pH-dependency of basic ligand binding to α_1-acid glycoprotein (orosomucoid)

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The binding interactions of a series of basic ligands with α_1-acid glycoprotein (AAG) were examined as a function of pH. The binding to AAG increased with increasing pH, and the binding data were satisfactorily fitted to a model that incorporates the effect of pH and discriminates the association constants of neutral (non-protonated) and protonated forms of ligands. It was shown that ligands in the neutral form have a markedly higher affinity for AAG than the protonated forms, resulting in a concomitant decrease in the pK_a of bound ligands. The u.v.–visible difference spectra generated upon binding of a representative ligand to AAG also showed that there was a contribution to the binding arising from the deprotonation of the ligand. It is suggested that all tested ligands bind similarly to AAG and that hydrophobic interactions dominate high-affinity binding to AAG.

INTRODUCTION

α-Acid glycoprotein (AAG) is a small acute-phase glycoprotein that is negatively charged at physiological pH and contains a large proportion of carbohydrates (40% by wt.) (Schmid, 1975). AAG interacts with a variety of ligands, e.g. acidic drugs (Urien *et al.*, 1982), steroids (Westphal, 1971) and particularly basic drugs (Piafsky, 1980; Routledge, 1986). A role for AAG as high-affinity carrier has been recognized for most basic drugs, including β-adrenergic-receptor blockers, antidepressants, neuroleptics and local anaesthetics. The binding of these drugs has been quantitatively studied and available data were collected in a recent review (Kremer *et al.*, 1988), but the molecular details of the AAG–ligand interaction remain poorly understood.

We now report a detailed analysis of the effect of pH on the binding of several basic drugs to AAG. This approach allows us to determine the ionization states and protonation equilibrium of AAG-bound ligands, and to measure the association constants of the ligands in ionized and un-ionized forms.

MATERIALS AND METHODS

α_1-Acid glycoprotein

AAG (Behringwerke, Marburg, Germany) was dissolved in Sörensen's phosphate buffer and used without further modification in the binding experiments.

Drugs

Radiolabelled and non-radiolabelled drugs were obtained from the following manufacturers: [^{14}C]binedaline (2.15 GBq/mmol) from Cassenne (Paris, France), dipyridamole from Boehringer (Ingelheim, Germany), loxapine from Lederle Laboratories (Pearl River, NY, U.S.A.), [^{14}C]imipramine (0.27 GBq/mmol) from Amersham International (Amersham, Bucks., U.K.), [^{14}C]nicardipine (0.935 GBq/mmol) from Sandoz (Basel, Switzerland), [^{3}H]propranolol (0.925 TBq/mmol) from Amersham International, [^{14}C]darodipine (0.396 GBq/mmol) from Sandoz and [^{14}C]isradipine (0.558 GBq/mmol) from Sandoz.

Binding experiments

Equilibrium dialysis, measurement of radioactivity and spectropolarimetric or differential u.v.–visible binding titrations were conducted as previously described (Urien *et al.*, 1982, 1984). Sörensen's phosphate buffer was used for all experiments, and the ionic strength varied from 0.09 at pH 6.0 to 0.20 at pH 8.2. The spectropolarimetric titrations of dipyridamole and loxapine binding to AAG were performed at 420 nm and 310 nm respectively, where substantial amplitudes were observed in the differential c.d. spectra of ligand–AAG complexes.

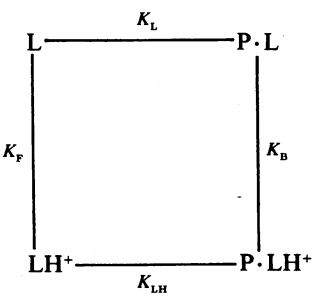
Data analysis

According to the Law of Mass Action, $K_A = [P \cdot L]/[L][P]$, the protein-bound ($B = [P \cdot L]$) and free ($F = [L]$) ligand concentrations from equilibrium-dialysis experiments are related by the following relationship:

$$B = \frac{n \cdot K_A \cdot F \cdot P_t}{1 + F \cdot K_A} \quad (1)$$

where P_t is the total protein concentration, and n and K_A are respectively the number of binding sites and association constant.

In the case of optical titrations, the change in the signal (S) at a given wavelength amounts to a measurement of the fractional



Scheme 1. Equilibria describing the binding of neutral (L) and protonated (LH^+) ligand to AAG (P)

Abbreviation used: AAG, α_1-acid glycoprotein.

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saturation of the protein with the ligand at a particular ligand concentration. Then we have:

$$S = k \cdot \frac{L_t + n \cdot P_t + 1/K_A - [(n \cdot P_t - L_t + 1/K_A)^2 + 4L_t/K_A]^{\frac{1}{2}}}{2} \quad (2)$$

where k is a proportionality factor relating S to the bound ligand concentration and L_t is the total ligand concentration.

When a basic ligand binds to a protein and if the affinity depends on the ionization state of the ligand, K_A is an apparent constant that relates protonated (LH^+) and neutral (L) bound ligand concentrations to free ligand species concentrations:

$$K_A = \frac{[P \cdot LH^+] + [P \cdot L]}{[P] \cdot ([LH^+] + [L])} \quad (3)$$

The equilibria describing the binding of a basic ligand to a protein are shown in Scheme 1 and obey the following equations:

$$K_L = [P \cdot L]/[P][L] \quad (4)$$

$$K_{LH} = [P \cdot LH^+]/[P][LH^+] \quad (5)$$

$$K_F = [L][H^+]/[LH^+] \quad (6)$$

$$K_B = [P \cdot L][H^+]/[P \cdot LH^+] \quad (7)$$

with $pK_a(F) = -\log(K_F)$ and $pK_a(B) = -\log(K_B)$. It follows that the observed association constant, K_A, can be expressed in terms of the binding constant of protonated ligand, K_{LH}, the binding constant of the neutral ligand, K_L, and the ionization constant of the ligand in the free state, K_F:

$$K_A = \frac{K_{LH} \cdot ([H^+]/K_F) + K_L}{[H^+]/K_F + 1} \quad (8)$$

In addition, we have, for the ionization constant of the bound ligand:

$$K_B = \frac{K_L \cdot K_F}{K_{LH}} \quad (9a)$$

which can also be written as:

$$pK_a(F) - pK_a(B) = \log(K_L) - \log(K_{LH}) \quad (9b)$$

Practically, the pH values were chosen in order to involve significant changes in the apparent association constant, K_A. Also, the choice of pH was constrained by the solubility properties of the ligands investigated. By combining eqn. (1) or (2) with eqn. (8), the binding data obtained at different pH values can be analysed together and described in terms of three unknowns, n, K_{LH} and K_L. These were estimated by a non-linear least-squares fit of at least 30 values of (F,B) or (L_t,S) at three different pH values to the above equations with a commercially available software (MicroPharm; I.N.S.E.R.M. 1990).

RESULTS

Table 1 summarizes the effect of pH variations on the apparent association constant of the studied ligand to AAG. The n values were generally in the range 0.8–1.2, indicating that the stoichiometry of the binding was 1:1. For all these basic ligands there was a net increase in the apparent association constant when the pH was increased, with no significant effect on the number of binding sites. The binding data obtained at different pH values were then analysed together with the model that incorporates the effect of pH and assumes different values for the binding of neutral and protonated forms of the ligand. Representative data analysed in this way are shown in Fig. 1. The results for all the ligands studied are shown in Table 2 and demonstrate

Table 1. Binding constants of various ligands to AAG as a function of pH

Ligand	pH	n	K_A (mM^{-1})	Temperature (°C)
Binedaline	6.0	0.84±0.02	804±87	37
	6.6	0.87±0.02	1505±192	37
	7.4	0.87±0.02	2304±297	37
Dipyridamole	7.0	0.76±0.06	654±223	20
	7.4	0.84±0.02	1002±157	20
	7.8	0.96±0.03	2860±1120	20
Imipramine	6.8	0.94±0.04	29±3	37
	7.4	0.92±0.04	63±7	37
	8.2	1.26±0.03	98±9	37
Loxapine	7.0	0.67±0.05	597±239	20
	7.4	0.75±0.04	945±362	20
	7.8	0.75±0.04	1107±490	20
Nicardipine	6.3	0.77±0.13	301±78	37
	6.7	0.78±0.08	783±134	37
	7.4	1.06±0.09	763±103	37
Propranolol	6.8	1.19±0.14	102±14	37
	7.4	1.23±0.13	113±18	37
	8.0	1.13±0.19	189±51	37
Darodipine	6.8	1.21±0.10	170±36	37
	7.4	1.20±0.11	168±42	37
	8.0	1.12±0.09	159±14	37
Isradipine	6.8	0.81±0.07	237±100	37
	7.4	0.90±0.02	217±21	37
	8.0	0.76±0.04	235±80	37

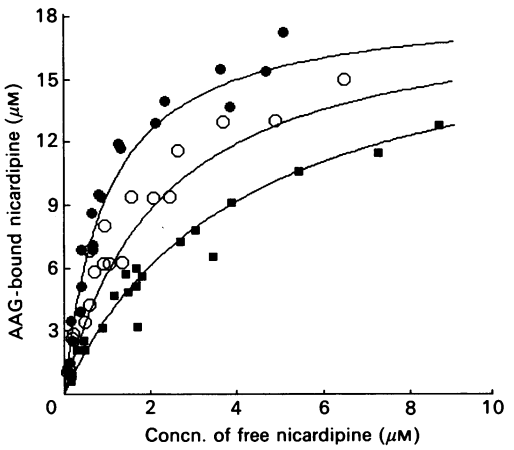

Fig. 1. Binding of nicardipine to AAG at 37 °C and pH 6.3 (■), pH 6.7 (○) and pH 7.4 (●)

Curves are drawn according to eqns. (1) and (8) according to the values in Table 2. AAG concentration is 25 μM in Sörensen's phosphate buffer.

Table 2. Calculated binding constants of AAG for the two ionic forms (neutral, K_L, and protonated, K_{LH}) of ligands

Ligand	$pK_a(F)$	$pK_a(B)$	n	K_{LH} (mM^{-1})	K_L (mM^{-1})
Binedaline	6.9	6.0	0.86±0.01	420±87	3500±370
Dipyridamole	6.4	5.7	0.89±0.03	272±155	1406±425
Imipramine	9.5	8.2	1.22±0.04	28±2	519±70
Loxapine	6.6	5.8	1.09±0.03	156±95	1050±360
Nicardipine	7.2	6.0	0.93±0.08	68±26	1640±239
Propranolol	9.4	8.1	1.20±0.09	98±27	2350±884

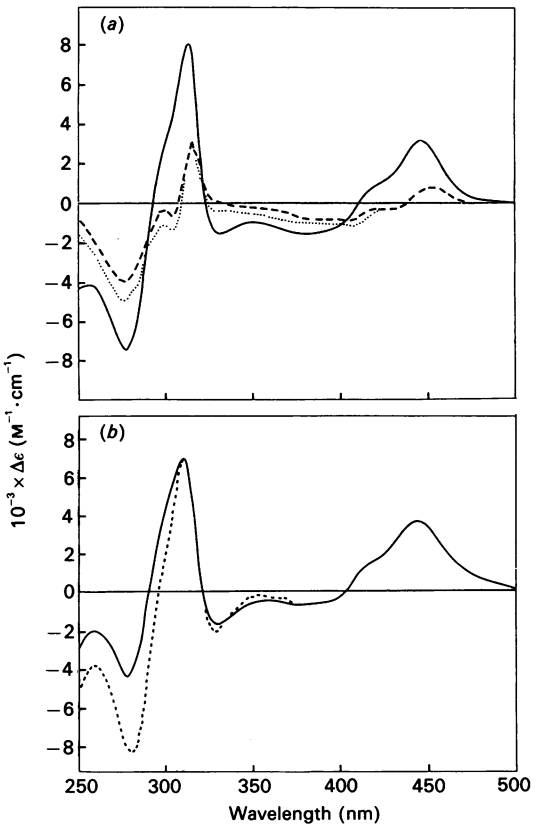

Fig. 2. pH-dependence of difference spectra generated upon dipyridamole binding to AAG at 20 °C

(*a*) Difference spectra at pH 5.8 (——), pH 7.4 (·····) and pH 8.2 (----). (*b*) Difference between the difference spectra for dipyridamole binding at pH 8.2 and pH 5.8 (——) is compared with the deprotonation spectrum of free dipyridamole (-----). The difference spectra are obtained by using matched quartz split-compartment cells with each compartment of path length 0.4375 cm. Background (unmixed compartments) is run first, then sample (mixed compartments) is run. The deprotonation spectrum of dipyridamole is the difference between spectra at pH 4.0 and pH 8.2.

that the association constants of the neutral forms of ligands are dramatically higher than those of the protonated forms of ligands. Moreover, there was a shift in the ionization constants of the bound ligands, the pK_a values of the bound ligands being about 1 unit lower than those of the free ligands. The bindings to AAG of two un-ionizable ligands, darodipine and isradipine, were not pH-dependent, as shown in Table 1, providing additional evidence that the pH-induced change in the binding of the basic ligands was essentially due to different affinities of the two ionization states, with no or insignificant contribution of pH-induced changes in the AAG macromolecule itself.

For a compound that exhibits different absorption spectra in two ionization states, and whose pK_a changes upon binding to AAG, the difference spectrum generated upon binding will contain a component arising from the change in the degree of protonation of the ligand. Fig. 2(*a*) shows the difference spectra produced by dipyridamole binding to AAG at different pH values. This ligand was chosen for its large-amplitude difference spectrum in the range 250–500 nm. The main features (pH 5.8) are negative bands at 277, 330 and 380 nm and positive bands at 313 and 445 nm with shoulders at 300 and 432 nm. For comparison, the difference spectrum between dipyridamole at pH 4 and pH 8.2 (i.e. between neutral and protonated forms, deprotonation spectrum) is shown in Fig. 2(*b*). Similarities are obvious between the difference spectra generated upon dipyridamole binding to AAG and the deprotonation spectrum.

The difference spectra associated with dipyridamole binding to AAG can be interpreted as being the sum of a deprotonation difference spectrum plus electronic changes in bound dipyridamole. The form of the difference spectrum and the band maxima do not change with pH, but the amplitude of the bands clearly does. This indicates unambiguously that the protonation of dipyridamole contributes to the difference spectrum. If the pH-dependence of the amplitude of the protonation contribution is the main source of the pH-dependence of the binding difference spectrum, then the difference between the difference spectra obtained at two pH values should resemble the deprotonation difference spectrum. Fig. 2(*b*) shows the difference between the spectra obtained at pH 5.8 and 8.2 and the deprotonation difference spectrum of dipyridamole. The two spectra are closely similar, the amplitude difference in the range 250–325 nm probably reflecting some contribution from the protein.

DISCUSSION

The six basic drugs examined here behave similarly, their affinity to AAG increasing with increasing pH, i.e. with increasing proportion of neutral molecules in solution. Previous studies have already documented this phenomenon, e.g. propranolol (Ravis *et al.*, 1987), phencyclidine (Owens *et al.*, 1983), gallopamil (Rutledge & Pieper, 1987) and prilocaine (Bachmann *et al.*, 1990). The possibility that the variation in ligand binding could be due to pH-induced changes in the protein is rendered unlikely by the behaviour of darodipine and isradipine, un-ionizable ligands whose affinities remain constant in the pH range 6.8–8.0.

The equilibrium model in Scheme 1 implies that K_B/K_F equals K_L/K_{LH} (eqn. 9a). In other words, the basicity of the bound ligand will be shifted to reflect the relative affinities of the neutral and protonated species. The goodness of fit of our experimental data to eqn. (1) or (2) and eqn. (8) can be seen when comparing $[pK_a(F) - pK_a(B)]$ values with $[\log(K_L) - \log(K_{LH})]$ values (Table 3). The concordance is rather good and demonstrates that the higher affinity of the neutral species causes a proportional decrease in the basicity of the bound ligand, i.e. an increase in the proportion of high-affinity species. Moreover, this indicates that the variations of the association constants are not related to the structure or ionization properties of the ligands, but they depend on the pH domain investigated and on the difference $[\log(K_L) - \log(K_{LH})]$, i.e. the difference between the affinities of the neutral and ionized (protonated) ligands. Also, the different pH domains investigated along with the goodness of fit of the data to the model demonstrate the applicability of the calculation method on a wide pH scale.

The general resemblance between the difference spectrum generated by deprotonation of dipyridamole and that observed when dipyridamole binds to AAG is additional support for

Table 3. pK_a shift between free and bound ligands as compared with the differential affinity between neutral and protonated ligands (see eqn. 9b)

Ligand	$pK_a(F) - pK_a(B)$	$\log(K_{LH}) - \log(K_L)$
Binedaline	0.9	0.9
Dipyridamole	0.7	0.7
Imipramine	1.3	1.3
Loxapine	0.8	0.8
Nicardipine	1.2	1.4
Propranolol	1.3	1.4

this conclusion. The difference observed in the range 250–310 nm is probably the sum of microenvironmental changes occurring in the region of the solvent-accessible aromatic amino acid residues. Indeed, pH-induced changes in the environment of tryptophan residues have been demonstrated by fluorescence-quenching studies (Friedman *et al.*, 1985), but such changes may also arise upon complex-formation from local perturbation of aromatic amino acid residue(s) in the binding domain.

The calculated K_L and K_{LH} values show that the neutral (unprotonated) ligands bind from 5 to 20 times more tightly to AAG than the protonated forms. This suggests that hydrophobic and van der Waals interactions dominate binding to AAG, and further supports the view that the binding domain of AAG includes several hydrophobic amino acid residues. Moreover, from a physicochemical point of view, the apolar complexing of neutral ligand with AAG is also favoured because neutral molecules are less hydrated than protonated ones (Smithrud & Diederich, 1990). Kute & Westphal (1976) have ascribed the hydrophobic amino acid sequence 21–31 of the AAG primary structure to an important part of the binding site for progesterone. This ligand-binding domain should also contain two relatively shielded tryptophan residues (Friedman *et al.*, 1985), indicating that it can be viewed as a crevice in the protein structure with limited accessibility for water molecules. There is, however, a weaker but significant binding to AAG of protonated ligands, which may result from various causes, e.g. greater dehydration upon binding, less favourable alignment in the binding site due to an ionic bond and/or simply decreased lipophilicity of the charged molecule. The suggestion that hydrophobic interactions are one of the dominant forces in ligand–AAG interactions could be tested in aqueous solutions of increased ionic strength or solvent mixtures of different polarities. In such experiments, however, the effect of the medium on AAG conformation must be taken into account and this calls for careful experimental design.

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