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Dibutyltin-3-hydroxyflavone bromide: a fluorescent inhibitor of F_1F_0-ATPase

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Dibutyltin-3-hydroxyflavone bromide [Bu_2SnBr-(of)] is a fluorescent inhibitor (excitation max, 395 nm; emission max., 450 nm) of mitochondrial F_1F_0–ATPase which does not inhibit F_1–ATPase. Bu_2SnBr(of) binding to mitochondria and submitochondrial particles results in a 10-fold fluorescence enhancement which correlates with the amount of F_1F_0–ATPase in the inner membrane. Enhancement is not affected by respiratory-chain substrates, ATP, uncoupling agents, ionophores or respiratory-chain inhibitors. It is reversed by tributyltin chloride (Bu_3SnCl), indicating competition for a common triorganotin-binding site on the F_0 segment of F_1F_0–ATPase. Enhancement is not reversed by dialkyltins, monoalkyltins, tributyl-lead acetate, efrapeptin or oligomycin. Bu_2SnBr(of) is thus a new class of fluorescent probe of the F_0 segment of F_1F_0–ATPase which titrates F_0.

Keywords: Dibutyltin-3-hydroxyflavone, fluorescence probe, F_1F_0–ATPase, oligomycin, tributyltin-binding site, mitochondria

INTRODUCTION

The effects of organotin compounds on mitochondrial and chloroplast energetics have been widely investigated[1–3] as also have their effects as environmental hazards.[4,5]

A major mitochondrial site of action of the trialkyltins (R_3SnX) and the dialkyltins (R_2SnX_2) is the F_0 segment of the mitochondrial F_1F_0–ATPase complex[1,6] but their site(s) of action have not been defined.[7,8] A recent survey of various fluorescent organotins indicates that organotinflavone complexes have properties suitable for their use as fluorescence probe inhibitors of mitochondrial ATPase.[9] One of these compounds, dibutyltin-3-hydroxyflavone bromide [Bu_2SnBr(of)], has been investigated further and this paper describes studies of its use as a fluorescent probe of mitochondrial F_1F_0–ATPase and its effects on mitochondrial energetics. It is shown that Bu_2SnBr(of), a five-coordinate tin complex,[10] is a specific inhibitor which reacts at the F_0 segment of F_1F_0–ATPase and has useful properties as a fluorescent probe which titrates mitochondrial F_1F_0–ATPase.

MATERIALS AND METHODS

3-Hydroxyflavone (of) and dibutyltin dibromide (Bu_2SnBr_2) were purchased from Aldrich Chemical Co. (UK) and were used without further purification. The sources of other organotin compounds and reagents were as previously described,[6,8,9] as also were the preparations of partially purified F_1–ATPase, liver mitochondria, heart mitochondria, and liver and heart submitochondrial particles (SMP).[6,8,9] Mitochondria and SMP were stored at −25 °C in HSE buffer (10 mM-Hepes, 0.25 M-sucrose, 0.5 mM-EGTA, pH 7.4). The assays for mitochondrial ATPase, mitochondrial respiration at the oxygen electrode and oxidative phosphorylation were as described previously.[6,8,9] Inorganic phosphate was assayed as described in Ref. 11. Mitochondrial membrane potential ($\Delta\psi$) was determined by the fluorimetric method of Mewes and Rafael[12] as previously described[13] using 2-(4′-dimethylaminostyryl)-1-methylpyridinium iodide as a fluorescent indicator. Spectroscopic and fluorimetric methods are described in Ref. 9 and additional details are presented in the legends to figures.

Preparation of Bu_2SnBr(of)

Bu_2SnBr(of) was prepared by the following modification of the method of Blunden and Smith[10] by mixing ethanolic solutions of 3-hydroxyflavone and dibutyltin dibromide: equal volumes of hot (>60°) ethanolic solutions of 3-hydroxyflavone

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(10 mM) and Bu_2SnBr_2 (10 mM) were mixed and stirred in the dark overnight at room temperature. The reaction could be followed by spectrophotometry and spectrofluorimetry by removing small samples and diluting in ethanol. On mixing, the absorption peak of 3-hydroxyflavone at 340 nm, $E_M = 1.2 \times 10^4$, declined to $E_M = 4 \times 10^3$ and a new absorption peak at 395 nm appeared, $E_M = 8 \times 10^3$. Concomitantly the 3-hydroxyflavone fluorescence emission peak at 535 nm (excitation, 350 nm) declined and was replaced by a new emission peak at 450 nm (excitation, 395 nm). The reaction was essentially complete in 30 min.

The resulting 5 mM solution of $Bu_2SnBr(of)$ was used directly in all experiments and was stable for at least two weeks. This simple method gave a product with reproducible properties which could be made shortly before use. This procedure avoids any variation due to dismutation reactions when solvent is removed by rotary evaporation and the resulting oil is redissolved in ethanol. The identity of the product was confirmed by mass spectrometry.

Concentrated ethanolic solutions of $Bu_2SnBr(of)$ disperse poorly in aqueous buffers so they should be diluted with ethanol to the lowest operational concentration before dispersion into HSE buffer. Plastic cuvettes and pipette tips should be pre-wetted with HSE buffer to avoid adsorption effects.

$Bu_2SnBr(of)$ has been used in all the experiments described below but equivalent results are obtained with $Bu_2SnCl(of)$ prepared in a similar fashion.

Fluorescence properties of $Bu_2SnBr(of)$

Ethanolic solutions of $Bu_2SnBr(of)$ have marked fluorescence (excitation max., 395 nm; emission max., 450 nm), which decreases markedly in aqueous solutions with a small shift in the emission maximum to 445 nm. The fluorescence is polarity-dependent being 30 times greater in ethanol than in water. The fluorescence enhancement which is observed on binding of $Bu_2SnBr(of)$ to mitochondrial membranes (see below) thus appears to be due to binding to a specific apolar site in the mitochondrial inner membrane.

Safety and toxicology

All organotin compounds should be handled with care. Many are neurotoxic and immunotoxic and can be absorbed through the skin. Handling of liquid and solid samples should be carried out in a fume cupboard with adequate glove and face-mask protection.

RESULTS

Inhibition of ADP-stimulated respiration by $Bu_2SnBr(of)$

Tributyltin chloride (Bu_3SnCl) is known to inhibit ADP-stimulated respiration in coupled mitochondria with an apparent I_{50} value of 0.4 nmol (mg protein)$^{-1}$.[3] In similar experiments $Bu_2SnBr(of)$ inhibits ADP-stimulated respiration (state 3) in liver mitochondria with an I_{50} value of approximately 1.5–2 nmol (mg protein)$^{-1}$ and appears to titrate a mitochondrial component in similar fashion to Bu_3SnCl in studies reported by Sone and Hagihara.[3] $Bu_3SnBr(of)$ does not inhibit uncoupler-stimulated respiration with succinate or pyruvate/malate as substrates.

Inhibition of mitochondrial F_1F_0–ATPase and oxidative phosphorylation

$Bu_2SnBr(of)$ inhibits mitochondrial F_1F_0–ATPase in liver and heart mitochondria [I_{50} values 0.9–1.0 nmol (mg protein)$^{-1}$] and liver and heart submitochondrial particles (SMP) with I_{50} values in the range of 0.7–0.9 nmol (mg protein)$^{-1}$, values which are 20–25% lower than those obtained for Bu_3SnCl. Figure 1 shows the results obtained with liver SMP and liver mitochondria and also demonstrates that $Bu_2SnBr(of)$ does not inhibit partially purified F_1–ATPase[14] at levels up to 20 nmol (mg protein)$^{-1}$, indicating that the site of action is on the F_0 segment of F_1F_0–ATPase, similarly to that of Bu_3SnCl. Figure 1 also shows that $Bu_2SnBr(of)$ inhibits oxidative phosphorylation in liver mitochondria with glutamate/malate as substrate [I_{50} 1–1.2 nmol (mg protein)$^{-1}$]. This I_{50} value is similar to that obtained with F_1F_0–ATPase and does not show the marked difference in sensitivity observed with Bu_3SnCl in previous studies.[15]

Effects of $Bu_2SnBr(of)$ on mitochondrial $\Delta\psi$

Studies of mitochondrial $\Delta\psi$ were made by the fluorimetric assay of the distribution of 2-(4′-dimethylaminostyryl)-1-methylpyridinium iodide

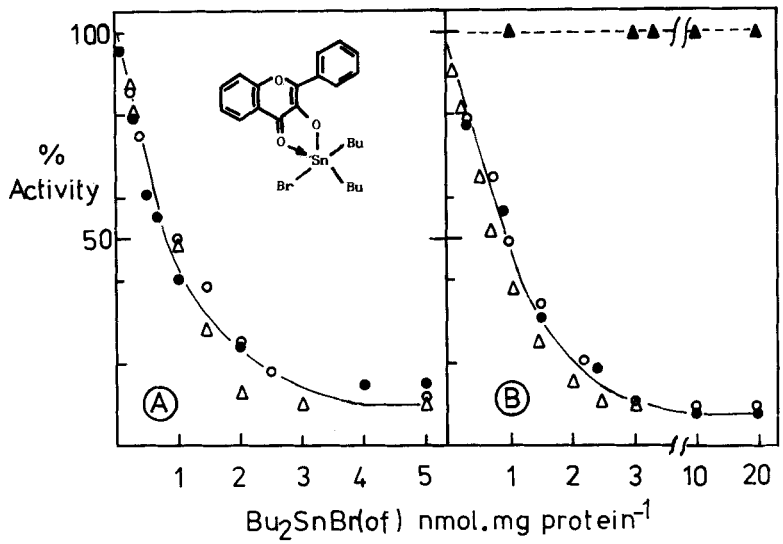

Figure 1 Inhibition of oxidative phosphorylation and ATPase by $Bu_2SnBr(of)$.

(A) Oxidative phosphorylation was assayed as described in Refs 13 and 15 in a glucose hexokinase trap system containing 250 mM-sucrose; 50 mM-Hepes–KOH, pH 7.4; 20 mM-glucose; 5 mM-potassium phosphate; 2 mM-$MgSO_4$; 2 mM-ADP; 0.5 mM-EDTA; 5 units of yeast hexokinase (Sigma type F 300); and 5 mM oxidizable substrate. ATP synthesis was measured as the disappearance of inorganic phosphate[11] in 20 min. temp., 25 °C. ○, Liver mitochondria (glutamate/malate), 100% = 120 nmol min^{-1} (mg protein^{-1}); ●, heart SMP (succinate), 100% = 94 nmol min^{-1} (mg protein)$^{-1}$; △, liver mitochondria (glutamate/malate), Bu_3SnCl used as inhibitor.
Insert: Structural formula of $Bu_2SnBr(of)$.

(B) ATPase was assayed as decribed in Refs 13 and 15 by the appearance of inorganic phosphate[11] in a system containing 250 mM-sucrose; 50 mM-Hepes–KOH, pH 7.4; 2 mM-$MgSO_4$ and 2 mM-ATP. ○, Liver mitochondria (+1 μM-CCCP), 100% = 130 nmol min^{-1} (mg protein); ●, liver SMP (+1 μM-CCCP), 100% = 810 nmol min^{-1} (mg protein); △, heart SMP (+1 μM-CCCP), 100% = 1420 min^{-1} (mg protein)$^{-1}$; ▲, Crude F_1-ATPase, 100% = 9.4 μmol min (mg protein)$^{-1}$.
Maximal inhibition of oxidative phosphorylation and ATPase was 85–90%. The remaining activity was inhibited by 5 μg cm^{-3} oligomycin.

(DASPMI).[12,13] Figure 2 shows that $Bu_2SnBr(of)$ causes a rapid decline in $\Delta\psi$ generated by MgATP due to inhibition of proton-translocating F_1F_0–ATPase [apparent I_{50} 2.5 nmol (mg protein)$^{-1}$]. In this assay it is markedly less effective than Bu_3SnCl and other F_0 proton channel inhibitors such as dicyclohexylcarbodiimide (DCCD) and oligomycin. $Bu_2SnBr(of)$ has little or no effect on $\Delta\psi$ generated by substrate oxidation at levels up to 20 nmol (mg protein)$^{-1}$. This is in marked contrast to Bu_3SnCl, which causes a marked decline in respiration-generated $\Delta\psi$ at levels greater than 3 nmol (mg protein)$^{-1}$ (Fig. 2A).

Fluorescence Studies

The effects of $Bu_2SnBr(of)$ on ATP-generated $\Delta\psi$, oxidative phosphorylation, ADP-stimulated respiration, mitochondrial F_1F_0–ATPase and the lack of inhibition of F_1–ATPase described above, all indicate that $Bu_2SnBr(of)$ interacts with the F_0 segment of F_1F_0–ATPase. The site of interaction is probably at the same site as the Bu_3SnCl interaction site on F_0, or at a related site. This conclusion is supported by fluorescence studies of the interaction.

Addition of $Bu_2SnBr(of)$ to mitochondria and submitochondrial particles (SMP) results in a

major increase in the fluorescence emission with a shift in the emission maximum from 450 nm to 445 nm. Binding of $Bu_2SnBr(of)$ to mitochondria and SMP results in an apparent 3-fold and a 6–7-fold fluorescence enhancement, respectively (Fig. 3A). However, the true fluorescence enhancement, after correction for light scattering, is approximately 10-fold for mitochondria and for SMP. Light scattering is maximal for mitochondria, markedly lower for SMP and minimal for solubilized F_1F_0–ATPase or F_0 preparations. Maximal fluorescence enhancement ($FE\Delta F$) is observed at 5 μM-$Bu_2SnBr(of)$, the concentration used in the following experiments (fig. 3).

Fluorescence enhancement (binding) is not affected by the energy state of the membrane generated by respiratory substrates or by ATP. In addition, uncoupling agents, ionophores and respiratory-chain inhibitors do not affect fluorescence enhancement, whether added after $Bu_2SnBr(of)$ or by preincubation before addition of $Bu_2SnBr(of)$ (Fig. 3A).

Figure 3(B) shows that addition of Bu_3SnCl (2:1 molar ratio) to $Bu_2SnBr(of)$ reversed the fluorescence enhancement obtained on addition of $Bu_2SnBr(of)$, indicating competition for a common binding site: 50% reversal by Bu_3SnCl was achieved at a molar ratio of 0.3:1 [$Bu_3SnCl/Bu_2SnBr(of)$] (Fig. 3B). Similar results were obtained with other R_3SnX compounds,

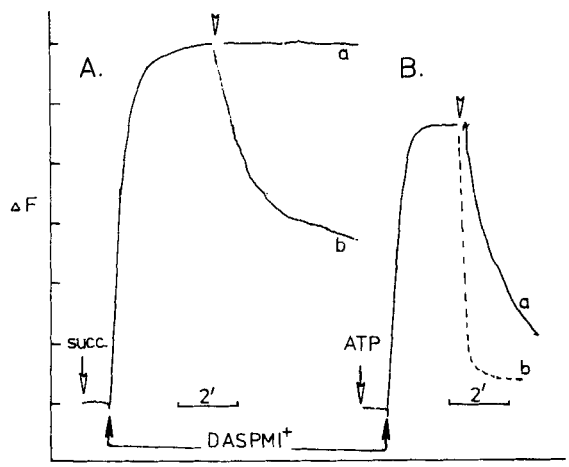

Figure 2 Effects of $Bu_2SnBr(of)$ on mitochondrial Δ. Mitochondrial $\Delta\psi$ was estimated as described in Refs 12 and 13. Conditions: 1 mg liver mitochondria; 1 μM-$DASPMI^+$; 1 μM-tetraphenylborate; 1 μM-rotenone; HSE buffer, pH 7.4. Total volume, 2.0 cm^3. Excitation, 480 nm; emission, 565 nm. Additions: (A) succinate, 5 mM final; a, 20 nmol $Bu_2SnBr(of)$; b, 3 nmol Bu_3SnCl. (B) ATP–Mg^{2+}, 5 mM final; a, 2.5 nmol $Bu_2SnBr(of)$; b, 2 nmol Bu_3SnCl.

(viz. Pr_3SnCl, Et_3SnCl, Me_3SnCl) but these compounds were markedly less effective than Bu_3SnCl, with molar ratios for 50% reversal of 2:1, 15:1 and 250:1, respectively. The effectiveness of reversal by R_3SnX compounds thus correlates with the I_{50} values for ATPase inhibition; i.e. $Bu_3SnCl < Pr_3SnCl < Et_3SnCl \ll Me_3SnCl$.[16] Figure 3(C) shows that preincubation with Bu_3SnCl at a 1:1 molar ratio and lower ratios was more effective than addition to membrane-bound $Bu_2SnBr(of)$, again indicating competition for a common binding site.

Reversal is specific for R_3SnX compounds, particularly Bu_3SnCl, as addition of Bu_2SnBr_2, $BuSnCl_3$ or Bu_3PbOAc to membrane-bound $Bu_2SnBr(of)$ at 2:1 molar ratios did not cause reversal. Similar negative results were obtained on addition of the F_0 inhibitor, oligomycin [5 μg (mg protein^{-1})], and the F_1–ATPase inhibitor, efrapeptin [2μg (mg protein^{-1})]; fig. 3B, trace d.

Titration of F_1F_0–ATPase by $Bu_2SnBr(of)$

Successive additions of $Bu_2SnBr(of)$ to mitochondrial membranes led to increases in fluorescence enhancement [$FE\Delta F$ (mg protein)$^{-1}$] until the binding site was titrated. Figure 4 shows that the differences in mitochondrial inner membrane content (cytochrome content and F_1F_0–ATPase content), which are seen in the electron microscope as the increases in number of cristae in liver mitochondria, kidney mitochondria and heart mitochondria,[17] were reflected in increasing titration values for fluorescence enhancement with $Bu_2SnBr(of)$. The increase in fluorescence enhancement in the series heart > kidney > liver correlates with the content of membrane-bound cytochromes (Fig. 4). The best correlation is seen with cytochrome *b* ($\Delta A_{560-575}$) and to a lesser extent with cytochrome aa_3 ($\Delta A_{605-575}$ and $\Delta A_{605-630}$). There is no correlation with the cytochrome *c* content, which probably reflects the variable degree of loss of cytochrome *c* during the isolation of liver, kidney and heart mitochondria. As cytochrome content is known to correlate with ATPase complex content, fluorescence enhancement on addition of $Bu_2SnBr(of)$ would appear to titrate the mitochondrial F_1F_0–ATPase.

Similar results have been obtained in studies with heart mitochondria and derived submitochondrial particles (SMP) which are inner membrane vesicles containing the mitochondrial membrane-bound cytochromes and ATPase complex. Similar titrations of heart mitochondria and

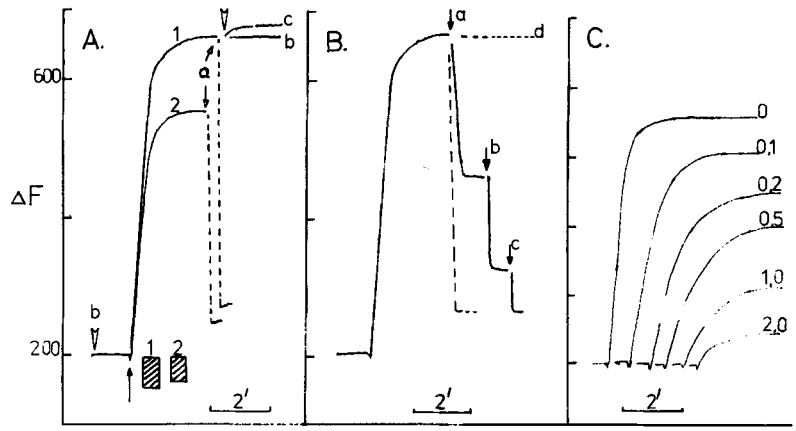

Figure 3 Fluorescence enhancement (binding) of $Bu_2SnBr(of)$.

(A) To 1 mg liver mitochondria in HSE buffer was added 1 or 2 µl of 5 mM-$Bu_2SnBr(of)$. Fluorescence was measured in a Perkin–Elmer LS5 spectrofluorimeter at room temp. (18–20°C). Excitation, 395 nm; emission, 450 nm. The hatched area indicates fluorescence due to $Bu_2SnBr(of)$ before addition of mitochondria. Total volume, 2.0 cm^3. Additions: 1, 10 nmol $Bu_2SnBr(of)$; 2, 5 nmol $Bu_2SnBr(of)$; (a) 20 nmol Bu_3SnCl; (b) glutamate/malate *or* succinate *or* ATP; rotenone *or* antimycin A; CCCP *or* gramicidin *or* valinomycin–K^+ added before or after $Bu_2SnBr(of)$; (c) 20 nmol Bu_2SnBr_2.

(B) Conditions as in A. Additions: (a) 3 nmol Bu_3SnCl; (b) 6 nmol Bu_3Sn Cl; (c) 6 nmol Bu_3SnCl.

(C) Conditions as in A. 5 nmol $Bu_2SnBr(of)$ added; 0 indicates no addition; 0.1–2.0 indicates preincubation with 0.1–2.0 nmol Bu_3SnCl for 2 min before addition of $Bu_2SnBr(of)$. $\Delta F1$, initial baseline reading; $\Delta F2$, final reading after addition of $Bu_2SnBr(of)$; $FE\Delta F = \Delta F2 - \Delta F1$. With 1 mg mitochondrial protein, the initial fluorescence reading ($\Delta F1$) is approximately 30% of the total fluorescence ($\Delta F2$) and is due to light scattering.

SMP with $Bu_2SnBr(of)$ showed that the fluorescence enhancement [$FE\Delta F$ (mg protein)$^{-1}$] obtained with heart SMP was approximately two-fold higher than that obtained with heart mitochondria. Table 1 shows that there is a parallel increase in membrane-bound cytochromes and a parallel increase in oligomycin-sensitive ATPase activity. Fluorescence enhancement [$FE\Delta F$ (mg protein)$^{-1}$] thus titrates a component of the mitochondrial inner membrane which appears to be a component of the F_0 segment of the F_1F_0–ATPase complex.

Location and nature of the $Bu_2SnBr(of)$ binding site

The interaction of $Bu_2SnBr(of)$ with mitochondrial inner membrane is of high affinity with an apparent $K_d < 1 \times 10^{-7}$. Very little bound $Bu_2SnBr(of)$ is removed by washing mitochondria or SMP by centrifugation and resuspension. Thus tight binding to an apolar site on the inner membrane, probably F_0, is indicated.

There is no information on the location of the interaction site of $Bu_2SnBr(of)$ with individual components of F_0 at present, although Factor B has been suggested as a possible interaction site for trialkyltins.[20] Identification of the binding components will require the preparation of radioactive $Bu_2SnBr(of)$ labelled in the n-butyl and/or the flavone moiety. However, in preliminary studies of the binding of $Bu_2SnBr(of)$-labelled mitochondrial membranes, the following observations have been made.

(1) During preparation of F_1-ATPase from $Bu_2SnBr(of)$-labelled particles by the chloroform extraction method,[14] little or no $Bu_2SnBr(of)$ was found in the F_1-ATPase-containing supernatant or the lipid-containing chloroform infranatant. almost all the fluorescent label is associated with the membrane protein located at the interface which contains F_0 and the cytochromes of the respiratory chain.

(2) The site for $Bu_2SnBr(of)$ binding survived treatment with mild detergents during the iso-

lation of purified F_1F_0–ATPase or F_0 complexes from liver mitochondria,[18] complex V from heart mitochondria[19] and lysolecithin-extracted ATP synthase from heart mitochondria.[20] As expected, the fluorescence enhancement ratios [FEΔF (mg protein)$^{-1}$] observed are in the order ATPase complex > SMP > mitochondria, and the ratio SMP/mitochondria correlates with cytochrome and ATPase content (Table 1). However, the observed fluorescence enhancement ratio for the isolated complexes[18–20] is 10–20% lower than expected for the degree of purification of these F-ATPase complexes. In addition, there are significant decreases in the response to addition of Bu_3SnCl (displacement) as compared with the membrane-bound ATPase complex.

This suggests that a structural feature of F_1F_0–ATPase, forming the trialkyltin-binding site, is modified during the fractionation procedure. Alternatively, variable amounts of a trialkyltin-binding component may be lost during the isolation procedure. In this context it is worthy of note that it is the lysolecithin-extracted ATP synthase complex containing Factor B[20] which shows the lowest degree of modification with respect to Bu_2SnBr(of) fluorescence properties on binding.

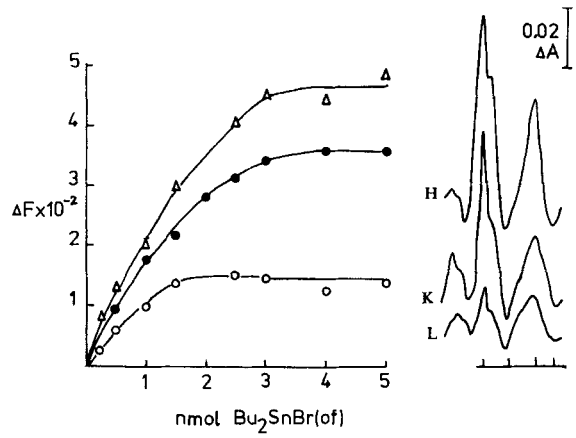

Figure 4 Titration of liver, kidney and heart mitochondria by Bu_2SnBr(of).

Mitochondria were prepared from homogenates of livers, kidneys and hearts from two rats. Titration was by additions of 1 µl of ethanolic 2 mM-Bu_2SnBr(of) solutions to mitochondria (0.55 mg protein) suspended in HSE buffer, pH 7.4. Total volume, 2.0 cm^3. Fluorescence estimation: excitation, 395 nm; emission, 450nm. The fluoresence emission values have been corrected for light scattering by mitochondria. Maximal ΔF is achieved at 4–5 nmol Bu_2SnBr(of). ○, Liver; ●, kidney; △, heart. The cytochrome spectra are difference spectra of 5.5 mg cm^{-3} mitochondrial protein cm^{-3}, dithionite reduced *minus* oxidized) solubilized with 0.5% w/v deoxycholate of liver, kidney and heart mitochondria. Wavelength markers, left to right: 560 nm, 575 nm, 605 nm, 630 nm. Spectra: L, liver; K = kidney; H = heart.

(3) The development of a simple method for the isolation of the F_0 proton channel from liver mitochondria by McEnery *et al.*[18] has made it possible to correlate directly the Bu_2SnBr(of) binding site with F_0.

Table 2 shows a specific increase in binding sites, as estimated by FEΔF (mg protein)$^{-1}$, during purification of F_0. Initial values of FEΔF in inner-membrane vesicles increase 3–4-fold after guanidine extraction of washed inner-membrane vesicles. The guanidine extract does not contain any component which binds Bu_2SnBr(of) leading to fluorescence enhancement. CHAPS extraction of the guanidine-extracted membranes leads to a further 2–3-fold increase in FEΔF (mg protein)$^{-1}$ in the purified F_0 preparation. The FEΔF data shown in Table 1 correlate well with the data shown in Table 1 of McEnery *et al.*[18] and firmly establish the location of the Bu_2SnBr(of) binding site in F_0.

The good correlation with the degree of purification of F_0, FEΔF values being within 85–90% of the expected values, indicates only minor modification of the organotin-binding site during the isolation procedure. However, the displacement response to addition of equimolar Bu_3SnCl is 30–40% lower as compared with membrane-bound F_0. This indicates a degree of modification of the binding site and this parameter and others under investigation illustrate the utility and versatility of this reagent in studies of F_0.

DISCUSSION

Bu_2SnBr(of) is a new type of fluorescent probe for the study of the F_0 segment of the F_1F_0–ATPase complex in mitochondrial membranes and isolated ATPase complexes. Inhibition of ADP-stimulated respiration, mitochondrial F_1F_0–ATPase, oxidative phosphorylation and the non-inhibition of F_1–ATPase all categorize Bu_2SnBr(of) as an F_0 inhibitor which probably acts at the same site as tributyltin and other trialkyltins. This conclusion is supported by

Table 1 Titration of F_1F_0-ATPase in mitochondria and SMP

Ox heart mitochondria (0.32 mg protein) and heart SMP (0.3 mg protein) were titrated with 5 mM-Bu_2SnBr(of) and the maximum fluorescence enhancement was obtained at 5 μM-Bu_2SnBr(of). Excitation, 395 nm; emission, 450 nm. HSE buffer, pH 7.4. Total volume, 2.0 cm^3. FEΔF was determined by subtraction of the initial baseline reading (ΔF_1) from the maximal fluorescence (ΔF_2) obtained after addition of 5 μM-Bu_2SnBr(of).

Difference spectra of cytochromes: 3.3 mg protein cm^{-3}; dithionite reduced *minus* oxidized. Solubilized with 0.5% w/v deoxycholate. Cyt *b*, $\Delta A_{560-575}$; Cyt aa_3, $\Delta A_{605-575}$; Cyt aa_3, $\Delta A_{605-630}$.

ATPase activity: Determined as in Refs 13 and 15 using the deoxycholate-solubilized preparations. 1 μM CCCP present.

	(*a*) Mitochondria	(*b*) SMP	*b*/*a*
FEΔF (mg protein^{-1})	1032 ± 34 (3)	1960 ± 25 (3)	1.90
Cyt. *b*, $\Delta A_{560-575}$ mg protein^{-1})	0.0135 ± 0.001 (3)	0.0265 ± 0.001 (3)	1.96
Cyt aa_3, $\Delta A_{605-575}$ (mg protein^{-1})	0.0106 ± 0.001 (3)	0.0204 ± 0.001 (3)	1.92
$\Delta A_{605-630}$ (mg protein^{-1})	0.0106 ± 0.001 (3)	0.023 ± 0.001 (3)	2.16
ATPase, nmol min^{-1} mg protein^{-1})	405 ± 25 (3)	903 ± 35 (3)	2.23

Table 2 Purification of F_0 from rat liver mitochondria

Membranes and fractions were prepared as described by McEnery *et al.*[18] and summarized below.

Fraction 1 (inner membrane vesicles) was prepared from rat liver mitochondria by use of digitonin.

Fraction 2 (3 × membranes) was prepared from Fraction 1 by washing three times with an extraction buffer to remove peripheral proteins from the inner membrane; 30–40% of the total protein is extracted.

Fraction 3 (3 × G membrane) was prepared from Fraction 2 by washing three times with buffered guanidine solution which extracts F_1-ATPase and other tightly bound peripheral proteins.

Fraction 4 (3 × G extract) is the guanidine-extracted F_1-ATPase and other peripheral proteins.

Fraction 5 (CHAPS extract) was obtained by extraction of Fraction 3 with the zwitterionic detergent 3-[(3-cholamidopropyl) dimethylammonio-1-propanesulphonate (CHAPS)]. The CHAPS extract is the pure preparation of F_0 described by McEnery *et al.*[18] The F_0 preparation showed a similar gel electrophoresis pattern to that described by McEnery *et al.*[18]

Each fraction was assayed under standard conditions to determine FEΔF (mg protein^{-1}). Protein is dispersed in HSE buffer, pH 7.4, 2 μl 10 mM-Bu_2SnBr(of) added and fluorescence enhancement, FEΔF (excitation 395 nm; emission 450 nm) determined. Total volume 2.0 cm^3.

Fraction	Protein in assay (μg)	FEΔF (mg protein^{-1})	Purification	
1. Inner membrane vesicles	300	1740	1	(1)
2. 3 × membranes	ND[a]	ND	ND	(1.4)
3. 3 × G membrane	40	6450	3.7	(3.2)
4. 3 × G extract	90	0	0	0
5. F_0 (CHAPS extract)	30	16 700	9.5	(10.4)

[a] ND, not determined. Values in parentheses are estimated from Table 1 in Ref. 18.

fluorescence probe studies which show that Bu_3SnCl displaces $Bu_2SnBr(of)$ from a binding site which is probably a common binding site for R_3SnX compounds. Displacement is specific and other tin compounds (Bu_2SnBr_2, $BuSnCl_3$, Bu_3Sn–imidazole and Bu_3PbAc) do not displace $Bu_2SnBr(of)$, indicating competition for a common triorganotin-binding site.

The fluorescence enhancement on binding indicates that a specific apolar binding site is involved and that it is correlated with the amount of cytochromes and number of cristae[17] in mitochondria (Fig. 4). As this also correlates with the amount of F_1F_0–ATPase, $Bu_2SnBr(of)$ can be used to titrate the F_0 segment in mitochondrial membranes. Specific displacement by Bu_3SnCl is a further parameter which can be used to define Bu_3SnCl binding in mitochondria. Detailed studies of these interactions by stopped-flow fluorimetry are necessary to evaluate binding constants and to evaluate the number of binding sites involved.

These properties can be used as a simple method to study organotin-sensitive sites in other F–ATPases, e.g. from *E. coli*, yeasts, mammalian mitochondria and plant mitochondria. Potential uses are in the titration of F_0 in yeast mitochondria from wild-type (*rho*$^+$), petite (*rho*$^-$) and *mit*$^-$ mutants. Other potential uses are in studies of mammalian mitochondrial mutations,[21] many of which are associated with human neurodegenerative disease states.

The titration properties of $Bu_2SnBr(of)$ can be applied to studies of the organotin-binding site in isolated F–ATPases (D. E. Griffiths, unpublished). The sensitivity to organotins is markedly modified in some isolated F–ATPases as compared with the membrane-bound enzyme complex. This may be due to loss of a component which binds Bu_3SnCl during the preparation of many types of F–ATPase complexes. This point has been addressed by Sanadi and co-workers[20] with respect to the presence or absence of Factor B and they infer that the majority of isolated F–ATPase preparations are not representative of the structure and function of ATP synthase. The use of $Bu_2SnBr(of)$ as a fluorescent probe of F–ATPases and ATP synthase preparations may thus provide an experimental system for the role of Factor B in oxidative phosphorylation.

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