Organotin–protein interactions

Binding of triethyltin bromide to cat haemoglobin

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Triethyltin binds to native cat and rat haemoglobin but not to their denatured forms or to other animal haemoglobins. Two molecules of the organotin bind to one molecule of R-state cat haemoglobin with affinity constants of about $1 \times 10^5 \text{ M}^{-1}$. Little or no triethyltin is bound to the deoxygenated (T-state) protein. Binding appears to be dependent upon the existence of a specific three-dimensional configuration of cysteine and histidine residues. The properties of the triethyltin–cat haemoglobin complex are consistent with those of a haemoglobin conformer whose allosteric equilibrium is displaced toward the R-state. Its oxygen affinity and rate of oxidation by nitrite is increased, and the rate of reduction of the methaemoglobin derivative by ascorbate is decreased. These effects of triethyltin are opposite and antagonistic to the effects of inositol hexaphosphate. They are exerted on the α - as well as β -haem groups, even though triethyltin is bound at sites on α -globin far removed from the haem groups.

INTRODUCTION

Organotin and organolead compounds are known to exert toxic effects in man and animals, but little information is available concerning the molecular basis for these effects. In efforts to elucidate their mode of action, Aldridge and his associates (Rose & Aldridge, 1968) examined the interaction of these compounds with a variety of proteins and found that they were bound to only a few. Included among these were cat and rat Hbs, which bound two molecules of triethyltin (Et_3Sn^+) or triethyl-lead per native Hb tetramer (Rose & Aldridge, 1968; Rose, 1969; Elliott & Aldridge, 1977). Other Hbs examined failed to bind these compounds, indicating that a highly specific binding site is required. Subsequent work on cat Hb established that one molecule of organometal is bound to each of the two α -globin subunits at a locus near the N-terminal region (Taketa *et al.*, 1980).

The stable Et_3Sn^+ –cat Hb complex demonstrates an increase in oxygen affinity over that of the free Hb (Taketa *et al.*, 1980). This suggests that the compound may bind preferentially to the high-affinity R-quaternary state of cat Hb, since conditions that enhance the stabilization of such a conformation lead invariably to an increase in the oxygen affinity of Hb (Perutz, 1970). Viewed from the perspective of allosteric theory, Et_3Sn^+ may thus be an effector that perturbs the RT equilibrium of Hb toward R. If so, the affinity of Et_3Sn^+ for cat Hb may depend upon the quaternary state of the protein, being higher for the oxy- or carbonmonoxy (CO)- than the deoxy-derivative. Previous binding studies conducted in Tris buffer, pH 8.0, at 4 °C have shown that Et_3Sn^+ binds to cat oxy- or CO-Hb with affinity constants of about $1 \times 10^5 \text{ M}^{-1}$ (Elliott & Aldridge, 1977). Binding to the protein in other states of haem ligation or under conditions that are known to affect the conformation of cat Hb has not been considered previously. Therefore, the present work was conducted to investigate the possibility

that binding may depend upon the quaternary structure of the protein and, thus, upon the existence of a highly specific three-dimensional configuration of ligands within the α -subunits. In addition, the allosteric effects of the interaction were analysed by examining changes in the spectra and reactivity of the haem groups.

MATERIALS AND METHODS

Chemicals

Et_3SnBr was purchased from the Ventron Corp., Danvers, MA, U.S.A. Appropriate amounts were dissolved in a small volume of ethanol (10%, v/v, final concn.) and adjusted to the desired concentration in a suitable buffer or in distilled water. InsP_6 was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. It was dissolved in water, neutralized to pH 7.0 with HCl, and adjusted to volume with distilled water. All other chemicals were of the highest purity available.

Preparation of Hb solutions

Fresh red cells from cat human blood were packed by centrifugation, freed of plasma and buffy coat, and washed three times with iso-osmotic NaCl. The packed cells were lysed by mixing with an equal volume of distilled water and 0.4 vol. of toluene, and then centrifuged at 27000 g for 20 min in a Sorvall refrigerated centrifuge to remove stroma. Hb solutions were used immediately, or bubbled with CO and stored at –70 °C. When needed, the CO-Hbs were converted into the O_2 derivatives as described by Kilmartin & Rossi-Bernardi (1971). Hb concentration was determined by the cyanmethaemoglobin procedure (Hainline, 1958). Met-haemoglobin (Met-Hb) was prepared by adding a 5-fold excess of $\text{K}_3\text{Fe}(\text{CN})_6$ or a 20-fold excess of NaNO_2 to the oxy-Hb (1 mM) solution and allowing the oxidation to

Abbreviations used: Et_3Sn^+ , triethyltin; Et_3SnBr , triethyltin bromide; InsP_6 , inositol hexaphosphate; Hb(s), haemoglobin(s); CP(A+B), carboxypeptidases A and B.

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proceed for at least 15 min at room temperature. The solution was then passed through a column (1 cm $\times$ 40 cm) of Sephadex G-25 equilibrated with 5 mM-Bistris buffer, pH 7.5, containing 100 mM-NaCl and 1 mM-EDTA. When ferricyanide was used as the oxidant, the gel-filtered Met-Hb solution was percolated through a column (1 cm $\times$ 10 cm) of Bio-Rad AG1-X8 anion-exchanger equilibrated with distilled water to remove bound $\text{Fe}(\text{CN})_6^{3-}$ ions (Hegesh & Avron, 1967). The fluoromet derivative was prepared by adding 0.1 M-NaF to a 20 μM -Met-Hb solution (Perutz *et al.*, 1974b). CP(A+B)-digested cat Hb was prepared by sequential digestion with each carboxypeptidase (Worthington) as described by Antonini *et al.* (1961) and by Lessard (1970). Generation of the expected digested product was verified by isoelectric focusing (Drysdale *et al.*, 1971), which showed a homogeneous band of Hb that migrated anodal to the undigested protein.

The half-oxidized products of cat and human Hbs $\alpha_2\beta_2^+$, were prepared by adding a 3 (cat)- or 6 (human)-fold molar excess of 0.1 M- CuSO_4 per oxy-Hb tetramer (1 mM) solution (Taketa & Antholine, 1982). After a few minutes of reaction at 24 °C, excess 20 mM-EDTA was added and the mixture was passed through a column (1 cm $\times$ 40 cm) of Sephadex G-25 equilibrated with 5 mM-Bistris buffer, pH 7.5, containing 100 mM-NaCl and 2 mM-EDTA to remove copper ions. Removal of more than 95% of the added copper was confirmed by monitoring the $g = 2.21$ and $g = 2.05$ signals for Cu^{2+} ions by e.s.r. spectroscopy. The other half-oxidized product, $\alpha_2\beta_2^0$, was prepared by reduction of 0.25 mM-Met-Hb with 6 mM-ascorbic acid, as described by Tomoda *et al.* (1978). Excess ascorbate and dehydroascorbate were then removed by Sephadex G-25 gel filtration with 5 mM-Bistris buffer, pH 7.5, containing 100 mM-NaCl and 1 mM-EDTA. This was verified by the analysis of the eluate with 2,4-dinitrophenylhydrazine (Roe & Oesterling, 1944; Bolin & Book, 1947). That $\alpha_2\beta_2^0$ and $\alpha_2\beta_2^+$ were the major products obtained from these reactions was confirmed by their presence in the expected positions in electrofocused polyacrylamide gels (Tomoda *et al.*, 1976, 1978).

Direct binding studies

Binding of Et_3Sn^+ to the various Hb derivatives was analysed by equilibrium dialysis at 25 °, a slight modification of the procedure described by Elliott & Aldridge (1977) being used. The Hb concentration was 20 μM (3 ml) in 0.1 M-Tris buffer, pH 8.0, and Et_3Sn^+ concentrations ranged from 5 to 100 μM (10 ml) in the same buffer. Control experiments indicated that equilibrium was attained within 5–7 h, but equilibration was continued for 18 h before the Et_3Sn^+ concentrations in the dialysis sac and the external buffer were analysed spectrophotometrically by a modification of the Dithizone (diphenylthiocarbazone) procedure of Aldridge & Street (1981) (described below). To determine the concentrations of Et_3Sn^+ within the sac, 1 ml of 20% (w/v) trichloroacetic acid was added to the contents and the denatured protein removed by centrifugation. Portions of the clear supernatant fluid were then assayed. The data were subjected to Scatchard (1949) analysis after making appropriate corrections for dilutions. Binding measurements with deoxy-Hb were made in a N_2 -filled glove box. Oxy-Hb was deoxygenated by repeated cycles of evacuation and flushing with N_2 . All other solutions,

including the dialysis sacs, were purged with N_2 before being transferred into the glove box. Mixtures of deoxy-Hb and Et_3Sn^+ solutions were then prepared, placed in dialysis sacs, and allowed to equilibrate for 18 h within the box. A slight positive pressure of N_2 was maintained by passing a gentle stream of N_2 through the box during this period. Under these conditions the Hb remained deoxygenated as determined from its visible spectrum. After equilibration, the sacs were removed from the surrounding buffer solution before they were brought out from the box for further analysis.

Dithizone assay

To 2 ml of the solutions containing Et_3Sn^+ , 6 ml of a buffer consisting of 1.33 M-diethanolamine, pH 9.0, 5.88 M-sodium perchlorate and 8 mM-EDTA were added. When necessary, the pH was adjusted back to 9.0 by the addition of small amounts of concentrated NaOH or HClO_4 . Chloroform (4 ml) and 2 ml of 117 μM -dithizone in chloroform were then added and the glass-stoppered tubes were vortex-mixed for 10 s. The layers were allowed to separate and the absorbance of the chloroform layer was determined in 1 cm-pathlength cells at 610 nm and corrected for the absorbance of a sample without Et_3Sn^+ . The assay was linear over the range 0–100 nmol and it was capable of detecting as little as 5 nmol of Et_3Sn^+ .

Release of triethyltin from the Et_3Sn^+ – cat oxy-Hb complex

The dissociation of Et_3Sn^+ from the oxy-Hb complex was analysed by measuring its rate of release from a 20 μM -Hb solution (3 ml) contained in a dialysis sac into an external medium containing 0.1 M-phosphate buffer, pH 6.0, with and without 1 mM- InsP_6 (10 ml). The initial concentration of Et_3Sn^+ within the sac was 80 μM . Replicate dialysis-sac systems were set up to permit the assay of sac contents and the external medium at selected intervals. To examine the effect of deoxygenation on the dissociation of Et_3Sn^+ from its oxy-Hb complex, the latter was deoxygenated, placed in dialysis sacs and equilibrated against deoxygenated buffer within a N_2 -filled glove box. Samples of dialysis-sac contents and external medium were analysed for Et_3Sn^+ at selected intervals of time.

Difference spectra

U.v./visible difference spectra were recorded in a Cary 210 spectrophotometer interfaced with an Apple II plus computer and a Cary 219–210 u.v./visible spectrophotometer wavelength-scanning program (Varian). Hb at 30 μM was used for the spectra recorded in the 450–700 nm region, and at 4.55 μM in the 240–450 nm range.

Effects on the reactivity of the α - and β -haem groups

The kinetics of oxidation of oxy-Hb and of the half-oxidized valency-hybrid Hbs by NaNO_2 were measured at 25 °C and monitored at 630 nm by using solutions containing 400 μM -oxidizable haem and 400 μM - NaNO_2 in 50 mM-potassium phosphate buffer, pH 7.4. When the effects of Et_3Sn^+ and InsP_6 on the reaction were tested, the reaction mixtures were made to contain up to 1 mM- Et_3Sn^+ and 500 μM - InsP_6 .

Rates of reduction of Met-Hb and of the valency hybrids by ascorbate were measured as described by Tomoda *et al.* (1976). A solution containing 23 μM -Met-Hb in 0.05 M-Bistris, pH 7.0, and 0.1 M-NaCl (1.5 ml total vol.) was used for the assay at 25 °C. The reaction was

Fig. 1. Scatchard plots for the binding of Et_3Sn^+ to cat Hb

The data were obtained from equilibrium dialysis as described in the Materials and methods section. ○, Cat CO-Hb; □, cat Met-Hb; △, cat oxy-Hb. The inset depicts the binding of Et_3Sn^+ to cat deoxy-Hb (■) and cat deoxy-Hb/ InsP_6 (1:2 molar ratio) (●). Affinity constants (Table 1) were determined from the slopes of the lines.

started by addition of sodium ascorbate (6 mM final concn.) and the rate of Met-Hb reduction was measured in a Cary 210 spectrophotometer at 576 nm. The experiments were performed in the presence and absence of InsP_6 (50 μM) and Et_3Sn^+ (20–115 μM).

RESULTS

Binding of Et_3Sn^+ to various forms of cat Hb

The results of studies on the binding of Et_3Sn^+ to the various forms of cat Hb are shown in Fig. 1 and Table 1. Two equivalents of Et_3Sn^+ are bound to the oxy-, CO- and Met-Hbs with affinity constants of about $9.5 \times 10^4 \text{ M}^{-1}$ – $9.7 \times 10^4 \text{ M}^{-1}$, whereas only about 0.30 equivalent is bound to cat deoxy-Hb, with an affinity constant that appears to be smaller by about one and one-half orders of magnitude. The calculated fractional value for the binding stoichiometry in the deoxygenated sample may be indicative of Et_3Sn^+ bound to a small fraction of Hb that remained in the oxygenated form due to incomplete deoxygenation, or to the fraction of the unliganded protein that is present in the R-quaternary form in the deoxygenated state of the protein. Alternatively it may be a reflection of the presence of a separate weaker binding site or of non-specific binding in the deoxygenated protein. No distinction between these possibilities can be made from the present analysis, since the conditions employed in the present work were not sufficiently sensitive to distinguish between them. In any case, it is clear that there is a substantial decrease in the binding of Et_3Sn^+ to cat Hb when the protein is converted from the oxygenated into the deoxygenated form. The influence of certain other parameters that affect the

Table 1. Binding of Et_3Sn^+ to various forms of cat Hb

Haemoglobin	$K_a \text{ (M}^{-1}\text{)}^*$	Stoichiometry (mol of Et_3Sn^+ / mol of Hb)
Oxy-Hb	9.5×10^4	2.01
CO-Hb	9.6×10^4	2.00
Met-Hb	9.7×10^4	2.03
Deoxy-Hb	1.6×10^3	0.30
Oxy-Hb/ InsP_6 (1:2)	2.3×10^4	1.59
Met-Hb/ InsP_6 (1:2)	2.3×10^4	1.21
Deoxy-Hb/ InsP_6 (1:2)	1.1×10^3	0.06
CO-CP(A+B)-Hb†	1.6×10^5	2.02
Deoxy-CP (A+B)-Hb	1.5×10^5	1.99
FluoroMet-Hb	3.8×10^4	1.47

* Binding studies were performed by equilibrium dialysis and the affinity constants and stoichiometry of binding were determined by the method of Scatchard (1949) as described in the Materials and methods section.

† CP(A+B)-Hb, carboxypeptidase A+B-digested haemoglobin.

quaternary structure of the protein were likewise analysed. These included the addition of the organic phosphate InsP_6 , which is known to perturb the allosteric equilibrium of Hb toward T (Perutz *et al.*, 1974a,b), and digestion with CP(A+B), which removes C-terminal residues to produce des-Arg-Tyr(α)-, des-His-Tyr(β)-Hb, whose allosteric equilibrium is biased toward the R-state (Antonini *et al.*, 1961; Kilmartin & Wootton, 1970; Kilmartin *et al.*, 1980). When Et_3Sn^+ binding to cat deoxy-Hb was measured in the presence of saturating

amounts of InsP_6 (molar ratio $\text{InsP}_6/\text{Hb} = 2:1$), little if any binding was observed (0.06 equiv./mol). On the other hand, two equivalents of Et_3Sn^+ were bound per mol of the CP(A+B)-digested CO-Hb with a slight, but significant, increase in affinity compared with the value for the undigested CO-Hb. Moreover, the same results were obtained even with the deoxygenated form of CP(A+B)-digested Hb, apparently because its RT equilibrium is shifted far toward the R-state. Addition of InsP_6 to Met-Hb or conversion of Met-Hb into its fluoromet derivative to shift the conformation of Met-Hb toward the T-state resulted in decreased affinity and stoichiometry of Et_3Sn^+ binding. Thus, the combined results of these analyses indicate that Et_3Sn^+ binds preferentially to the R-conformation of cat Hb.

Dissociation of Et_3Sn^+ from its oxy-Hb complex upon deoxygenation

The effect of deoxygenation on the dissociation of the Et_3Sn^+ from the oxy-Hb complex and its subsequent efflux from the contents of a dialysis sac is shown in Fig. 2. That deoxygenation causes the release of Et_3Sn^+ from the complex is indicated by the significantly greater decrease in the Et_3Sn^+ concentration within the sac for the deoxygenated sample compared with the oxygenated sample. Under the conditions employed, equilibrium distribution of Et_3Sn^+ between the sac and external buffer was attained at about 6 h, and practically all of the compound was accounted to be in the free form when the

Fig. 2. Dissociation of Et_3Sn^+ from its complex with cat Hb upon deoxygenation

The cat Hb- Et_3Sn^+ complex was prepared and the measurements made as described in the Materials and methods section. ○, Cat oxy-Hb; ●, cat deoxy-Hb; □, cat oxy-Hb/ InsP_6 (1:2 molar ratio); ■, cat deoxy-Hb/ InsP_6 (1:2 molar ratio).

sample was deoxygenated. In contrast, the distribution of Et_3Sn^+ for the oxygenated sample was consistent with a 2:1 Et_3Sn^+ -Hb complex remaining at equilibrium. These results indicate that the compound is rapidly dissociated from cat oxy-Hb when the latter is deoxygenated.

Properties of the triethyltin - cat Hb complex

Difference spectra. The interaction of Et_3Sn^+ with cat Met-Hb was also examined by analysis of difference spectra. Figs 3(a) and 3(b) show the visible and u.v. difference spectra generated when increasing amounts of Et_3Sn^+ were added to the Met-Hb solution. Three negative-difference peaks in the regions of 500, 611 and 655 nm are dominant features of the visible spectrum, indicative of perturbations in the environment of the haem groups. Comparison of these peaks with those that appear in the difference spectrum of the InsP_6 -Met-Hb complex reveals that the corresponding peaks occur in opposite directions. The positive difference peaks in the region of 512, 600 and 649 nm in the spectrum of the latter have been ascribed to an InsP_6 -induced shift in the quaternary structure of the Hb toward the T-conformation (Perutz *et al.*, 1974a,b). The opposing influence of Et_3Sn^+ and InsP_6 reflected in the visible difference spectra of cat Met-Hb is in agreement with the observations that InsP_6 binds with higher affinity to the deoxy(T) (Mauk, 1974), and Et_3Sn^+ to the oxy(R) conformation of cat Hb. Similar differences between the Met-Hb complexes of Et_3Sn^+ and InsP_6 can also be seen in the u.v. region of the difference spectra. The positive peaks at 294 and 302 nm characteristic of the spectrum for the InsP_6 -Met-Hb complex are lacking in the spectrum for the Et_3Sn^+ -Met-Hb complex (Fig. 3b).

Reactivity of the haem groups. Previous work (Taketa *et al.*, 1980) showed that Et_3Sn^+ raises the O_2 affinity of cat Hb, presumably via an allosteric mechanism. It also exerts an apparent allosteric effect on the autoxidation of the haem groups in cat oxy-Hb. The time required for 50% conversion into Met-Hb at 25 °C (120 μM total haem concn.) was increased from about 95 h in its absence to 112 and 127 h when Et_3Sn^+ was provided at molar ratios ($\text{Et}_3\text{Sn}^+/\text{Hb}$) of 2:1 and 5:1 respectively (results not shown). This rate-retarding effect of Et_3Sn^+ can be explained by its preferential binding to oxy-Hb and stabilizing the haem iron- O_2 bond, since the autoxidation evidently proceeds through a mechanism involving dissociation of oxygen and generation of deoxy-Hb (Wallace *et al.*, 1982).

When NaNO_2 was used to promote the oxidation, the rate of conversion of cat oxy-Hb to Met-Hb was markedly enhanced by Et_3Sn^+ (Fig. 4). This effect is related to the specific interaction of Et_3Sn^+ with cat oxy-Hb, since there was no influence of the compound on the oxidation of human or other animal oxy-Hbs. The increase in rate was associated with a shortening of the lag phase that is characteristic of the kinetics of the reaction (Wallace & Caughey, 1975), and it was maximal in the presence of about 2–4 mol of Et_3Sn^+ /mol of cat oxy-Hb. It was in contrast with the effect of InsP_6 , which prolonged the lag phase of the reaction and diminished the overall rate of oxidation (Fig. 4). The rate-retarding influence of saturating amounts of InsP_6 was diminished when small increments of Et_3Sn^+ were added to the reaction mixture, and it was completely overcome when

Fig. 3. Difference spectra generated by the interaction of Et_3Sn^+ with cat Met-Hb

(a) Cat Met-Hb ($30 \mu\text{M}$) in 20 mM-Bistris buffer, pH 7.0, was incubated with the indicated concentrations (μM) of Et_3Sn^+ for 5 min at room temperature before the spectra from 460 to 680 nm were recorded. Molar ratios of triethyltin/Hb ranged from 0.5 to 10.0. The broken line is the difference spectra recorded for $30 \mu\text{M}$ -cat Met-Hb with $150 \mu\text{M}$ - InsP_6 . (b) Cat Met-Hb ($4.55 \mu\text{M}$) in 20 mM-Bistris buffer, pH 7.0, was incubated with $20 \mu\text{M}$ - InsP_6 or $20 \mu\text{M}$ - InsP_6 and $20 \mu\text{M}$ - Et_3Sn^+ for 5 min at room temperature before the spectra from 250 to 330 nm were recorded. Curve 1, cat Met-Hb plus InsP_6 ; curve 2, cat Met-Hb plus InsP_6 and Et_3Sn^+ .

Fig. 4. Kinetics of oxidation of cat oxy-Hb by NaNO_2 in the presence or absence of Et_3Sn^+ and InsP_6

Cat oxy-Hb ($100 \mu\text{M}$, $400 \mu\text{M}$ in oxidizable haem) was oxidized with NaNO_2 as described in the text. Curve 1, cat oxy-Hb; curve 2, cat oxy-Hb plus $500 \mu\text{M}$ - Et_3Sn^+ ; curve 3, cat oxy-Hb plus $500 \mu\text{M}$ - InsP_6 ; curve 4, cat oxy-Hb plus $500 \mu\text{M}$ - Et_3Sn^+ and $500 \mu\text{M}$ - InsP_6 .

equimolar amounts of Et_3Sn^+ and InsP_6 were present, confirming that Et_3Sn^+ is bound with higher affinity to cat oxy-Hb than is InsP_6 (Mauk & Taketa, 1972; Mauk, 1974).

To evaluate the allosteric effect of Et_3Sn^+ on the oxidation of the α - and β -haem groups separately, the oxidation by NO_2^- of the cat valency hybrids $\alpha_2^+\beta_2^{\text{O}_2}$ and $\alpha_2^{\text{O}_2}\beta_2^+$ was examined. Analysis of the former gave information on the influence of Et_3Sn^+ on the β -haems, and the latter on the α -haem groups. The results (not

shown) indicated that, in the presence of saturating amounts of Et_3Sn^+ , the rates of oxidation of both hybrids were enhanced to about the same degree, indicating that the compound exerts its influence equally at the α - as well as at the β -haem group, even though it is bound only to the subunits.

Tomoda *et al.* (1976, 1978) demonstrated that the reduction by ascorbate of the β -haem groups in human Met-Hb occurs much more rapidly than that of the α -haem groups, such that the valency hybrids $\alpha_2^+\beta_2^{\text{O}_2}$ is

Fig. 5. (a) Reduction of cat Met-Hb (23 μ M) by ascorbate (6.0 mM) in the presence or absence of Et_3Sn^+ and (b) the kinetics of reduction of the cat Hb valency hybrid $\alpha_2\beta_2^+$ by ascorbate in the presence or absence of Et_3Sn^+ .

(a) The course of the reduction at room temperature was monitored for 20 min at 576 nm. Curve 1, cat Met-Hb; curve 2, cat Met-Hb plus 200 μ M- InsP_6 ; curve 3, cat Met-Hb plus 200 μ M- Et_3Sn^+ ; curve 4, cat Met-Hb plus 200 μ M- InsP_6 and 200 μ M- Et_3Sn^+ . (b) The conditions for the reaction were identical with those described in (a). Curve 1, $\alpha_2\beta_2^+$; curve 2, $\alpha_2\beta_2^+$ with 200 μ M- Et_3Sn^+ .

found as the major component in the mixture when the rapid phase of the biphasic reaction is completed. They showed that InsP_6 stimulated the rate of this reaction and concluded that an allosteric effect on the haem was mediated via a shift toward the quaternary T-conformation of Met-Hb. The reduction of cat Met-Hb by ascorbate proceeds similarly, with the preferential reaction at the β -haem groups, and it is also enhanced by InsP_6 . The results shown in Fig. 5(a) indicate that Et_3Sn^+ retards the rate of reduction of cat Met-Hb by ascorbate and reverses the stimulation in rate due to InsP_6 . Fig. 5(b) shows that the reduction of the valency hybrid, $\alpha_2\beta_2^+$, by ascorbate was similarly affected by Et_3Sn^+ . Thus the α -subunit-bound Et_3Sn^+ clearly affects the reactivity of the β -haem groups.

DISCUSSION

The higher affinity of Et_3Sn^+ for liganded than for unliganded forms of cat Hb, coupled with the nature of its effects on the functional properties of the haem groups, suggests that the compound binds more strongly to the R- than to the T-quaternary form of the protein and shifts the equilibrium between the two forms towards the R-form. That a quaternary structure change is induced

is suggested by the observation that Et_3Sn^+ exerts functional effects that are opposite and antagonistic to those that are produced by InsP_6 , an effector that is known to bind more strongly to the T-form of these proteins and to shift the RT equilibrium towards the T-form (Perutz & Ten Eyck, 1971; Perutz *et al.*, 1974a; Tomoda *et al.*, 1977). This is also indicated by the fact that the reactivities of both α - and β -haem groups are affected, even though Et_3Sn^+ binding occurs exclusively on the α -subunits of the protein. However, the stereochemical mechanism by which these effects are produced is not known, since the Et_3Sn^+ binding sites (discussed below) appear to occur in regions of the protein that have not been implicated in the allosteric mechanism of the protein (Baldwin & Chothia, 1979).

The binding sites are evidently composed of a highly specific three-dimensional arrangement of donor ligands that become disrupted when the protein is deoxygenated. From the results of the effects of chemical modification and from analysis of Mossbauer spectra of the Et_3Sn^+ -cat Hb complex, Aldridge and his associates (Elliott & Aldridge, 1977; Elliott *et al.*, 1979) concluded that the tin atom of Et_3Sn^+ is pentaco-ordinated, axial donor ligands being provided by cysteine and imidazole residues of globin. Evidence presented in our previous studies suggests that one of the donor ligands is provided by Cys-13 of the α -globin subunits (Taketa *et al.*, 1980).

Involvement of its -SH group is suggested by the fact that reactivity of the group is masked by Et_3Sn^+ . Previously, His-20 α had been proposed as a possible second donor ligand on the basis of its proximity in the protein sequence to Cys-13 (Taketa *et al.*, 1980).

However, recent examination of a simulated three-dimensional structure of cat Hb, based on the known high-resolution structures of human Hb, rules out involvement of this residue, since it is separated from the sulphur atom of Cys-13 by the adjacent peptide backbone of the A-helix (Chu *et al.*, 1985). Instead, it appears that the imidazole group of His-113 α is the second donor ligand, since this is the only functional group that is in close juxtaposition with the -SH group of Cys-13. Significantly, the sequences of cat and rat Hbs are unique in that both possess Cys-13 α and His-113 α (Dayhoff, 1976; Brimhall *et al.*, 1977). The absence of either residue evidently eliminates the ability of the protein to bind trialkyltin complexes. Alanine occurs at position 13 α and leucine at position 113 α in the sequences of horse and human Hbs (Dayhoff, 1976), molecules for which detailed knowledge about the three-dimensional structures obtained by X-ray-crystallographic analyses is available (Fermi, 1975; Ladner *et al.*, 1977; Shaanan, 1984; Fermi *et al.*, 1984). Residue 13 α (A11) occupies a position within the A-helix and leucine-113 α (GH1) is at the end of the G-helix and the beginning of the non-helical bend between the G- and H-helices. Both of these residues are at the external surface of the protein. These regions of the A- and G-helices are juxtaposed in such a way that the side chains of A11 and GH1 approach each other. Insertion of cysteine at A11 and histidine at GH1 provides potential side-chain tin ligands in close apposition to permit binding of Et_3Sn^+ . Such an assignment places the Et_3Sn^+ as a bridge between the A- and G-helices of the cat α -globin subunit, at the surface of the protein, far removed from the haem groups and the $\alpha\beta$ -dimer contacts.

From the simulated three-dimensional structures of the

deoxy- and carbonyl-derivatives of cat Hb, Chu *et al.* (1985) have been able to tentatively explain the decrease in affinity of Et_3Sn^+ for the T-state Hb molecule. They found that in the R-state of the protein, the SG sulphur atom of Cys-13 and the NE2 nitrogen atom of His-113 are separated by about 0.6 nm (6 Å), an ideal separation for potential tin ligands. On changing to the deoxy state, the A- and G-helices rotate with respect to one another. The overall distance between the side chains of these donor amino acids remains approximately the same; however, the tin atom can now interact only through the ND1 nitrogen atom of His-113 α . Such an interaction is less sterically favourable and less stable from an energetic point of view (Reynolds & Tzeng, 1976) than the interaction with the NE2 atom in the R-state of the protein. The low affinity of Et_3Sn^+ for cat deoxy-Hb can be explained by this change in geometry.

Clearly, Et_3Sn^+ exerts its effects on the haem groups of cat Hb by an allosteric mechanism. Presumably, structural alterations initiated at the binding site on each α -subunit are transmitted to the $\alpha_1\beta_2$ interface of the Hb tetramer through tertiary structural changes that affect the contacts at the FG corners of the $\alpha\beta$ dimers (Baldwin & Chothia, 1979). That a structural change in the Et_3Sn^+ -binding region can affect the function of the Hb is indicated by the observation that Hb Hopkins-2, a human variant with a His $\rightarrow$ Asp substitution at position 112 (G19), adjacent to residue 113 α (GH1), demonstrates increased oxygen affinity relative to normal human adult Hb (Charache & Ostertag, 1970). However, the functional consequence of amino acid substitutions at either 13 α (A11) or 113 α (GH1) is not known, since such human variants have not been found. Examination of the α -chain sequences of other animal Hbs (Dayhoff, 1976) reveals that alanine and leucine are found most commonly at positions A11 and GH1 respectively. Exceptions include the presence of threonine in dog, isoleucine in kangaroo, and cysteine in cat and rat at A11 and of valine in rabbit and histidine in echidna, dog, cat, and rat at GH1. Thus, only cat and rat α -globins contain the polar residues, cysteine and histidine, at these loci. Since cat and rat Hbs demonstrate relatively low oxygen affinity in comparison with other animal Hbs (Taketa & Morrell, 1966; Baumann *et al.*, 1971), the polar character imparted to this region of the protein may be functionally significant. Binding of the somewhat non-polar Et_3Sn^+ to the side chains of Cys-13 α and His-113 α confers a less polar character to this region. This may be a factor in the triggering of a tertiary-structure change that leads to the observed increase in O_2 affinity.

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