Binding of aromatic isonitriles to haemoglobin and myoglobin

Matthew A. WOOD, Keith DICKINSON, Gerald R. WILLEY and George H. DODD*

Olfaction Research Group, Department of Chemistry, University of Warwick, Coventry CV4 7AL, U.K.

A series of aromatic isonitriles were synthesized and their binding to sheep haemoglobin and horse heart myoglobin was investigated. The disubstituted ligands 2,6-dimethylphenylisonitrile and 2,6-diethylphenylisonitrile were found to bind to horse-heart myoglobin with affinities ranging from 500 to 5000 times greater than that of ethylisonitrile (4.6×10^{-6} M) which has been the tightest binding isonitrile ligand for myoglobin thus far reported. The tight binding was not found to vary significantly with pH or temperature. An explanation for the unexpectedly high affinity is offered in terms of the electronic structure of aromatic isonitriles.

INTRODUCTION

Isonitriles have been suggested as suitable probes for the haemoglobin binding site since 1951 (St. George & Pauling, 1951). St. George & Pauling examined the binding of ethylisonitrile, isopropylisonitrile and *tert*-butylisonitriles to both haemoglobin and a model system comprising ferrihaem chloride in aqueous solution. The affinity of the model haem system for the isonitriles was effectively constant for all the ligands. From this it was concluded that the strength of the iron–isonitrile bond was constant regardless of the aliphatic group and hence, any variation in affinity of the protein for the ligand can be attributed solely to interactions between the ligand and amino acid residues surrounding the distal site of the protein. Such effects are termed ‘distal effects’.

However, the observation that the affinities of bovine haemoglobin for ethylisonitrile, isopropylisonitrile and *tert*-butylisonitrile varied greatly (K_D values 0.1 mM, 0.3 mM and 22.0 mM respectively) supported Pauling’s proposal that the haem group was buried within the haemoglobin molecule and that incoming ligands were subject to steric interaction.

Reisberg & Olson (1980) in a study using a series of 13 aliphatic isonitrile probes estimated the favourable hydrophobic contribution for ligand binding to haemoglobin by calculating the chemical potential of ligands in aqueous solution assuming the chemical potential of the same ligand in the organic phase to be zero. This was achieved using the method of Harris *et al.* (1973) which demonstrates a linear relationship between the surface area of a molecule and the free energy of partition from aqueous to organic phase. In this way Reisberg & Olson transformed the data from simple equilibrium binding to component factors of favourable hydrophobic interaction and unfavourable steric hindrance and so were able to compile a free-energy steric map of the sixth coordinate site of haemoglobin.

To our knowledge, the binding of aromatic isonitriles to haemoglobin or myoglobin has not been reported in the literature. We have synthesized a series of substituted aromatic isonitriles. The 2,6-disubstituted aromatic isonitriles are more stable than the monosubstituted ligands (i.e. they show neither heavy pigmentation associated

with polymerization, nor nitrile impurities resulting from isomerization). We report here the unexpectedly tight binding of these novel probes to both haemoglobin and myoglobin.

EXPERIMENTAL

Materials

Aliphatic and aromatic isonitriles were synthesized from the amine precursors (as obtained from Aldrich) by the phase-transfer-catalysed Hoffman carbylamine reaction (Gokel *et al.*, 1976). The following aromatic isonitriles were synthesized; phenylisonitrile (b.p. 55 °C/2 mmHg), methylphenylisonitrile (*ortho*, b.p. 90 °C/10 mmHg; *meta*, b.p. 46 °C/1 mmHg; *para*, b.p. 24 °C/0.3 mmHg), 2,6-dimethylphenylisonitrile (DIMPI; m.p. 72–73 °C), 2,6-diethylphenylisonitrile (DEPI; b.p. 70–73 °C/0.4 mmHg) and 2,6-di-isopropylphenylisonitrile (DIPI; b.p. 94–96 °C/0.7 mmHg). DIMPI was a white crystalline solid, the others were clear liquids at room temperature. All isonitriles were purified by vacuum distillation. Purities were estimated using g.l.c. (SE30 and OV225 columns), and were greater than 99%. Characteristic stretching frequencies of 2120 cm⁻¹ were observed for the aromatic isonitriles.

Sheep haemoglobin, horse-heart myoglobin (type III) and Hepes were supplied by Sigma and were used as supplied. Sodium dithionite was obtained from Vine Chemicals, Widnes, Cheshire. Nitric oxide was made according to the method of Keilin & Hartree (1937). Carbon monoxide (purity 95%) and dimethylformamide (which was distilled prior to use to remove coloured impurities), were obtained from BDH, and oxygen (purity 95%) from BOC.

Ligand binding experiments

Protein solutions (approx. 50 µM according to haem) were made up each day in 0.1 M-Hepes buffer. The solutions were degassed under vacuum and then flushed with nitrogen.

Measurements were made in Sarstedt 4 ml gas-sealable plastic cuvettes sealed with Suba-Seals (size 29). Titration additions were made using a 1 µl Hamilton microsyringe.

Abbreviations used: DIMPI, 2,6-dimethylphenylisonitrile; DEPI, 2,6-diethylphenylisonitrile; DIPI, 2,6-di-isopropylphenylisonitrile.

* To whom correspondence should be addressed.

Spectrophotometric measurements were obtained using a Shimadzu UV/VIS 365 spectrophotometer.

A few grains of dithionite were added to cuvettes prior to addition of the protein solutions in order to scavenge any residual oxygen and to convert the ferric form of the protein to the deoxy ferrous form.

Titration were performed by making sequential additions (typically 0.2–1.0 μ l) of the isonitrile stock solution to the cuvettes. Titrations were followed by absorption peak and isosbestic point measurements after each addition. Changes in isosbestic points were used to correct base-line shift, but such adjustments were rarely necessary.

Attempts to displace ligands with carbon monoxide or nitric oxide were performed by titrating the protein to saturation with the isonitrile ligand and then adding a quantity of buffer saturated with the appropriate competitor. The reverse experiments were also performed by first saturating the protein with carbon monoxide or nitric oxide and then adding a saturating quantity of isonitrile.

Estimates of affinity were made using the relationship:

$$n = \frac{[\text{CO}]_{\text{free}} \cdot K_D(\text{RNC})}{[\text{RNC}]_{\text{free}} \cdot K_D(\text{CO})} \quad \text{where } n = \frac{[\text{MbCO}]}{[\text{MbRNC}]}$$

RESULTS

All seven phenylisonitrile probes were found to bind to both proteins giving the usual two-peak spectrum scanning from 450 nm to 650 nm. The wavelengths of the peaks for all ligands were 531 nm and 561 nm for haemoglobin and 532 nm and 567 nm for myoglobin. Fig. 1(a) shows the spectral change occurring during the course of a titration of myoglobin with DIMPI. In view of the instability of the monosubstituted ligands, further investigations were conducted using only the disubstituted ligands.

Fig. 1(b) shows a direct binding plot (i.e. fractional saturation versus total ligand concentration) comparing an aliphatic isonitrile (ethylisonitrile) with DIMPI. The affinity with which these aromatic ligands are bound is so high that normal binding plots were not possible: values of free ligand concentration could not be calculated. Phenylisonitrile and the monosubstituted ligands were not stable at room temperature and were excluded from any further investigation. The appearance of the binding plot for all ligands was not affected by temperature (between 20 and 40 °C) or pH (between 4 and 9).

Further investigations were conducted using only myoglobin as this avoided complications due to co-operative binding and the lability of the haemoglobin tetramer. Other methods of estimating the affinity of the aromatic isonitriles were sought. The affinities of oxygen, carbon monoxide and nitric oxide for horse heart myoglobin are 7.5×10^{-7} M, 3.4×10^{-8} M and 1×10^{-11} M (pH 7.0 at 25 °C; Mims *et al.*, 1983; Wittenburg & Wittenburg, 1981) respectively and they were suggested as suitable competitors.

Displacement of DIMPI with oxygen, carbon monoxide and nitric oxide was slow and the results were somewhat variable. Ethyl isonitrile, which has the highest reported affinity of the aliphatic isonitriles (3.6×10^{-6} M; Mims *et al.*, 1983), underwent immediate 100 % displacement by nitric oxide.

Attempts were made to obtain quantitative measure-

Fig. 1. (a) Repeated scan spectra made during the course of a titration of haemoglobin (50 μ M-haem in 0.1 M-Hepes, pH 7.0, 20 °C) with 5 μ M increments of DIMPI and (b) plot of fractional saturation versus total ligand concentration using data obtained from the titration shown in (a) and additional data from a titration of ethylisonitrile with haemoglobin

ments using carefully monitored levels of competitor to displace isonitriles. Using this approach and measuring the proportion of each complex present at equilibrium yielded data which can then be used to get estimates of K_D .

This approach was attempted several times using either carbon monoxide or oxygen to displace both DIMPI and DEPI. The results showed a high degree of variation and were somewhat dependent on the competitor used. K values for DIMPI determined using oxygen varied from 4 to 25 nM whereas carbon monoxide gave values ranging from 3 to 13 nM. For DEPI, only two determinations were attempted, giving values of 0.19 and 2.8 nM (using carbon monoxide). Attempts to estimate the K_D of DIPI by this method failed due to solubility problems which caused erratic baseline drift.

Fig. 2. Theoretical projection of DIMPI onto the schematic free-energy map of the haemoglobin binding site (Reisberg & Olson, 1980)

DISCUSSION

The tightest binding aliphatic isonitrile for myoglobin so far reported is ethylisonitrile (K_D 3.6×10^{-6} M) and the weakest binding is *tert*-butyl isonitrile (K_D 5.0×10^{-4} M) (Mims *et al.*, 1983).

The high affinity of aromatic isonitriles observed in the present study is surprising especially when viewed in the light of the present model for human haemoglobin (Reisberg & Olson, 1980). Considering the size and positioning of the bulky alkyl substituents on the aromatic ligands it is reasonable to expect that considerable structural disruption must occur during binding to both haemoglobin and myoglobin. Ideally, the work described here should have been performed using the same protein as used by Reisberg & Olson (1980); however, for reasons described earlier, our estimates of affinity were conducted using the simpler protein myoglobin. Strictly speaking, the Reisberg & Olson model is not fully applicable to our system. There is, however, sufficient structural homology between the two proteins to make some comparisons reasonable.

According to the model proposed by Reisberg & Olson (1980), the disubstituted aromatic isonitriles would be bound with their aliphatic side arms situated in the areas of high steric interaction (Fig. 2), suggesting that the ligand must meet with considerable steric hindrance at the binding site. What then is the driving force behind this unusually strong binding?

Reisberg & Olson conclude from their investigation that as ligand size is increased there is a roughly equal play-off between steric hindrance and hydrophobic attraction. But even if such a play-off were equal, the degree of affinity would, nevertheless, still be expected to be of the order of μ M and not nM.

The overall free energy change resulting from ligand binding (ΔG) is taken to be (Reisberg & Olson, 1980) the difference between the chemical potential of the bound ligand [$G(\text{Hbx})$] and the chemical potential of the ligand in aqueous solution, $G(x)$: $\Delta G = G(x) - G(\text{Hbx})$.

Using the model of Reisberg & Olson (1980), the parameters ΔG , $G(x)$ and $G(\text{Hbx})$ can be estimated. For example, the calculated value of $G(x)$ for DIMPI is +1.27 kJ/mol. This can be calculated given that DIMPI has a relative surface area of approx. 1.82 (benzyl, 1.31; methyl, 0.51) using the Harris *et al.* (1973) proportionality coefficient (0.97 kJ/mol per unit surface area) and subtracting 0.49 kJ/mol [which is the $G(x)$ value of methyl isonitrile which has been arbitrarily assigned a value of zero].

A theoretical value of the bound potential, $G(\text{Hbx})$, of DIMPI in the model of Reisberg & Olson, is estimated by superimposing the structure of DIMPI on to the free-energy map of the haemoglobin binding site, and assuming that seven carbon atoms are likely to occupy the area of high steric interaction (0.4 kJ/C atom). Allowing for the $G(\text{Hbx})$ value of methylisonitrile (−1.12 kJ/mol) which represents the negative contribution from the formation of the metal–ligand bond, a value of +5.84 kJ/mol is obtained. This is an unreasonably large bound potential and would imply that, according to the model, DIMPI should not bind. Finally, given the relationship $\Delta G = G(\text{Hbx}) - G(x)$, the theoretical value for ΔG can be estimated to be +0.13 kJ/mol.

An observed value of ΔG and $G(\text{Hbx})$ can be obtained given the mean K_D for DIMPI (10×10^{-9} M) and the relationship $\Delta G = RT \cdot \ln K$. Hence, in our system, ΔG for DIMPI is −2.6 kJ/mol and $G(\text{Hbx})$ accordingly is −1.32 kJ/mol. Comparing theoretical and observed ΔG values reveals a major discrepancy of 2.72 kJ/mol.

Similar calculations for DEPI (relative surface area 2.45; K 1×10^{-9} M) can be performed assuming that the terminal methyl groups of the alkyl side chains are pointing out of the area of highest steric interaction. Comparing theoretical and observed values of ΔG for DEPI reveals a discrepancy of 2.35 kJ/mol. This is

Table 1. Comparison of theoretical and observed free-energy parameters related to the binding of disubstituted aromatic isonitriles to myoglobin

R.S.A. is relative surface area (Reisberg & Olson, 1980).

	R.S.A.	Value (kJ/mol)			K_D (nM)
		$G(x)$	$G(\text{Hbx})$	ΔG	
DIMPI					
Theoretical	1.82	+1.26	+1.40	+0.13	—
Observed	1.82	+1.26	−1.32	−2.58	10.0
DEPI					
Theoretical	2.54	+1.96	+1.40	−0.56	—
Observed	2.54	+1.96	−0.96	−2.92	1.0

Fig. 3. Schematic representations of (a) metal-alkyl isonitrile bonding involving sigma and pi components and (b) metal-aromatic isonitrile bonding to show pi-delocalization between $M[d(t_{2g})]$, $NC(\pi^*)$, and aromatic ring $\pi^*(e_{2u})$ orbitals

comparable with the discrepancy seen for DIMPI (2.72 kJ/mol). Results for both ligands are shown in Table 1.

Accepting that the Reisberg & Olson model is extensive enough to account for the binding of large ligands, the $G(\text{Hbx})$ term is the most likely source from which the discrepancy in ΔG values may arise. The $G(\text{Hbx})$ term is calculated assuming that the isonitrile-iron bond strength is constant and is unaffected by the nature of the attached group. Whilst this assumption is reasonable when applied to aliphatic isonitriles, it may not be so for aromatic isonitriles, where there is substantial evidence for pi-interactions between the aromatic ring and the functional group; for the inorganic iron(II) aromatic isonitrile complexes $\text{FeCl}_2(\text{DIMPI})_4$ and $[\text{FeCl}(\text{DIMPI})_5]\text{FeCl}_4$, X-ray structural data show mean iron-carbon bond distances of 1.895×10^{-1} nm and 1.906×10^{-1} nm respectively which are significantly shorter (5–10%) than those in corresponding iron(II) alkyl isonitrile systems. (Drew *et al.*, 1986).

When aliphatic isonitriles bind to metal ions they act

both as sigma donors, $(\text{NC})\sigma^2$ metal $d(e_g)$, and as pi acceptors, metal $d_{\pi^2}(t_{2g})$ $(\text{NC})(\pi^*)$; the latter relieves the build-up of negative charge on the metal ion via empty accessible π^* orbitals on the ligand (Fig. 3a). These effects are synergic and lead to a strong metal-ligand linkage. Any sigma inductive effects between the aliphatic group (R) and the functional group (NC) are considered to be negligible and hence have little influence over the strength of the metal ligand binding. However, in considering aromatic isonitrile binding to metal ions there is a significant difference, i.e. the proximity of the aromatic framework. In this case vacant ring orbitals of suitable energy and symmetry (π^*e_{2u}) can interact with the $\pi^*(\text{NC})$ orbitals of the isonitrile group and hence facilitate extensive delocalization of charge into the aromatic ring (Fig. 3b). Thus the ring becomes an effective electron sink, thereby increasing the pi-acceptor capacity of the ligand. Such an arrangement reinforces the strength of the metal-isonitrile bond.

It seems likely that this enhanced metal-ligand bond is responsible for the unexpectedly high affinity observed for aromatic isonitriles with both myoglobin and haemoglobin, and thereby accounts for the 10 kJ/mol discrepancy between predicted and observed $G(\text{Hbx})$ values.

This work was supported by a grant from the S.E.R.C. K. D. was supported by the Research and Innovation Fund of the University of Warwick. We thank Dr. M. McKillen, Trinity College Dublin, for helpful discussions.

REFERENCES

- Drew, M. G., Dodd, G. H., Williamson, J. & Willey, G. (1986) *J. Organomet. Chem.* **314**, 163–172
- Gokel, G. W., Widera, W. P. & Webber, W. P. (1976) *Org. Synth.* **55**, 96–100
- Harris, M. J., Higuchi, T. & Rytting, J. H. (1973) *J. Phys. Chem.* **77**, 2694–2703
- Keilin, D. & Hartree, E. F. (1937) *Nature (London)* **139**, 548–550
- Mims, M. P., Arturo, G. P. & Olson, J. S. (1983) *J. Biol. Chem.* **258**, 14219–14231
- Reisberg, P. I. & Olson, J. S. (1980) *J. Biol. Chem.* **255**, 4144–4150
- St. George, R. C. C. & Pauling, L. (1951) *Science* **114**, 629–634
- Wittenburg, J. & Wittenburg, B. (1981) *Methods Enzymol.* **76**, 29–54

Received 2 March 1987/5 May 1987; accepted 23 July 1987