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THE BINDING OF SPIN-LABELED PROPRANOLOL AND SPIN-LABELED PROGESTERONE BY OROSOMUCOID

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The binding of spin-labeled propranolol and spin-labeled progesterone to human orosomucoid has been studied as a function of temperature by electron spin resonance (ESR) techniques. At 20°C the association constants are 1.9×10^6 and 4.9×10^5 M^{-1}, respectively. In each case, the binding is competitive with unlabeled ligand. Above about 50°C the apparent association constant for both ligands decreases rapidly with increasing temperature. This is due to thermal denaturation of the orosomucoid, as was shown independently by ultraviolet absorption spectroscopy and differential scanning calorimetry. Below the denaturation region the number of binding sites per orosomucoid molecule remains constant at approx. 1. Examination of the thermodynamic parameters shows the progesterone binding at 37°C to be essentially enthalpically driven, while the propranolol binding at 37°C has a substantial entropic component.

1. Introduction

Orosomucoid (α_1-acid glycoprotein) is an acute phase serum protein which, although very widely studied, has yet to be assigned a physiological function. It is a small glycoprotein (mol. wt. 41000), very negatively charged at physiological pH, and contains 40% carbohydrate and up to 16 sialic acid residues (10–14% by weight). It is the most soluble plasma protein, and has been thought to be highly resistant to denaturation [1], although recent work in this laboratory has shown otherwise [2,3].

Orosomucoid undergoes a variety of interactions, associated with the polypeptide or carbohydrate portions of the molecule. Thus, it binds many Δ_4 ketosteroids [4], interacts with collagen [5] and thrombin [6], and has been implicated in immune reactions [7]. Orosomucoid also binds a number of basic drugs [8], including propranolol [9]. It appears to be the only high-affinity carrier for most of these basic drugs in serum, and therefore, since the pharmacological effects of most drugs are dependent on the unbound concentrations of the drugs [10], knowledge of the mechanism and extent of binding become important in their clinical use. Such information should also prove useful in the design of analogues yielding the same therapeutic effect but with lower association constants (K_{ass}) for orosomucoid.

Propranolol is a β-adrenergic blocker enjoying wide use in humans. Recent work indicates that most of the bound fraction in serum resides with orosomucoid and has a K_{ass} of 1.3×10^6 M^{-1} at 20°C for a single binding site [11]. No thermodynamic data of binding or physicochemical data pertaining to the binding site have been reported. The purpose of the work reported here was to evaluate the thermodynamics of binding of spin-labeled propranolol to orosomucoid, and relate this to the physical state of the macromolecule. The techniques developed have also been applied to the binding of progesterone to orosomucoid, for which some limited binding data are available [4,12,13].

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Abbreviation: TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

2. Materials and methods

2.1. Materials

Orosomucoid was prepared from nephrotic urine as reported earlier [14]. Lipid depletion of the product was done according to Ganguly et al. [15]. Orosomucoid prepared in this manner was homogeneous by SDS-polyacrylamide gel electrophoresis, immunoelectrophoresis, Ouchterlony immunodiffusion, analytical gel chromatography and analytical ultracentrifugation.

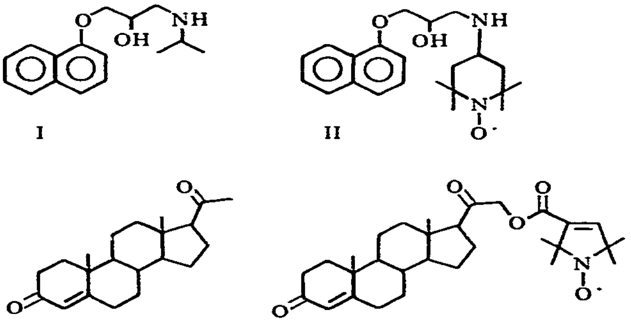
Spin-labeled propranolol was purchased from Molecular Probes, Inc., Plano, TX, as the HCl salt of TEMPO-propranolol and used without further purification. Degradation was noted both by changes in the ESR spectrum and by TLC when solutions were stored in the cold for several months. Periodic checks were therefore made for purity.

Propranolol was obtained from Sigma Chemicals, St. Louis, and used without further purification.

Spin-labeled 21-hydroxyprogesterone was synthesized as reported previously [16] as the 21-ester of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid. All other chemicals were of reagent grade or better.

2.2. ESR

ESR measurements were performed on a Varian E-4 spectrometer under the control of a microcomputer data acquisition system [17]. Care was taken with all instrumental parameters to avoid distortion or power saturation of the signals. Samples were contained in a low-temperature aqueous sample cell in the presence of an external chromium standard. Sample temperature was controlled by a Varian variable temperature accessory, which was calibrated with a Doric 410 thermocouple thermometer. Under the conditions of these experiments, the sensitivity of the instrument did not allow precise measurement of the signal due to bound ligand. All of the ESR results are therefore based on the intensity of the low-field line in the three-line spectrum of free ligand, which was measured as a function of temperature in the presence and absence of orosomucoid.



Scheme 1. Ligands used: I, propranolol; II, TEMPO-propranolol; III, progesterone; IV, spin-labeled progesterone.

2.3. Scatchard analysis

Some of the binding data were evaluated according to Scatchard [18]. In this case, the free ligand ESR signals were converted to molar concentrations using a calibration curve. The concentration of bound ligand was taken as the difference between the total ligand and free ligand values. Concentrations of spin-labeled propranolol, spin-labeled progesterone and orosomucoid were determined spectrophotometrically, using molar extinction coefficients of 5800 M^{-1} cm^{-1} (289 nm; this work), 20260 M^{-1} cm^{-1} (236 nm, 95% ethanol; this work) and 36600 M^{-1} cm^{-1} (278 nm [19]), respectively.

2.4. Van't Hoff analysis

The statistical evaluation of alternative models for nonlinear van't Hoff plots of $\ln K$ versus $1/T$ was performed in a manner similar to that described previously [13]. The most significant change was the writing of the fitting equations for the models in terms of the actual free ligand signal heights and total concentrations of protein and ligand, rather than in terms of association constants. This facilitated the input of data and, particularly, of estimates of their uncertainties, ensuring the appropriate weighting of the experimental points in the fitting. Since the models are not linear in the parameters to be determined, nonlinear least-squares calculations are required. Linear estimates of the standard deviations in the parameters are determined by the fitting program. They reflect only the random errors in the measurements in a particular experiment. The calculations make use of the total ligand and total protein concentrations as constants, without uncertainty. Alterations of these constants within their estimated standard deviations (2 and 1%, respectively) led to variations in the calculated parameters. These variations have been combined with the uncertainties from a single calculation to give the uncertainty estimates (standard deviations) tabulated with the parameters. The resultant increase was by a factor of approx. 2, except for ln $K_{\mathrm{ass}}^{30^\circ\mathrm{C}}$, for which the factor was nearly 10.

2.5. Differential scanning calorimetry

DSC was performed on a Microcal MC-1 apparatus (Microcal Inc., Amherst, MA) with a heating rate of 1°C/min. Known equal volumes of deaerated sample and blank solutions were delivered to the precooled twin platinum cells of the calorimeter with a calibrated microsyringe, and allowed to temperature equilibrate. Scans were in the range of 0–90°C.

2.6. Ultraviolet absorption spectroscopy

Ultraviolet absorption spectroscopy used a Gilford model 240 spectrophotometer fitted with a model 2527 thermoprogrammer. The heating rate was 1°C/min, and absorption was monitored continuously at 285 nm.

3. Results

The apparent binding constant for the association of orosomucoid and spin-labeled propranolol exhibits a marked decrease with increasing temperature above about 50°C. The resulting van't Hoff plot (fig. 1) is strongly nonlinear. Unsuccessful attempts were made to rationalize these results in terms of the two simple models discussed earlier for nonlinear van't Hoff plots [13]. The first is the biphasic model:

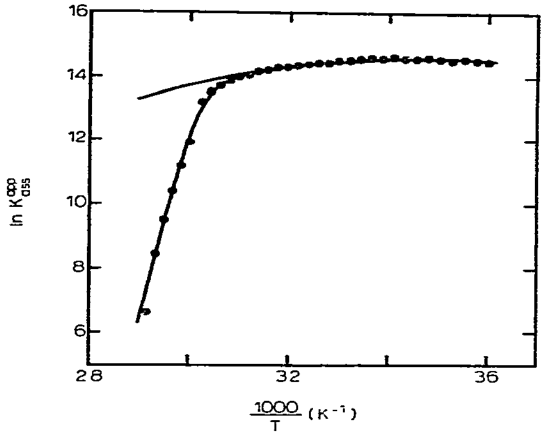


Fig. 1. Van't Hoff plot for the association of TEMPO-propranolol and orosomucoid in 0.01 M phosphate/0.14 M NaCl/0.001 M EDTA, pH 7.4. (●) Experimental points. Line through points is best fit to the ΔC_p-denaturation model. Upper extrapolation is related to K_{ass} in the absence of denaturation. See section 3 for explanation of model.

$$\mathrm{P_1 + L \overset{K_1}{\rightleftharpoons} P_1L}$$

$$\mathrm{P_2 + L \overset{K_2}{\rightleftharpoons} P_2L}$$

$$\mathrm{P_1 \overset{K_3}{\rightleftharpoons} P_2} \qquad (1)$$

where there is a temperature-dependent equilibrium between the two forms of the protein, P_1 and P_2. A single ligand, L, can bind to either form. The standard enthalpies of the first two reactions are restricted to constant values, and that of third reaction is assumed to be large enough that the protein changes sharply from form P_1 to form P_2 at a critical temperature. The second is the ΔC_p model:

$$\mathrm{P + L \overset{K_{ass}}{\rightleftharpoons} PL} \qquad (2)$$

where a single ligand can bind to a single form of the protein, P. The heat capacity of PL is different from the sum of the heat capacities of P and L. This difference, ΔC_p, is restricted to a constant value over the range of temperature of the experiment. With these models either the fits to the data

Table 1

Binding parameters for the association of TEMPO-propranolol and spin-labeled progesterone with human orosomucoid determined from fits to various models

A description of the models is given in section 3.

Ligand (2% ethanol)	Model fit	ln $K_{\mathrm{ass}}^{30°\mathrm{C}}$	$\Delta H_{\mathrm{ass}}^{30°\mathrm{C}}$ (kcal mol^{-1})	$\Delta C_{p_{\mathrm{ass}}}^{30°\mathrm{C}}$ (cal mol^{-1} K^{-1})	ln $K_{\mathrm{D}}^{60°\mathrm{C}}$	$\Delta H_{\mathrm{D}}^{60°\mathrm{C}}$ (kcal mol)	$\Delta C_{p_{\mathrm{D}}}$ (kcal mol^{-1} K^{-1})
Propranolol (−)	ΔC_p-denaturation	14.48±0.21	−2.4 ±0.5	−170± 60	1.51±0.25	100± 5	1.6±1.5
Propranolol (+)	ΔC_p-denaturation	14.20±0.42	−4.1 ±1.4	−310± 12.0	1.61±0.11	90±17	1.5±1.3
Propranolol (−)	ΔC_p	14.49±0.22	−2.5 ±0.5	−200± 50	-	-	-
Propranolol (+)	ΔC_p	14.20±0.44	−4.2 ±1.4	−320±120	-	-	-
Progesterone (+)	ΔC_p	12.74±0.04	−7.46±0.17	−145± 12	-	-	-

were very poor, or the parameters determined were physically unreasonable.

The observed behavior can, however, be quantitatively explained according to the following model:

$$N + L \overset{K_{ass}}{\rightleftharpoons} NL \qquad (3)$$

$$N \overset{K_D}{\rightleftharpoons} D$$

where N and D are the 'native' and 'denatured' states of orosomucoid, and L is the spin-labeled ligand. This is just the ΔC_p model, extended by the inclusion of a simple, two-state denaturation process. It will be referred to as the ΔC_p-denaturation model. Note that the ligand does not bind to the denatured protein, D, in this model. Nonlinear least-squares calculations [13] yielded the best fit of this model to the experimental data. Parameters determined in this way are presented in table 1. Values of K_{ass} and K_{ass} (apparent) $= K_{ass}/(1 + K_D)$ were calculated from these parameters and are plotted as the solid lines in fig. 1. The fit of the ΔC_p-denaturation model to the data is very good. Application of the F(rel) test introduced earlier [13] allowed rejection of both the biphasic and ΔC_p models with greater than 95% confidence.

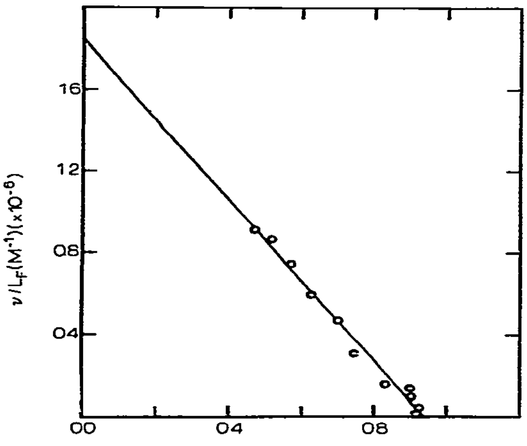


Fig. 2. Scatchard plot for the association of TEMPO-propranolol and orosomucoid in 0.01 M phosphate/0.14 M NaCl/0.001 M EDTA, pH 7.4, at 40°C.

Support for the essential correctness of the very simple model presented above comes from a variety of experiments. Scatchard analysis [18] of binding data from ESR measurements (fig. 2) shows orosomucoid to have indeed a single ($n = 0.94 \pm 0.01$), high-affinity binding site for spin-labeled propranolol over the temperature range 4–46°C. At 20°C, the average value of K_{ass} from several experiments is 1.9×10^6 M^{-1}, only a little higher than that for unlabeled propranolol at the same temperature (1.3×10^6 M^{-1} [11]). In addition, the binding of the spin-labeled propranolol was found to be competitive with that of the unlabeled substrate (% ligand bound decreased from 81 to 10 upon addition of a 30-fold molar excess of unlabeled propranolol). The rapid drop in the apparent binding constant above 50°C (fig. 1) leads unfortunately to extreme imprecision in data for Scatchard analysis in that range. Since nearly all the ligand is free, the concentration of bound ligand, which is determined from the free and total ligand concentrations, becomes very uncertain. Thus, direct evidence for the denaturation of

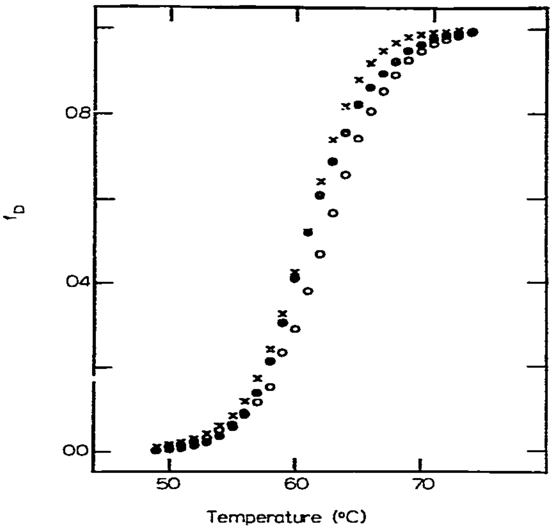


Fig. 3. Fraction denatured of orosomucoid as a function of temperature in 0.01 M phosphate/0.14 M NaCl/0.001 M EDTA, pH 7.4, by several techniques. (●) Differential scanning calorimetry, (○) absorbance, (x) calculated from the parameters of the ΔC_p-denaturation model fit shown in fig. 1.

orosomucoid (decreasing *n* values) cannot be obtained with confidence from these data.

The denaturation of orosomucoid is readily observed, however, by means of ultraviolet melting experiments. The results of a typical measurement are presented in fig. 3 where the fraction of the orosomucoid denatured is plotted versus the temperature. Differential scanning calorimetry has also been employed to detect the denaturation process [3]. The curve determined from a typical experiment is included in fig. 3. Also presented in fig. 3 is the analogous curve calculated from the least-squares parameters for K_D in the first row of table 1. There is relatively good agreement among these results from various, independent experiments.

The association of orosomucoid and spin-labeled propranolol in aqueous solution containing 2% ethanol was examined in a fashion analogous to that described above, and the results were very similar. The parameters are tabulated in table 1.

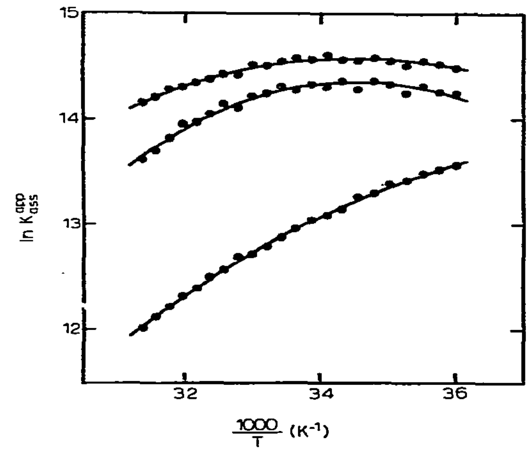


Fig. 4. Van't Hoff plot for the association of ligands with orosomucoid in 0.01 M phosphate/0.14 M NaCl/0.001 M EDTA, pH 7.4, in the predenaturation region. (●) Experimental points. Line through points is best fit to the ΔC_p model, see section 3 for explanation of model. Upper, TEMPO-propranolol; middle, TEMPO-propranolol in 2% ethanol; lower, spin-labeled progesterone in 2% ethanol.

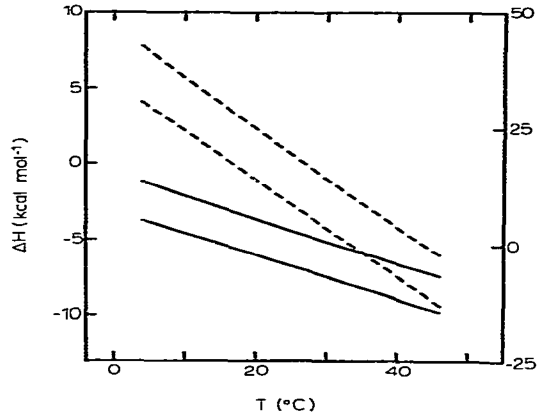


Fig. 5. Enthalpy and entropy changes for the association of orosomucoid in 0.01 M phosphate/0.14 M NaCl/0.001 M EDTA/2% ethanol, pH 7.4. (------) TEMPO-propranolol. (————) spin-labeled progesterone. The curves were computed from the parameters of the ΔC_p model fit (see section 3 for explanation of model). The upper curve in each pair is ΔS.

The association of orosomucoid and spin-labeled progesterone in aqueous solution with 2% ethanol (required because of the sparing solubility of the steroid) was also studied by means of analogous ESR measurements. The temperature range did not extend sufficiently far into the denaturation region, however, to allow application of the full ΔC_p-denaturation model. Therefore, the simple ΔC_p model [13] was fitted instead to the data up to 46°C. The effects of denaturation are negligible in this region, which includes the physiologically important temperature of 37°C. The resulting parameters are included in table 1. It should be noted that simple ΔC_p fits to the data over this temperature range for the propranolol binding yielded parameters for the association constant in agreement with those from the ΔC_p-denaturation fits within the uncertainty estimates listed (see table 1). In fig. 4, the experimental data (4–46°C) and calculated K_{ass} values are plotted for each of the three kinds of binding experiments. The spin-labeled progresterone could be displaced by the parent ligand (% bound from 68 to 49 by 1:1 molar ratio) and by propranolol (% bound from 57 to 11 by 31:1 molar ratio).

4. Discussion

Several workers have concerned themselves with the interpretation of nonlinear van't Hoff and Arrhenius plots for assorted macromolecular phenomena, including conformational transitions [20] and ligand binding [13,21,22]. Eftink and Biltonen [21] have recently provided a most thorough discussion of the thermodynamic changes associated with ligand binding. For the present work, the van't Hoff plot for ligand binding to orosomucoid, when considered over a wide temperature range, can be reduced to three components: two extended regions of slight curvature and a region of rapidly changing slope over a narrow temperature range (fig. 1). There is obviously a multitude of mechanistic models which could be devised to explain this result, but prior experience suggests those tested here. Of the three, that which consists of a nonzero heat capacity change for ligand binding coupled to a denaturation reaction with a different heat capacity change represents the experimental data best (fig. 1). Independent data from differential scanning calorimetry and absorption spectroscopy confirm that orosomucoid does indeed undergo a large endothermic, cooperative transition in the region where the apparent association constant for ligand binding falls dramatically. Further, the parameters calculated from the fit for the denaturation process are physically reasonable and compare very well with the independent, experimental values. A degree of caution should be exercised, however, since the calorimetric data do suggest that the denaturation transition is not a simple two-state event [2,3], as our simple model assumes.

Analysis of the van't Hoff plots prior to the onset of denaturation reveals curvature for both propranolol and progesterone binding (fig. 4). This indicates that the change in heat capacity for the ligand binding reaction is nonzero. The degree of curvature of the plot yields this value in principle, but errors in the apparent equilibrium constant are generally so large that little credence can be given to this method [21]. The approach used here for the determination of the apparent equilibrium constant was chosen for its high precision, the large number of points it yielded, and the ease with which it allowed error propagation for statistical analysis. To some degree the aim was achieved for the standard deviations on parameters on a single run are quite low, however, as expected, the differences between runs for the change in heat capacity are larger than these internal errors. This only allows two comparisons to be made: (1) how the heat capacity changes for propranolol and progesterone compare relatively, and (2) how both of these values compare with the binding of hydrophobic ligands to proteins in general. If this is done, then we see that the heat capacity changes at 30°C for both ligands are approximately the same and not distinguishable because of the associated uncertainties. The magnitude of the change is comparable to that of the binding of many small ligands to macromolecules which lie in the range of -50 to -300 cal K^{-1} mol^{-1} [23]. Comparison of these values with model studies [24] shows that propranolol should be much less hydrophobic in character than progesterone, because of the interaction of its ring π-electrons with water. Consequently, it is to be expected that if the binding interactions are due only to this feature of the ligands, then the unitary entropy change would be higher for progesterone than propranolol. This is not the case, and examination of the variation in ΔS and ΔH with temperature (fig. 5) reveals that propranolol binding has a much larger entropic component than progesterone binding. In the latter case, a relatively large (21 e.u. for unit mole fraction standard state) entropic contribution at 4°C is rapidly lost until at 37°C it is 5 e.u. The trends suggest then that propranolol binding is mainly an entropic process, and progesterone binding an enthalpic process at physiological temperatures. This is a somewhat surprising result in view of the hydrophobic character of both ligands, but may be rationalized on the basis of the contribution of vibrational entropy to the total entropy [25]. The number of vibrational degrees of freedom available to progesterone are greater than for propranolol at all temperatures, and the loss of some of these, therefore, will have a deleterious effect on the total entropy for the binding event. Attempting to translate the magnitudes of the ΔS and ΔH values for the two ligands into molecular events is risky and must be speculative. Any model that is constructed, however, must take account of the following points:

(1) Orosomucoid is known to consist of polypeptide variants [19] whose interactions with the ligands may not all be described by the same thermodynamic parameters.

(2) Progesterone and propranolol possess some structural similarity between the A- and B-ring pair of the steroid and the naphthyl function of the drug.

(3) Although the propranolol side chain is moderately hydrophilic, it contains a hydrophobic end in the form of -$CH(CH_3)_2$ which may allow a second hydrophobic interaction to occur.

(4) Progesterone and propranolol binding are competitive, although the form of the competition is unknown.

On the basis of the current data above, it is not possible to decide whether the differences seen between propranolol binding in the absence and presence of 2% ethanol are significant. We do find, however that in calorimetric experiments a quite large (≈ 50 kcal/mol) endothermic event is induced in orosomucoid by 2% ethanol [3]. A change is also observed spectroscopically at 285 nm [3], suggesting that a conformation alteration occurs. It seems reasonable to expect this to be reflected in the thermodynamic parameters of ligand binding, particularly since the polypeptide portion of orosomucoid is quite small (mol. wt. 24600).

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