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FLUORESCENCE STUDIES OF β-ADRENERGIC LIGAND BINDING TO α_1-ACID GLYCOPROTEIN WITH 1-ANILINO-8-NAPHTHALENE SULFONATE, ISOPRENALINE, ADRENALINE AND PROPRANOLOL

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Abstract—The present study shows that ANS (1-anilino-8-naphthalene sulfonate), propranolol, isoprenaline, adrenaline and dopamine have common binding sites on AAG (α_1-acid glycoprotein). A fluorescence technique was employed to characterize the interaction between the ligands and AAG at 20–22°. The binding of ANS to AAG caused increased fluorescence intensity at emission and excitation wavelengths of 400 and 470 nm. In this situation, propranolol displaced ANS in a concentration-dependent mode with an apparent dissociation constant of 6.2 ± 0.01 μM, whereas isoprenaline did not reduce the ANS–AAG fluorescence. However, in the presence of AAG, catecholamines caused a marked increase of fluorescence at excitation and emission wavelengths of 250 and 325 nm, respectively. These wavelengths were employed to characterize the binding of isoprenaline, adrenaline and propranolol to AAG. Two subsets of binding sites were demonstrated. The K_d values were 0.87 ± 0.03 and 25.1 ± 10.7 μM for ANS, 0.76 ± 0.09 and 133 ± 30.4 μM for propranolol, 140 ± 14 and 2.18 ± 0.58 mM for isoprenaline, 137 ± 24 and 14.8 ± 0.1 mM for adrenaline, respectively. AAG had identical high affinity binding capacity for these ligands ($n \approx 1$). However, the second class of binding sites showed ligand-dependent binding capacity: $n = 1$ for ANS, $n \approx 10$ for propranolol, $n \approx 15$ for adrenaline, $n \approx 20$ for isoprenaline, respectively. ANS, propranolol, dopamine and adrenaline caused concentration-dependent inhibition of isoprenaline binding to AAG with apparent dissociation constants of 5.1 ± 1.8 μM, 6.4 ± 1.1 μM, 0.57 ± 0.13 mM and 1.5 ± 0.46 mM, respectively.

α_1-Acid glycoprotein (AAG§) has binding capacity for β-adrenoceptor blockers and stimulators. The binding of propranolol to the serum protein fraction including AAG indicated that AAG bound propranolol with high affinity and low capacity ($K_d \approx 1$ μM and $B_{max} \approx 1$ site/molecule) [1]. This was confirmed in a study with purified AAG [2]. The maximum capacity of the high affinity binding site has been reported to be: 0.2–1 site per AAG molecule [2–8]. With the exception of the reported K_d value of 33 μM [2], the reported K_d values are very similar (1–4 μM) [3–8]. A secondary class of low affinity binding sites on AAG (K_d = 35–200 μM, $B_{max} \approx 1$ site per AAG molecule) has been reported [6, 8]. The secondary binding component has also been interpreted according to a non-saturable interaction [7].

The observation that the β-adrenergic agonists isoprenaline, adrenaline and noradrenaline displaced propranolol which was bound with high affinity in serum [9], suggested that AAG possessed a common β-adrenergic binding site or domain. In agreement with this hypothesis, catecholamine binding sites on AAG have been identified and characterized [10, 11]. In a recent study, ANS was used as a fluorescence probe to characterize the interaction between propranolol and AAG [12]. These observations led us to employ fluorescence techniques to study β-adrenergic ligand binding to AAG.

MATERIALS AND METHODS

Chemicals. The following substances were used: (±)-propranolol hydrochloride and (±)-isoprenaline sulphate (The Norwegian Drug Monopoly, Harstad, Norway), (±)-dopamine hydrochloride (Janssen Chemica, Beerse, Belgium), (−)-noradrenaline hydrochloride, (−)-adrenaline bitartrate, ANS, (−)-ascorbic acid (Sigma Chemical Co., St Louis, MO, U.S.A.) and DEAE Sepharose Fast Flow PhastGel, gradient 8–25 (Pharmacia AB, Uppsala, Sweden). All other chemicals were of analytical grade.

Incubation buffer. A modified Krebs–Ringer phosphate buffer comprised: NaCl 122 mM, KCl 4.9 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.3 mM and Na_2HPO_4 15.9 mM, pH 7.4.

Isolation and purification of AAG. AAG was purified from human plasma by the addition of ammonium sulphate (360 g/L) and by carrying out the pH adjustments described by Weimer *et al.* [13]. The final product was dialysed against 25 mM sodium acetate buffer (pH 4.1) and applied to a DEAE-Sepharose Fast Flow column (2.6 × 20 cm), previously equilibrated against this buffer. The glycoprotein was finally eluted from the column using a gradient of NaCl (0–0.3 M) in 25 mM sodium acetate buffer, pH 4.1. The fractions containing

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§ Abbreviations: AAG, α_1-acid glycoprotein; ANS, 1-anilino-8-naphthalene sulfonate.

AAG were pooled, concentrated and dialysed against double distilled water. The final product was freeze dried and stored frozen at −20° until required. The final product appeared homogenous in SDS–PAGE (Phastsystem, Pharmacia AB, Uppsala, Sweden) under reducing conditions.

Fluorescence spectroscopy. Fluorescence measurements were made in a Fluorescence Spectrophotometer MPF-3 (Perkin-Elmer Ltd, Norwalk, CC) or a Luminescence Spectrophotometer LS-50 (Perkin-Elmer Ltd, Beaconsfield, U.K.). Two mL of AAG/buffer solution were titrated with successive 2 μL additions of fluorescence probes and/or displacing ligands. The fluorescence was measured at 20–22° at pH 7.4 and the relative intensities were obtained directly from fluorometer readings at excitation and emission wavelengths of the ligand–protein complex.

Data analysis. The data were analysed by the PC-computer program Enz-Fitter[R] (Biosoft, Cambridge, U.K.).

RESULTS

Effect of β-adrenergic ligands on the AAG–ANS fluorescence

The fluorescence, given as relative fluorescence (%F), increased successively with increasing concentrations of ANS in a solution of AAG (10 μM) at excitation and emission wavelengths of 400 and 470 nm. Scatchard analysis [14] showed two classes of binding sites; $K_{d1} = 0.87 \pm 0.03$ μM and $B_{max1} = 387 \pm 56$%F, $K_{d2} = 25.1 \pm 10.7$ μM and $B_{max2} = 325 \pm 65$%F (mean value ± SD, N = 6). When the data were transformed according to Weber and Young [15], the respective binding capacities were 1.06 ± 0.13 and 0.89 ± 0.11 sites per AAG molecule.

Propranolol quenched the extrinsic fluorescence of the AAG–ANS complex in a concentration-dependent mode at these wavelengths (Fig. 1). The apparent dissociation constant (K_i) for propranolol was 6.23 ± 0.01 μM (mean ± SD, N = 6). In contrast to the results with propranolol, isoprenaline gave a concentration-dependent increase in fluorescence intensity of the AAG–ANS mixture at excitation and emission wavelengths of 400 and 470 nm (Fig. 1).

Intrinsic and extrinsic fluorescence of catecholamines, propranolol, AAG and ascorbic acid

Spectrum analysis showed that isoprenaline, adrenaline, noradrenaline and dopamine in the presence of AAG, caused a marked increase in the fluorescence intensity at excitation and emission wavelengths of 250 and 325 nm, respectively. However, all catecholamines and propranolol showed considerable intrinsic fluorescence in the incubation medium, but markedly lower than in the presence of AAG (Table 1). In addition, noradrenaline and dopamine demonstrated quenching of the extrinsic fluorescence. The maximum emission of AAG in buffer was found at 340 nm and contributed to considerable intrinsic fluorescence at 325 nm (Table 1). In Krebs–Ringer phosphate buffer, pH 7.4, ascorbic acid showed minimal fluorescence, but caused quenching of the intrinsic fluorescence of AAG (Table 1).

Concentration-dependent binding of isoprenaline, adrenaline and propranolol to AAG

In the AAG solution, isoprenaline concentrations less than 10 μM gave no additional fluorescence above background. However, for concentrations above 10 μM, the fluorescence intensity increased in a concentration-dependent manner. The Scatchard plot (Fig. 2a) suggested that isoprenaline interacted with two different classes of binding sites. The binding characteristics obtained by non-linear curve fitting, showed that isoprenaline was bound to two sets of binding sites, one with high ($K_d = 140 \pm 14$ μM) and another with low affinity ($K_d = 2.18 \pm 0.58$ mM, mean ± SD, N = 6). The maximal binding, expressed as relative fluorescence, was 2.75 ± 0.28 and 58.6 ± 6.3%F, respectively (mean value ± SD, N = 6). Using the method of Weber and Young [15], the respective binding capacities were 1.04 ± 0.02 and 22.4 ± 2.4 sites per AAG molecule (mean ± SD, N = 6).

Figure 2b shows a Scatchard plot of adrenaline binding to AAG. The non-linear plot was decomposed into two classes of binding sites, one with high ($K_d = 137 \pm 24$ μM) and another with low affinity (14.8 ± 0.1 mM, mean ± SD, N = 3). The maximal binding expressed as relative fluorescence was 19.2 ± 1.5 and 292 ± 13.4%F, respectively. The average number of high and low affinity binding sites per AAG molecule was 0.94 ± 0.02 and 15.8 ± 2.5, respectively.

The fluorescence of propranolol, expressed as relative fluorescence (%F), increased concentration-dependently from 1 μM. The Scatchard plot suggested two classes of binding sites (Fig. 2c). Non-linear curve fitting gave the following binding characteristics: $K_{d1} = 0.76 \pm 0.09$ μM, $B_{max1} = 6.7 \pm 1.5$%F and $K_{d2} = 133 \pm 30.4$ μM, $B_{max2} = 62.6 \pm 5.0$%F (mean ± SD, N = 3). Using the method of Weber and Young [15], the respective binding capacities were 1.02 ± 0.002 and 9.91 ± 2.59 sites per AAG molecule (mean ± SD, N = 3).

Displacement of isoprenaline by ANS, propranolol, dopamine and adrenaline

ANS showed small extrinsic fluorescence in the AAG solution at the excitation and emission wavelengths of 250 and 325 nm (Table 1), and reduced the extrinsic fluorescence of isoprenaline bound to AAG, in a concentration-dependent manner (Fig. 3) with an apparent dissociation constant (K_i) of 5.11 ± 1.80 μM (mean ± SD, N = 3). Propranolol bound to AAG showed considerable extrinsic fluorescence (Table 1). However, the difference in extrinsic fluorescence in the presence and absence of isoprenaline, showed a concentration-dependent displacement of isoprenaline (Fig. 3). The apparent dissociation constant (K_i) for propranolol was 6.43 ± 1.09 μM (mean value ± SD, N = 3). Both dopamine and adrenaline displaced isoprenaline bound to AAG. After correction for the extrinsic fluorescence of the agonists in the presence of AAG (Table 1), the fraction of isoprenaline bound to AAG decreased in a

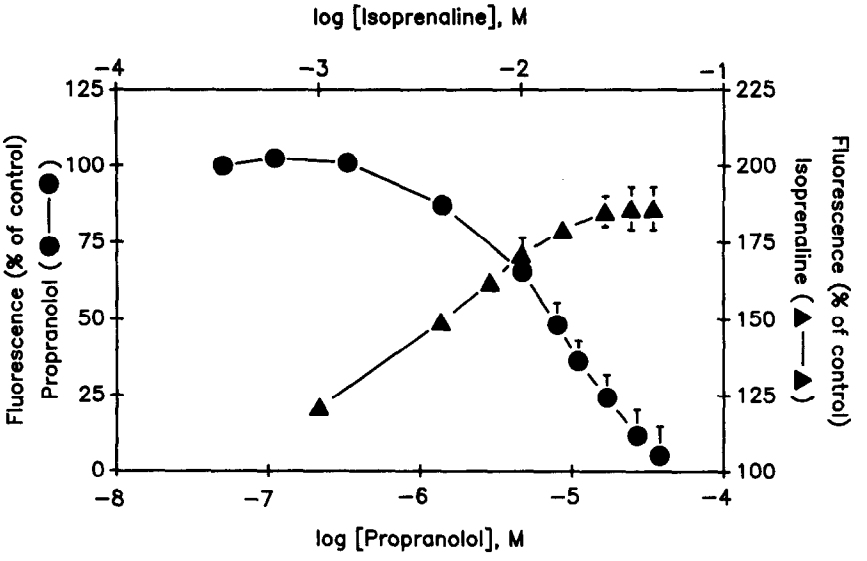

Fig. 1. The effect of propranolol (●) and isoprenaline (▲) on the extrinsic fluorescence of ANS (5 μM) in a solution of AAG (10 μM) at emission and excitation wavelengths of 400 and 470 nm. The fluorescence intensity was measured before and after the addition of increasing concentrations of propranolol (0.05–39 μM, N = 6) and isoprenaline (1.0–45 mM, N = 3). Ascorbic acid was added to the isoprenaline solution as antioxidant (0.1 mM). The results are presented as means ± SD.

Table 1. Relative fluorescence of α_1-acid glycoprotein (AAG), ascorbic acid (ASC), isoprenaline (ISO), adrenaline (ADR), noradrenaline (NA), dopamine (DA) and propranolol (PRO) in Krebs–Ringer phosphate buffer (KRPB), pH 7.4 at 20–22° or in 10 μM AAG dissolved in KRPB, pH 7.4 at 20–22° at excitation and emission wavelengths of 250 and 325 nm, respectively

Substance	Relative fluorescence (%F)		Comments on fluorescence	
	In KRPB	In AAG	Intrinsic	Extrinsic
AAG (10 μM)	17–19	—	High	
ASC (0.1 mM)	≪1	4–6*	Low	Low
ANS (10 μM)	≪1	11–12	Low	Low
ISO (1 mM)†	19–22	46–50	High	
ADR (1 mM)†	20–22	54–58	High	
NA (0.5 mM)†	20–22	32–37	High	Quenching > 0.5 mM
DA (0.5 mM)†	18–20	32–36	High	Quenching > 2.0 mM
PRO (12 μM)	8–11	24–30	High	

* Quenching of intrinsic AAG fluorescence.
† In the presence of ascorbic acid (0.1 mM).

competitive manner (Fig. 3). The apparent dissociation constants (K_i) for dopamine and adrenaline were 0.57 ± 0.13 mM (N = 6) and 1.5 ± 0.46 mM (mean value ± SD, N = 4), respectively.

DISCUSSION

AAG has binding capacity for a large number of drugs [16]. Recent results indicate that AAG possesses one wide binding area with overlapping binding sites for basic and acidic drugs [17]. Such a binding domain may also account for the fact that some drugs have more than one class of binding sites. High as well as low affinity binding sites have been identified on AAG for propranolol [6, 8] and catecholamines [10].

Fluorescence techniques have been widely employed in the study of drug binding to AAG. Dipyridamole and chlorpromazine cause quenching of the intrinsic fluorescence of AAG [18–20]. On the other hand, a number of ligands shows extrinsic fluorescence after binding to AAG [12, 16, 21–24].

For excitation and emission wavelengths of 400 and 470 nm, the present study showed that ANS was bound to two classes of binding sites and interacted with propranolol with characteristics in agreement with previous reports [12, 22]. In contrast to propranolol that quenched the extrinsic fluorescence

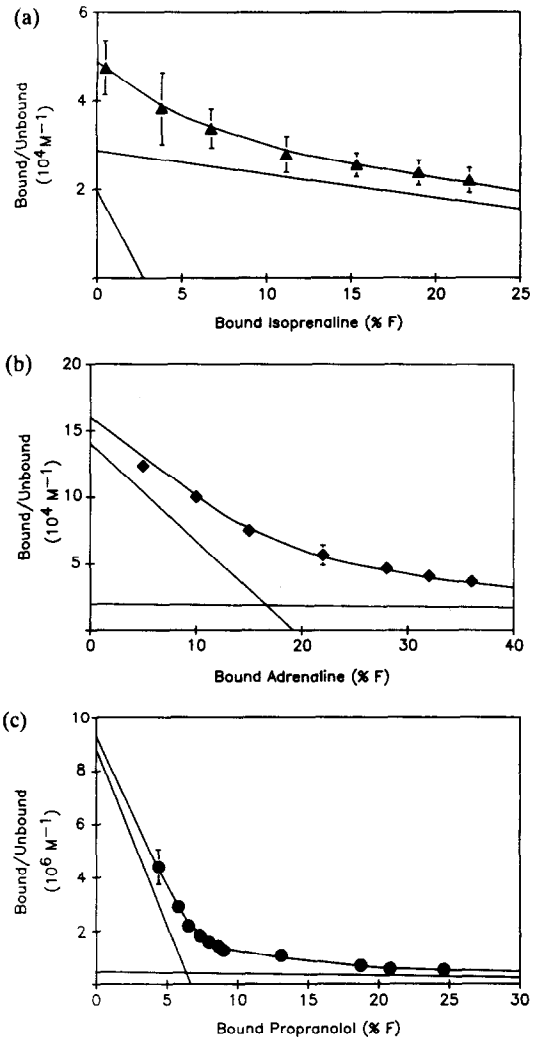

Fig. 2. The binding of isoprenaline, adrenaline and propranolol to AAG is presented in Scatchard plots. AAG (10 μM) was successively titrated with isoprenaline (10 μM–1 mM), adrenaline (0.05–1 mM) or propranolol (0.1–73 μM), respectively. The catecholamine mixture contained ascorbic acid (0.1 mM) to prevent oxidation. The difference between extrinsic and intrinsic fluorescence was determined at excitation and emission wavelengths of 250 and 325 nm, respectively. The results are presented as mean value ± SD for (a) isoprenaline (▲), N = 6; (b) adrenaline (◆), N = 3 and (c) propranolol (●), N = 3. The experimental data were analysed by non-linear curve fitting. The binding lines for each class of binding sites and the resultant total binding curve are shown.

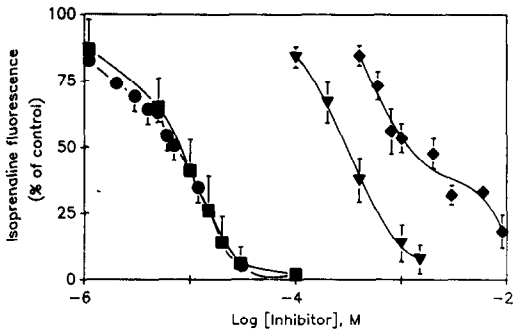

Fig. 3. Displacement of bound isoprenaline (1 mM) to AAG (10 μM) by ANS (1.1–100 μM), propranolol (1.1–100 μM), dopamine (0.4–9.0 mM) and adrenaline (0.1–1.5 mM). The difference between extrinsic and intrinsic fluorescence was determined at excitation and emission wavelengths of 250 and 325 nm, respectively. The results are presented as means ± SD for ANS (■), N = 3; propranolol (●), N = 3; dopamine (▼), N = 6; and adrenaline (◆), N = 4.

of ANS at 400 and 470 nm, isoprenaline caused a concentration-dependent increase of fluorescence intensity. This can be explained by an emission peak with maximum at 490 nm of intrinsic isoprenaline fluorescence.

The addition of the catecholamines as well as the β-adrenoceptor blocker propranolol to the AAG solution caused marked increase in fluorescence intensity. In contrast to ANS, these compounds had high intrinsic fluorescence. This property, which is greatly enhanced in acidic solutions, has been employed to measure the concentrations of these substances [25, 26]. The magnitude of intrinsic fluorescence makes the catecholamines and propranolol unfit as fluorescence markers, but the difference between extrinsic and intrinsic fluorescence was sufficient to study the binding in both concentration saturation as well as displacement experiments.

For the experimental situation with emission and excitation wavelengths of 250 and 325 nm, respectively, the fluorescence data revealed two classes of apparently independent binding sites for isoprenaline, adrenaline and propranolol. The high affinity binding dissociation constants were in close agreement with previously reported values for isoprenaline and adrenaline [10] and propranolol [1, 3–8]. The present results suggested that AAG possessed one high affinity binding site per molecule for isoprenaline, adrenaline and propranolol, in close correspondence with the previous reported values; 0.6–1.1 sites/molecule [2–8, 10].

The secondary class of binding sites had K_d values similar to previously reported values for isoprenaline, adrenaline [10] and propranolol [6, 8]. The maximum binding capacity showed a marked difference between propranolol, adrenaline and isoprenaline. For isoprenaline and adrenaline, the binding capacity was 22 and 16 sites per AAG molecule, slightly higher than the value (10 sites per AAG molecule) reported previously [10]. The present study with 10 low affinity sites for propranolol per AAG molecule appears to reflect an intermediate situation of the previously reported single saturable low affinity site [6, 8] and the non-saturable binding component [7]. A variable number and distribution of negative charges within the binding domain of AAG within the binding domain of AAG [16], due to differences in the experimental conditions, may account for these discrepancies.

The displacement experiments showed that ANS, propranolol, isoprenaline, adrenaline and dopamine

have common binding sites or binding domains on AAG due to the facts that (1) propranolol showed competitive reduction of the extrinsic fluorescence of ANS at 400 and 480 nm, (2) ANS, propranolol, dopamine and adrenaline reduced the extrinsic fluorescence of isoprenaline at 250 and 325 nm in a concentration-dependent mode. The binding affinities (K_i values) obtained from the displacement curves, appeared to reflect an intermediate of the high and low binding affinities, respectively, obtained in the saturation binding curves. This suggests a mutual competition for both high and low affinity binding sites on AAG.

As far as we know, this is the very first report on AAG as a binding protein for dopamine with an affinity similar to that of isoprenaline. Concerning the binding of noradrenaline to AAG [10, 11], the combined effect of high intrinsic fluorescence and quenching of intrinsic fluorescence made the data difficult to interpret.

This study shows that several adrenergic ligands exhibit an extrinsic fluorescence in combination with AAG that is distinctly higher than the intrinsic fluorescence. This property has been used to characterize their binding sites on AAG. However, the significant degree of intrinsic fluorescence makes these compounds not suitable as fluorescence probes.

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