments in Figs 6 and 7 indicate that input from NADH (site 1), succinate (site 2) and ascorbate–TMPD (site 3), i.e. all three 'energy conservation sites' of the respiratory chain, can lead to the reduction of the T-pool. However, the pathways of electron transfer and proton transfer have not been established. In this context, it should be noted that all of the complexes of the respiratory chain have apparently redundant protein components whose function in electron transfer and proton transfer is not known, especially with respect to 'reversal' reactions.

The nature of the $Bu_2Sn(of)$-titratable component (T-pool)

The $Bu_2Sn(of)$-titratable component (T-pool) has not been identified, so discussion on its role in ATP synthase must remain speculative, however, some conclusions can be drawn from the available evidence, as follows.

(a) $Bu_2Sn(of)$ is an energy-transfer inhibitor with little or no effect at 20 nmol $Bu_2Sn(of)$ $(mg\ protein)^{-1}$ on respiration or succinate oxi-

dation generated $\Delta\psi$.[17] Thus it is unlikely that it inhibits by direct interaction with ubiquinone.

(b) There is extensive evidence for a pool of thiols [>30 nmol (mg protein)$^{-1}$] in the mitochondrial inner membrane, part of which may be specifically associated with the mechanism of oxidative phosphorylation.[29–33] The experimental problem is to distinguish a pool of thiols which is specifically involved in the mechanism of oxidative phosphorylation from a much larger thiol pool which is present in the inner membrane. Interpretation of titration data, usually obtained by the Ellman method,[34] is difficult, as modification of a particular thiol pool by a chemical reagent or a change in physiological state which modifies its redox status may influence the redox status of another thiol pool which has little or no relevance to the mechanism of oxidative phosphorylation. Binding studies (Fig. 1B) indicate the presence of apparently only one class of binding site with K_d values in the range 0.1–0.2 μM. However, the presence of more than one binding species, in rapid equilibration, is not precluded by this analysis. The possible presence of more than one type of binding site is indicated in back-titration studies[25] with oligomycin, venturicidin and trialkyltins (Fig. 3).

(c) The high reactivity of dithiols with organotins and their known capacity to reverse organotin inhibition of ATPase and oxidative phosphorylation[13] make dithiol residues the preferred candidate for the Bu_2Sn(of) interaction site. However, specific site active thiol residues, thioester residues and novel meraptohistidine residues which have been demonstrated in chloroplasts[35] have not been excluded from consideration. The isolation, identification and characterization of the Bu_2Sn(of) interaction site (T-pool) is thus of prime importance as the presence of a redox component in ATP synthase, a presumptive dithiol component, has major implications for the mechanism of oxidative phosphorylation.

Implications for the mechanism of oxidative phosphorylation

The findings presented in this paper and summarized in Fig. 8 indicate a direct link between the respiratory chain and ATP synthase and they thus have implications for current studies of the mechanism of ATP synthase which are devoted exclusively to considering how an electrochemical gradient, $\Delta\mu_{H^+}$, drives ATP synthesis.[36] The demonstration of a redox cycle associated with ATP synthase and the presumptive involvement of a thiol component make possible a different formulation of the mechanism of F_0 induction of conformational changes in F_1-ATPase.

As a working hypothesis, it is proposed that the ATP synthase mechanism contains a cyclic reaction sequence whose function is to catalyse the required conformational changes in F_1 via dithiol–disulphide interchanges. The proton translocation events ($3H^+$/ATP) which are claimed to accompany ATP synthesis may thus represent proton transfers at individual reaction steps in the catalytic cycle carried out by the tripartite enzyme system. This aspect of the proposed hypothesis is similar to a mechanism proposed previously by Robillard and Konings[37] involving dithiol–disulphide interchanges and a proton-translocating dithiol–disulphide cycle. In addition, many features derived from current studies, especially the sites of action of inhibitors,[25] point up several similarities to the Q-cycle system associated with Complex III.[38] The linkage of the T-pool to the Q-pool demonstrated in this paper provides the reducing equivalents and driving force which are required for this reaction cycle. If a series of reactions analogous to the Q-cycle is operative (T-cycle), the system should contain a species which links different dithiol sites in similar fashion to the role of cytochromes b_H and b_L in linking UQ_i centres and UQ_0 centres. F_B may fulfil such a role but a non-haem iron or UQ–non-haem iron complex may also fulfil this role. Detailed analysis of the thiol, non-haem iron and ubiquinone content of various isolated ATP synthase preparations may be informative.

The overall reaction sequence thus involves membrane-bound enzyme reactions which are consistent with the random collision hypothesis[6] and with established reactions in protein chemistry and do not involve novel mechanisms to explain the transmutation of an electrochemical gradient into a conformation change as required in current concepts of ATP synthesis driven by $\Delta\mu_{H^+}$. Detailed information on the location of thiol residues in F_1 and F_0 segments of ATP synthase is now available[39] which can be utilized to examine the feasibility of thiol–disulphide interchanges as part of the reaction mechanism. Evidence for dithiol–disulphide interchanges is already available as a result of the extensive studies on CF_1. CF_0, mainly by McCarty and co-workers.[40]

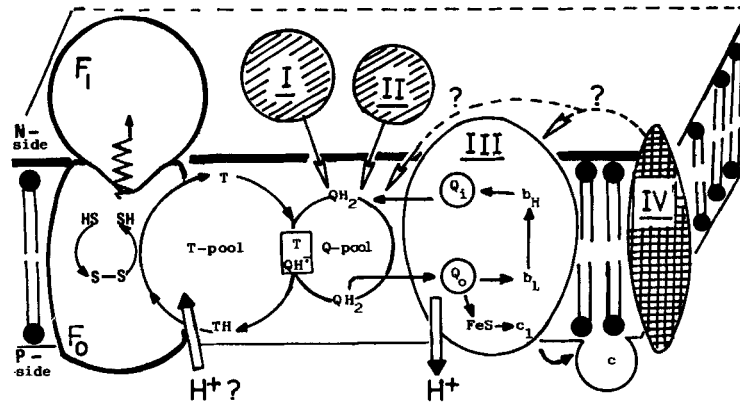

Figure 8 Interaction of the Q-pool with the $Bu_2Sn(of)$-titratable component (T-pool); the Q–T cycle. The diagram summarizes the data in Figs 5, 6 and 7 which show that the T-pool component, a component of ATP synthase F_0, is oxidized and reduced in a redox cycle involving the Q-pool. Reducing equivalents from the respiratory chain enter via the Q-pool, probably via a Q-cycle-generated ubisemiquinone species. A non-haem iron species is a possible candidate for interaction of ubisemiquinone with the T-pool component.

The interactions with F_0 are speculative. It is proposed that the unidentified T-pool component, a presumptive dithiol component, is involved in a series of thiol–disulphide interchanges leading to conformational changes in F_1.

The Q-cycle in complex III is shown as it provides a possible model for T_i and T_0 reaction centres in F_0 which are analogous to the Q_i and Q_0 centres of the Q-cycle. The different interaction centres for oligomycin and venturicidin[25] may be diagnostic of such reaction centres in F_0, analogous to the diagnostic use of antimycin A and myxothiazole in Complex III. The orientation of thiol–disulphide centres in the membrane is also a feature of the proton-translocating thiol–disulphide interchange mechanism of Robillard and Konings.[37] This mechanism also requires a species for redox interchange analogous to the role of cytochromes b_H and b_L. Factor B (F_B) may fulfil such a role but a non-haem iron species is a more likely possibility.

The working hypothesis outlined above which is based on current investigations provides a framework for further investigations utilizing $Bu_2Sn(of)$ and other organotin fluorophores. It also provides a framework for investigations of the control and regulation of oxidative phosphorylation. However, the isolation and characterization of the $Bu_2Sn(of)$ interaction site (T-pool) must remain a prime objective for further detailed studies of the proposed reaction mechanism.

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