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ORGANOTIN-FLAVONE COMPLEXES: A NEW CLASS OF FLUORESCENT PROBES FOR F_1F_0ATPASE

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SUMMARY: Fluorescent 5-coordinate organotin-flavone complexes of 3-hydroxy-flavone (Hof) and 3,5,7,2',4',-pentahydroxyflavone (morin) are good inhibitors of mitochondrial F_1F_0ATPase but do not inhibit F_1-ATPase and they have been examined as possible fluorescent probes of F_1F_0ATPase. R_2SnX (morin) complexes exhibit low fluorescence enhancement on binding to mitochondrial membranes with no displacement by equimolar tributyltin.
In contrast R_2SnX (of) complexes exhibit high fluorescence enhancement whose extent is variable and is displacable by equimolar tributyltin. Fluorescence enhancement by R_2SnX (of) complexes correlates with the ATPase I_{50} values. Dialkyltin-3-hydroxy flavone, R_2SnX(of), complexes act as a new class of fluorescent probes which titrate the F_0 segment of F_1F_0ATPase.

Trialkyl and triaryltins inhibit mitochondrial oxidative phosphorylation showing different modes of action depending on concentration (1). The major effect is the inhibition of the mitochondrial F_1F_0ATPase complex as a result of an interaction with the F_0 component (2,3). The site of action of organotin inhibitors on the F_0 component has not been established (4,5) but functional studies as to the nature of the triorganotin binding site suggest that dithiols may play an important role in the conduction of protons through the F_0 proton channel (6,7,8). As 5-coordinate triorganotins are highly specific ATPase inhibitors with little or no effect on respiration generated membrane potential (9,10), a specific high affinity site on the F_0 segment of mitochondrial ATPase is indicated. Analysis of this binding site would be facilitated by the development of 5-coordinate triorganotin inhibitors which would serve as highly specific fluorescent probes.
This paper describes the fluorescence properties of several 5-coordinate organotin-flavone complexes first described by Blunden & Smith (11) which are derivatives

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of 3-hydroxyflavone (Hof) and 3,5,7,2'4' pentahydroxy flavone (morin) and their interaction with mitochondrial membranes. Of several compounds investigated, 5-coordinate $R_2SnX(of)$ complexes (Figure 1) have properties which make them suitable fluorescence probes of the mitochondrial ATPase complex.

MATERIALS AND METHODS

3-hydroxyflavone (Hof), 3,4,7,2',4' pentahydroxyflavone (morin), dibutyltin dibromide, dibutyltindichloride, dimethyltin dichloride, diethyltindichloride and diphenyltin dichloride were purchased from Aldrich Chemical Company and were used without further purification. Dioctyltin dichloride was a gift from Dr. Peter Smith, International Tin Research Institute, Uxbridge, Middlesex. The sources of other chemicals used: trialkyltins and triaryltins, uncouplers, and reagents for mitochondrial assays have been described previously (6,7,9).

Preparation of organotin-flavone complexes. $Bu_2SnBr(morin)$, $Et_2SnCl(morin)$, $Ph_2SnCl(morin)$ and $Me_2SnCl(morin)$ were prepared by mixing equimolar quantities of the equivalent organotin dihalides with morin in ethanol and standing in the dark overnight, essentially as described by Blunden & Smith (11) for the synthesis of $Ph_2SnCl(of)$. $Bu_2SnBr(of)$, $Bu_2SnCl(of)$, $Et_2SnCl(of)$, $Ph_2SnCl(of)$ $Me_2SnCl(of)$ and $Oct_2SnCl(of)$ were prepared in similar fashion with the equivalent organotin dihalides and 3-hydroxyflavone. Ph_2SnCl (morin) and Ph_2SnCl (of) were obtained in a crystalline state by evaporating the solvent. The other compounds were not crystallized and were obtained as oils after rotary evaporation of the solvent, dissolved in ethanol or dimethylsulphoxide, and the molarity calculated assuming the reactions had gone to completion. $Bu_3Sn(of)$ and $Bu_2Sn(of)_2$ were prepared as described previously (11).

Fluorescence analysis. All fluorescence analyses were carried out in the Perkin Elmer LS-5 spectrofluorimeter at room temperature (18-21°). The uncorrected excitation and emission spectra of organotin-flavone complexes were determined in ethanol. Interaction of flavone complexes with mitochondrial membranes were determined in aqueous 10 mM Hepes 0.25 M Sucrose, 1mM EGTA buffer, pH 7.4 (HSE). Approximately 1 mg liver mitochondrial protein plus 2-5 μM organotin-flavone complex were used in a total volume of 2.0 ml HSE buffer. Displacement of the organotin-flavone was studied by addition of 5-10 μM Bu_3SnAc or Bu_3SnCl. Lower amounts of mitochondrial proteins were used in experiments with sub-mitochondrial particles and heart mitochondria.

Enzyme assays. The assay systems for mitochondrial ATPase, protein, inorganic phosphate, ATPase I_{50} values, mitochondrial respiration in the oxygen electrode and the preparation of rat liver and rat heart mitochondria and submitochondrial particles (SMP) have been described previously (6,7,9) Crude F_1ATPase was prepared from rat liver mitochondria by the chloroform extraction method (12).

RESULTS

Several organotin-flavone complexes were synthesised by mixing equimolar amounts of the flavone with the appropriate diorganotindihalide in ethanol.

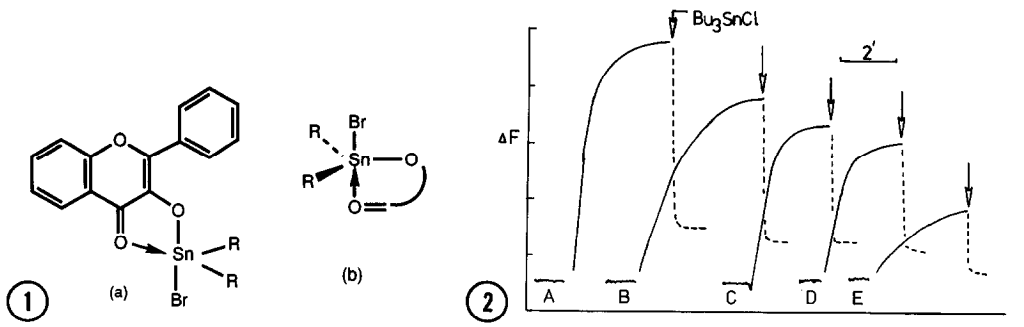

Figure 1. Dialkyltin-3-hydroxyflavone bromide, $R_2SnBr(of)$.

Figure 2. Fluorescence enhancement on binding of $R_2SnX(of)$ complexes to SMP. Fluorescence analysis was as described in the methods section: $R_2SnX(of)$ complexes (5μM) were added to 0.3 mg SMP in 2.0 ml HSE buffer, pH 7.4. Excitation, 395 nm; emission, 450 nm. A = Bu_2 SnBr(of); B = $Ph_2SnCl(of)$; C = $Et_2SnCl(of)$; D= $Me_2SnCl(of)$; E = $Oct_2SnCl(of)$. The ΔF values have not been corrected for light scattering.

Fluorescent products were obtained in all cases but only Ph_2SnCl (morin) and $Ph_2SnCl(of)$ were obtained in a crystalline state. The other compounds were obtained as oils after evaporation of the solvent, redissolved in a known volume of ethanol and the molarity calculated assuming the reaction had gone to completion. The reaction is rapid and can be monitored spectrophotometrically and spectrofluorometrically in ethanol solutions. In the case of 3-hydroxflavone complexes the absorption peak of 3-hydroxflavone at 350 nm is replaced by a new peak at 390-400 nm with decreased absorption. The fluorescence emission peak of 3-hydroxy flavone at 535 nm (excitation 350 nm) disappears and a new emission peak appears at ~ 450 nm (excitation 395 nm).

The fluorescence properties of organotin-flavone complexes are listed in Table 1 which also includes data of mitochondrial fluorescence enhancement on binding and the fluorescence decrease due to displacement on addition of Bu_3SnCl or Bu_3SnAc which are illustrated in Fig.2.

Diorganotin morin complexes. eg. Bu_2SnBr (morin), have a yellow green fluorescence with emission maxima in the range 505-510 nm. (Table 1). They exhibit a relatively low fluorescence enhancement on addition of mitochondrial membranes (Table 1). Also, addition of excess Bu_3SnCl does not cause a fluorescence decrease due to displacement of the organotin morin complex from the mitochondrial binding site(s). The one exception is Ph_2SnCl(morin) which shows significant fluorescence enhancement and a 30% displacement by Bu_3SnCl.

Diorganotin-3-hydroxyflavone complexes. e.g. $Bu_2SnX(of)$, have a marked yellow fluorescence with emission maxima around 450 nm. They exhibit a low basic fluorescence in aqueous media, as do organotin morin complexes, but all $R_2SnX(of)$ complexes tested show marked fluorescence enhancement (Table 1 and Fig. 2.) on interaction with mitochondrial membranes. The most marked fluor-

TABLE 1 Organotin-flavone complexes: Fluorescence properties and I_{50} values for ATPase inhibition

Compound*	(a) Excit./ Emiss. (nm)	(b) Fluorescence Enhancement	(c) Displacement by Bu_3SnCl	(d) ATPase I_{50}
Me_2SnCl(of)	392/455	++	> 80%	12-13
Et_2SnCl(of)	390/450	++	> 80%	1.5
Bu_2SnBr(of)	395/455	++++	> 80%	0.7-0.9
Ph_2SnCl(of)	393/450	+++ (slow)	> 80%	1.5
Oct_2SnCl(of)	394/445	(+)(slow)	> 80%	12-13
Me_2SnCl(morin)	415/510	minor	no effect	> 100
Et_2SnCl(morin)	415/510	(+)	no effect	5-6
Bu_2SnBr(morin)	415/510	+	no effect	0.6-0.8
Ph_2SnCl(morin)	415/505	++	30%	3.5-4
Bu_3Sn(of)	395/450	nil	nil	1.5-2.0
$Bu_2Sn(of)_2$	395/450	nil	nil	1.5-2.0
morin	395/535	nil	nil	>> 200
3-hydroxy flavone (Hof)	350/535	nil	nil	>> 200

(a) Fluorescence and emission spectra are uncorrected and were determined on 0.5 μM solutions in ethanol.
(b) Enhancements were determined in HSE buffer pH 7.4 with 0.3 mg SMP and 5 μM R_2SnX(mor) or 5 μM R_2SnX(of). + = approx. 150% increase.
(c) Displacement = decrease in fluorescence enhancement on addition of 10 μM Bu_3SnCl (2:1 molar ratio)
(d) ATPase; I_{50} values, nmole inhibitor/mg protein. 100% activity (0.81 μ mole/min/mg protein) 0.2 mg liver SMP in the assay. The I_{50} value for Bu_3SnCl was 1.0 nmol/mg protein.
*The abbreviations for the organotin-flavone complexes are described in (11).

escence enhancement on binding is shown by Bu_2SnBr(of). The fluorescence enhancement is decreased by addition of a 2-fold excess of Bu_3SnCl indicating displacement by competition for a common binding site (Figure 2) Similar results are obtained with other R_2SnX(of) complexes and these findings are consistent with the binding of R_2SnX(of) complexes to a specific binding site on mitochondrial ATPase which is competed for by the known F_0 inhibitor, Bu_3SnCl.

Inhibition of ATPase. The effects of the organotin-flavone complexes on mitochondrial F_1F_0ATPase is shown in Table 1. In the series, Me_2SnCl (of), Et_2SnCl(of), Ph_2SnCl(of) Bu_2SnBr(of), Oct_2SnCl(of); the I_{50} values for ATPase inhibition are 12-13, 1.5, 1.5, 0.7-0.9 and 12-13 nmole/mg protein respectively. These I_{50} values are broadly similar to results with the equivalent triorganotins (1,9,10). The I_{50} values for Bu_2SnBr(of) are significantly lower than those obtained for Bu_3SnCl or Bu_3SnAc (~ 1.0 nmole/mg protein). The range of I_{50} values obtained for ATP inhibition are also related to the degree of fluorescence enhancement observed with the series of R_2SnX(of) complexes (Figure 2). This suggests that the inhibitory site on F_1F_0ATPase is related to, and is probably

identical with, the binding site involved in fluorescence enhancement.
In the series Me_2SnCl(morin), Et_2SnCl(morin) Ph_2SnCl(morin) and Bu_2SnBr (morin), the I_{50} values for ATPase inhibition are > 100, 5-6, 3.5-4 and 0.6-0.8 nmole/ mg protein, respectively. In contrast to the R_2SnX(of) series, there appears to be no general correlation with ATPase I_{50} values and fluorescence enhancement; e.g. Bu_2SnCl (morin) is a good inhibitor of ATPase despite exhibiting a low fluorescence enhancement and no apparent displacement by Bu_3SnCl. (Table 1) . Similarly the pentacoordinate Bu_3Sn(of) and the hexaco-ordinate $Bu_2Sn(of)_2$ complexes (11) exhibit no fluorescence enhancement on binding to mitochondrial membranes but are fairly good ATPase inhibitors (I_{50} values 1.5-2.0 nmol/mg protein, respectively).
Fluorescence enhancement with concomitant displacement by Bu_3SnCl thus appears to be a property of R_2SnX(of) complexes which makes them particularly suitable fluorescent probes of the mitochondrial F_1F_0ATPase.
R_2SnX(of) complexes and R_2SnX(morin) complexes also do not inhibit mitochondrial F_1ATPase prepared by the method of Beechey et al (12) at 20 times the level which inhibits F_1F_0ATPase (higher levels were not investigated). The flavones, 3-hydroxyflavone, morin and quercetin do not inhibit F_1ATPase at 150, 50 and 50 nmol/mg protein, respectively. It is known that quercetin inhibits F_1ATPase at ~ 1500 nmol/mg protein (13). This indicates that the interaction site is on the F_0 segment of the mitochondrial F_1F_0ATPase complex and is similar to that of the trialkyltins and of the triaryltins. R_2SnX(of) complexes also do not inhibit mitochondrial NADH oxidase or succinoxidase indicating that there is no inhibition of the electron transfer chain (data not presented).
Titration of mitochondrial ATPase. R_2SnX(of) complexes interact with mitochondrial membranes at a specific interaction site on F_1F_0ATPase and the fluorescence enhancement appears to titrate this site. The fluorescence enhancement is greater per mg protein in SMP than in mitochondria and appears to correlate with the amount of ATPase present (Fig 3). Similarly the fluorescence enhancement is greater in heart mitochondria than in liver mitochondria and thus correlates with the higher inner membrane content of heart mitochondria. The interaction (fluorescence enhancement) is unaffected by the energy state of the membrane as it is not affected by oxidisable substrates and by ATP nor by respiratory chain inhibitors and uncouplers.

DISCUSSION

Organotin hydroxyflavone and organotin morin complexes have been used previously in the fluorimetric analysis of organotins (14,15) but the present studies show that they have potential as fluorescent probes of the F_0 segment of the mitochondrial F_1F_0ATPase complex. The organotin hydroxyflavone complexes

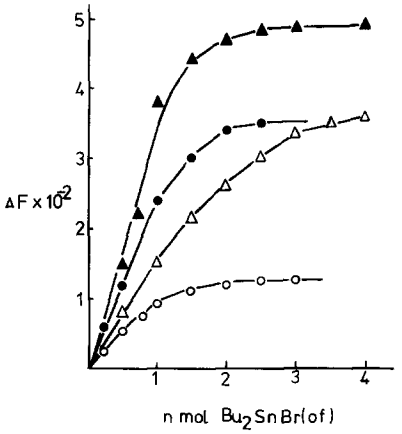

Figure 3. Titration of mitochondrial F_1F_0 by $Bu_2SnBr(of)$.
Experimental conditions are as described in Figure 2. Aliquots (1µl) of ethanolic solutions of $Bu_2SnBr(of)$ were added to suspensions (0.33 mg protein in 2.0 ml HSE buffer). The ΔF values have been corrected for light scattering.
(O) liver mitochondria; (Δ) liver SMP; (●) heart mitochondria; (▲) heart SMP.

$R_2SnX(of)$, especially Bu_2SnBr (of) seem to be particularly suitable for use as fluorescent probes.

(a) They inhibit mitochondrial F_1F_0ATPase at levels similar to or lower than the equivalent trialkyltin compounds and do not inhibit F_1F_0ATPase.

(b) There is a specific fluorescent enhancement on addition of $R_2SnX(of)$ complexes to all types of mitochondrial membranes which appears to correlate with the F-ATPase content of the membrane. $R_2SnX(of)$ complexes thus appear to titrate the mitochondrial membrane bound F_0 segment of F_1F_0ATPase.

(c) Fluorescence enhancement (binding) of $R_2SnX(of)$ complexes correlates with the I_{50} values for ATPase inhibition which in turn, correlate broadly with the I_{50} values of the equivalent trialkyltin compounds. $Bu_3SnX < Ph_3SnX < Et_3SnX << Me_3SnX$.

(d) Fluorescent enhancement is reversed by Bu_3SnCl, a F_0 inhibitor by displacement from the binding site. For a particular $R_2SnX(of)$ complex the fluorescence enhancement is reversed by the equivalent R_3SnCl and also by the non-equivalent R_3SnCl compounds. However in all cases displacement is most effective with the non-equivalent R_3SnCl compounds with the lowest I_{50} values e.g. $Bu_3SnCl >> Me_3SnCl$ (data not presented).

Points (a)-(d) indicate that fluorescence enhancement observed on binding of $R_2SnX(of)$ to mitochondrial membranes make this class of compounds a suitable fluorescence probe of the trialkyltin binding site located on the F_0 segment of F_1F_0ATPase. A particularly useful property of $R_2SnX(of)$ complexes is the capacity to titrate the F_0 component in mitochondrial membranes (Figure 3) as well as isolated F_1F_0 complexes (D.E. Griffiths and J. Usta, unpublished studies). The

interaction of Bu_2SnBr(of) with F-ATPases in bacterial plasma membranes and yeast mitochondria as well as interactions with V-ATPases (16) is under investigation. This class of fluorescent probes should be of particular value in studies of E.coli and yeast mutants with known defects in F_0 components. For equivalent mutations in human mitochondria screening techniques using flow cytometry of white cells are feasible.

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