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The F_1F_0-ATPase binding site of dibutyltin-3-hydroxyflavone: interactions with venturicidin, oligomycin and DCCD

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Dibutyltin-3-hydroxyflavone bromide, $Bu_2Sn(of)$, is a fluorescent probe inhibitor of mitochondrial F_1F_0ATPase which reacts with and titrates a component of F_0 with marked fluorescence enhancement and reacts similarly with chloroplast CF_1CF_0 and V-ATPases. Its use to monitor the interactions of other F_0 inhibitors (venturicidin, oligomycin, DCCD) with F_1F_0ATPase, both membrane-bound and purified by solubilization is described. Trialkyltins (Bu_3SnCl) back-titrate *all* $Bu_2Sn(of)$ interaction sites; whereas the macrolide inhibitor venturicidin backtitrates 60±5% and oligomycin only 30±3% of $Bu_2Sn(of)$ interaction sites. Bafilomycin, the macrolide inhibitor of V-ATPases, is inactive in this assay. DCCD acts in a different fashion from the other inhibitors. Current and potential applications of this fluorescent probe in mitochondrial bioenergetics and biogenesis are discussed.

Keywords: Dibutyltin-3-hydroxyflavone, fluorescence probe, F_1F_0ATPase, tributyltin, venturicidin, oligomycin, DCCD, VEN^R-TET^R mutants

INTRODUCTION

Dibutyltin-3-hydroxyflavone bromide, $Bu_2Sn(of)$, is a fluorescent probe inhibitor of mitochondrial F_1F_0ATPase which reacts with and titrates a component of F_0 with marked fluorescence enhancement.[1,2] It reacts similarly with chloroplast CF_1CF_0[3] and with V-ATPases.[4] The interaction site in mitochondria appears to be identical to the trialkyltin binding site on F_0 as trialkyltins (R_3SnX) appear to displace $Bu_2Sn(of)$ selectively with reversal of fluorescence enhancement.[1,2] This paper shows that it is also a novel fluorescent probe of F_0 which can be used for titration studies and to monitor the interactions of other F_0 inhibitors (venturicidin, oligomycin, dicyclohexylcarbodiimide (DCCD)) with F_1F_0ATPase, both membrane-bound and solubilized, by simple fluorescence assays.

Previous work on F_0 inhibitor interaction has relied on studies of modification of the labelling of subunit c by ^{14}C-DCCD, and kinetic studies are technically difficult. Effects on DCCD labelling of subunit c by oligomycin, venturicidin and dibutyl chloromethyltin (DBCT) have been reported,[5] and similar studies have been published for DBCT[6] and for venturicidin.[7] In contrast, equilibrium binding studies[8] using radioactive triethyltin have shown that oligomycin and venturicidin do not affect high-affinity binding of triethyltin whereas this site is competed for by other trialkyltin compounds, thus providing support for biochemical genetic studies[9,10] which have indicated that oligomycin, venturicidin and trialkyltins may have separate interaction sites.

In this paper it is shown that trialkyltins (R_3SnX) and venturicidin (VEN) interact rapidly and markedly with the $Bu_2Sn(of)$ binding site at maximal and submaximal fluorophore concentrations. In addition, it is shown that both oligomycin and DCCD can modify the interaction of mitochondrial membranes with $Bu_2Sn(of)$ under appropriate preincubation conditions and fluorophore concentrations, but appear to act in different fashions.

These effects are observed in mitochondria and submitochondrial particles and also in solubilized F_1F_0ATPase preparations with varying degrees of effectiveness. The results are discussed in terms of (a) venturicidin–trialkyltin binding-site interactions previously inferred from studies of yeast VEN^RTET^R mutants;[11] (b) potential applications in studies of the composition and mechanism of F_1F_0ATPase, ATPase F_0 mutants and F_1F_0ATPase biogenesis.

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MATERIALS AND METHODS

The preparation of heart mitochondria (BHM), submitochondrial particles (SMP), the sources of reagents and protein estimation were described previously.[1,2] Bafilomycin A_1 was obtained from Dr K Altendorf, University of Osnabrück. BHM and SMP were suspended in 50 mM Hepes, 0.25 M sucrose, 0.5 mM EGTA, pH 7.4, (HSE buffer) and stored frozen at −30 °C. Additional details are given in the legends to figures.

Dibutyltin-3-hydroxyflavone bromide, $Bu_2Sn(of)$, was prepared as described previously[2] and stored in the dark as a 5mM ethanolic solution. Dilutions were made in ethanol prior to use and protected from light. The conditions for fluorescence studies were as described previously.[1,2] $Bu_2Sn(of)$ and inhibitors were added as ethanolic solutions and control experiments showed that ethanol (≤0.5%, v/v) had no effect on the assay. All experiments were at room temperature (18–20 °C). $FE\Delta F$. mg protein^{-1} values, an index of $Bu_2Sn(of)$ binding site content, were determined as in Ref. 2, using excess $Bu_2Sn(of)$.

ATPsynthase was prepared as described by Sanadi and co-workers[12] by extraction with lysolecithin. The preparation was used prior to the sucrose-gradient purification step and had an $FE\Delta F$. mg protein^{-1} value of 8600. Complex V from beef heart mitochondria[13] was a gift from Dr Youssef Hatefi and was the ammonium sulphate precipitate prior to the final chromatography step. This preparation was assumed to be 60% pure, based on the data in Ref. 13 and had an $FE\Delta F$. mg protein^{-1} value of 4200. A highly purified sample of F_1F_0ATPase obtained from Dr J. E. Walker had an $FE\Delta F$. mg protein^{-1} value of 5850. Under our experimental and instrumental conditions, BHM and SMP have $FE\Delta F$. mg protein^{-1} values of 1100 ± 15% and 2000 ± 10%, respectively.[2] Thus, purified F_1F_0ATPases, which are 5–6-fold purified over SMP, should have $FE\Delta F$. mg protein^{-1} values of 10 000–12 000. There is a variable loss of $Bu_2Sn(of)$ binding sites during preparative procedures for F_1F_0ATPase (D.E. Griffiths, unpublished work).

RESULTS

In previous studies[2] with liver and heart mitochondria using supramaximal levels of the fluorophore, 3–5 μM $Bu_2Sn(of)$ was back-titrated by equimolar trialkyltins and triaryltins whose ATPase I_{50} values were similar to $Bu_2Sn(of)$, and titration was markedly enhanced by preincubation. In subsequent studies, venturicidin also showed a marked effect and the maximal back-titration with venturicidin at high fluorophore concentrations was approximately 50–60% of that observed with trialkyltins (data not shown). In contrast, at these excessively high fluorophore levels, oligomycin and DCCD had no effect.

Further investigation showed that the effects of other F_0 inhibitors, especially oligomycin and DCCD, were dependent on fluorophore concentration, were readily observable at submaximal fluorophore concentrations (0.25–1 μM) and could be enhanced by preincubation.

Titration studies with R_3SnX, venturicidin and oligomycin

Figure 1 shows (a) back-titration and (b) preincubation/titration experiments with 0.32 mg beef heart mitochondria at 0.5 μM $Bu_2Sn(of)$, which gave 85–90% of maximal fluorescence enhancement. Figure 1A(a) shows a typical back-titration by tributyltin (Bu_3SnX), which restores the fluorescence to close to baseline levels (~100% back titration). Venturicidin (4 nmol) also readily back-titrates the $Bu_2Sn(of)$ fluorescence enhancement to 60 ± 5% of the level obtained with excess Bu_3SnX [Figure 1B(a)], the remainder being titratable by Bu_3SnX. Significant effects are observed at 0.13 nmol venturicidin, indicating again a high affinity for the $Bu_2Sn(of)$ site.

Oligomycin shows slower small-scale changes in back-titration experiments [Fig. 1C(a)] and is slightly less effective than venturicidin, although it appears to be as effective as venturicidin in preincubation/titration experiments (Fig. 1C(b)]. The final changes at high oligomycin concentrations with BHM are less than 40% of the changes seen with venturicidin, although they can be as high as 50%. Detailed titration studies of R_3SnX, venturicidin and oligomycin interactions can thus provide information on the relative binding affinities of these F_0 inhibitors in different types of F_1F_0ATPase preparations.

Titration studies with DCCD

In Fig. 1D(a), addition of DCCD leads to a low but continuous decline in fluorescence which is dependent on DCCD concentration. The fluores-

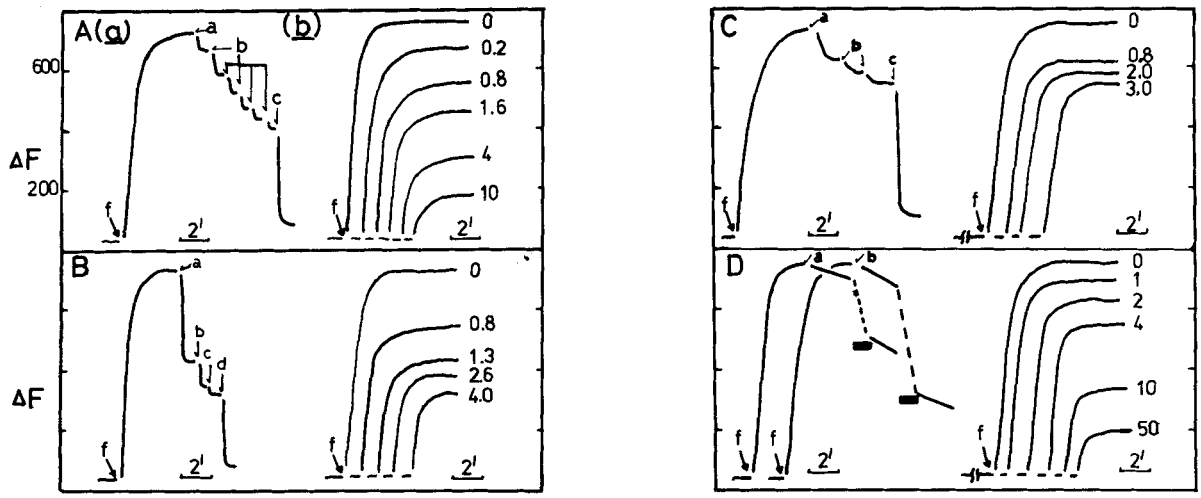

Figure 1. Effects of venturicidin, oligomycin, DCCD and tributyltin acetate on $Bu_2Sn(of)$ interaction with mitochondria. BHM (0.32 mg) in HSE buffer, pH 7.4, and $Bu_2Sn(of)$ were added (1 µl of a 1 mM solution). ΔF values (arbitrary units) were estimated in a Perkin–Elmer spectrofluorimeter: excitation, 400 nm; emission, 450 nm. total volume 2.0 cm^3. Temp. 18–20 °C. (a) The other F_0 inhibitors were added after maximum fluorescence enhancement was attained (back-titration). (b) The other F_0 inhibitors were preincubated with F_1F_0ATPase for the indicated times at room temperature before addition of $Bu_2Sn(of)$, (pre-incubation–titration). The initial blank values have been adjusted and are one-half the true experimental values.

Experiment A: Tributyltin acetate (Bu_3SnAc). (a) Additions: f, 1 µl of 1 mM $Bu_2Sn(of)$; a, 1 nmol Bu_3SnAc; b, 2,2,2,2,2 nmol Bu_3SnAc; 10 nmol Bu_3SnAc. (b) Preincubated for 2 min with indicated amounts (nmol) of Bu_3SnAc before addition of $Bu_2Sn(of)$, 1 nmol.

Experiment B: Venturicidin. (a) Additions: a, b, c, 2,1,1 nmol venturicidin; d, 10 nmol Bu_3SnCl. (b) Preincubated for 2 min with indicated amounts (nmol) of venturicidin.

Experiment C: Oligomycin. (a) Additions: a, b, c, 1,2,2 nmol oligomycin. (b) Preincubated for 10 min with indicated amounts (nmol) of oligomycin.

Experiment D: DCCD. (a) Additions: a, 5 nmol DCCD; b, 10 nmol DCCD. (b) Preincubation for 10 min with indicated amounts (nmol) of DCCD. Filled blocks indicate 30 min time span.

cence eventually decreases to ~30% of the maximal. This value is similar to that obtained with venturicidin, and the remaining fluorescence is back-titratable by tributyltin. Preincubation with DCCD enhances its activity as a modifier of $Bu_2Sn(of)$ binding (Fig. 1D(b)]. It markedly affects $Bu_2Sn(of)$ binding at low levels (1–4 nmol) which are known to inhibit ATPase and oxidative phosphorylation by reaction with Glu-61 on subunit c. DCCD preincubation [Fig. 1D(b)], can modify $Bu_2Sn(of)$ interaction by up to 85% of the maximal effect obtained with trialkyltins.

It should be noted that, in back-titration experiments, the effects of DCCD indicate a continuous modification (or destruction) of the $Bu_2Sn(of)$ site [Fig. 1D(a)], whereas preincubation with DCCD leads to a stable modification of the $Bu_2Sn(of)$ site.

Titration studies with SMP and solubilized F_1F_0ATPases

Back-titration experiments with SMP (Fig. 2) show qualitatively similar effects to those seen in BHM, although minor changes in trialkyltin back-titration and venturicidin back-titration have been observed. DCCD is equally effective in modifying the $Bu_2Sn(of)$ interaction site in SMP. This finding excludes the possibility that the DCCD effects observed with mitochondria are due to reduced uptake of $Bu_2Sn(of)$ by inhibition of mitochondrial porin.

The interactions of F_0 inhibitors with the $Bu_2Sn(of)$ binding site observed in mitochondria and SMP are also observed in solubilized ATPsynthase[12] and solubilized F_1F_0ATPase, Complex V[13] and a highly purified F_1F_0ATPase.

Figure 3 shows similar fluorescence enhancement and back-titration studies with partially purified preparations of Complex V[13] and a highly purified F_1F_0ATPase. The same general features as seen in mitochondria—total back-titration by trialkyltins, partial back-titration by the macrolide inhibitors venturicidin and oligomycin, and modification by DCCD—are retained to varying degrees in various types of solubilized F_1F_0ATPase preparations. This is a finding of particular significance for future studies of fractionation and reconstruction of the F_1F_0 ATPase complex. It should be noted that the response to oligomycin is lower in many solubilized preparations whereas the response to venturicidin is largely retained. This may be due to loss of lipid or a structural rearrangement during the isolation process. Bu_2Sn(of) interactions and F_0 inhibitor interactions in solubilized preparations are faster than in BHM and SMP due to the lack of a membrane permeability barrier or membrane partition barrier.

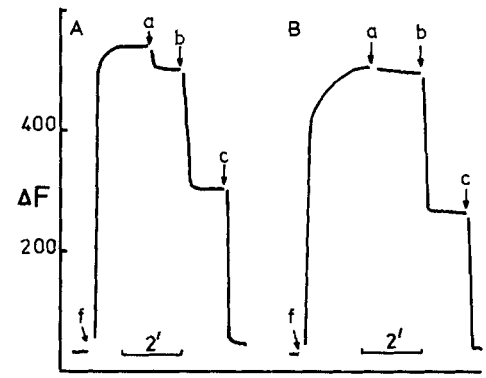

Figure 3 Effect of F_0 inhibitors on F_1F_0ATPase preparations. Experimental conditions as in Fig. 1.

Experiment A: 125 μg Complex V. Additions: f, 1 nmol Bu_2Sn(of); a, 2 nmol oligomycin; b, 4 nmol venturicidin; c, 10 nmol Bu_3SnCl.

Experiment B: 75 μg purified F_1F_0ATPase. Additions: as in A.

DCCD (5 nmol) caused a continuous decline in fluorescence in both types of preparation similar to that observed in Fig. 2B (data not shown).

DISCUSSION

These studies indicate that Bu_2Sn(of) is a versatile fluorescent probe of the F_1F_0ATPase complex, both in the membrane-bound and solubilized form. Figures 1–3 indicate some of the experiments that are now possible using this reagent for titration studies and studies of interaction with other F_0 inhibitors. These experiments, utilizing submaximal levels of fluorophore (2× and 4× I_{50} values) show that:

(a) The binding sites of Bu_2Sn(of) present in BHM and SMP are also present in solubilized and highly purified F_1F_0ATPases, albeit in reduced concentration.

(b) The binding sites for Bu_2Sn(of) are back-titrated by Bu_3SnX and other R_3SnX compounds. Thus, simple displacement of Bu_2Sn(of) by R_3SnX in competition for a common binding site is a possible explanation for the effects of Bu_3SnX in Fig. 1A(a) and this conclusion is supported by preincubation experiments [Fig. 1A(b)], as reported previously.[2]

R_3SnX compounds such as Bu_3SnX titrate *all* the Bu_2Sn(of) sites in mitochondrial membranes and solubilized F_1F_0ATPases. This suggests that the Bu_2Sn(of) interaction sites may be identical molecular species or related species of similar chemical reactivity which are present in the inner mitochondrial membrane.

(c) The binding site for Bu_2Sn(of) is readily back-titrated by venturicidin, a macrolide antibiotic, in all types of F_1F_0ATPase preparation (Fig. 1B), and at low and high levels

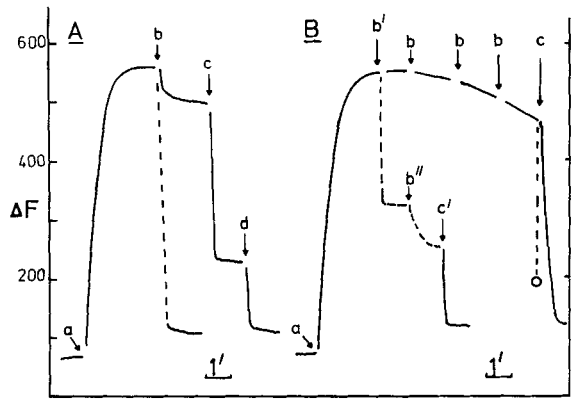

Figure 2. Effect of F_0 inhibitors on Bu_2Sn(of) interaction with SMP (0.25 mg). Experimental conditions as in Fig. 1.

Experiment A: Additions: a, 1 nmol Bu_2Sn(of); b, 1 nmol oligomycin; c, 4 nmol venturicidin; d, 10 nmol Bu_3SnCl.

Experiment B: Additions: a, 1 nmol Bu_2Sn(of); b, 5 nmol DCCD; c, 10 nmol Bu_3SnCl; b', 2 nmol venturicidin; b'', 4 nmol oligomycin; c', 10 nmol Bu_3SnCl. DCCD trace after 30 min, ---o.

of fluorophore. A maximal back-titration of $60 \pm 5\%$ by venturicidin is observed in membrane preparations and to lesser extents in solubilized F_1F_0 preparations. The results (Fig. 1B and Fig. 2) indicate that venturicidin has a high affinity for the Bu_2Sn(of) binding site and that it reacts rapidly with this site.

(d) In contrast, the other macrolide inhibitor, oligomycin, shows little or no effect on Bu_2Sn(of) binding at high fluorophore concentrations.[2] However, at low fluorophore concentrations, low levels of oligomycin elicit a response in back-titration studies and a slightly increased response on preincubation (Fig. 1C). These effects are the result of slow interactions and finally amount to less than 30% of the effects observed with trialkyltins and less than 50% of the effects observed with venturicidin. Thus oligomycin may act at a different interaction site from venturicidin or by a different reaction mechanism. Similar studies of other macrolide inhibitors of F_1F_0ATPase (botrycidin, peliomycin, ossamycin, venturicidin aglycone) are now feasible. Bafilomycin, the macrolide inhibitor of V-ATPases, was shown to be completely inactive when tested with BHM at levels up to 7.5 nmol mg protein^{-1}.

Figures 1–3 show that up to 35% of the Bu_2Sn(of) interaction sites are not back-titratable by venturicidin but are still titratable by Bu_3SnX compounds. Also, the effects of maximal oligomycin followed by maximal venturicidin (Fig. 2), or vice versa, are not additive, as only total of about 65% of the Bu_2Sn(of) sites are titrated by the macrolide inhibitors. A facile explanation of these results is that there are two classes of Bu_2Sn(of) sites; one 'free' and representing up to 35% of the total, and the other 'bound' to ATPsynthase and modifiable by the macrolide inhibitors, particularly venturicidin, which acts with high affinity at the same site, and by oligomycin, which acts with slightly lower affinity at a different but related site.

A common interaction site of venturicidin and trialkyltins, separate from the oligomycin binding site in F_0, has been inferred from previous studies in this laboratory on yeast venturicidin-resistant and yeast triethyltin-resistant mutants, the large majority of which are cross-resistant (VEN^RTET^R). This class of mutants is not isogenic with other mitochondrial genes[11] and thus cannot involve modifications of subunit c (DCCD binding protein). This differentiates the VEN^RTET^R interaction site from mitochondrially coded VEN^R and VEN^ROLI^R mutants which are modified in subunit c[10,14] and mitochondrially coded OLI^R mutants which are modified in subunit c and/or subunit b.[9,10,15] Studies of mitochondria from these four classes of mutants using Bu_2Sn(of) as a fluorescence probe can give useful information on the relationship between venturicidin, oligomycin and R_3SnX binding sites on F_1F_0ATPase.

The titration of Bu_2Sn(of) interaction sites in mitochondria, SMP and various purified F_1F_0ATPase complexes is a further facility of this reagent which allows studies of the distribution of Bu_2Sn(of) interaction sites during fractionation and purification of the complexes of the mitochondrial inner membrane (D. E. Griffiths, unpublished observations). Studies are under way to establish the identity, location and distribution of Bu_2Sn(of) interaction sites in the mitochondrial inner membrane and to establish whether they have a specific and unique association with the F_1F_0ATPase complex.

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REFERENCES

1. Usta, J and Griffiths, D E *Biochem. Biophys. Res. Comm.*, 1992, 188: 365
2. Usta, J and Griffiths, D E *Appl. Organomet. Chem.*, 1993, 7: 193
3. Minkov, I, Griffiths, D E and Strotmann, H unpublished studies
4. Webster, L, Griffiths, D E and Apps, D K *Biochem. Soc. Trans.*, 1993, 21: (in press)
5. Kiehl, R and Hatefi, Y *Biochemistry*, 1980 19: 541
6. Partis, M D, Bertoli, E, Griffiths, D E and Azzi, A *Biochem. Biophys. Res. Commun.*, 1980, 96: 1103
7. Ruder, F J and Kayser, H *Pesticide Biochem. Physiol.*, 1992, 42: 248
8. Cain, K and Griffiths, D E *Biochem. J.*, 1977, 162: 593
9. Griffiths, D E, Houghton, R L, Lancashire, W E and Meadows, P A *Eur. J. Biochem.*, 1975, 51: 393
10. Lancashire, W E and Griffiths, D E *Eur. J. Biochem.*, 1975, 51: 403

11. Lancashire, W E and Griffiths, D E *Eur. J. Biochem.*, 1975, 51: 377
12. Hughes, J, Joshi, S, Torok, K and Sanadi, D A *J. Bioenerg. Biomembr.*, 1982, 14: 287
13. Stiggall, D L, Galante, Y M and Hatefi, Y *J. Biol. Chem.*, 1978, 264: 12029
14. Galanis, M, Mattoon, J R and Nagley, P *FEBS Lett.*, 1989, 249: 333
15. Ray, M K, Connerton, I F and Griffiths, D E *Biochim. Biophys. Acta*, 1988, 951: 213