Binding of Triethyltin to Cat Haemoglobin and Modification of the Binding Sites by Diethyl Pyrocarbonate

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Cat haemoglobin binds 2 mol of triethyltin/mol of haemoglobin. Pretreatment of the haemoglobin with diethyl pyrocarbonate at pH 6.0 prevents binding to one site only, whereas photo-oxidation with Methylene Blue removes both sites. Pretreatment of rat haemoglobin with diethyl pyrocarbonate also leads to the loss of one binding site. The possibility is discussed that the two binding sites for triethyltin on both cat and rat haemoglobin have a different chemical nature.

Triethyltin binds to mitochondria from rat liver and brown adipose tissue (Aldridge & Street, 1970) and to a limited number of tissue proteins: rat haemoglobin (Rose, 1969), myelin from the rat central nervous system (Lock & Aldridge, 1975) and a soluble fraction from the cytosol of rat and guinea-pig liver (Rose & Lock, 1970). In contrast, triethyltin has little affinity for other proteins, including some haemoglobins (Rose & Aldridge, 1968).

Trialkyltin salts are known to inhibit mitochondrial function in three mechanistically different ways (Selwyn *et al.*, 1970; Rose & Aldridge, 1972; Aldridge, 1977), one of which involves binding of the tin compound to a component of the energy-conservation system. Since the chemistry of the energy-conservation system is not understood, it is clearly of interest to establish the chemical basis of the affinity of triethyltin for proteins.

Rose (1969) proposed that the binding site in rat haemoglobin consists of a pair of histidine residues. The low solubility of rat haemoglobin has restricted further work, and in the present paper the more soluble cat haemoglobin is shown to bind triethyltin. Pretreatment of cat haemoglobin with diethyl pyrocarbonate (ethoxyformic anhydride), as a specific reagent for histidine, has been investigated and the results show that the two sites are different.

Materials and Methods

Materials

Triethyltin sulphate was prepared from triethyltin hydroxide, supplied by the Tin Research Institute, Greenford, Middx., U.K., as described by Aldridge & Cremer (1955). Triethyl[^{113}Sn]tin chloride (8.6 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and stored as a 100 mM stock solution in ethanol. This was used in

the binding studies undiluted or diluted with unlabelled triethyltin sulphate. Synacthen, a pure synthetic polypeptide comprising the first 24 amino acid residues of human adrenocorticotropin, was the gift of Dr. D. Elliott of C.I.B.A., Horsham, Sussex, U.K. Triethyl- and tripropyl-lead acetate were purchased from Alfa Chemicals, Beverley, MA, U.S.A.; *N*^ε-acetylhistidine and diethyl pyrocarbonate were from Sigma Chemical Co., St. Louis, MO, U.S.A.; diethyl pyrocarbonate, FeCl_3 solution (a standard solution for atomic absorption spectroscopy) and all other chemicals were from BDH Chemicals, Poole, Dorset, U.K.

Cat and rat haemoglobins were prepared by lysing in an equal volume of water erythrocytes that had been previously washed three times with 0.9% NaCl. The stroma and intact cells were removed by centrifugation at approx. 10000g for 15 min. The lysate (2–3 mM-haemoglobin) was left at 4°C and crystals of oxyhaemoglobin were allowed to form over several weeks. These were rinsed once in cold water, dissolved in 0.1 M- KH_2PO_4 adjusted with NaOH to pH 6.0 and stored at 4°C.

Methods

Equilibrium dialysis (triethyltin concentrations 5–100 μM), measurement of the triethyl[^{113}Sn]tin and the photo-oxidation of the haemoglobin were carried out as described by Rose (1969).

The purity of the diethyl pyrocarbonate was estimated by standardization against *N*^ε-acetylhistidine (Holbrook & Ingram, 1973) and found to be 80–85% pure. Since one of the batches of diethylpyrocarbonate was inactive, the above assessment of purity should always be carried out.

Reaction of diethyl pyrocarbonate with haemoglobin. Diethyl pyrocarbonate (5 mM final concn.; freshly prepared in acetonitrile or ethanol) was added to the haemoglobin (20 μM) in 0.1 M- KH_2PO_4

adjusted to pH 6.0 with NaOH. The volumes of the two solutions were such that the concentration of organic solvent was less than 1% (v/v). After incubation at 20–22°C for the required time the reaction was stopped by the addition of imidazole to 10 mM and the pH adjusted to 7.3. This, together with a control solution containing the same concentrations of imidazole and solvent, was used for the dialysis experiments.

Spectrophotometric determination of ethoxycarbonylhistidine. The reaction of diethyl pyrocarbonate with haemoglobin in 0.1 M-KH₂PO₄ at pH 6.0 was followed spectrophotometrically at 240 nm (Ovadi *et al.*, 1967) on a Pye– Unicam SP. 1800 recording spectrophotometer. Before the addition of diethyl pyrocarbonate to the test cuvette an equivalent volume of solvent was added to the reference cuvette.

Determination of the molar extinction coefficient for oxyhaemoglobin solutions. The concentrations of oxyhaemoglobin solutions were derived from their A_{540} by using a molar extinction coefficient based on iron determination, by using 1,10-phenanthroline for bivalent iron, the latter being liberated from the haemoglobin by digestion with HClO₄/H₂SO₄ followed by reduction with hydroxylamine.

Results

Binding of triethyltin to cat haemoglobin

Extinction coefficients for human, rat and cat haemoglobin have been determined (Table 1). That for human haemoglobin agrees with published values, but that for rat does not. Also, the values for these three species are not the same.

Scatchard (1949) analysis of the binding of triethyltin to cat haemoglobin is in Fig. 1. The plot is linear, indicating a single class of site with an affinity constant of $3.5 \times 10^4 \text{ M}^{-1}$. The number of binding sites per molecule of haemoglobin is 2.0. The previously published value of 2.2 for rat haemoglobin (Rose, 1969) also becomes 2.0 when the correct molar

extinction coefficient for rat haemoglobin is used (cf. Table 1). The affinity constant for triethyltin and cat haemoglobin ($3.5 \times 10^4 \text{ M}^{-1}$) is lower than the published value for rat haemoglobin ($3.2 \times 10^5 \text{ M}^{-1}$; Rose, 1969). This difference is mainly due to the complexing of triethyltin by phosphate (Rose, 1969). Other experiments indicate that the affinity constant for triethyltin and cat haemoglobin determined in 0.1 M-Tris/HCl buffer, pH 8, is $1.0 \times 10^5 \text{ M}^{-1}$.

Molar extinction coefficient for ethoxycarbonylhistidine

Values of $3200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Ovadi *et al.*, 1967) and $3600 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Holbrook & Ingram, 1973) derived from the reaction of diethyl pyrocarbonate with *N*^α-acetylhistidine have frequently been used for the

Fig. 1. *Binding of triethyltin to cat haemoglobin* The results of two identical experiments (●, ▲) are plotted using a molar extinction coefficient of $4.84 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for cat haemoglobin: the number of binding sites per molecule of haemoglobin (n) is 2.0 and the affinity constant K is $3.5 \times 10^4 \text{ M}^{-1}$ in phosphate buffer.

Table 1. *Molar extinction coefficients for haemoglobin solutions*

Values are given $\pm$ s.d. for the numbers of observations in parentheses where available. The absorbance of carboxy- and oxy-haemoglobin solution is the same at 537 and 540 nm respectively.

Species	Haemoglobin	Wavelength (nm)	$10^{-4} \times \epsilon (\text{M}^{-1} \cdot \text{cm}^{-1})$	Reference
Human	Carboxy-	538	5.96	Allison & Cecil (1958)
	Oxy-	540	5.72	
	Oxy-	540	5.84	Van Assendelft & Zijlstra (1975)
	Oxy-	540	$5.83 \pm 0.07 (5)$	
Rat	Carboxy-	537	5.96	Present paper
	Oxy-	540	$5.34 \pm 0.18 (9)$	Rose (1969)
Cat	Oxy-	540	$4.84 \pm 0.27 (12)$	Present paper

Table 2. *Molar extinction coefficients for ethoxycarbonyl derivatives*

The maximum and minimum values of ϵ at 240nm (with the s.d. and number of readings) are given with the time when measured. Concentrations of reactants are as in Fig. 2.

	$\epsilon_{(\max.)}$ ($M^{-1} \cdot cm^{-1}$)	t (min)	$\epsilon_{(\min.)}$ ($M^{-1} \cdot cm^{-1}$)	t (min)
Imidazole	7680 ± 282 (3)	7	3930 ± 80 (3)	45
<i>N</i> ² -Acetylhistidine	4250 ± 89 (4)	12	3710 ± 132 (4)	60
L-Histidine	3780 ± 111 (4)	16	3540 ± 111 (4)	100
L-His-L-Tyr	4250 ± 117 (2)	11	3680 ± 78 (2)	65
Synacthen	3570 ± 47 (3)	13	3390 ± 37 (3)	50

Fig. 2. Dependence of the molar extinction coefficient of ethoxycarbonyl derivatives on time of reaction. Reaction of diethyl pyrocarbonate (5mm) with imidazole (1), *N*²-acetylhistidine (2) and Synacthen (3) (0.035–0.2mm). The curves represent the mean of three recorded spectra. The ϵ values (Table 2) are derived from the maximum and plateau regions.

Fig. 3. Triethyltin binding to cat haemoglobin pretreated with diethyl pyrocarbonate

Haemoglobin was pretreated for 1 (Δ), 2 ($\circ$) and 5 ($\blacktriangle$) min, and for two consecutive treatments of 15 min ($\square$); control+imidazole ($\bullet$) (see under 'Methods').

determination of the number of histidine residues in proteins. More recently a value of $3720 M^{-1} \cdot cm^{-1}$ has been obtained for the dipeptide His-Tyr (York & Roberts, 1976).

In the present work, however, the molar extinction coefficients for the products of the reaction of diethyl pyrocarbonate with imidazole and other model compounds, including the synthetic peptide Synacthen, were found to vary considerably (Table 2) and, in some cases, to change with time (Fig. 2). The reaction of diethyl pyrocarbonate with various imidazole derivatives is therefore not straightforward, a conclusion that is supported by the observation that reactions secondary to the formation of monoethoxycarbonylhistidine from *N*²-acetylhistidine occur in the presence of excess of diethyl pyrocarbonate (Avaeva & Krasnova, 1975).

From the limited number of compounds examined it appears that the reaction may be more straightforward the greater the molecular weight. Consequently the value of $3570 M^{-1} \cdot cm^{-1}$ (Table 2) has been

used in the present paper to calculate the number of histidine residues in haemoglobin.

Tyrosine residues can be modified by diethyl pyrocarbonate in proteins with different reaction conditions (Burstein *et al.*, 1974). Our experiments show that the A_{280} was always small (<3% of the A_{240}), indicating low reactivity of tyrosine residues in Synacthen and cat haemoglobin.

Modification of cat haemoglobin by reaction with diethyl pyrocarbonate or photo-oxidation

Treatment of cat haemoglobin with diethyl pyrocarbonate for increasing time-periods progressively decreases the number of triethyltin-binding sites per molecule to half (Fig. 3) after 5 min incubation, and incubation for longer times has no further effect.

Fig. 4. Rate of loss of the diethyl pyrocarbonate-sensitive site compared with the rate of loss of histidine residues from cat haemoglobin

Histidine residues calculated using $\epsilon = 3570 \text{ M}^{-1} \cdot \text{cm}^{-1}$ were for ethoxycarbonylhistidine. Rates measured over the first 3 min were for loss of binding (●) 0.275 min^{-1} and loss of histidine residues (○) 0.057 min^{-1} .

The affinity of the sites after incubation for 1 min is decreased to approximately half, but further incubation has little effect.

Diethyl pyrocarbonate decomposes in aqueous solution at different rates, depending on pH and buffer (Berger, 1975). Binding of triethyltin to cat haemoglobin after two consecutive incubations for 15 min with diethyl pyrocarbonate (Fig. 3) indicates that the inability to remove more than one binding site is not due to breakdown of the diethyl pyrocarbonate. Breakdown of the ethoxycarbonyl derivative is possible during the 17 h dialysis period. However, incubation of cat haemoglobin with diethyl pyrocarbonate for 15 min, followed by storage of the haemoglobin at 4°C for 24 or 48 h before measurement of triethyltin binding, gave results identical with those obtained when binding was measured immediately after incubation with diethyl pyrocarbonate (Fig. 3). Therefore unless such a breakdown from one site only occurs very rapidly, our experimental results cannot be caused by this re-activation.

The formation of ethoxycarbonylhistidine was measured spectrophotometrically at 240 nm after reaction of cat haemoglobin with diethyl pyrocarbonate, and the number of histidine residues modified after different times of incubation was calculated by using a molar extinction coefficient of $3570 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The results relating to total histidine are expressed in Fig. 4, along with the corresponding

Fig. 5. Effect of photo-oxidation on binding of triethyltin to cat haemoglobin

Haemoglobin with 0.01% Methylene Blue was exposed to light for 0 (□), 45 (●) and 90 (○) min.

loss of binding of triethyltin to the diethyl pyrocarbonate-sensitive site. The rate of loss of binding is clearly greater than the rate of loss of histidine residues, and values derived from the first 3 min of reaction show the initial rate of loss of binding to be approximately five times the initial rate of loss of histidine.

In contrast with the results obtained with diethyl pyrocarbonate, photo-oxidation of cat haemoglobin with 0.01% Methylene Blue for periods up to 90 min (Fig. 5) decreased the triethyltin-binding sites to 35% of the control value. Both binding sites in cat haemoglobin and rat haemoglobin (Rose, 1969) can therefore be destroyed by photo-oxidation. The rate of loss of binding is not the same for these two species of haemoglobin. This effect of photo-oxidation cannot be due to oxidation of the haemoglobin to ferrihaemoglobin, since this also binds triorganotins (Rose, 1969).

Modification of rat haemoglobin with diethyl pyrocarbonate

Preincubation of rat haemoglobin with diethyl pyrocarbonate decreases the number of triethyltin-binding sites without changing their affinity (Fig. 6). Binding ability is lost at a slower rate than with cat haemoglobin (Fig. 3), and even after two consecutive 30 min incubations only approx. 50% of the sites are lost. Interpretation of the results of incubation for longer time-periods or with higher concentrations of

Fig. 6. Triethyltin binding to rat haemoglobin pretreated with diethyl pyrocarbonate

Haemoglobin was pretreated for 1 (○), 7 (▲) and 45 (△) min, and for two consecutive treatments of 30 min (□); control+imidazole (●) (see under 'Methods'). The derived affinity constant for the control was $3.8 \times 10^4 \text{ M}^{-1}$.

Fig. 7. Rate of loss of diethyl pyrocarbonate-sensitive site compared with the rate of loss of histidine residues from rat haemoglobin

Histidine residues were calculated using $\epsilon = 3570 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for ethoxycarbonylhistidine. ●, Loss of binding; ○, loss of histidine.

Fig. 8. Effect of tripropyl- and triethyl-lead on triethyltin binding to cat haemoglobin

[Trialkyl-lead]/[triethyltin] = 1:1 and both compounds were added simultaneously to the haemoglobin. The different experiments are triethyltin alone (●), triethyltin and tri-*n*-propyl-lead (○) and triethyltin and triethyl-lead (△).

4). Fig. 7 shows that, in contrast with cat haemoglobin, the initial rate of loss of triethyltin binding to the diethyl pyrocarbonate-sensitive site in rat haemoglobin is similar to the initial rate of loss of total histidine.

Competition with triethyltin by triethyl- and tripropyl-lead for cat haemoglobin

Triethyl- and tripropyl-lead compete effectively with triethyltin for the binding sites on cat haemoglobin (Fig. 8). Triethyltin binding still occurs in the presence of equimolar concentrations of trialkyl-lead, with an apparent affinity constant for triethyl-lead of $9.9 \times 10^4 \text{ M}^{-1}$, and for tripropyl-lead of $3.6 \times 10^4 \text{ M}^{-1}$ (Lock & Aldridge, 1975). Triethyltin binding to cat haemoglobin may be almost prevented by concentrations of trialkyl-lead ten times higher than of triethyltin; thus both triethyl- and tripropyl-lead have access to both triethyltin-binding sites.

Discussion

Triethyltin binds to a limited number of proteins, which suggests that the binding is due to the three-dimensional structure of the protein rather than to a single chemical group. Trialkyltin compounds form penta-co-ordinate complexes with certain donor ligands (Luijten *et al.*, 1962; Poller, 1965) and it seems likely that such penta-co-ordination of tin

diethyl pyrocarbonate is difficult, owing to the formation of a precipitate of modified haemoglobin.

The initial rate of loss of histidine residues in rat haemoglobin measured spectrophotometrically (Fig. 7) is comparable with that in cat haemoglobin (Fig.

between two groups with a defined spatial relationship is involved in the selective binding of triethyltin to certain proteins.

Two molecules of triethyltin bind to one molecule of cat haemoglobin (Fig. 1) with an affinity constant similar to that for rat haemoglobin (Rose, 1969). The number of binding sites for triethyltin on rat haemoglobin is now 2.0 per molecule when the correct molar extinction coefficient for rat oxyhaemoglobin is used (Table 1). Therefore there is no need to postulate co-operativity (Rose, 1969) between the two sites.

The higher affinity of triethyl-lead for the triethyltin-binding site in haemoglobin and other proteins (Fig. 8; Lock & Aldridge, 1975) is consistent with the greater capacity of triorganoleads to form penta-coordinate complexes. Previous attempts to determine the chemical identity of the binding site were based on photo-oxidation studies (Rose, 1969; Rose & Lock, 1970); both sites were affected and the loss of binding could be correlated with a loss of bulk histidine residues. The pH-dependence of the binding to rat haemoglobin (Rose, 1969) and the characterization of penta-co-ordinate trialkyltin-imidazole complexes (Luijten *et al.*, 1962) supported the hypothesis that each of the two binding sites consisted of two histidine residues (Rose, 1969). Photo-oxidation of cat haemoglobin (Fig. 5) also removes both binding sites. However, after diethyl pyrocarbonate pre-treatment of both cat and rat haemoglobins (Figs. 3 and 6) only one of the two triethyltin-binding sites is lost; therefore histidine appears to be involved in only one of the binding sites and the possibility of a chemically distinct site must be considered. The demonstrated ability of tripropyl-lead to compete with triethyltin for both binding sites on cat haemoglobin (Fig. 8) suggests that diethyl pyrocarbonate can reach both sites.

The rates of reaction of both cat and rat haemoglobin with diethyl pyrocarbonate are not first order, and histidine residues are lost at different rates. Thus the rate of loss of those histidine residues relevant to binding cannot be measured, and no interpretation of the histidine/triethyltin ratio at one binding site can be made (Figs. 4 and 7).

The photo-oxidation studies of Rose (1969) led to the reasonable hypothesis that both sites consisted of two histidine residues. From the current studies it seems likely that only one site involves histidine, and we cannot say whether it contains one or two histidine residues. To answer these questions techniques must be developed to measure reaction at the relevant histidine site.

The known chemical reactivity of triethyltin makes the prediction of the amino acids of the second site difficult, but penta-co-ordinate complexes of trialkyltin compounds with ligands other than histidine have been reported (Van der Kerk *et al.*, 1962; Poller, 1965; Evans & Smith, 1975).

Our results may therefore be explained in two ways.

1. Both of the binding sites for triethyltin on cat and rat haemoglobin contain histidine residues. If this is true, one site either does not react with diethyl pyrocarbonate or does react to yield a labile product (i.e. the original histidine residue is regenerated before binding can be determined by equilibrium dialysis).

2. Only one site contains histidine residues. If this is true, the other site consists of different amino acids unreactive with diethyl pyrocarbonate but whose ability to bind triethyltin is destroyed by photo-oxidation.

We recognise that there is another possibility, i.e. diethyl pyrocarbonate does not interact with residues in the binding site(s) (whether they contain histidine or not), but instead reacts with histidine residues vital to the structural integrity of the sites. We think this is unlikely, because 1.5 M-guanidine, which is known to dissociate haemoglobin (into $\alpha\beta$ dimers), does not influence binding (B. M. Elliott & W. N. Aldridge, unpublished work).

Neither point (1) nor (2) is a completely satisfactory explanation, but although the 'two-histidine' hypothesis is attractive, the results in the present paper show that we must now consider in rat and cat haemoglobin one binding site for triethyltin containing one or two histidine residues and another which is chemically distinct.

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