Biochimica et Biophysica Acta, 626 (1980) 291—298

BBA 38578

THE ROLE OF ALBUMIN CONFORMATION IN THE BINDING OF DIAZEPAM TO HUMAN SERUM ALBUMIN

JAAP WILTING [a,*], BETSY J. 'T HART [b] and JOHAN J. DE GIER [b]

[a] *Department of Pharmaceutical Chemistry, Subfaculty of Pharmacy, State University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht (The Netherlands) and* [b] *Department of Pharmacotherapy, Subfaculty of Pharmacy, State University of Utrecht, Vondellaan 14, 3521 GE Utrecht (The Netherlands)*

(Received June 2nd, 1980)

Key words: Albumin conformation; Diazepam binding; pH dependence; Circular dichroism; (Human)

Summary

The effect of hydrogen, chloride and calcium ions on the binding of diazepam to human serum albumin has been studied by circular dichroism and equilibrium dialysis. In all cases the molar ellipticity of the diazepam-albumin complex increases with pH over the pH range 5 to 9. Under these conditions the free concentration of diazepam at a constant low drug to protein ratio decreases with pH. This free concentration is higher in the presence of chloride and calcium ions. With a two state conformational model for albumin it was shown, that the pH dependences of molar ellipticity of the diazepam-albumin complex and of the free concentration of diazepam are linked. It was demonstrated that the N-B transition of albumin is involved in the pH dependent binding of diazepam. The consequences of these findings for equilibrium dialysis procedures in determining free plasma levels of diazepam are discussed.

Introduction

Around neutral pH the conformational state of human serum albumin is dependent on the pH [1—5]. In this pH region the so-called neutral to base or N-B transition occurs [1—7]. It has been established that the binding of the anticoagulant drug warfarin to albumin * is affected by this N-B transition [2]. Müller and Wollert [8], Fehske et al. [9], Brodersen et al. [10] and Sudlow et

* To whom correspondence should be addressed.
* With albumin is meant human serum albumin unless otherwise stated.

al. [11] have demonstrated at least two very specific and selective binding sites to be present on the albumin molecule for a large number of highly bound drugs. These sites are the so-called warfarin and diazepam binding site, also denoted as site I and site II by Sudlow et al. [11]. Recently Sjöholm et al. [12] found evidence for the existence of a third specific site, the digitoxin binding site. However the drug tamoxifen was found to bind to a fourth site, the tamoxifen binding site [13]. But Sjöholm et al. [12] showed, that a large number of drugs investigated bind to the warfarin or to the diazepam binding site. Since previously [2] the linkage between the binding of warfarin and the N-B transition was found, it is interesting to know whether or not this transition also affects the diazepam binding site. Therefore in this paper a study on a possible linkage between the N-B transition and the binding of diazepam is described.

Materials and Methods

Albumin, crystallized and lyophilized, lot number 18C-0519, was obtained from Sigma Chemical Company, St. Louis, MO. The albumin was treated before use and concentration of albumin determined as described elsewhere [2]. This treated albumin has less than 0.2 mole fatty acid bound per albumin molecule, as was measured by the method of Novák [14]. In all experiments an albumin concentration of $6 \cdot 10^{-4}$ M was used. Diazepam (kindly supplied by Hoffmann La Roche Nederland, Mijdrecht) was used without further purification. All other chemicals were of analytical grade (Merck, Darmstadt, G.F.R. and J.T. Baker, Deventer, The Netherlands).

Equilibrium dialysis experiments were performed as described previously [2]. Adsorption of diazepam onto the membranes (Diachema, type 10.14, molecular weight cut off of 5000) was negligible. Equilibrium was achieved within 2 h and the effect of sodium, phosphate and borate on the binding of diazepam to albumin was negligible. Free concentrations of diazepam were determined by means of a gaschromatograph (Varian Model 1400) equipped with a ^{63}Ni electron-capture detector following the analysing procedure as described by de Gier and 't Hart [15].

The circular dichroic spectra of the diazepam-albumin complexes were obtained between 360 and 300 nm using a dichrograph III (Jobin Yvon, Long Jumeau, France). The slit was programmed for a half-band width of 2 nm (sensitivity 10^{-6} degree $\cdot$ mm^{-1}, scanning speed 3 nm $\cdot$ min^{-1}, time constant 10 s). After dialysis the contents containing albumin and diazepam were used for the CD measurements. The total drug to protein ratio r followed from dialysis of the dialysate. A rectangular cell (Hellma) of 1 cm pathlength was used. The observed ellipticities (θ_{obs}) are the differences between the CD spectra of the drug-albumin mixtures and the albumin alone at a given wavelength [16]. Molar ellipticities ($[\theta]$) were calculated from θ_{obs} assuming complete binding of diazepam to one binding site and using the equation $[\theta] = 100 \cdot \theta_{obs}/l \cdot c$ where l is the pathlength in cm and c the molar concentration of diazepam-albumin complex.

Results and Discussion

CD experiments

The albumin molecule has only a limited number of binding sites for most drugs, the warfarin and diazepam binding site. The first one was shown to be sensitive on the N-B transition [1,2]. The aim of this investigation is to demonstrate such a dependency for the diazepam binding site. Therefore we performed a number of CD experiments on the binding of diazepam to albumin. Müller and Wollert [17] and Sjöholm and Sjödin [18] already showed that the binding of diazepam to albumin give rise to an induced Cotton effect near 320 nm. This signal has been reported to be pH dependent [19]. We measured the CD signal of diazepam-albumin mixtures ($r = 2.34 \cdot 10^{-3}$) mainly at 330 nm in phosphate buffer, $I = 0.1$. The results are presented in Fig. 1. The molar ellipticity $[\theta]$ increases with pH between pH 6 and 7.5, whereas $[\theta]$ is constant out of this pH range. The pH range, over which $[\theta]$ increases is at somewhat lower pH than the pH range, where the N-B transition is observed by means of CD using bound warfarin as a label [1,2]. The change in $[\theta]$ with pH cannot be attributed to a change in physical chemical properties of the diazepam molecule, since the pK_a of diazepam is 3.3 [20]. Therefore a structural change in the protein must be responsible for the observed effects, and it is likely that this is the N-B transition. To find additional support for this we studied the effect of the chloride and the calcium ions on the pH dependence of $[\theta]$. The results are presented in Fig. 2. It is remarkable, that $[\theta]$ of the albumin complex is not affected by Ca^{2+} and/or Cl^- for pH > 8, whereas for pH < 6 this quantity changes considerably especially in the presence of Ca^{2+}. Previously we showed [21] that $[\theta]$ of the warfarin-albumin complex is very sensitive to Cl^-, when albumin is in the N conformation, whereas no or little change in $[\theta]$ could be observed when albumin is in the B form. Preliminary experiments (results will be reported elsewhere) showed, that the affinity of warfarin to albumin is very dependent on the commercial origin of the albumin when the albumin is in the N form, which is not the case for the B conformation. The

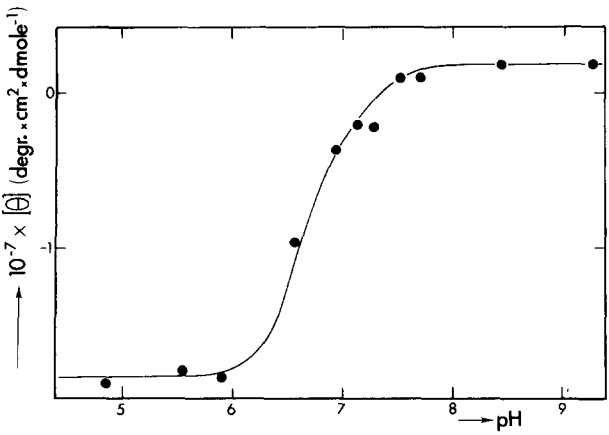
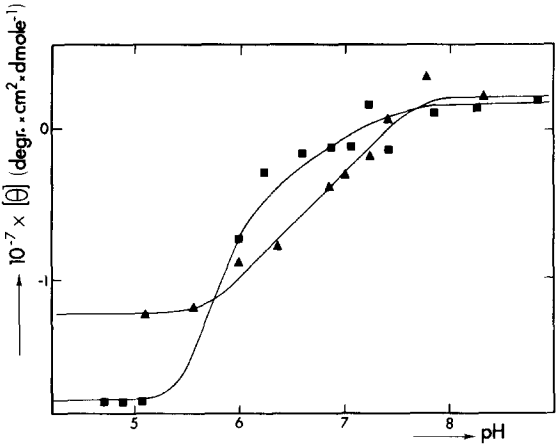


Fig. 1. The molar ellipticity $[\theta]_{330}$ of diazepam-albumin complex as a function of pH. Conditions: [albumin] = $6 \cdot 10^{-4}$ M, $r = 2.34 \cdot 10^{-3}$, pH ≤ 8.2 phosphate buffer, pH ≥ 8.2 borate buffer, $I = 0.1$, temperature, 25°C.



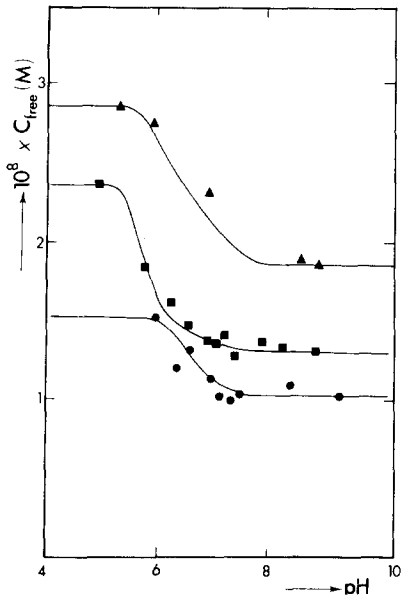


Fig. 2. $[\theta]_{330}$ of diazepam-albumin complex as a function of pH. 0.1 M Cl^- (■) and 0.1 M Cl^- + 2.5 · 10^{-3} M Ca^{2+} (▲). pH was adjusted with NaOH. Other conditions as in Fig. 1.

Fig. 3. Free concentration of diazepam (c_{free}) as a function of pH, phosphate or borate, I = 0.1 (●), 0.1 M Cl^- (■) and 0.1 M Cl^- + 2.5 · 10^{-3} M Ca^{2+} (▲). Other conditions as in Figs. 1 and 2.

findings that $[\theta]$ of the diazepam-albumin complex is sensitive to the composition of the solution for pH < 6, will indicate, that indeed the N-B transition is involved here. Moreover the specific effects of Cl^- and Ca^{2+} on the pH dependence of $[\theta]$ point in such a direction.

Dialysis experiments

The effect of H^+, Cl^- and Ca^{2+} on the binding of diazepam to albumin will be now of interest. Therefore we determined the free concentrations of diazepam as a function of pH at a constant low drug to protein ratio (r = 2.34 · 10^{-3}). This low ratio was chosen, since it was published that diazepam has one high affinity binding site [19,22—25] and more binding sites of considerably lower affinity [23] and at such a low ratio the contribution of these low affinity sites to the total binding proces can be neglected. It is worthwhile to mention

TABLE I

THE HILL COEFFICIENT n AND THE MIDPOINT pH, pH_{50}, UNDER VARIOUS CONDITIONS

Accuracy is about 0.05 for pH_{50} and 0.1 for n.

Condition	n	pH_{50}
Phosphate or borate, I = 0.1	1.9	6.8
0.1 M Cl^-	2.8	6.0
0.1 M Cl^- + 2.5 · 10^{-3} M Ca^{2+}	1.8	6.7

that the diazepam concentration used (400 ng/ml = $1.4 \cdot 10^{-6}$ M) is within the therapeutic range (200—600 ng/ml) and that the albumin has the physiological concentration (4 g/100 ml = $6 \cdot 10^{-4}$ M). The results of the dialysis experiments are given in Fig. 3, where are plotted the free concentration (c_{free}) vs. pH. The figure shows, that in all cases c_{free} decreases with pH. This decrease, however, is only limited to a small pH interval. In the presence of buffer this range is at about 6 to 7.5, for chloride this is 5.2 to 6.2 and in the presence of both chloride and calcium 5.6 to 7.6.

Treatment of results with two state model

To elucidate a possible linkage between changes in spectroscopic properties of the diazepam-albumin complex and changes in c_{free}, we treated the obtained results in a similar way as described previously [2]. Assuming a two state conformational model to be applicable here the fraction α of the protein in the basic (B) conformation as a function of pH follows from the $[\theta]$-pH curves. When $\log(\alpha/(1-\alpha))$ is plotted versus pH, the Hill coefficient n can be obtained from the slope at pH = pH_{50}, where $\alpha = 0.5$. The calculated values of n and pH_{50} are summarized in Table I. In the presence of buffer $n = 1.9$, which points to a strong cooperative effect of diazepam on proton binding to albumin. This observation is in strong contrast with the found n value of 0.8—0.9 in the case of warfarin-albumin in buffer, which means little or no cooperativity of warfarin on proton binding. Furthermore in the case of diazepam the pH_{50} is at 6.8, which was at 7.4 for warfarin. As can be seen from Table I, the chloride ion shifts the pH_{50} from 6.8 to 6.0 whereas the Hill coefficient increases markedly. In the presence of both chloride and calcium the pH_{50} is at 6.7 and $n = 1.8$. It should be noticed, that calcium has the opposite effect on the Hill coefficient when comparing this for diazepam-albumin complex and for warfarin-albumin complex.

We will calculate the free concentration as a function of pH ($c_{free,pH}$) from α and the experimentally found c_{free} values at the extreme pH ($c_{free,N}$ when the albumin is in the N form, $c_{free,B}$ when the albumin is in the B form) with the equation [2]

$$\frac{1}{c_{free,pH}} = \frac{1-\alpha}{c_{free,N}} + \frac{\alpha}{c_{free,B}} \tag{1}$$

This equation is only valid, when $K \cdot c_{free} << 1$ [2], where K is the binding constant. Diazepam has only one high affinity site to albumin with reported values for the binding constant of $2 \cdot 10^5$—$5 \cdot 10^5$ M^{-1} at pH 7.4 [19,22—25]. Since all experiments are carried out at a very low drug to protein ratio K can be obtained from v/c, where v is molar ratio of bound drug and albumin. The K values at some selected pH values under various conditions are summarized in Table II. It should be noticed, that the found binding constants are in very good agreement with the reported values, when the sensitivity of this constant to the composition of the solution is taken into account. The free concentration was on the average $2 \cdot 10^{-8}$ M. This means, that $K \cdot c_{free}$ is about $4 \cdot 10^{-3}$ which is much smaller than one. So Eqn. 1 can be applied in calculating c_{free} values between pH 5.5 and pH 8.5. Taking α from the $[\theta]$-pH curves and $c_{free,N}$ and $c_{free,B}$ from equilibrium dialysis at appropriate pH values, and using Eqn. 1,

TABLE II

THE (APPARENT) BINDING CONSTANT K OF DIAZEPAM TO ALBUMIN AT pH 6.2, 7.4 AND 8.2 UNDER VARIOUS CONDITIONS

Condition	K (M^{-1}) × 10^5		
	pH 6.2	pH 7.4	pH 8.2
Phosphate or borate, $I = 0.1$	1.7	2.2	2.3
0.1 M Cl^-	1.5	1.8	1.8
0.1 M Cl^- + $2.5 \cdot 10^{-3}$ M Ca^{2+}	1.0	1.1	1.3

the drawn lines in Fig. 3 will be obtained. The theoretical curves fit well the experimental points. This means, that in all cases the pH dependence of c_{free} can be explained by assuming two conformational states N and B for the protein with pH independent binding constants K_N and K_B for diazepam to albumin. As far this concerns, the same conclusions can be drawn for diazepam and warfarin. Clearly both the diazepam and the warfarin binding site are sensitive to the N-B transition. Some authors reported on the effect of pH on the binding constant of benzodiazepines [19,24]. Guilband et al. [24] suggested, that for diazepam this pH dependence is due to a deprotonation of amino acid residues decreasing the number of positive charges on the protein and to the N-B transition. Müller and Wollert [19] assumed the N-B transition to be involved in a process of creating a new binding site which does not contribute to the Cotton effect. However, Guilband et al. [24] found that the binding constant of diazepam at the high affinity site increases with pH over the pH range 5.5—8.5. From our results it is clear, that the pH dependence of c_{free} can be fully explained by the two state conformational model.

The observed opposite effect of Ca^{2+} on the Hill coefficient when warfarin [2] or diazepam are bound in the presence of Cl^- can reasonably be explained by the two state model. Considering the model of Monod et al. [26] which was applied to explain the cooperative binding of oxygen to hemoglobin [27,28], the R and T forms correspond with the N and B forms of albumin. The R-state has the highest affinity to oxygen, the N form to protons, acting as the ligand. The drug (warfarin or diazepam) and Ca^{2+} should be interpreted as the affector compounds, having the highest affinity for the B conformation, which is a necessary condition for cooperativity. Rubin and Changeux [29] reported on the relation between the Hill coefficient and the apparent allosteric constant L', in our case being the molar ratio B to N in the presence of drug and Ca^{2+}. They showed, that for low values of L' $d \log L'/dn > 0$, whereas for larger L' this slope is negative. It is obvious that in terms of this model the L' value for warfarin corresponds with a positive slope and for diazepam with a negative slope. Ca^{2+} increases the value of L', causing a decrease in n for diazepam-albumin complex and an increase in n for warfarin-albumin complex.

In spite of the fact, that even in the presence of both chloride and calcium the pH dependence of c_{free} can be reasonably explained by a two state model, it should be remarked that Ca^{2+} increases c_{free} over the whole pH range under investigation. In the case of warfarin, calcium induced changes in c_{free} of war-

farin could be explained simply by an effect of calcium on the N ⇌ B equilibrium, favouring the B conformation. Besides this effect of Ca^{2+} in the case of diazepam an additional effect of Ca^{2+} has to be assumed, since even at the extreme pH values, when the albumin is in the N or B conformation, c_{free} is calcium dependent. It is not likely that this additional calcium effect is due to the N-B transition, since at the lowest pH under investigation Ca^{2+} has only a negligible affinity to albumin and this affinity strongly increases with pH [30, 31]. The nature of this effect is a subject of current investigations. In the presence of chloride alone c_{free} of diazepam is higher than in phosphate buffer. This was also observed for the binding of warfarin to albumin [2]. It was shown [21] that chloride and warfarin compete for the warfarin-binding site. In the case of diazepam at yet no such conclusions can be drawn. An effect of ionic strength can be ruled out since in all experiments $I = 0.1$.

Consequences for dialysis of diazepam containing plasma

When Fig. 3 is studied in more detail, it can be remarked, that at pH 7.4 c_{free} in the presence of Ca^{2+} and Cl^- is about twice the c_{free} value in phosphate buffer. This result may be of importance for the choice of the buffer composition for the buffer compartment in equilibrium dialysis, when diazepam containing plasma is dialysed in order to determine free plasma levels of diazepam. When no Ca^{2+} and Cl^- is present in the buffer, a dilution of the Ca^{2+} and Cl^- in the plasma will be expected due to the dialysing procedure. This may lead to erroneous free concentrations of diazepam in plasma. When for example 1 ml of plasma is dialysed against 5 ml of buffer (1 ml plasma, a minimum amount of plasma is necessary, 5 ml buffer in order to collect a reasonable amount of free drug for analysis), than the calcium and the chloride in the plasma will be diluted by a factor of 6 and so a value for c_{free} near the c_{free} in phosphate has to be expected, which is nearly half the value of the actual c_{free} in the plasma. Even in cases an isotonic buffer with chloride is used a lower c_{free} will be found due to the large Ca^{2+} effect in the case of diazepam-protein binding. As a consequence of our results it seems important to pay more attention to the composition of the buffer in the buffer compartment. It follows also from Fig. 3, that the free concentration depends on the pH. Therefore it is necessary to keep the pH of the plasma constant at the pH of the blood in the particular patient during sample handling and dialysis. Studies showing the importance of buffer composition in dialysis of diazepam containing plasma in determining the free concentrations of diazepam in plasma are in progress.

References

1 Wilting, J., Weideman, M.M., Roomer, A.C.J. and Perrin, J.H. (1979) Biochim. Biophys. Acta 579, 469—473

2 Wilting, J., van der Giesen, W.F., Janssen, L.H.M., Weideman, M.M., Otagiri, M. and Perrin, J.H. (1980) J. Biol. Chem. 255, 3032—3037

3 Wallevik, K. (1973) J. Biol. Chem. 248, 2650—2655

4 Leonard, W.J., Vijai, K.K. and Foster, J.F. (1963) J. Biol. Chem. 238, 1984—1988

5 Goldsack, D.E. and Waern, P.M. (1971) Can. J. Biochem. 49, 1267—1275

6 Harmsen, B.J.M., de Bruin, S.H., Janssen, L.H.M., Rodrigues de Miranda, J.F. and van Os, G.A.J. (1971) Biochemistry 10, 3217—3221

7 Zurawski, V.R. and Foster, J.F. (1974) Biochemistry 13, 3465—3471
8 Müller, W.E. and Wollert, U. (1979) Pharmacology 19, 59—67
9 Fehske, K.J., Müller, W.E. and Wollert, U. (1979) Biochim. Biophys. Acta 577, 346—359
10 Brodersen, R., Sjödin, T. and Sjöholm, I. (1977) J. Biol. Chem. 252, 5067—5072
11 Sudlow, G., Birkett, D.H. and Wade, D.N. (1976) Mol. Pharmacol. 12, 1052—1061
12 Sjöholm, I., Ekman, B., Kober, A., Ljungstedt-Palman, I. Serving, B. and Sjödin, T. (1979) Mol. Pharmacol. 16, 767—777
13 Sjöholm, I. (1980) Acta Pharm. Suec. 17, 76—77
14 Novák, M. (1965) J. Lipid Res. 6, 431—433
15 De Gier, J.J. and 't Hart, B.J. (1979) J. Chromatogr. 163, 304—309
16 Chignell, C.F. (1976) in Methods in Pharmacology (Chignell, C. F., ed.), Vol. 2, pp. 138—140, Meredith Corporation, New York
17 Müller, W. and Wollert, U. (1973) Naumyn-Schmiedeberg's Arch. Pharmacol. 278, 301—312
18 Sjöholm, J. and Sjödin, T. (1972) Biochem. Pharmacol. 21, 3041—3052
19 Müller, W.E. and Wollert, U. (1974) Naumyn-Schmiedeberg's Arch. Pharmacol. 283, 67—82
20 Barrett, J., Franklin Smith, W. and Davidson, J. (1973) J. Pharm. Pharmac. 25, 387—393
21 Wilting, J., van der Giesen, W.F. and Janssen, L.H.M. (1980) Biochem. Pharmacol., in the press
22 Müller, W.E. and Wollert, U. (1973) Naumyn-Schmiedeberg's Arch. Pharmacol. 280, 229—237
23 Sjödin, T., Roosdorp, N. and Sjöholm, I. (1976) Biochem. Pharmacol. 25, 2131—2140
24 Guilband, P., Bourdeaux-Pontier, M., Sari, J.C. and Briand, C. (1978) Stud. Biophys. 69, 193—208
25 Tsutsumi, E., Inaba, T., Mahon, W.A. and Kalow, W. (1975) Biochem. Pharmacol. 24, 1361—1362
26 Monod, J., Wyman, J. and Changeux, J.P. (1965) J. Biol. Chem. 12, 88—118
27 Wyman, J. (1968) Q. Rev. Biophys. 1, 35—80
28 Kilmartin, J.V. and Rossi-Bernardi, L. (1973) Physiol. Rev. 53, 836—890
29 Rubin, M.M. and Changeux, J.P. (1966) J. Mol. Biol. 21, 265—274
30 Pederson, K.O. (1972) Scand. J. Clin. Lab. Invest. 29, 75—83
31 Katz, S. and Klotz, J.M. (1953) Arch. Biochem. Biophys. 44, 351—361