Biochemical and Biophysical Research Communications 478 (2016) 462–466

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A fluorescent reporter detects details of aromatic ligand interference in drug-binding sites of human serum albumin

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ARTICLE INFO

Article history:
Received 8 June 2016
Accepted 14 June 2016
Available online 16 June 2016

Keywords:
Human serum albumin
Phenylbutazone
Fluorescent reporter CAPIDAN
Interference of aromatic ligands

ABSTRACT

Human serum albumin (HSA) transports many ligands including small aromatic molecules: metabolites, drugs etc. Phenylbutazone is an anti-inflammatory drug, which binds to the drug-binding site I of HSA. Its interaction with this site has been studied using a fluorescent dye, CAPIDAN, whose fluorescence in serum originates from HSA and is sensitive to the changes in HSA site I in some diseases. Its fluorescence in HSA solutions is strongly suppressed by phenylbutazone. This phenomenon seems to be a basic sign of a simple drug-dye competition. However, a more detailed study of the time-resolved fluorescence decay of CAPIDAN has shown that phenylbutazone lowers fluorescence without changing the total amount of bound dye. In brief, the HSA-bound dye forms three populations due to three types of environment at the binding sites. The first two populations probably have a rather strong Coulomb interaction with the positive charge of residues Arginine 218 or Arginine 222 in site I and are responsible for approximately 90% of the total fluorescence. Phenylbutazone blocks this interaction and therefore lowers this fluorescence. At the same time the binding of the third population increases considerably in the presence of phenylbutazone, and, as a result, the actual number of bound dye molecules remains almost unchanged despite the ligand competition. So, time resolved fluorescence of the reporter allows to observe details of interactions and interference of aromatic ligands in drug binding site I of HSA both in isolated HSA and in serum.

1. Introduction

Phenylbutazone (Fig. 1) is a well-known anti-inflammatory drug that binds to site I of human serum albumin (HSA) [1–6]. The drug localization in site I of crystallized HSA has been described in detail [5,6]. However, a detailed description of the complex in water and, more importantly, in serum is absent. We have tried to develop a picture of the phenylbutazone-HSA interaction. An approach proposed many years ago has been used, i.e., the competition of a drug with a fluorescent reporter that is able to bind to HSA [7]. A fluorescent probe, CAPIDAN (Fig. 1; [8–10]), has been used. This dye is very sensitive to the changes in HSA binding sites that accompany some diseases [8–11]. The drug and the dye have rather similar structural elements, i.e., aromatic rings and aromatic ketone groups. The HSA-bound dye has a complex fluorescence decay, which provides much more information on events in the binding site than does steady-state fluorescence.

2. Materials and methods

Phenylbutazone was purchased from Sigma-Aldrich.

The fluorescent probe CAPIDAN, *N*-(*p*-carboxyphenyl)imide of 4-(*N*,*N*-dimethylamino)naphthalic acid, was synthesized and was a kind gift of B.M. Krasovitsky and colleagues (Institute of Monocrystals, Kharkov, Ukraine).

Lyophilized powders of human serum albumin (cat. A 9511) (below it is named as “natural” HSA) and fatty acid-free HSA (cat. A 1887) were purchased from Sigma-Aldrich. HSA was dissolved in a buffer of 0.01 M sodium phosphate at pH 7.4 (ionic strength 0.02 M), and NaCl was added to obtain an ionic strength greater than 0.02 M. Serum from a healthy human donor was obtained via

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http://dx.doi.org/10.1016/j.bbrc.2016.06.072
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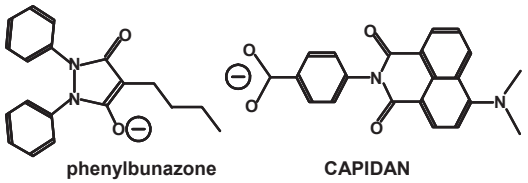

Fig. 1. Phenylbutazone [14–17] and CAPIDAN [8–10] in their anionic forms.

the standard method. The subject gave informed consent for this study.

Steady-state fluorescence spectra were measured using a Hitachi F 4000 spectrofluorometer, at excitation wavelength 450 nm and emission wavelength range 480–580 nm. Time-resolved fluorescence decays were measured using a Pico-Quant instrument; the excitation source was a pulsed diode with a spectral maximum at 455 nm; the instrument response function, IRF, was close to 0.6 ns. The CAPIDAN fluorescence decay was detected at 530 nm and approximated as a sum of three exponentials using the chi-squared criterion [8,9]:

$$F(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + A_3 \exp(-t/\tau_3) \qquad (1)$$

where t is time after excitation. These three exponentials reflect the actual existence of three different fluorescent species with different dye-environment interactions inside the HSA binding sites [8–10]. The value of τ_1 is close to 9 ns, τ_2 is almost 3 ns, and τ_3 is approximately 1 ns. Two first species are responsible for approximately 90% of the total fluorescence intensity of HSA-bound CAPIDAN. The amplitudes of these exponentials, A_1, A_2 and A_3, respectively, can be converted into the absolute concentrations of the species as described earlier [12,13]; at used conditions the fraction of the bound dye was about 20% or lesser.

3. Results

3.1. Phenylbutazone/CAPIDAN interference: steady-state fluorescence detection

In water, the CAPIDAN fluorescence intensity is very low (not shown in Fig. 2) [8]. The addition of HSA at the physiological ionic strength, $I = 0.16$ M, increases this intensity by many times (the left point of the curve (triangles) in Fig. 2). The lower the ionic strength is, the greater the intensity (Fig. 2). This behaviour was assumed [9] to be caused by the Coulomb interaction of the negatively charged carboxyl of the dye (Fig. 1) with the positive charges on the surface of HSA drug-binding site I, probably Arginine 218 or Arginine 222 [18]. The important role of the negative charge of dyes in HSA binding was noticed a long time ago [19,20].

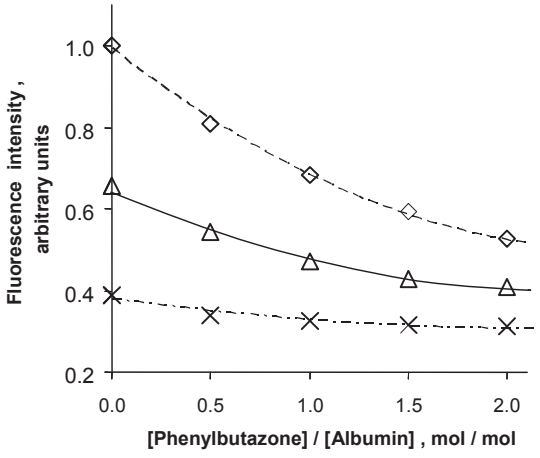

Fig. 2. Phenylbutazone and ionic strength influence on the steady-state fluorescence intensity of CAPIDAN (12 μM) in HSA solution (12 μM), pH 7.4. HSA is fatty acid free. Ionic strength: $I = 0.02$ M (rhombs), 0.16 M (triangles) and 0.63 M (crosses). All intensities are normalized to the maximal value at $I = 0.02$ M.

The presence of phenylbutazone in HSA + CAPIDAN solutions suppresses the fluorescence intensity (Fig. 2). Most of this decrease occurs at when the drug/HSA molar ratio = 1. Thus, phenylbutazone interferes in some manner with the dye at some drug-binding sites of HSA. Phenylbutazone specifically binds with the so-called first drug-binding site of HSA, and the binding constant, K, is approximately $(0.3–1.5)\cdot 10^6$ M^{-1} [3,5]; in contrast, for CAPIDAN, the binding constant is much lower, $(1–5)\cdot 10^4$ M^{-1} [8,9]. In other words, phenylbutazone should be significantly preferred during this competition and therefore should displace the dye from its "naturally occupied" site.

It is interesting that suppressing the HSA-bound dye fluorescence by a high ionic strength is similar to the suppression by phenylbutazone (Fig. 2). In particular, in the presence of $I = 0.63$ M, the drug can no longer cause a strong decrease in the fluorescence (the lower curve in Fig. 2). It can be assumed that the drug and I affect the dye fluorescence in the same manner. The presence of many ions prevents the negatively charged carboxyl of dye from interacting with the positive charge of Arginine 218 or Arginine 222 in site I [18]. Therefore, phenylbutazone probably hinders this charge-charge interaction.

3.2. Phenylbutazone/CAPIDAN interference: time-resolved fluorescence detection

The phenylbutazone/dye interference can be analysed in more detail by considering the time-resolved fluorescence decay data in accordance with the idea of three species of HSA-bound dye (Equation (1) in section 2 – Methods). The intensity, F, of the steady-state fluorescence described above is a sum of the partial intensities of these three species:

$$F = b_1(A_1\tau_1 + A_2\tau_2 + A_3\tau_3) \qquad (2)$$

where b_1 is an instrument constant. As has been shown earlier [9], the three decay times remain the same, and they almost are unchanged (Table 1) in the presence of either phenylbutazone (variations are less than 4%) or increase in ionic strength (not shown); in contrast, the amplitudes are sensitive to the drug and I (Table 1). Thus, the F changes described above (Fig. 2) cannot be attributed to decay time changes (Table 1) and are a result of the effect of I and phenylbutazone on the amplitudes A_i.

These amplitudes are proportional to the absolute concentrations of the species [9,12,13]. The presence of phenylbutazone at physiological ionic strength leads to decreases in A_1 and A_2 ([9] and Table 1); these two species responsible for approximately 90% of the total fluorescence intensity F; therefore, this total intensity decreases upon the drug addition (triangles in Fig. 2). This A_1 and A_2 decrease might be assumed to be a sign of exclusion of the dye from HSA into water, which quenches the fluorescence. However, the third amplitude, A_3, does not decrease; moreover, A_3 increases approximately to the same extent as the sum $A_1 + A_2$ decrease (Table 1). As a result, the total number of HSA-bound dye molecules, i.e., a value proportional to the sum $A_1 + A_2 + A_3$, remains approximately independent of the presence of phenylbutazone. More precisely, one phenylbutazone molecule per one HSA molecule leads to decreases in A_1 by 38% and A_2 by 15% while A_3 simultaneously increases by 13%; thus, the sum $A_1 + A_2 + A_3$