Studies on the nature of the high-affinity trialkyltin binding site of rat liver mitochondria

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1. The proteolipid fraction isolated from rat liver mitochondria pretreated with [^3H]triphenyltin chloride is enriched in triphenyltin compared with the original mitochondria. 2. Part of this [^3H]triphenyltin is eluted with a protein of M_r 5000–6000 on Sephadex LH20 chromatography. 3. Mössbauer spectra of the proteolipid fraction treated with ^{119}Sn -enriched triethyltin chloride show a doublet which corresponds closely with that assigned previously [Farrow & Dawson (1978) *Eur. J. Biochem.* **86**, 85–95] to the absorption of triethyltin bound to the high-affinity binding site of the mitochondrial ATPase.

The major effects of trialkyltin compounds on mitochondria can be explained on the basis of a combination of two different modes of action. The first of these is the catalysis of an anion–hydroxide exchange across the mitochondrial membrane (Selwyn *et al.*, 1970; Stockdale *et al.*, 1970) which results in uncoupling, perturbation of intra-mitochondrial pH (Dawson & Selwyn, 1974) and expulsion of respiratory substrates (Manger, 1969; Skilleter, 1975). The second action is an oligomycin-like effect, resulting in inhibition of phosphorylation and of the mitochondrial ATPase (Stockdale *et al.*, 1970; Rose & Aldridge, 1972). Like oligomycin, trialkyltins appear to act by blocking proton conduction through the membrane sector of the ATPase (Dawson & Selwyn, 1975).

Study of the binding of trialkyltin compounds to rat liver mitochondria has yielded rather complex binding curves which have been interpreted as showing the existence of either one high-affinity and one low-affinity class of binding site (Aldridge & Street, 1970) or one high-affinity component, one low-affinity component and a component due to the partition of lipophilic trialkyltin species into the membrane (Farrow & Dawson, 1978). In either case, there is general agreement that binding to the high-affinity site results in inhibition of the ATPase (Rose & Aldridge, 1972; Farrow & Dawson, 1978). ^{119}Sn Mössbauer spectroscopy revealed the presence of three different triethyltin-binding environments in rat liver mitochondria (Farrow & Dawson, 1978), which were assigned to triethyltin binding to the mitochondrial ATPase (with high affinity), to an unidentified *N*-ethylmaleimide-sensitive component (with low affinity) and to triethyltin partitioned into the mitochondrial membrane.

The identification of a Mössbauer absorption doublet associated with binding of triethyltin to a component of the mitochondrial ATPase suggested a possible means of monitoring the isolation of this component which would be more specific than measurement of binding of radiolabelled trialkyltin. However, early attempts to do this proved unsuccessful, since the natural abundance of ^{119}Sn , the Mössbauer absorbing isotope of tin, is only 8.6% and was too low to produce a detectable spectrum of triethyltin in mitochondrial extracts. To overcome this problem we have synthesized 84%-enriched triethyl[^{119}Sn]tin chloride. Even using this compound, the spectra take a long time to collect, so that Mössbauer spectroscopy would be unsatisfactory as a routine means of following an isolation procedure. We have therefore adopted a compromise strategy of firstly developing an isolation procedure using radiolabelled triphenyltin chloride as a marker for trialkyltin binding components and then comparing their triethyltin complex Mössbauer spectra with that assigned to the trialkyltin binding component of the ATPase complex. Triphenyltin was chosen as a marker because its affinity for the high-affinity site is ten times higher than that of triethyltin (Farrow, 1978).

Following the observations of Cattell *et al.* (1971) that the dicyclohexylcarbodi-imide binding site was a proteolipid, as defined by Folch & Lees (1951), we used a similar procedure to see whether the trialkyltin binding site also resided in a proteolipid. It appears that this is the case, since radioactivity from mitochondria pretreated with [^3H]triphenyltin is extracted by chloroform/methanol and precipitated by diethyl ether. Binding of triethyl[^{119}Sn]tin to this fraction yields a component in the Mössbauer

spectrum which is compatible with that originating from the high-affinity triethyltin binding site of whole mitochondria.

Experimental

Materials

Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] was obtained from Hopkin and Williams. [^3H]Triphenyltin chloride ($32\text{ mCi}\cdot\text{mmol}^{-1}$) was from The Radiochemical Centre, Amersham. The 10 mCi calcium [$^{119\text{m}}\text{Sn}$]stannate Mössbauer source, and [^{119}Sn]tin metal, 84% enriched, were from New England Nuclear. Chlorosulphonic acid was from Aldrich and triethylaluminium (25% in hexane) was from Alfa (Lancaster Synthesis, Lancaster, U.K.). Azolectin, soya bean phospholipid, was obtained from Sigma as type II_s phosphatidylcholine. This was then partially purified by the method of Kagawa & Racker (1971). All other materials were of AnalaR or highest available grade.

Preparation of rat liver mitochondria

Mitochondria were prepared from the livers of albino Wistar rats by the method of Selwyn *et al.* (1970). The final suspension medium was 250 mM -sucrose/ 5 mM -Hepes/KOH, pH 7.6.

Analytical methods

Mitochondrial protein concentration was determined by the biuret method (Gornall *et al.*, 1949) after clarification with Triton X-100. Protein concentrations of chloroform/methanol extracts were measured by evaporating the solvent, solubilizing the residue in 1% (w/v) sodium dodecyl sulphate and estimating the protein present by the method of Lowry *et al.* (1951).

For measurements of ^3H -labelled triphenyltin in chloroform/methanol mixtures, a similar procedure was adopted to avoid quenching by chloroform. The organic solvent was removed by evaporation, the residue solubilized in 1% (w/v) sodium dodecyl sulphate and the radioactivity in aliquots of the solution was determined by liquid-scintillation counting in 15 ml of scintillant (4.67 g of 2,5-diphenyl-oxazole in 660 ml of toluene and 340 ml of Triton X-100). Counts were recorded on an Intertechnique SL30 liquid-scintillation counter. The counting efficiency was 38%.

Phospholipid content of fractions was determined by digestion of dried residues with sulphuric acid, followed by perchloric acid. The inorganic phosphate released was measured by the method of Fiske & SubbaRow (1925).

Synthesis of triethyl[^{119}Sn]tin chloride

All steps were carried out under an atmosphere of dry nitrogen. Chlorosulphonic acid ($455\mu\text{l}$) was

added dropwise to 200 mg of ^{119}Sn metal powder in a microdistillation apparatus. When the reaction was complete the fraction distilling at $92\text{--}100^\circ\text{C}$ was collected. The yield of $^{119}\text{SnCl}_4$ was 184 mg (42%). Triethyltin chloride was then made by a modification of the method of Neumann (1962). Triethylaluminium ($405\mu\text{l}$ of a 25% solution in hexane) was slowly added to the $^{119}\text{SnCl}_4$ obtained from the previous step, followed by $89\mu\text{l}$ of dry diethyl ether. The reaction was stirred for 1 h at 70°C , followed by 1 h at 102°C . The reaction mixture was cooled and dissolved in 3 ml of dry chloroform. The triethyltin chloride was separated from the reaction mixture by using preparative t.l.c. The chromatogram was developed with chloroform/methanol/water (55:1:3, by vol.) and the product was eluted from the chromatogram by scraping off the required region of the silica plate into 25 ml of chloroform/methanol (2:1, v/v). The yield was $174\mu\text{mol}$ of triethyl[^{119}Sn]tin chloride. The product was homogeneous on t.l.c. and had a Mössbauer spectrum showing one absorption doublet: quadrupole splitting (Δ) $3.66 \pm 0.1\text{ mm}\cdot\text{s}^{-1}$, isomer shift (δ) $1.49 \pm 0.05\text{ mm}\cdot\text{s}^{-1}$ (literature $\Delta = 3.75$, $\delta = 1.61\text{ mm}\cdot\text{s}^{-1}$; Smith, 1970).

Mössbauer spectroscopy

The Mössbauer spectrometer used was an NM700 series instrument from J & P Engineering, Reading, U.K. The velocity of the drive was calibrated by using the room temperature spectra of calcium stannate ($0\text{ mm}\cdot\text{s}^{-1}$) and β -tin foil ($2.56\text{ mm}\cdot\text{s}^{-1}$; Ho & Zuckermann, 1973). Apart from the calibration runs, all spectra were obtained at a sample temperature of 77 K.

Preparation of the proteolipid fraction

This was carried out according to the method of Fillingame (1976), with some modifications. Frozen-thawed rat liver mitochondria (8 g of protein), either with or without the addition of triethyl[^{119}Sn]tin chloride or [^3H]triphenyltin chloride were centrifuged at $90000g$ for 50 min and the pellets taken up to 136 ml final volume with water. The suspension was added slowly with stirring to 2700 ml of chloroform/methanol (2:1, v/v) and left stirring for 24 h at 4°C . The suspension was filtered through glass-fibre paper and 500 ml of water was mixed with the filtrate. The resulting emulsion was allowed to stand for 24 h at 4°C and then was warmed to room temperature. The upper aqueous phase was removed by aspiration and the emulsified lower layer was centrifuged for 10 min at $10000g$, after which the remaining aqueous phase was removed by aspiration. Chloroform (1 vol.) was added to the chloroform phase, which was then evaporated to dryness, with the addition of methanol on the appearance of cloudiness. The residue was

taken up in 40 ml of chloroform/methanol (2:1, v/v), filtered and cooled to -20°C . Diethyl ether (160 ml), pre-cooled to -20°C , was slowly added, and the mixture was left overnight at -20°C . The precipitate was recovered by centrifugation at 12000g for 20 min at 0°C , after which the supernatant was decanted and the precipitate was dried in a stream of nitrogen. The pellet was dissolved in 10 ml of chloroform/methanol (2:1, v/v). The final yield was between 15 and 30 mg of protein.

Sephadex LH-20 chromatography

An aliquot (1 ml) of the chloroform/methanol solution obtained in the previous step was loaded on to a column (9 cm $\times$ 2 cm) of Sephadex LH-20 equilibrated with chloroform. Elution was firstly with chloroform and then with step changes to chloroform/methanol mixtures containing increasing proportions of methanol. Details are given in the Figure legend.

Results

Extraction of the triphenyltin-binding component

Table 1 shows that following extraction of mitochondria pretreated with [^3H]triphenyltin with chloroform/methanol (2:1, v/v), about 2% of the total protein is present in the washed chloroform extract, in good agreement with the equivalent data for beef heart mitochondria (Cattell *et al.*, 1971). However, about 40% of the original triphenyltin is present at this stage. Since triphenyltin chloride is very soluble in chloroform (Selwyn, 1976) this is not surprising. Most of it will have originated from triphenyltin species either partitioned into the mitochondrial membrane or loosely bound to the low affinity sites. The value given in Table 1 for the ratio of triphenyltin to protein at this stage will therefore certainly not be an accurate reflection of the amount of triphenyltin actually bound to extracted components of the mitochondrial membrane. However, triphenyltin chloride is also very soluble in chloroform/diethyl ether mixtures, and the radioactivity precipitated by ether can only be due to binding to one or more components in the proteolipid fraction.

Furthermore, the figure of 21.5 nmol of triphenyltin bound per mg of protein shows a very significant enrichment over the original mitochondrial suspension. In an attempt to purify the binding component further, a portion of the proteolipid fraction was chromatographed on a column of Sephadex LH-20. The results are shown in Fig. 1. A large peak of radioactivity is eluted with chloroform, running slightly behind the first protein peak. A much smaller radioactive peak, associated with a protein peak is eluted with chloroform/methanol (8:1, v/v). The first peak of radioactivity corresponds precisely with the elution profile of triphenyltin chloride applied to the column in the absence of mitochondrial extract, and it seems likely that it represents triphenyltin which has dissociated from the proteolipid fraction before and during the chromatographic procedure. However, the second, small peak appears to be still associated with protein and phospholipid and therefore presumably indicates the presence of a trialkyltin binding component in the original ether precipitate. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the protein fraction eluting with the radioactive peak showed one protein band with a mobility corresponding to M_r 5000–6000.

Mössbauer spectroscopy

Fig. 2 shows the Mössbauer spectrum of triethyl[^{119}Sn]tin bound to mitochondria. It is very similar to the spectrum reported previously (Farrow & Dawson, 1978) for the binding of non-enriched triethyltin, and is composed of at least three rather poorly resolved doublets. It should be noted that in our earlier report, although the data shown in the Figures is correct, there was a calculation error in working out the isomer shift and quadrupole splitting of the high affinity-site. The correct values, as shown in Fig. 6 of Farrow & Dawson (1978) are $\Delta = 1.67 \pm 0.1 \text{ mm} \cdot \text{s}^{-1}$; $\delta = 1.22 \pm 0.05 \text{ mm} \cdot \text{s}^{-1}$. The corresponding values for the inner doublet, marked (1) in Fig. 2 are $\Delta = 1.67 \pm 0.1 \text{ mm} \cdot \text{s}^{-1}$; $\delta = 1.25 \pm 0.05 \text{ mm} \cdot \text{s}^{-1}$.

The proteolipid fraction obtained by diethyl ether precipitation of chloroform/methanol extracts, while

Table 1. *Fractionation of mitochondria in the presence of [^3H]triphenyltin chloride*

Rat liver mitochondria (8.0 g) in 100 ml of 250 mM-sucrose/5 mM-Hepes, pH 7.6, were treated with 25 μmol of triphenyltin chloride containing 16 μCi of [^3H]triphenyltin chloride. After 30 min at 0°C the mitochondria were sedimented and fractionation was carried out as described in the Experimental section.

	Total protein (mg)	Total triphenyltin (nmol)	Triphenyltin (nmol/mg)
1. Mitochondrial suspension	8000	25 500	3.19
2. After centrifugation	—	24 000	—
3. In washed chloroform extract	144	9800	68
4. In ether precipitate from chloroform extract	15	323	21.5

Fig. 1. *Sephadex LH-20 chromatography of the proteolipid fraction from [^3H]triphenyltin-treated mitochondria* Part (1.0 ml) of the proteolipid fraction from the experiment shown in Table 1, containing 1.5 mg of protein and 32.3 nmol of [^3H]triphenyltin (21 nCi of ^3H) was applied to a column (9 cm $\times$ 2 cm) of Sephadex LH-20. The column was developed with the solvent mixtures shown in the figure, and 5 ml fractions were collected. (O) protein, (●) radioactivity, (Δ) phospholipid phosphate.

Fig. 2. *Mössbauer spectrum of triethyl[^{119}Sn]tin species bound to mitochondria*

Rat liver mitochondria (2.0 g) suspended in 100 ml of 250 mM-sucrose/5 mM-Hepes/KOH, pH 7.6, were treated with 12 μmol of triethyl[^{119}Sn]tin chloride for 30 min at 4°C. The mitochondria were then sedimented at 90 000 g for 60 min, and the pellet loaded into the Mössbauer cell. The cell had a diameter of 2 cm and a volume of 3.0 ml.

soluble in chloroform or chloroform/methanol mixtures, appears to be insoluble in other organic solvents that we have tried. Unfortunately, chloroform when frozen completely attenuates the 23.9 keV

γ rays emitted from $^{119\text{m}}\text{Sn}$, so that Mössbauer spectroscopy on extracts dissolved in chloroform is not practicable. Accordingly, the method we adopted was to incorporate the chloroform/methanol soluble fraction into soya bean phospholipid liposomes, on the basis that provision of a lipid environment should protect the proteolipid from denaturation in aqueous suspension. The proteolipid fraction (30 mg of protein), dissolved in chloroform/methanol (2:1, v/v) was mixed with 77 mg of soya bean phospholipid in 50 ml of chloroform. After evaporation in a rotary evaporator, liposomes were made by shaking the residue with glass beads in 2.8 ml of 250 mM-sucrose/5 mM-Hepes/KOH, pH 7.6. The Mössbauer spectra arising from liposomes incorporating mitochondrial proteolipid are shown in Fig. 3. Fig. 3(a) shows the spectrum obtained when triethyl[^{119}Sn]tin (11 nmol·mg of protein $^{-1}$) was added to mitochondria, followed by isolation of the proteolipid fraction. Fig. 3(b) shows the spectrum obtained after the addition of triethyl[^{119}Sn]tin (58 nmol·mg of protein $^{-1}$) to the proteolipid fraction prepared from untreated mitochondria. In both cases there is a clearly visible inner doublet [$\Delta = 1.41 \pm 0.1 \text{ mm} \cdot \text{s}^{-1}$, $\delta = 1.12 \pm 0.05 \text{ mm} \cdot \text{s}^{-1}$ for Fig. 3(a); $\Delta = 1.57 \pm 0.1 \text{ mm} \cdot \text{s}^{-1}$, $\delta = 1.25 \pm 0.05 \text{ mm} \cdot \text{s}^{-1}$ for Fig. 3(b)]. The isomer shift and quadrupole splitting of these doublets are sufficiently close to those of the high-affinity site ($\Delta = 1.67 \pm 0.1 \text{ mm} \cdot \text{s}^{-1}$, $\delta = 1.22 \pm \text{mm} \cdot \text{s}^{-1}$) as to make it very likely that the

Fig. 3. Mössbauer spectra of triethyl[^{119}Sn]tin species bound to proteolipid liposomes

(a) Mitochondria (8 g of protein) were suspended in a total volume of 100 ml with 250 mM-sucrose/5 mM-Hepes/KOH, pH 7.6. Triethyl[^{119}Sn]tin chloride (87 μmol), dissolved in 2 ml of ethanol, was added slowly with stirring. After 30 min at 4°C the mitochondria were sedimented and the proteolipid fraction (30 mg of protein) was isolated and incorporated into liposomes as described in the text. (b) The proteolipid fraction (34 mg of protein) isolated from 8 g of mitochondria was dissolved in 10 ml of chloroform/methanol (2:1, v/v). After the addition of 2 μmol of triethyl[^{119}Sn]tin, the sample was incubated for 1 h at room temperature and the proteolipid was incorporated into liposomes.

same triethyltin co-ordination state is responsible for all three of these spectra.

The outer doublets observed when triethyltin interacts with proteoliposomes may indicate two additional triethyltin environments, since the left-hand component of the doublet is rather broad and

Fig. 4. Mössbauer spectrum of triethyl[^{119}Sn]tin species in soya bean phospholipid liposomes

Liposomes were prepared as described in the text from 77 mg of soya bean phospholipids in the presence of 4.3 μmol of triethyl[^{119}Sn]tin.

may be comprised of two absorption peaks. Fig. 4 shows the spectrum from the interaction of triethyl[^{119}Sn]tin with soya bean phospholipid liposomes in the absence of proteolipid. The spectrum is very similar to that of the outer doublet in Fig. 3. The left-hand component is broad and probably double, while the right-hand component appears to be single. Thus if there are two components in this doublet, the right-hand sectors of the spectra must be coincident. These components of the spectrum must arise from triethyltin species partitioned into the phospholipid bilayer. However, the inner doublet observed in Fig. 3 is absent from Fig. 4, reinforcing the suggestion that it arises from the interaction of triethyltin with the proteolipid.

Discussion

In order to investigate the binding site for triorganotin compounds, Cain *et al.* (1977) used ^3H -labelled dibutylchloromethyltin chloride, which was expected to bind at the same site as trialkyltins but to form a stable covalent link by reaction of the chloromethyl group with an adjacent part of the binding site. They found that after reaction of this compound with yeast submitochondrial particles most of the radioactivity could be extracted with chloroform/methanol, but only a very small percentage (2%) was precipitated by diethyl ether. Their further investigations were mainly on the ether-soluble fraction, although they did report that a radioactively labelled component of M_r 6000–8000

was present in the chloroform/methanol extract. The original inhibitor-to-protein ratio used by Cain *et al.* (1977) was about $8\text{ nmol}\cdot\text{mg}$ of protein⁻¹ and on the basis of the 1:1 stoichiometry found between the high-affinity triethyltin binding site and the mitochondrial ATPase (Farrow & Dawson, 1978) it is possible that quite a high proportion of dibutylchloromethyltin label was attached to components other than the ATPase.

The results reported here by using [³H]triphenyltin chloride as the label show a very similar pattern to that found by Cain *et al.* (1977), in that only about 1% of the radioactivity originally associated with the mitochondria is precipitated in the proteolipid fraction. However, since the concentration of the F1 ATPase in rat liver mitochondria is about $0.12\text{ nmol}\cdot\text{mg}$ of protein⁻¹ (Bertina *et al.*, 1973), only about 4% of the original triphenyltin chloride ($3.19\text{ nmol}\cdot\text{mg}$ of protein⁻¹) is likely to be bound to the ATPase. The binding of trialkyltin compounds to mitochondria is slowly reversible (Cain *et al.*, 1977) so that a 1% recovery of triphenyltin in the proteolipid fraction is compatible with the involvement of this species in the inhibition of the ATPase. Furthermore, there is considerable enrichment of [³H]triphenyltin in the proteolipid fraction, compared with the original mitochondria. The radioactivity was associated with a protein component with apparent M_r 5000–6000 in agreement with the results of Cain *et al.* (1977) for yeast mitochondria. The results are also compatible with the findings of Aldridge & Street (1970), who found that the high-affinity triethyltin binding site was not extracted from rat liver mitochondria by 1% Triton X-100, suggesting that it was either very hydrophobic or tightly bound in a high-molecular-weight complex.

Since the bond formed between trialkyltins and a ligand attached to the fourth co-ordination position is very susceptible to ligand exchange or hydrolysis, the distribution of radioactivity during a fractionation procedure cannot be taken as conclusive evidence of the distribution of radioactivity in the original mitochondria. However, the results using Mössbauer spectroscopy on triethyl[¹¹⁹Sn]tin binding strongly suggest that the proteolipid fraction, after ether precipitation, contains a component which binds triethyltin in a very similar environment to that which causes inhibition of the ATPase activity of whole mitochondria. The observation that a very similar spectrum is obtained whether triethyl[¹¹⁹Sn]tin was added before or after the isolation of the proteolipid fraction indicates that the ligand does not have to be attached to maintain the binding component in its native state during isolation, in contrast with the adenine nucleotide transporter (Kramer *et al.*, 1977) which is very unstable unless isolated in the presence of inhibitor.

The relationship between the sites of inhibition of the ATPase by triethyltin chloride and dicyclohexylcarbodi-imide is not yet clear. It appears that in both cases binding to a low-molecular-weight proteolipid causes inhibition, but there is as yet no evidence that the proteolipid is the same in both cases. The prior reaction of mitochondria with dicyclohexylcarbodi-imide does not prevent the binding of either triethyltin chloride (Farrow, 1978) or dibutylchloromethyltin chloride (Partis *et al.*, 1980), so that the binding sites are not identical. However, Partis *et al.* (1980) showed that addition of dibutylchloromethyltin chloride modified the e.s.r. spectrum of a nitroxide analogue of dicyclohexylcarbodi-imide bound to the mitochondria. The modification of the spectrum was consistent with the dicyclohexylcarbodi-imide analogue either becoming more immobile or entering a more polar environment. In either case, it would appear that the binding sites for the two inhibitors are likely to be quite close together.

An associated problem is the nature of the ligand to which the trialkyltin compounds bind when they inhibit the ATPase. Elliott *et al.* (1979) studied the binding of triethyltin chloride to cat haemoglobin, which also has a high affinity for trialkyltin compounds. They reported a Mössbauer spectrum (quadrupole splitting $1.74\text{ mm}\cdot\text{s}^{-1}$, isomer shift $1.48\text{ mm}\cdot\text{s}^{-1}$) which is quite similar to that for triethyltin bound to the mitochondrial ATPase. However, in the case of cat haemoglobin, Elliott *et al.* (1979) by the use of chemical modification methods and internally co-ordinated trialkyltin compounds, produced convincing evidence that triethyltin binds as a *cis*-pentaco-ordinate species, probably bridging a histidine and a cysteine residue. In the case of the ATPase inhibition site, Farrow & Dawson (1978) found evidence that triethyltin bound as a four co-ordinate species. Internally co-ordinated trialkyltin derivatives were potent inhibitors of the ATPase as long as one co-ordination position was free, suggesting that only one bond is formed between triethyltin and its inhibitory site. We have, however, so far failed to match the Mössbauer spectrum obtained from the mitochondrial high affinity binding site with either published data for triethyltin derivatives (Smith, 1970) or model compounds prepared in this laboratory. It seems likely that a more fruitful approach will be an investigation of the nature of the proteolipid responsible for the binding of trialkyltin compounds.

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