Alkyl and aromatic isocyanide binding to haem complexes

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Equilibrium constants for the binding of ethyl (EIC), *n*-butyl (BIC), *p*-toluenesulphonylmethyl (TMIC) and 2,6-dimethylphenyl isocyanides (DIMPI) to an imidazole–haem complex in toluene and aqueous detergent micelle solutions were determined. In contrast to an earlier study, which indicated that the large affinities of myoglobin for binding DIMPI and 2,6-diethylphenylisocyanide (DEPI) relative to EIC were due to an electronic effect, the present study shows a similarity in binding constants for EIC, BIC, and DIMPI to the imidazole–haem complex in toluene, suggesting no such electronic effect is present. The measured hydrophobic effect ($K^{\text{DIMPI}}/K^{\text{EIC}} = 11$) cannot account for the large binding constant reported for DIMPI relative to the binding of EIC to myoglobin. Based on the results of these model studies, the equilibrium binding constant for DIMPI to myoglobin has been re-measured and the standard free energy of binding has been analysed by a more recent method.

INTRODUCTION

Alkyl isocyanides with alkyl groups of different size have been used as probes for assessing the steric accessibility of the haem iron in haemoproteins (St. George & Pauling, 1951; Ainsworth *et al.*, 1960; Brunori *et al.*, 1972; Stetzkowski *et al.*, 1979; Reisberg & Olson, 1980*a–c*; Mims *et al.*, 1983). Differences in the binding constants of alkyl isocyanides to a model haem complex in detergent micelle and haemoproteins have also indicated a partitioning effect of the hydrophobic haem environment on binding (Olson *et al.*, 1983).

Recently, Wood *et al.* (1987) reported the binding of aromatic isocyanides to haemoglobin and myoglobin and observed a 500- and 5000-fold higher binding constant for 2,6-dimethylphenylisocyanide (DIMPI) and 2,6-diethylphenylisocyanide (DEPI) relative to ethylisocyanide (EIC), respectively. Based on calculated steric and partitioning effects of these alkyl isocyanides, it was concluded that the greater binding constants for DIMPI and DEPI were due to an electronic effect. (The standard free energies calculated were in error owing to misuse of the factor for converting calories to joules.)

To determine whether the observed difference between the DIMPI and EIC binding constants is due to such an electronic effect and also to quantify this effect, we report the direct measurement of equilibrium binding constants of alkyl and aromatic isocyanides to a sterically unconstrained model, imidazole protohaem dimethyl ester (Im-Haem) in toluene. Equilibrium constants were also measured for the binding of both alkyl and aromatic isocyanides to the Im-Haem complex embedded in an aqueous suspension of soap micelle, to evaluate the partitioning effect of the aromatic isocyanide.

Results obtained from model studies in toluene and detergent showed no electronic effect and a difference in the partitioning effect of only 11-fold between DIMPI and EIC. Based on these observations, the equilibrium binding constant for DIMPI to myoglobin has been re-

measured and the effect of the protein on the free energy of binding re-calculated by a more recent method of analysis (Mims *et al.*, 1983).

EXPERIMENTAL

Materials

Methyl and ethyl isocyanides were synthesized according to the procedure of Casanova *et al.* (1963). DIMPI was synthesized according to the procedure of Gokel *et al.* (1976). White crystals were obtained for DIMPI following sublimation, m.p. 86 °C; ¹H n.m.r. (60 MHz, CDCl₃) δ (p.p.m.) 2.55 (s, 6H), 7.1–7.5 (m, 3H); i.r. (KBr) 2123 cm⁻¹. Horse heart myoglobin (type III) was purchased from Sigma and solutions of myoglobin in 0.1 M-phosphate buffer were filtered (0.45 μm membrane filter) before use. Protohaemin-chloride dimethylester was dissolved in toluene with an imidazole concentration of 3.5 × 10⁻³ M. Protohaemin dimethylester was incorporated into the detergent micelle by co-dissolving the haemin, cetyltrimethylammonium bromide (CTAB), and imidazole in a chloroform/methanol solution (1:10, v/v) (Traylor *et al.*, 1979). The solvent was then evaporated to dryness and 0.1 M-phosphate buffer at pH 7.0 was added to the residue to obtain solubilized haemin in detergent micelle [2% (w/v) CTAB] with an imidazole concentration of 3.5 × 10⁻³ M.

Ligand binding to Im-Haem solutions

All equilibrium measurements were carried out in rectangular bottle cuvettes fitted with septa. Aliquots (2.0 ml) of each haem-plus-imidazole solution were added to the cuvette and CO was either bubbled through (toluene) or passed over (detergent) the surface of the solution with stirring and intermittent shaking until saturation was achieved. The ferric haem in toluene was reduced with 10 μl of 0.1 M-NaBH₄ in methanol. The ferric haem in the aqueous micelle solution was reduced

Abbreviations used: EIC, ethylisocyanide; BIC, *n*-butylisocyanide; TMIC, *p*-toluenesulphonylmethylisocyanide; DIMPI, 2,6-dimethylphenylisocyanide; DEPI, 2,6-diethylphenylisocyanide; Im-Haem, imidazole protohaem dimethyl ester; CTAB, cetyltrimethylammonium bromide; RIC, alkyl and aromatic isocyanides.

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with an aqueous sodium dithionite solution. The resulting solutions containing CO at atmospheric pressure and an imidazole concentration of 3.5×10^{-3} M contain essentially pure monocarbonyl-monoimidazolehaem (Rougee & Brault, 1975). The Im-Haem-CO complex solutions were then titrated with alkyl and aromatic isocyanides (RIC) using a 25 μ l gas-tight syringe with a repeating dispenser attachment (Hamilton). All spectral measurements were made in the visible region (650–350 nm) on a Cary 14 or Cary 17 spectrophotometer after equilibrium was achieved.

Absorption changes measured at the Soret absorption maximum for the Im-Haem-RIC complex were used to determine the observed competitive binding constant, K_{obs} , from the slope of a plot of A versus $(A - A_0)/[L]$ and the concentration of CO, consistent with the following expression:

$$K_{\text{obs}} = \frac{[\text{Im-Haem-RIC}][\text{CO}]}{[\text{Im-Haem-CO}][\text{RIC}]}$$

$$\frac{[\text{Im-Haem-RIC}]}{[\text{Im-Haem-CO}]} = \frac{A - A_0}{A_{100} - A}$$

$$K_{\text{obs}} = \frac{(A - A_0)[\text{CO}]}{(A_{100} - A)[\text{RIC}]}$$

$$A = \frac{(A_0 - A)[\text{CO}]}{[L]K_{\text{obs}}} + A_{100}$$

where A_0 and A_{100} are absorbances at 0% and 100% Im-Haem-RIC complex formation, respectively; $[L]$ is the isocyanide concentration and A is the absorbance corresponding to an intermediate RIC concentration. The concentration of CO was calculated from the solubility of CO and the pressure. Equilibrium constants (K^{RIC}) for the binding of isocyanides to the Im-Haem complex were determined from the observed competitive binding constant and reported equilibrium constants for CO binding to an imidazole-haem complex according to the following equations:

$$K^{\text{RIC}} = K_{\text{obs}} K^{\text{CO}}$$

Ligand binding to myoglobin

Aliquots (2.0 ml) of a solution of ferric myoglobin in 0.1 M-phosphate buffer at pH 7.0 were added to a rectangular bottle cuvette sealed with a septum. The cuvette was de-aerated by bubbling with nitrogen and the ferric myoglobin reduced with sodium dithionite. Pure CO gas (10–20 μ l) was then injected into the cuvette using a gas-tight syringe (Hamilton) to obtain 100% myoglobin-CO complex. The concentration of CO in solution was calculated from the partial pressure of CO in the gas phase of the cuvette and the solubility of CO in water [9.6×10^{-4} M/atm (1 atm = 101325 Pa); Olson *et al.*, 1983]. The partial pressure of CO in the gas phase was computed from the ratio of the volume of pure CO gas

injected to the volume of the gas phase in the sealed cuvette bottle. The myoglobin-CO complex was then titrated with a DIMPI stock solution using a 25 μ l gas-tight syringe with a repeating dispenser attachment. The isocyanide binding constant was calculated from K_{obs} , and the reported binding constant for CO to myoglobin (Mims *et al.*, 1983) as described above for isocyanide binding to model haem complexes.

RESULTS

Fig. 1 shows a typical spectrophotometric titration of the Im-Haem-CO complex in toluene with DIMPI under 1 atm of CO. The substitution of alkyl and aromatic isocyanides for CO in the Im-Haem-CO complex is associated with a shift in the Soret absorption maximum from 418 nm to 430 nm. K_{obs} was calculated from the slope of a plot of A versus $(A - A_0)/[L]$ (Fig. 2) and the concentration of CO. Equilibrium constants for

Fig. 1. Spectrophotometric titration of Im-Haem-CO complex with DIMPI in toluene at 1 atm CO and 3.5×10^{-3} M imidazole

DIMPI concentrations correspond to 0.0, 2.15×10^{-3} , 5.37×10^{-3} , 1.07×10^{-2} , 1.59×10^{-2} , 2.62×10^{-2} , 3.62×10^{-2} , 5.56×10^{-2} M.

Fig. 2. Plot of A versus $(A - A_0)/[L]$ for the spectrophotometric titration of Im-Haem-CO complex with DIMPI as described in Fig. 1

Table 1. Comparison of equilibrium constants for the binding of isocyanides to haems

Haem complex	Ligand	$K^{\text{RIC}} (\text{M}^{-1})$
Im-Haem*	EIC	$1.6 \times 10^8 \dagger$ ($1.3 \times 10^8 \ddagger$)
	BIC	$2.2 \times 10^8 \dagger$ ($1.9 \times 10^8 \ddagger$)
	DMPI	$1.8 \times 10^8 \dagger$ ($1.5 \times 10^8 \ddagger$)
	TMIC	$4.7 \times 10^9 \dagger$ ($3.9 \times 10^9 \ddagger$)
Chelated protohaem§	EIC	$1.6 \times 10^8 \parallel$
	BIC	$2.6 \times 10^8 \parallel$
		$4.4 \times 10^8 \P$
	TMIC	$7 \times 10^9 \P$

* In toluene at 25 °C and 3.5×10^{-3} M-imidazole.

† Calculated using $K^{\text{CO}} = 3.3 \times 10^8 \text{ M}^{-1}$ for chelated protohaem ester and a solubility of CO = 11.7×10^{-3} M/atm (Olson *et al.*, 1983).

‡ Calculated using $K^{\text{CO}} = 4.4 \times 10^8 \text{ M}^{-1}$ for chelated protohaem ester (Traylor & Stynes, 1980) and a solubility of CO = 7.5×10^{-3} M/atm (White *et al.*, 1979).

§ Chelated protohaem is protohaem-*N*-[mono-3-(1-imidazole)propylamide] monomethyl ester in benzene (Traylor *et al.*, 1979).

|| Olson *et al.* (1983).

¶ Traylor & Stynes (1979).

isocyanide binding to the Im-Haem complex, K^{RIC} , were calculated from the values of K_{obs} and reported equilibrium constants for the binding of CO, K^{CO} , to a chelated imidazole-protohaem complex. Table 1 compares the measured isocyanide binding constants, K^{RIC} , for Im-Haem in toluene with constants for the chelated imidazole-protohaem ester (Olson *et al.*, 1983; Traylor & Stynes, 1980). The binding constants for EIC, BIC, and DIMPI to Im-Haem in toluene are similar to each other. The binding constants for EIC and BIC to Im-Haem are also in good agreement with those reported for the chelated protohaem ester (Olson *et al.*, 1983).

Table 2 shows the equilibrium constants for isocyanide binding to the Im-Haem complex and chelated protohaem in detergent micelle (Olson *et al.*, 1983). The binding constants for EIC and *n*-butyl isocyanide (BIC) to the Im-Haem complex are somewhat larger than those reported for chelated protohaem. However, the ratio of

Table 2. Comparison of equilibrium constants for the binding of isocyanides to haems in detergent micelles

Haem complex	Ligand	$K^{\text{RIC}} (\text{M}^{-1})$
Im-protohaem*	EIC	0.76×10^8
	BIC	6.4×10^8
	DIMPI	8.3×10^8
Chelated protohaem†	EIC	0.34×10^8
	BIC	2.8×10^8

* In CTAB (0.1 M-phosphate buffer, pH 7.0, 25 °C).

† In myristyltrimethylammonium bromide, (0.1 M-phosphate buffer, pH 7.0, 20 °C; Olson *et al.*, 1983).

Fig. 3. Spectrophotometric titration of myoglobin-CO complex with DIMPI in 0.1 M-phosphate buffer at pH 7.0

DIMPI concentrations correspond to 0.0, 1.4×10^{-5} , 2.8×10^{-5} , 4.2×10^{-5} , 5.6×10^{-5} , 7.0×10^{-5} , 9.8×10^{-5} M.

$K^{\text{BIC}}/K^{\text{EIC}}$ is similar for both model systems (8.4 for Im-Haem and 8.2 for chelated protohaem).

Fig. 3 shows a typical spectrophotometric titration of the myoglobin-CO complex with DIMPI. K_{obs} was calculated from the slope of a plot of A versus $(A - A_0)/[L]$ and the CO concentration. The equilibrium constant for DIMPI binding to myoglobin, K^{DIMPI} , was calculated from K_{obs} and the reported equilibrium constant for CO

Table 3. Equilibrium constants (K_{eq}) and $\Delta G_{prot.}$ for the binding of isocyanides to myoglobin

Ligand	K_{eq} (M^{-1})	$\Delta G_{prot.}$ (kcal/mol)*
EIC	$0.216 \times 10^6 \dagger$	3.41
BIC	$0.061 \times 10^6 \dagger$	5.38
DIMPI	0.76×10^6	4.08

* $\Delta G_{prot.}$ calculated using the measured K_{soap} for Im-Haem in CTAB (Table 2).
 † Mims *et al.* (1983).

binding to myoglobin, K^{CO} . Table 3 shows binding constants for EIC, BIC, and DIMPI binding to myoglobin. The equilibrium constant for DIMPI binding to myoglobin showed a value of $7.6 \times 10^5 \pm 1.6 \times 10^5 M^{-1}$. The K^{DIMPI}/K^{EIC} is 3.5 compared with a ratio of 500 observed by Wood *et al.* (1987).

DISCUSSION

Equilibrium constants for the binding of EIC, BIC, and DIMPI to the sterically unconstrained Im-Haem model in toluene have been measured to evaluate directly differences in electronic properties of these aliphatic and aromatic isocyanides. Olson *et al.* (1983) have previously observed that differences in the basicity of the aliphatic isocyanides, which may result from differences in the alkyl group, do not significantly affect the equilibrium binding constant to chelated protohaem. The similarity of equilibrium constants for EIC and BIC binding to the Im-Haem complex in toluene is in agreement with this observation. Moreover, the EIC and BIC binding constants are also similar to those reported for the chelated protohaem (Table 1). The binding constant for TMIC is 20-fold higher than that for BIC, which is in good agreement with the value reported for chelated protohaem (Traylor & Stynes, 1980). Traylor & Stynes (1980) attributed the greater binding constant to a stronger metal–ligand bond resulting from an increase in the π -acceptor properties of the isocyanide due to the electron-withdrawing tosylmethyl group. Wood *et al.* (1987) also suggested that the higher binding constants observed for DIMPI and DEPI to myoglobin relative to EIC were due to the greater π -acceptor capacity of these aromatic ligands. They argued that the ring facilitates delocalization of charge and an increase in π -acceptor properties. However, the present results indicate that the binding constant for DIMPI to Im-Haem is similar to constants for EIC and BIC, suggesting that no such electronic effect is present or that any differences in the π -accepting properties are compensated by differences in the sigma-bonding properties.

Factors other than an electronic effect may account for the large binding constants previously observed for DIMPI and DEPI to myoglobin. One possibility is that the partitioning effect of a hydrophobic haem environment may be larger than predicted. The equilibrium constants for binding alkyl and aromatic isocyanides to the Im-Haem complex in detergent micelles were measured to determine the hydrophobic effect (Table 2). The equilibrium constant for DIMPI binding to Im-Haem is 11-fold higher than the EIC binding constant,

which suggests that the hydrophobic effect is not large enough to account for all of the 500-fold higher binding constant observed for DIMPI relative to EIC binding to myoglobin.

Based on the results of these model studies, which suggest that neither the electronic nor the hydrophobic effect can account for the large binding constant observed for DIMPI to myoglobin, the equilibrium constant for DIMPI binding to myoglobin has been re-measured. The equilibrium constant for DIMPI binding to myoglobin is $7.6 \times 10^5 M^{-1}$, which is much less than the value ($1 \times 10^8 M^{-1}$) reported by Wood *et al.* (1987). The ratio of K^{DIMPI}/K^{EIC} for myoglobin is only 3.5 compared with that for the model which is $K^{DIMPI}/K^{EIC} = 11$, which would indicate that DIMPI is being sterically hindered. To evaluate the extent of the steric effect, standard free energies of binding were analysed by a more recent method of Mims *et al.* (1983).

Mims *et al.* (1983) evaluated the extent of steric interaction between the alkyl isocyanide and protein in haem proteins by considering the differences between the free energies of ligand binding to a sterically unconstrained chelated protohaem model in a soap micelle and to the haem protein according to the following expression:

$$\Delta G_{prot.} = RT \ln(K_{soap}/K_{obs.})$$

$\Delta G_{prot.}$ is a measure of both the steric interaction between the ligand and the protein at the sixth coordination site and the proximal effect at the fifth coordination site. This type of analysis is based on the assumption that the hydrophobic environment in the micelle is similar to that of the protein and that the homologous series of alkyl and aromatic isocyanides will equally be affected by any proximal effect. Traylor *et al.* (1979) and Traylor (1981) have demonstrated that the penta-co-ordinated haem model in micelles exhibits spectral properties as well as CO and O₂ rate and equilibrium constants very similar to those of R-state haemoglobin. Based on a study of the effect of solvent on the absorption maxima of Im-haem complexes, it has further been suggested that the haem environment in myoglobin is similar to the hydrophobic environment of a detergent micelle (Romberg & Kassner, 1982). It has been shown in model studies in benzene that the homologous series of alkyl isocyanides exhibit equivalent binding constants and likewise that myoglobin and the R- and T-states of haemoglobin show no differences in binding among the homologous series of alkyl isocyanides as a result of proximal differences (Olson *et al.*, 1983; Mims *et al.*, 1983). Therefore, differences in $\Delta G_{prot.}$ between CO and alkyl isocyanides are a measure of steric effects between the alkyl isocyanides and the protein at the sixth coordination site. Table 3 shows the $\Delta G_{prot.}$ values for the binding of alkyl and aromatic isocyanides to myoglobin. In the absence of any steric effect, $\Delta G_{prot.}$ would be constant for each of the isocyanides. An increase in $\Delta G_{prot.}$ values as the size of the ligand increases is consistent with an increase in steric interaction as previously observed for haemoglobin and myoglobin (Mims *et al.* 1983).

An increase in the value of $\Delta G_{prot.}$ of 0.7 kcal/mol is observed for DIMPI relative to EIC. This increase is associated with steric hindrance between the protein and the ligand. Comparison of $\Delta G_{prot.}$ for DIMPI and BIC suggests that the binding of DIMPI is more favourable

by 1.3 kcal/mol, even though DIMPI is more bulky than BIC. This more favourable binding of DIMPI relative to BIC can perhaps be attributed to favourable π -interactions between the aromatic ligand and aromatic amino acid residues (histidine and phenylalanine) in the haem-ligand environment of the protein, as previously indicated for haemoglobin and myoglobin from the relative binding of benzyl and cyclohexyl isocyanides which have similar surface areas [$K^{\text{benzyl}}/K^{\text{c-hexyl}} = 10$ for haemoglobin (Reisberg & Olson, 1980a) and $K^{\text{benzyl}}/K^{\text{c-hexyl}} = 20$ for myoglobin (Mims *et al.*, 1983)]. π - π Interactions could provide a secondary bonding interaction between the ligand and the protein analogous to the H-bonding interaction between O_2 and the distal histidine in haemoglobin and myoglobin. Alternatively, the π - π interactions could increase the iron-ligand bond strength by inductively affecting the electronic properties of the ligand. The absence of a significant difference between the binding constants of DIMPI and alkyl isocyanides in toluene would suggest that this latter effect is less likely.

The results indicate that there is no intrinsic electronic effect on ligand binding associated with differences between alkyl and aromatic isocyanides as previously indicated. Furthermore, the results show that differences between alkyl and aromatic isocyanide binding constants to myoglobin are much smaller than those previously reported.

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