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SHORT COMMUNICATION

Characterization of a Specific Drug Binding Site of Human Serum Albumin

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SUMMARY

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The rapid reaction of p-nitrophenyl acetate with human serum albumin is stoichiometrically inhibited by the binding of ligands in a specific apolar anion binding site. Inhibition of the reaction by certain ligands, including many drugs, thus affords a convenient and sensitive means to detect and study their interactions with that site. Procedures for the general qualitative and quantitative characterization of interactions of ligands with the p-nitrophenyl acetate reactive site are described and preliminary results obtained with six well known drugs are presented.

INTRODUCTION

Albumin, the most abundant protein in human serum, has the unusual capacity to interact strongly with a wide variety of apolar compounds. A relatively large number of essentially independent binding sites appear to account for the diversity of ligands with which it associates. Binding to albumin is an important factor in the solubilization and subsequent transport of many drugs in serum. Due to its relative abundance and its high affinity for many compounds, serum albumin is also a major reservoir and, in such cases, by competing with tissue receptors may seriously influence the distribution to target tissues and organs.

The binding of drugs and other ligands (e.g., fatty acids (1), tryptophan (2), bilirubin (3), etc.) by serum albumin has been studied by a variety of techniques including equilibrium partitioning methods (4, 5), gel filtration (6), differential spectrophotometry (7), ultrafiltration (8), circular dichroism (9), fluorescence (10), and electron spin resonance (11). The results using the last three techniques in combination with specific probes have been used recently to study the binding at particular sites. Detailed qualitative and quantitative information on the binding of drugs and their degradation products to individual sites, however, is still lacking. Methods to monitor such interactions would be useful for identifying ligands that might compete for the same site and are necessary for detailed characterization of structural and conformational requirements of binding.

In this communication we will extend our studies of the reaction of human serum albumin with p-nitrophenyl acetate (12) as a probe to monitor the binding of several drugs at a specific small apolar anion binding site.

Reactions of p-nitrophenyl acetate (recrystallized Aldrich, Lot #081167) with defatted human serum albumin (Fraction V, "Essentially Fatty Acid Free," Sigma) were

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followed by monitoring formation of the bright yellow p-nitrophenolate ion using a Cary 118C recording spectrophotometer equipped with a 25° thermostatted cell holder under pseudo first-order conditions with albumin in at least ten-fold molar excess over p-nitrophenyl acetate, as described previously (12).[1] Appropriate quantities of ligand were mixed with albumin in a cuvette and incubated several minutes to allow the temperature to equilibrate. Reactions were initiated by the addition of p-nitrophenyl acetate through a stoppered hole in the lid of the cell compartment. Useful absorbance readings were obtained within three seconds of addition. First-order rate constants were calculated as the linear least squares slopes of plots of $\log(A_\infty - A_t)$ versus reaction time where A_∞ and A_t are absorptions due to p-nitrophenolate anion at the completion of the reaction (at least ten half-lives) and at various times during the reaction, respectively.

The rapid reaction between p-nitrophenyl acetate and albumin results in the irreversible acetylation of a single amino acid residue (12). Stoichiometric inhibition of the reaction by strongly bound ligands, L, appears to reflect the formation of unreactive 1:1 ligand-albumin complexes, HSA·L,

$$\underset{\text{(Reactive)}}{\text{HSA} + \text{L}} \rightleftarrows \underset{\text{(Unreactive)}}{\text{HSA·L}} \qquad (1)$$

Figure 1 shows the decrease in observed first order rate constants with increasing concentration of several ligands. Chlordiazepoxide (Hoffmann LaRoche) and naproxen (Syntex) give results which appear to represent simple binding isotherms, where complete inhibition is obtained at 1:1 ratios of drug to albumin, as expected for binding primarily at the p-nitrophenyl acetate reaction site. Salicylic acid (Fisher Scientific) and indomethacin (Sigma Chemical Co.) exhibit unusual binding properties. Indomethacin does not inhibit p-nitrophenyl acetate hydrolysis until one molar equivalent is added (Fig. 1) indicating that it is

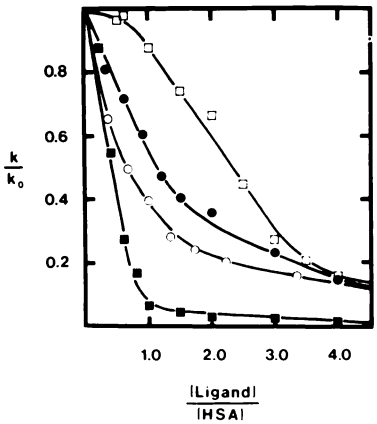

FIG. 1. *Inhibition of the reaction between human serum albumin and p-nitrophenyl acetate by increasing concentrations of ligands*

Reactivities in the presence of increasing amounts of naproxen (■), chlordiazepoxide (○), salicylic acid (●) and indomethacin (□) expressed in terms of observed first-order rate constants, k, as a ratio to that in their absence, k_0 are plotted versus the mole ratio of ligand to human serum albumin (HSA) at 6.5×10^{-5} M under conditions given in Table 1.

bound preferentially at another site and not at the p-nitrophenyl acetate site until the primary site is saturated. Salicylic acid causes a gradual loss of reactivity consistent with its binding competitively at more than one site. Still other drugs have little or no effect on reactivity with p-nitrophenyl acetate even when in large excess, thus demonstrating their low affinity for the reactive site. Dissociation constants,

$$K_d = \frac{[\text{HSA}][\text{L}]}{[\text{HSA·L}]} \qquad (2)$$

for those ligands that interact primarily with the p-nitrophenyl acetate reactive site can be determined from the observed inhibition, as previously described (13)[2] according to the relationship:

$$\frac{[\text{HSA}]_{\text{free}}}{[\text{HSA}]_{\text{total}}} = \frac{k - k_B}{k_0 - k_B} \qquad (3)$$

where k and k_0 are the first-order rate constants in the presence and absence of inhib-

[1] Best results are obtained at albumin concentrations close to K_d. In our hands, concentrations from approximately 10 times to 0.1 times K_d are satisfactory.

[2] A preliminary account of this work was presented at the 174th National Meeting of the American Chemical Society, Chicago, Ill. August 29, 1977, Division of Biological Chemistry Abstract No. 64.

itory ligand, respectively, and k_B is that in the presence of a large excess of ligand due to other reactions of p-nitrophenyl acetate (12), according to the relationship

$$\frac{[\text{Ligand}]_{\text{total}}}{1 - \frac{k - k_B}{k_0 - k_B}} = \frac{K_d}{\frac{k - k_B}{k_0 - k_B}} + [\text{HSA}]_{\text{total}} \quad (4)$$

Thus, as shown in Fig. 2, plots of $[\text{Ligand}]_{\text{total}}/1 - (k - k_B/k - k_B)$ versus $k_0 - k_B/k - k_B)$ give straight lines, the slopes of which are equal to K_d and with intercepts equal to the concentration of binding sites (14–16).

Values of dissociation constants determined using Eq. 4 for the drugs ibuprofen (Upjohn), chlorodiazepoxide, naproxen, and α(o-chlorophenoxy)-isobutyric acid (Sigma Chemical Co.), all of which appear to follow simple 1:1 binding isotherms, are shown in Table 1.

These data are presented as an example of the power of this method to examine ligand binding at a specific site of albumin. That site appears to be an important binding site for many small apolar anions including many drugs (see Table 1) and for straight chain saturated fatty acids with ten or fewer carbons (13).[2] The described method can be used to determine dissociation constants for ligands interacting primarily with that site and to identify qualitatively ligands subject to competition for that binding site. Ligands which do not bind at the same site result in no inhibition while those interacting simultaneously at other sites give complex isotherms which cannot be quantitatively evaluated by the presently described procedure. The technique is quite simple, requires very small quantities of albumin and ligand, and avoids difficult analytical techniques for each ligand since it is based on a property of the protein and not that of individual compounds. In addition to dissociation constants, the method also provides a measure of binding site concentrations or, in the presence of competing ligands, a measure of binding site availability.

Finally it should be mentioned that the same reaction and its inhibition can be observed similarly in human serum (13).[2] By studying the interaction of a series of systematically varied compounds with this site, we hope to determine its size, shape, and general composition.

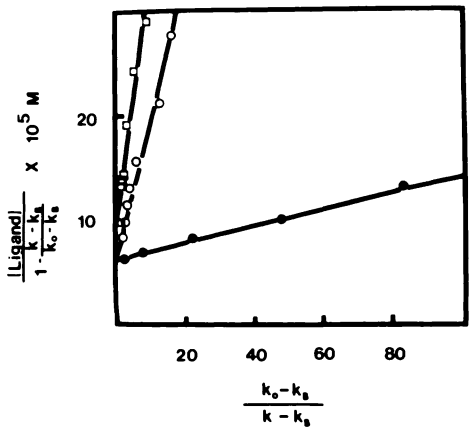

FIG. 2. *Inhibition of the reaction between human serum albumin and p-nitrophenyl acetate plotted according to Eq. 4*

Data for naproxen (●) and salicylic acid (□) taken from Fig. 1 and for α(o-chlorophenoxy)-isobutyric acid (○) under the same conditions, plotted according to Eq. 4. Dissociation constants obtained as the linear least squares best fit slopes are given in Table 1, intercepts indicating binding site concentrations are 6.5×10^{-5}, 9.6×10^{-5} and 6.6×10^{-5} M, respectively.

TABLE 1

Dissociation constants calculated for several drugs

Dissociation constants (K_d) obtained by monitoring the inhibition of the reaction between human serum albumin and p-nitrophenyl acetate under pseudo first-order conditions in 0.06 M triethanolamine/HCl buffer pH 8.0, μ = 0.02 M at 25°.

Compound	k_d
Ibuprofen	$7.3 \pm 2.0 \times 10^{-6}$ M
Chlordiazepoxide	$2.5 \pm 0.2 \times 10^{-5}$ M
Naproxen	$8.5 \pm 2.0 \times 10^{-7}$ M
α(o-Chlorophenoxy) isobutyric acid	$1.3 \pm 0.2 \times 10^{-5}$ M

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REFERENCES

1. Ashbrook, J. D., Spector, A. A. & Fletcher, J. E. (1972) Medium chain fatty acid binding to human plasma albumin. *J. Biol. Chem.*, **247**, 7038–7042.
2. McMenamy, R. H. & Oncley, J. L. (1958) The specific binding of L-tryptophan to serum albumin. *J. Biol. Chem.*, **233**, 1436–1447.
3. Wooley, P. I. & Hunter, M. J. (1970) Binding and circular dichroism data on bilirubin-albumin in the presence of oleate and salicylate. *Arch. Biochem. Biophys.*, **140**, 197–209.
4. Krazner, J., Giacoia, G. P. & Yaffe, S. J. (1973) Drug-protein binding in newborn infant. *Ann. N. Y. Acad. Sci.*, **226**, 101–114.
5. Anton, A. H. (1973) Increasing activity of sulfonamides with displacing agents: a review. *Ann. N. Y. Acad. Sci.*, **226**, 273–292.
6. Muller, W. E. & Wollert, J. (1973) Characterization of the binding of benzodiazepines to human serum albumin. *Naunyn-Schmiedeberg's Arch. Pharmac.* **280**, 229–237.
7. Coutinho, C. B., Lucek, R. W., Cheripko, J. A. & R. Kuntzman. (1973) A new approach to the determination of protein-bound bilirubin displacement and its applications. *Ann. N. Y. Acad. Sci.*, **226**, 238–246.
8. Parke, D. V. & Kindup, W. E. (1973) Quantitative and qualitative aspects of the plasma protein binding of carbenoxolone, an ulcer-healing drug. *Ann. N. Y. Acad. Sci.*, **226**, 200–213.
9. Sjodin, T., Roosdorp, N. & Sjoholm, I. (1976) Studies on the binding of benzodiazepines to human serum albumin by circular dichroism measurements. *Biochem. Pharmacol.*, **25**, 2131–2140.
10. Sudlow, G., Birkett, D. J. & Wade, D. N. (1976) Further characterization of specific drug binding sites on human serum albumin. *Mol. Pharmacol.*, **12**, 1052–1061.
11. Basset, M., Chambaz, E. M. & DeFaye, G. (1977) Study of steroid-protein interactions by electron spin resonance spectroscopy. Binding of a spin-labeled dihydrotestosterone to bovine serum albumin. *Biochim. Biophys. Acta*, **491**, 434–446.
12. Means, G. E. & Bender, M. L. (1975) Acetylation of human serum albumin by p-nitrophenyl acetate. *Biochemistry*, **14**, 4989–4994.
13. Koh, S. W. & Means, G. E. (1979) Characterization of a small apolar anion binding site on human serum albumin. *Arch. Biochem. Biophys.*, **192**, 73.
14. Bieth, J. Some kinetic consequences of the tight binding of protein-proteinase-inhibitors to proteolytic enzymes and their application to the determination of dissociation constants, in *Bayer-Symposium V "Proteinase Inhibitors"* (H. Fritz, H. Tschesche, L. V. Greere, and E. Truscheit, editors), Springer-Verlag, Berlin, 1974, 463–469.
15. Webb, J. L. Inhibition kinetics in mutual depletion systems, in *Enzyme and Metabolic Inhibitors*, Vol. I, Academic Press, New York, 1963, 66–82.
16. Easson, L. H. & Stedman, E. (1936) The absolute activity of choline esterase. *Proc. Roy. Soc.*, **B121**, 142–164.