pH-dependence of warfarin binding to α_1 -acid glycoprotein (orosomucoid)

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The binding of warfarin to α_1 -acid glycoprotein (AAG) was found to increase with decreasing pH. The u.v.-visible difference spectra generated upon binding to AAG at pH 5.0 or 7.4 showed warfarin to bind as the anion. Warfarin-binding data were satisfactorily fitted to a model that incorporates the effect of pH and discriminates the association constants of the non-protonated and protonated binding site of the protein. It

was shown that AAG-binding site in the protonated form had a markedly higher affinity for warfarin than the non-protonated form, with a pK value of 7.7 ± 0.1 , which is likely to be a histidine residue. Among other possible interactions, it is suggested that ligand binding to AAG involves a reinforced hydrogen bond.

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% w/w). AAG interacts with a variety of ligands, e.g. acidic drugs, steroids and, particularly, basic drugs (Kremer et al., 1988). In a recent study we showed that the non-protonated (neutral) form of basic ligands was more tightly AAG-bound than the protonated form, with a decrease in the pK of the bound ligand reflecting a higher proportion of non-protonated species relative to the unbound state (Urien et al., 1991). Accordingly, ligand binding to AAG could result from hydrophobic interactions between an apolar region of the protein and an un-ionized ligand. However, the binding may also be viewed as the result of hydrogen-bond(s) formation between ligand (H-donor) and protein (H-acceptor), explaining the enhanced ligand deprotonation in the bound state. Since the binding site of basic ligands on AAG is also shared by some acidic drugs (Urien et al., 1982), some H-bond formation could also be involved in the binding of acidic ligands to AAG.

We now report a detailed analysis of the effect of pH on the binding of an acidic drug, warfarin, to AAG. This approach allows us to characterize the protonation equilibrium of the AAG-warfarin complex, and to measure the association constants of the ligand to the protonated and non-protonated form of the protein binding site.

MATERIAL AND METHODS

AAG

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

Warfarin

Radiolabelled [14 C]warfarin (1.9 GBq/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.).

Binding experiments

Equilibrium dialysis, measurement of radioactivity and

differential u.v.-visible spectra recordings were conducted as previously described (Urien et al., 1982, 1984). All solutions were made in 0.066 M Sørensen's phosphate buffer and adjusted to pH values > 8 with 1 M NaOH.

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 respectively.

When a ligand assumed to be in a constant electrical form binds to a protein and if the affinity depends on the protonation state of the binding site, K_A is an apparent constant which relates the ligand binding to the protonated (PH^+) and non-protonated (P) binding site to the free ligand concentration:

$$K_A = \frac{[PH^+ \cdot L] + [P \cdot L]}{[L] \cdot ([PH^+] + [P])} \quad (2)$$

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

$$K_P = [P \cdot L]/[P][L] \quad (3)$$

$$K_{PH} = [PH^+ \cdot L]/[L][PH^+] \quad (4)$$

$$K_F = [P][H^+]/[PH^+] \quad (5)$$

$$K_B = [P \cdot L][H^+]/[PH^+ \cdot L] \quad (6)$$

with $pK_F = -\log(K_F)$ and $pK_B = -\log(K_B)$. It follows that the observed association constant, K_A , can be expressed in terms of the binding constant of protonated protein, K_{PH} , the binding constant of the non-protonated protein, K_P , and the ionization constant of the protein binding site in the free state, K_F :

Abbreviation used: AAG, α_1 -acid glycoprotein.

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Scheme 1 Equilibria describing the binding of a ligand (L) to the non-protonated (P) and protonated (PH⁺) binding site of AAG

$$K_A = \frac{K_{pH} \cdot ([H^+]/K_f) + K_p}{[H^+]/K_p + 1} \quad (7)$$

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

$$K_B = \frac{K_p \cdot K_f}{K_{pH}} \quad (8)$$

If, in contrast, the affinity of the ligand–protein complex depends on the ionization state of the ligand, but not of the binding site, then LH and L substitute to PH and P in eqns. (2)–(8), and K_f and K_B refer to the ionization constants of the ligand [see Urien et al (1991) for a detailed description].

Practically, the pH values were chosen in order to involve significant changes in the apparent association constant. First, it was necessary to demonstrate that, over the pH range investigated, warfarin binds to AAG in a single, constant, electrical form. Then the K_A values obtained over a large range of pH values (5–10) were analysed as a function of pH according to eqn. (7) and provided estimates for K_{pH} , K_p and K_f . By combining eqn. (1) with eqn. (7), the binding data obtained at three different pH values can be analysed together and described in terms of three unknowns, n , K_{pH} and K_p . The parameters were estimated by a non-linear least-squares fit with commercially available software (MicroPharm; Urien, 1990).

RESULTS

Table 1 summarizes the effect of pH variations on the apparent association constant of the AAG–warfarin complex. The n values were in the range 0.8–0.9, indicating that the stoichiometry of the binding was 1:1. As can be seen, there was a net increase in the apparent association constant when the pH decreased, with no significant effect on the number of binding sites. Figure 1 shows the pH effect on the apparent association constant of the AAG–warfarin complex over the pH range 5–10.

Differential u.v.-absorption spectroscopy was used to examine the ionization state of warfarin when bound to AAG over the pH range 5–8. Warfarin exhibits two different absorption spectra in its two ionization states: neutral and anionic. If there is a pH-related change in the binding to AAG, the difference spectrum generated upon binding will contain a component arising from the change in the degree of ionization of either the ligand or the protein or both. Figure 2 shows the difference spectra produced by warfarin binding to AAG at pH 5.0 and 7.4. For comparison, the difference spectrum between warfarin at pH 3.0 and pH 7.4 (i.e. between neutral and anionic form, deprotonation or anion formation spectrum) is also shown. Similarities are obvious between the difference spectra generated upon warfarin binding

to AAG at pH 5.0 and the deprotonation spectrum. At pH 5.0, free warfarin (pK 5.0) is equally distributed between its anionic and neutral forms, and the resemblance between the deprotonation spectrum and the difference spectrum generated on binding indicates that warfarin undergoes extensive deprotonation upon binding to AAG. In other words, AAG-bound warfarin is markedly more acidic (i.e., has a smaller pK) than free warfarin. At pH 7.4, free warfarin is completely ionized

Table 1 Binding constants of warfarin to AAG as a function of pH

pH	n	K_A (mM ⁻¹)
5.4	0.86 ± 0.02	1059 ± 136
6.4	0.82 ± 0.02	797 ± 111
7.9	0.84 ± 0.01	402 ± 25

Figure 1 The apparent association constant [$K(\text{app.})$] of AAG–warfarin complex as a function of pH

The curve is drawn according to eqn. (7) with K_{pH} 689, K_p 108 mM⁻¹ and pK_f 7.71.

Figure 2 pH-dependence of difference spectra generated upon warfarin binding to AAG at 20 °C

Difference spectra at pH 5.0 (—), pH 7.4 (---), deprotonation spectrum of free warfarin (.....). 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 warfarin is the difference between spectra at pH 3.0 and 7.4.

Figure 3 Binding of warfarin to AAG at 37 °C and pH 7.9 (■), pH 6.4 (○) and pH 5.4 (●)

(a) The binding is assumed to depend on the ionization of the binding site. (b) The binding is assumed to depend on the ionization of warfarin. Curves are drawn according to eqns. (1) and (7). The AAG concentration was 20 μ M in Sørensen's phosphate buffer.

Table 2 Estimated binding constants for the interaction of anionic warfarin with the two states of the binding site of AAG (non-protonated, P, and protonated, PH)

pK_F	pK_B	n	K_{PH} (mM^{-1})	K_P (mM^{-1})
7.71	8.93	0.84 ± 0.01	937 ± 83	55 ± 74

(deprotonated), and the very weak amplitude of the difference spectrum generated on binding, without any noticeable similarity with the deprotonation spectrum, indicates that free and AAG-bound warfarin are essentially in the same ionization state, deprotonated.

Having established that at pH 5.0 and above warfarin bound to AAG is in a constant ionic state, eqns. (2)–(8) become applicable. The data were fitted according to eqn. (7), and the analysis demonstrated that no satisfactory curve fitting could be obtained when the pK_F value was set to the ionization constant of warfarin (5.0). The curve-fitting depicted in Figure 1 was then obtained by estimating three parameters: K_{PH} , K_P and K_F . The K_F estimate, 7.71 ± 0.10 , was significantly different from the free-warfarin pK_F and even more different from the bound-warfarin pK (< 5.0 ; see above). The binding data obtained at three 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 either the protein binding site or the ligand. As shown in Figure 3, the best curve-fitting was obtained when the protonation equilibrium of

the unoccupied binding site was assumed to occur with a pK_F value of 7.71. Accordingly, the parameter estimates in Table 2 indicate that the association constant of the protonated binding site is dramatically higher than that of the non-protonated binding site. Moreover, there was a shift in the ionization constant of the binding site, the pK_F value of the bound site being 1.2 unit higher than that of the free binding site.

DISCUSSION

The results of the present study show that warfarin binding to AAG is sensitive to pH variations. The affinity of the warfarin–AAG complex increased with decreasing pH, i.e. with an increasing proportion of protonated molecules, ligand or protein, in solution. A previous study of pH effect on phenprocoumon interaction with AAG showed similar results (Otagiri et al., 1987).

The spectroscopic experiments show that warfarin is essentially in the anionic state when bound to AAG; even at acidic pH that should favour the neutral form. This is indicative of a decrease in the pK of AAG-bound warfarin. Therefore, the pH-induced variation in the warfarin–AAG binding does not result from a change in the ionization state of bound warfarin, but from a change in the protonation state of an amino acid residue in the binding site. The analysis of the pH titration of the apparent association constant is an additional support to this conclusion, the ionization constant (pK_F) of the free AAG species involved in binding had to be set to 7.71 (instead of 5.0) to obtain a satisfactory fit.

The equilibrium model in Scheme 1 implies that, from eqn. (8), $pK_B - pK_F (= 1.22)$ equals $\log(K_{PH}) - \log(K_P) (= 1.23)$. In other words, the basicity of the bound protein site will be shifted to reflect the relative affinities of the non-protonated and protonated protein. The agreement is rather good and demonstrates that the higher affinity of the protonated binding site causes a proportional increase in the basicity of the binding site, i.e. an increase in the proportion of high-affinity species.

The estimated K_P and K_{PH} values show that the protonated binding site binds warfarin 20 times more tightly than the non-protonated site. As the warfarin bound form is the anion, this suggests a hydrogen bond may be involved in its binding to AAG. Kute and Westphal (1976) have ascribed the hydrophobic amino acid sequence 21–31 of the AAG primary structure to an important part of the binding site of progesterone. This sequence includes one lysine, one tryptophan and one tyrosine residue. Using a fluorescence quenching technique, Friedmann et al. (1985) have proposed a model in which the binding domain of AAG includes two tryptophan residues, with the third tryptophan residue of AAG on the periphery of the domain. The pK_F estimated for the residue involved in the binding, 7.71, ranges between the expected pK of the imidazole moiety of histidine in protein, 6.3 (values of 7.10 and 7.40 reported for alkaline phosphatase and lactoglobulin), and pK values of tyrosine (9.6) and lysine (10.4) side chains (Steinhardt and Reynolds, 1969). The histidine pK values are the closest. These residues are located at positions 127, 130 and 171 (Schmid, 1975), and one of them might come close to the binding domain in the three-dimensional structure of AAG (note that His-171 is relatively close to Trp-160). If a protonated imidazole ring is indeed the H-bond donor of AAG, then the interaction is a *reinforced H-bond* of the form $N^+ \cdots \cdots O^-$.

The present results, along with our study of pH effects on basic ligand binding, indicates that one of the intermolecular forces by which AAG binds its ligands (be they basic or acidic) is the donation of a hydrogen bond. This mechanism occurs with an

increase in the pK of AAG, and a decrease in the pK of the ligand. This *mutually induced* 'pK fit' is in itself a phenomenon deserving further study.

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