# Mechanism of ligand binding to $\alpha_1$-acid glycoprotein (orosomucoid): correlated thermodynamic factors and molecular parameters of polarity

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Eight ligands were used in this study, four basic, three neutral and one acidic. Their binding to serum $\alpha_1$-acid glycoprotein (orosomucoid) was measured at several temperatures, and the data were analysed together by a general model with three unknowns, number of binding sites, $\Delta H^0$ and $\Delta S^0$. The partition coefficients of the ligands were measured in octanol/water and heptane/water systems ($\log P_{oct.}$ and $\log P_{hep.}$), and their molecular volumes were calculated by molecular modelling techniques. These structural properties allow determination of polarity parameters ($\Delta\log P_{oct.-hep.}$, $\Lambda_{oct.}$ and $\Lambda_{hep.}$) which encode in different proportions the various polar interactions between the solute and the aqueous and organic phases, i.e. hydrogen-bonding capacity and dipolarity/polarizability. This study shows that good correlations exist between $\Delta H^0$ or $\Delta S^0$ and polarity parameters, such that the enthalpic contribution to binding increases with increasing polarity of the ligands, mainly hydrogen-bond-donor acidity, whereas their entropic contribution to binding decreases.

## INTRODUCTION

$\alpha_1$-Acid glycoprotein (AAG; orosomucoid) is a small acute-phase glycoprotein which is negatively charged at physiological pH and contains a large proportion of carbohydrates (40% by weight). It interacts with a variety of ligands including acids, neutral compounds and bases. A role for AAG as a high-affinity carrier for most basic ligands, including $\beta$-blockers, antidepressants, neuroleptics and local anaesthetics, has been recognized [for a review, see Kremer et al. (1988)]. In recent studies, we have shown that the binding of ionizable ligands to AAG involves a hydrogen bond with a $pK_a$ shift of the ligand and/or an amino acid residue of the protein-binding site (Urien et al., 1991, 1993).

We now report a detailed analysis of the effect of temperature on the binding to AAG of several ligands. The enthalpic and entropic components of the interaction of a given ligand were estimated directly from the measured binding data at various temperatures using an integrated model. This analysis, which incorporates the information from all the binding data (at least 45 experimental points), allows the precision and significance of the parameters to be determined.

Physicochemical investigations were performed in parallel to characterize relevant properties of the ligands ($pK_a$, $\log P_{oct}$, $\log P_{hep.}$, $\Delta\log P_{oct.-hep.}$, $\Lambda_{hep.}$). Relating thermodynamic and molecular parameters should shed light on structure–affinity relationships and the intermolecular forces controlling ligand binding.

## MATERIALS AND METHODS

### Materials

AAG (99% pure; Behring) was dissolved in Sörensen's phosphate buffer (0.066 M $KH_2PO_4$/0.066 M $Na_2HPO_4$; 19.7:80.3, v/v) and used without further modification in the binding experiments.

Radiolabelled ligands were obtained from the following manufacturers: [$^{14}$C]binedaline (Cassenne, Paris, France; 2.15 GBq/mmol; 98% pure), [$^{14}$C]bornaprolol (CEA, Gif/Yvette, France; 1.63 GBq/mmol; 98.5% pure), [$^{14}$C]darodipine (Sandoz, Basel, Switzerland; 0.396 GBq/mmol; 98% pure), [$^3$H]propranolol (Amersham, Gif/Yvette, France; 0.925 TBq/mmol; 96% pure), [$^{14}$C]isradipine (Sandoz; 0.558 GBq/mmol; 98% pure), [$^3$H]-progesterone (Amersham; 1.85 TBq/mmol, 97% pure), [$^3$H]-tertatolol (IRIS; 1.1 TBq/mmol; 99% pure) and [$^{14}$C]warfarin (Amersham; 1.85 GBq/mmol; 98.5% pure). Their chemical structures are given in Figure 1.

### Binding experiments

Equilibrium dialysis and measurement of radioactivity were conducted at pH 7.4 as previously described (Urien et al., 1991, 1993).

### Estimation of thermodynamic parameters

According to the law of mass action, $K_A = [PL]/[L][P]$, the protein-bound ($B = [PL]$) and free ($F = [L]$) ligand concentrations from equilibrium dialysis experiments are related by the following relationship:

$$B = \left(\frac{nK_AF}{1+FK_A}\right)P_t \quad (1)$$

where $P_t$ is the total protein concentration, and $n$ and $K_A$ are respectively the number of binding sites and association constant. The equilibrium constant, $K_A$, is related to the difference in equilibrium free energy between the free and bound states ($\Delta G^0$) as follows:

$$K_A = e^{-\Delta G^0/RT} \quad (2)$$

where $R$ is the gas constant (1.987 cal/mol·K) and $T$ the temperature in degrees Kelvin.

Enthalpy ($\Delta H^0$) and entropy ($\Delta S^0$) are related to $\Delta G^0$:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (3)$$

Abbreviation used: AAG, $\alpha_1$-acid glycoprotein.

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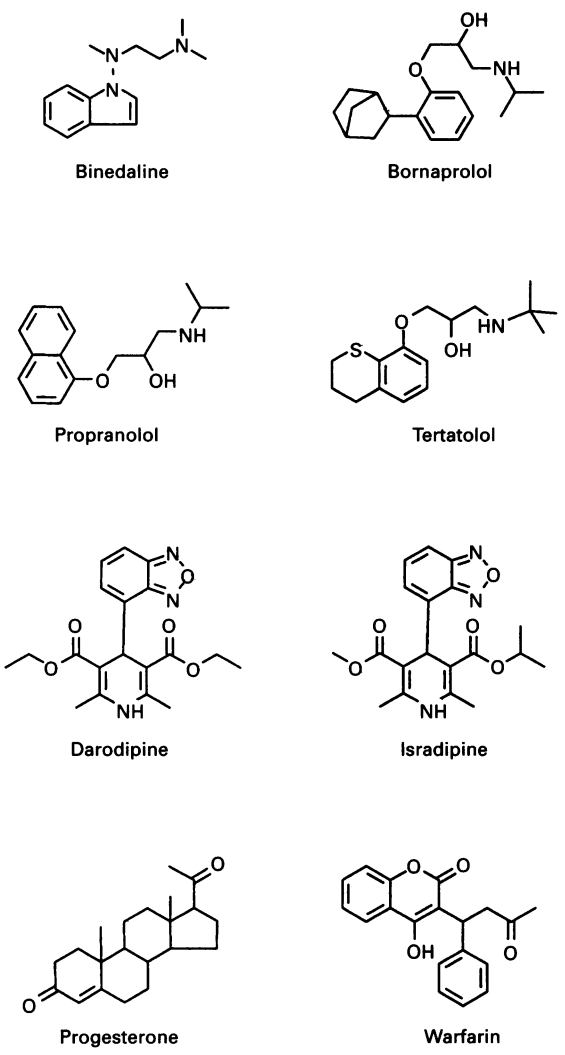


**Figure 1 Chemical structures of ligands investigated**

Combining eqns. (2) and (3) yields:

$$K_A = e^{-[(\Delta H^0/RT)-(\Delta S^0/R)]} \quad (4)$$

It follows that the observed association constant, $K_A$, can be expressed in terms of its enthalpic and entropic contributions. These thermodynamic parameters are usually determined from the slope and intercept of the ln $K_a$ versus $1/T$ plot (van't Hoff plot). Here, these parameters were directly determined from the equilibrium dialysis data, i.e. by combining eqn. (1) with eqn. (4), providing an integrated model:

$$B = \left\{ \frac{n e^{-[(\Delta H^0/RT)-(\Delta S^0/R)]} F}{1 + F e^{-[(\Delta H^0/RT)-(\Delta S^0/R)]}} \right\} P_t \quad (5)$$

Thus the binding data $\{F, B\}$ obtained at different temperatures can be analysed together and described in terms of three unknowns, $n$, $\Delta H^0$ and $\Delta S^0$. These were estimated by a non-linear least-squares fit of at least 45 values of $\{F, B\}$ at three or four different temperatures.

### Potentiometric determination of p$K_a$ values

p$K_a$ values of bornaprolol, tertatolol and binedaline in water were measured by potentiometry using a Dosimat Metrohm type 665 (Herisau). The measuring cell was kept at 26±1 °C and a constant flow of $N_2$ was maintained through it. The ionic strength of the samples was adjusted to 0.1 M with KCl, and titration of the compounds as hydrochlorides was conducted with 0.01 M NaOH.

The titration curves were registered with a Metrohm titroprocessor type 670. The p$K_a$ values were calculated by a non-logarithmic linearization of the titration curve with correction for dilution (Benet and Goyan, 1967; Leeson and Brown, 1966).

The very weak acidity and low water solubility of darodipine made it necessary to use another approach to determine its p$K_a$ in water. Solutions of the hydrochloride ($6 \times 10^{-4}$–$9 \times 10^{-4}$ M) were prepared in methanol/water mixtures (26:74, 33:67 and 40:60, v/v) and titrated with 0.5 M KOH using a Sirius PCA 101 titrator (Sirius, Forest Row, East Sussex, U.K.). The apparent p$K_a$ values thus determined were extrapolated to 0% methanol using the built-in option of the Yasuda–Shedlovsky equation (Avdeef et al., 1993). The p$K_a$ of isradipine could not be measured because of the small amounts of material available; instead, it was estimated to be identical with that of darodipine, considering the great structural similarity between the two compounds.

### Determination of distribution and partition coefficients

Distribution coefficients in octan-1-ol/buffer and *n*-heptane/buffer systems were measured by horizontal flow-through centrifugal partition chromatography using a coil-planet-type centrifuge (Vallat et al., 1990; El Tayar et al., 1991b). The design of the instrument has previously been described (Ito, 1986). The apparatus (models CCC-1000 and CCC-3000; Pharma-Tech Research Corp., Baltimore, MD, U.S.A) used three columns. Zwitterionic buffers composed of Mes, pH 4 and 6, and Mops, pH 7.4, were used for the measurement of distribution coefficients (log $D$) at different pH values. log $P$ of the neutral form could thus be calculated in combination with p$K_a$ using eqns. (6) and (7) for acids and bases respectively:

$$\log P = \log D + \log(1 + 10^{pH - pK_a}) \quad (6)$$

$$\log P = \log D + \log(1 + 10^{pK_a - pH}) \quad (7)$$

log $P_{oct.}$ of darodipine and isradipine could not be determined by this method because of their highly lipophilic nature (log $P_{oct.} > 3$). An alternative, using a capacity factor (log $k$) derived from reversed-phase h.p.l.c., was used to estimate log $P_{oct.}$ of these two compounds. The experimental procedures have been described elsewhere (El Tayar et al., 1985). Using a large set of compounds, it has been found that log $P_{oct.}$ is highly correlated with reversed-phase-h.p.l.c. capacity factor using methanol/buffer (40:60, v/v) (log $k_{40}$) as the mobile phase:

$$\log P_{oct} = 1.622 \log k_{40} + 0.812 \quad (8)$$

Eqn. (8) was thus used to estimate log $P_{oct.}$ of darodipine and isradipine.

### Determination of the molecular volume, *V*

For all compounds, the conformational space was explored by using the systematic search option in the molecular modelling software SYBYL (version 6.0; Tripos Associates, St. Louis, MO, U.S.A.) running on a Silicon Graphics Indigo R4000 workstation. The geometry of each global minimum was fully optimized with the Tripos force field containing electrostatic terms. Molecular-volume calculations were performed with the MOLSV program (QCPE program no. 509) using atomic radii described by Gavezzoti (1983).

**Table 1 Binding constants of the ligands investigated to AAG as a function of temperature**

Results are estimates ± S.D.

| Ligand | $K_A$ ($mM^{-1}$) 20 °C | 30 °C | 37 °C | 42 °C |
|---|---|---|---|---|
| Basic | | | | |
| Binedaline | 424 ± 85 | – | 410 ± 77 | 390 ± 59 |
| Bornaprolol | 223 ± 35 | 222 ± 43 | 217 ± 27 | 297 ± 42 |
| Propranolol | 298 ± 110 | 237 ± 82 | – | 171 ± 54 |
| Tertatolol | 355 ± 21 | 264 ± 10 | 260 ± 39 | 218 ± 16 |
| Neutral | | | | |
| Darodipine | 419 ± 160 | – | 265 ± 30 | 198 ± 18 |
| Isradipine | 340 ± 91 | – | 164 ± 30 | 134 ± 14 |
| Progesterone | 100 ± 7 | 70 ± 6 | – | 50 ± 3 |
| Acidic | | | | |
| Warfarin | 1086 ± 22 | 702 ± 20 | 474 ± 30 | 372 ± 21 |

### Determination of polarity parameters ($\Delta\log P_{oct.-hep.}$ and $\Lambda_{hep.}$)

Lipophilicity is a physicochemical property expressed as log $P$ which results from the various solute–solvent interactions in both the aqueous and organic phases, usually octanol (log $P_{oct.}$). As such, lipophilicity varies with the organic solvent. El Tayar et al. (1992) have shown that log $P$ can be factorized into a volume term $V$, expressing hydrophobic interactions, and a polarity term $\Lambda$, which encodes polar interactions such as dipole–dipole interactions and hydrogen bonds:

$$\log P = a_1 V - \Lambda \quad (9)$$

In fact, the $\Lambda$ term can be factorized into dipolarity/polarizability ($\pi^*$), hydrogen-bond-donor capacity ($\alpha$) and hydrogen-bond-acceptor capacity ($\beta$) (El Tayar et al., 1992; Abraham, 1993):

$$\Lambda = a_2\pi^* + a_3\alpha + a_4\beta \quad (10)$$

Although $a_1$, the coefficient of the $V_{term}$, varies little from one solvent to another, large differences are seen in the coefficients $a_2$, $a_3$ and $a_4$. In the heptane/water system, the polar term $\Lambda_{hep}$ reflects exclusively solute–water interactions, whereas in the octanol/water system the $\Lambda_{oct.}$ term reflects the balance of solute–octanol and solute–water interactions. As for the difference log $P_{oct.}$ − log $P_{hep.}$ (i.e. $\Delta\log P_{oct.-hep.}$), it is mainly a function of $\alpha$, and to a lesser extent also of $\pi^*$ and $\beta$ (El Tayar et al., 1991a). Actually, $\Delta\log P_{oct.-hep}$ reflects mainly the polar interactions between the solute and octanol.

In this study, $\Delta\log P_{oct.-hep.}$ was obtained by simply subtracting the experimentally obtained log $P_{hep.}$ from log $P_{oct.}$. In analogy with previous work (El Tayar et al., 1992), $\Lambda$ was calculated by the equation:

$$\Lambda = \log P_{est} - \log P_{exp.} \quad (11)$$

where log $P_{est.}$ is defined as the partition coefficient of an alkane having the same molecular volume as the investigated compound and log $P_{exp.}$ is an experimental value. log $P_{est.}$ can be calculated by the following calibration equations, which are derived from the measured partition coefficient of simple alkanes and their molecular volume:

$$\log P_{est.}(\text{octanol}) = (3.087 \times 10^{-2})V + 0.346 \quad (12)$$

$$\log P_{est.}(\text{heptane}) = (3.760 \times 10^{-2})V + 0.346 \quad (13)$$

### Statistical analysis

Statistical analysis was performed with the QSAR module of the software SYBYL in combination with additional home-made programs.

## RESULTS

### Binding and thermodynamic parameters

Table 1 summarizes the effect of temperature variation on the association constant of the investigated ligands to AAG. For the basic ligands, affinity was generally poorly influenced by temperature, except for propranolol where a slight and continuous decrease in affinity was observed when temperature increased. In the case of the acidic and neutral ligands, there was a net and regular decrease in the association constant when the temperature increased.

When the binding data obtained at various temperatures were fitted to the integrated model [eqn. (5)], the number of binding sites and thermodynamic parameters were satisfactorily estimated for each ligand (Table 2). The number of binding sites was between 0.66 and 1.22, indicating that the stoichiometry of ligand binding to AAG was 1:1. To illustrate the validity of the

**Table 2 Estimated thermodynamic parameters for ligand–AAG interaction at 37 °C**

Results for $n$, $\Delta H^0$ and $\Delta S^0$ are estimates ± S.D. $\Delta G^0$ values were calculated using the relation $\Delta G^0 = -RT\ln K_A$ ($R$ = 1.987 cal/mol · K). Note: 1 cal ≡ 4.184 J (S.I. unit).

| Ligand | $n$ | $\Delta H^0$ (kcal/mol) | $\Delta S^0$ (cal/mol · K) | $\Delta G^0$ (kcal/mol) |
|---|---|---|---|---|
| Basic | | | | |
| Binedaline | 0.73 ± 0.03 | −0.04 ± 1.26* | 27.0 ± 4.0 | −3.71 |
| Bornaprolol | 0.66 ± 0.02 | −1.96 ± 1.77* | 20.2 ± 5.8 | −3.45 |
| Propranolol | 1.22 ± 0.03 | −4.09 ± 1.70 | 10.1 ± 5.6* | −3.27 |
| Tertatolol | 1.00 ± 0.01 | −3.48 ± 0.30 | 13.4 ± 1.0 | −3.43 |
| Neutral | | | | |
| Darodipine | 1.00 ± 0.01 | −5.76 ± 1.34 | 6.1 ± 4.5* | −3.44 |
| Isradipine | 0.80 ± 0.04 | −8.51 ± 1.66 | −2.6 ± 5.5* | −3.14 |
| Progesterone | 0.91 ± 0.09 | −5.39 ± 0.78 | 4.5 ± 2.6* | −2.51 |
| Acidic | | | | |
| Warfarin | 0.82 ± 0.01 | −9.00 ± 0.27 | −3.0 ± 1.0 | −3.80 |

* Value not significantly different from zero.

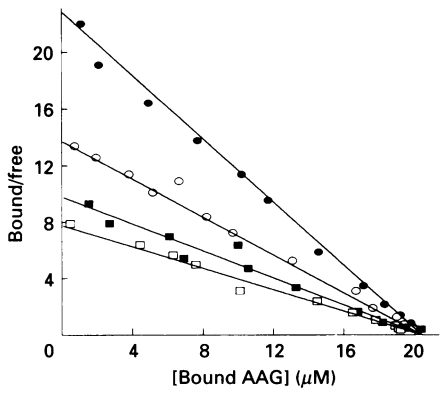


**Figure 2 Binding of warfarin to AAG at pH 7.4 and 20 °C (●), 30 °C (○), 37 °C (■) and 42 °C (□)**

Curves are drawn using eqn. (5) and the values in Table 2. AAG concentration is 20 μM in Sörensen's phosphate buffer.

integrated model, Figure 2 depicts the curve-fitting of warfarin-binding data obtained at four temperatures using the thermodynamic parameters estimated in Table 2.

As shown in Table 2, the enthalpic and entropic components differ between the basic ligands on the one hand, and the neutral and acidic ligands on the other. Binding of the basic ligands binedaline and bornaprolol is relatively insensitive to temperature as previously reported (Kirley et al., 1982; Shaw et al., 1985) and hence markedly entropy-driven, with a small or non-existent enthalpic contribution. As for propranolol and tertatolol, their enthalpic and entropic contributions are comparable. In contrast, binding of the neutral and acidic ligands is almost exclusively enthalpy-driven. As this stage, however, no further rationalization is apparent.

### Physicochemical parameters

Calculated or experimentally determined physicochemical and structural parameters of the investigated ligands are compiled in Table 3. All compounds are lipophilic as judged by their log $P_{oct.}$ values, whereas the log $P_{hep.}$ values show much larger differences.

What is clear from Table 3 is the greater polarity of the neutral and acidic ligands than the basic ligands. This difference is seen in both polarity parameters, i.e. $\Delta\log P_{oct.-hep}$ and $\Lambda_{hep.}$, indicating marked attractive interactions with octanol and water respectively (see the Materials and methods section). We interpret these data to indicate that darodipine, isradipine and warfarin are good hydrogen-bond donors and acceptors, and that progesterone must be a very good hydrogen-bond acceptor. In contrast, the basic ligands must have a somewhat smaller capacity to donate and/or to accept hydrogen-bonds.

## DISCUSSION

The stoichiometry of the ligand–AAG interactions was generally not significantly different from unity, indicating that all ligands bind to one site on AAG. For binedaline and bornaprolol, however, $n$ values of 0.71 and 0.66 were estimated, indicating that these ligands could be preferentially bound to one AAG variant, F1S or A (Hervé et al., 1993). Binedaline affinity for the F1S variant was ten times that measured for the A variant (analogous to warfarin), and bornaprolol (like all the other ligands studied here) had similar affinity for each variant (Hervé et al., 1994; F. Hervé, personal communication). As the commercial AAG preparation we used is a mixture of the F1S and A variants and the proportion of F1S is 70%, binding of all the ligands to this preparation reflects in all cases binding to the F1S fraction.

The main objective of this study was to search for structure–binding relationships and their mechanistic interpretation. A correlation matrix (not shown) for all biological parameters (Tables 1 and 2), distribution coefficients (log $D$), partition coefficients (log $P$) and polarity parameters reveal some interesting facts. First, no good correlation (i.e. $r \geqslant 0.8$) exists between any biological parameter and log $D_{oct.}$, log $D_{hep.}$, log $P_{oct.}$ or $\Lambda_{oct.}$. It is quite intriguing that the affinity (expressed by $\Delta G^0$ or $K_A$) does not correlate ($r < 0.6$) with any of the physicochemical or structural parameters investigated here (e.g. Figure 3), in contrast with other studies (Testa et al., 1987; Brée et al., 1986). This means that no structure–affinity relationship exists in

**Table 3 Structural and physicochemical parameters of the ligands investigated**

$V$ is the calculated van der Waals volume (see the Materials and methods section). p$K_a$ data were taken from Hansch and Leo (1993). log $P$ is that of the neutral form. log $P_{hep.}$ and $\Delta\log P_{oct.-hep.}$ are calculated polarity parameters (see the Materials and methods section).

| Ligand | $V$ | p$K_a$ | log $P_{oct.}$ | log $P_{hep.}$ | $\Delta\log P_{oct.-hep.}$ | $\Lambda_{hep.}$ |
|---|---|---|---|---|---|---|
| Basic | | | | | | |
| Binedaline | 229.4 | 7.89* | 3.78 | 3.14 | 0.64 | 5.8 |
| Bornaprolol | 317.6 | 9.0* | 4.46 | 3.37 | 1.09 | 8.9 |
| Propranolol | 262.9 | 9.45 | 3.31 | 1.56 | 1.75 | 8.7 |
| Tertatolol | 293.0 | 9.82* | 3.24 | 1.75 | 1.49 | 9.6 |
| Neutral | | | | | | |
| Darodipine | 328.4 | 11.4* | 3.65† | 1.04 | 2.61 | 11.7 |
| Isradipine | 329.0 | 11.4* | 3.58† | 0.98 | 2.60 | 11.7 |
| Progesterone | 325.3 | – | 3.87‡ | 1.23‡§ | 2.64 | 11.3 |
| Acid | | | | | | |
| Warfarin | 279.5 | 5.1 | 3.06 | 0.59 | 2.47 | 10.3 |

* Determined in this work (see the Materials and methods section).
† Estimated from the experimental log $k_{40}$ using eqn. (8).
‡ Taken from Hansch and Leo (1993).
§ Partition coefficient in hexadecane/water.

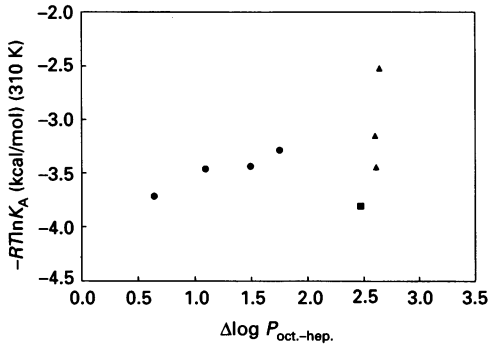


**Figure 3 Poor relationship ($r = 0.46$) between $-RT\ln K_A$ ($\Delta G^0$) and a polarity parameter ($\Delta\log P_{oct.-hep.}$) for basic (●), acidic (■) and neutral (▲) ligands**

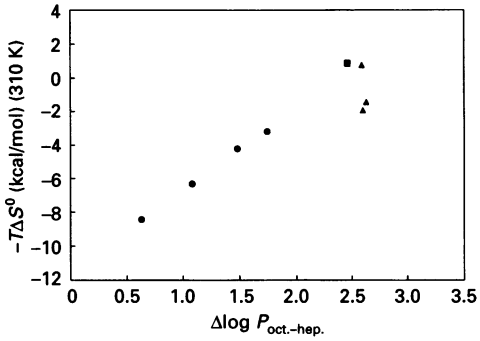


**Figure 5 Linear relationship ($r = 0.94$) between $-T\Delta S^0$ and a polarity parameter ($\Delta\log P_{oct.-hep.}$) for basic (●), acidic (■) and neutral (▲) ligands**

**Table 4 Correlation matrix ($r$) of thermodynamic (37 °C) and physicochemical parameters**

| | $V$ | $\Delta\log P_{oct.-hep.}$ | $\Lambda_{hep.}$ | $\Delta H^0$ | $\Delta S^0$ |
|---|---|---|---|---|---|
| $\Delta\log P_{oct.-hep.}$ | 0.656 | | | | |
| $\lambda_{hep.}$ | 0.875 | 0.924 | | | |
| $\Delta H^0$ | −0.493 | −0.896 | −0.808 | | |
| $\Delta S^0$ | −0.528 | −0.937 | −0.847 | 0.988 | |
| $\Delta G^0$ | 0.547 | 0.459 | 0.491 | −0.158 | −0.288 |

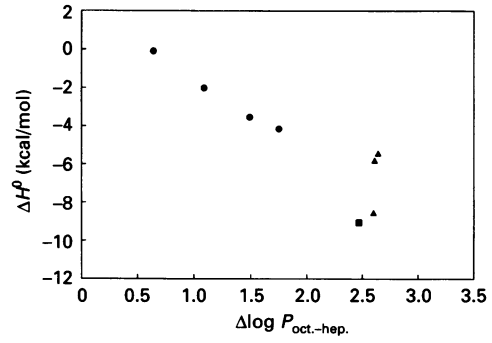


**Figure 4 Linear relationship ($r = -0.90$) between $\Delta H^0$ and a polarity parameter ($\Delta\log P_{oct.-hep.}$) for basic (●), acidic (■) and neutral (▲) ligands**

this series if affinity is expressed only as $\Delta G^0$ or a binding constant.

In contrast, significant and meaningful relationships emerge when $\Delta G^0$ is broken down into its enthalpic and entropic components (Table 4). These two terms, which are highly intercorrelated ($r = 0.988$), show a good correlation with the two polarity terms, particularly $\Delta\log P_{oct.-hep.}$. As discussed above, these two parameters reflect the polar interactions elicited by a solute: mainly its hydrogen-bond-donor acidity, but also its hydrogen-bond-acceptor basicity and dipolarity/polarizability. As shown in Figures 4 and 5, the higher the global polarity of a ligand, the greater its enthalpic component in binding to AAG and the smaller its entropic component.

In conclusion, this study demonstrates in a quantitative manner (i.e. Figures 4 and 5) that the enthalpic drive to AAG binding increases with the global polarity of the ligands, whereas the entropic drive increases with decreasing polarity. To the best of our knowledge, this is the first time that such relationships have been demonstrated with experimentally determined polarity parameters.

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