Modification of the free sulphydryl groups of bovine serum albumin to probe conformational transitions in the neutral region

Lambert H. M. Janssen* and Anton C. J. Roomer

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, State University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands

(Received 28 November 1984)

The free SH group in bovine serum albumin has been modified by covalent coupling with 2-chloromercuri-4-nitrophenol and 2-chloromercuri-2,4-dinitrophenol. The ionization of the phenolic OH group of the former label when bound to albumin can be followed spectrophotometrically. The pK of this group was influenced by the presence of Ca^{2+}. This is not a direct effect but proceeds via an effect of Ca^{2+} on the protein conformation. Similar results were obtained by following the c.d. signal of this label. This conformational change seems to be different from the one which can be detected by measuring the induced c.d. of a non-covalently bound ligand like diazepam.

Keywords: Bovine serum albumin; SH groups; conformational change

Introduction

Serum albumin is known to undergo pH dependent conformational changes. One such change occurs at pH 2 to 5 and the other is encountered in the neutral range[1,2]. The pH region in which this latter conformational change occurs suggests that imidazole groups from histidyl residues are involved. On the basis of model calculations Harmsen *et al.*[3] reached the conclusion that up to ten imidazole groups have a high p*K* in the N conformation and a normal lower value in the B conformation. They also made the interesting observation that Ca^{2+} ions strongly influence this N–B conformational change.

This N–B transition might have a physiological significance. The role of this conformational change in drug–albumin interaction has been documented by Wilting *et al.*[4] for human albumin. They found that pH dependent binding phenomena could be explained by a model in which the affinity of drugs is different for the N and B conformation. Many other studies have appeared since then in which attention has been drawn to the linkage between N–B transition and drug binding.

A more detailed study of this transition seems warranted. Several methods have been described to follow this conformational change. Leonard *et al.*[2] and Harmsen *et al.*[3] used the optical rotation of the protein itself. Wilting *et al.*[4,5] used the molar ellipticity of the albumin alone and of the warfarin–albumin complex to monitor this transition. Similar studies using fluorescence and the induced circular dichroic (c.d.) signal of other drugs have been reported. In all these studies the reporter groups were not covalently bound to the albumin. It might be preferable to have such a group covalently bound to the albumin at a site different from one of the drug binding sites. Modifications of the one free SH group in albumin belonging to Cys-34, effected using several reagents, have been reported. The signal of the attached label was followed using induced optical activity[6,7], e.s.r.[8], n.m.r.[9] and fluorescence[10]. The study of Zurawski and Forster[9] is especially noteworthy in this connection. They demonstrated that the magnitude of the chemical shift of the ^{19}F probe coupled to the free SH group paralleled the proportion of the N and B forms of the protein as judged by optical rotation measurements at 300 nm.

All these studies point to the fact that the environment of the SH groups is somehow sensitive to the N–B conformational change. So far there do not seem to be any derivatives which can be studied by simple u.v. or visible spectroscopy. We have undertaken this study because of the simplicity of the measuring technique. For this purpose we used several organomercurials described earlier[11] and studied the coupling to the free SH group of bovine serum albumin.

Experimental

Crystalline bovine serum albumin was obtained from Povite (Poviet Produkten BV, Boxtel, The Netherlands), 100% pure. A molecular weight of 66 200 was used in the calculations. Concentrated albumin solutions (approx. 12%) were prepared in water and subsequently deionized. By this procedure the amount of fatty acid is reduced to a reproducible ratio[12]. The number of free SH groups on the protein was determined using the method described by Brocklehurst[13].

The two organomercurials used, 2-chloromercuri-4-nitrophenol (compound I) and 2-chloromercuri-2,4-dinitrophenol (compound II), were obtained from Whatman Biochemicals Ltd, Maidstone, Kent, England and used without further purification. The behaviour of these compounds as coupling agents for SH groups has been

* To whom correspondence should be addressed.

0141-8130/85/030141-04$03.00

described by McMurray and Trentham[11]. Compounds I and II were coupled with mercaptoethanol and cysteine by reacting equimolar amounts dissolved in water at room temperature.

The reaction between I and albumin was performed under several conditions. Excess of I could not be removed by simple dialysis or column chromatography. Dialysis in 6 M urea of 2 M guanidine–HCl did not release the excess reagent either. Suzukida *et al.*[10] recently made similar observations. However titration of protein solutions at a given pH with increments of a solution of I showed sharp equivalent points, as will be demonstrated in this paper. For the preparation of covalent complexes between albumin and I or II, therefore, equivalent amounts of solutions of I and II were allowed to react with concentrated deionized albumin solutions.

Iodoacetamide and albumin were coupled by adding a tenfold excess of reagent at pH 8 for 1 h, keeping the pH constant by the addition of NaOH. Excess reagent was removed by dialysis.

Spectrophotometric titrations of the phenolic OH group of I or II, either free or coupled to albumin, were performed by measuring the absorbance of solutions of adequate concentration at 405 nm in 1 cm cells. The pH was changed either by titration with 0.1 M NaOH or by the addition of solid Tris. No difference between the two methods could be detected.

Circular dichroic (c.d.) experiments as a function of pH were performed essentially as described previously[4,5]. The complex between I and albumin proved to have an optically active absorption band at 405 nm. The c.d. spectrum of the modified albumin was also measured in the presence of diazepam (at a diazepam to albumin ratio of 0.4), following the diazepam induced signal at 320 nm. In both cases a 0.4% albumin solution in 2 cm cuvettes was measured using the signal at 500 nm as a reference. All spectrophotometric measurements were performed at 25°C, at an ionic strength of 0.15, which was attained by the addition of either KCl or $CaCl_2$.

Compound I was also titrated titrimetrically with NaOH using the method described earlier[14].

The measured absorbance A as a function of pH can be given by

$$A=(A_0H^n+A_1K^n)/(H^n+K^n) \quad (1)$$

In this equation A_0, A_1 represent the absorbance at low and high pH respectively, H is the hydrogen ion concentration, K is the dissociation constant and n is the Hill coefficient. In the case of a simple ionization process, n will be one. Equation (1) is a non-logarithmic form of the equation $n\text{pH}=n\text{p}K+\log(\alpha/(1-\alpha))$ where $\alpha=(A-A_0)/(A_1-A_0)$. The measured absorbance values as a function of pH were analysed according to Equation (1) using a non-linear least squares programme[15]. A_0, A_1, K and n were considered as adjustable parameters. A direct analysis of the absorbance *versus* pH values seems more reliable than an analysis in the form of a Hill plot, namely $\log(A-A_0)/(A_1-A)$ *versus* pH. Using the standard errors in the parameters and the number of data points, the 95% confidence interval was calculated using tables of Student t values.

Results and discussion

Reaction of organomercurials with SH groups

Acid–base titration curves showed that 2-chloromercuri-4-nitrophenol had two pK values in the neutral region, namely 6.6 and 8.3. Spectrophotometrically (at 405 nm) only one pK of 6.6 was found. This value clearly represents the dissociation of the phenolic group, in accordance with the literature[11]. It was further established that Ca^{2+} did not influence this phenolic pK value significantly (see also *Table 1*, No. 1 and 2). The higher pK of 8.3 can be attributed to the binding of OH^- by the chloromercuri group. This pK however, is of no importance in the remainder of this study.

Coupling of 2-chloromercuri-4-nitrophenol to small SH containing molecules did raise the pK of the phenolic

Table 1 Results for spectrophotometric and c.d. measurements on 2-chloromercuri-4-nitrophenol modified bovine serum albumin

Compound[a]		[b]	[c]	pK[d]	n[e]
1.	I	spec	Cl^-	6.65 (6.58–6.72)	0.90 (0.78–1.01)
2.	I	spec	Ca^{2+}	6.57 (6.55–6.58)	0.99 (0.95–1.03)
3.	I-BSA	spec	Cl^-	7.86 (7.83–7.88)	0.81 (0.77–0.85)
4.	I-BSA	spec	Ca^{2+}	7.59 (7.52–7.66)	0.73 (0.63–0.82)
5.	I-BSA-IAA	spec	Cl^-	7.53 (7.50–7.55)	0.70 (0.68–0.75)
6.	I-BSA-IAA	spec	Ca^{2+}	7.44 (7.41–7.47)	0.74 (0.71–0.78)
7.	I-BSA	c.d.	Cl^-	7.95 (7.83–8.06)	1.03 (0.76–1.30)
8.	I-BSA	c.d.	Ca^{2+}	7.71 (7.63–7.78)	1.24 (1.01–1.47)
9.	D-BSA	c.d.	Cl^-	7.83 (7.80–7.87)	1.45 (1.31–1.59)
10.	D-BSA	c.d.	Ca^{2+}	7.14 (7.00–7.27)	1.24 (0.79–1.71)

[a] The following abbreviations are used: I: 2-chloromercuri-4-nitrophenol; BSA, bovine serum albumin; IAA, iodoacetamide; D, diazepam.
[b] Spec or c.d. designates the spectrophotometric or c.d. measurements as indicated in the text.
[c] Cl^- or Ca^{2+} designates measurements in 0.15 M KCl or 0.05 M $CaCl_2$.
[d] pK values as explained under Experimental. The values in parentheses represent the 95% confidence interval.
[e] Value of the Hill coefficient. The values in parentheses represent the 95% confidence interval.

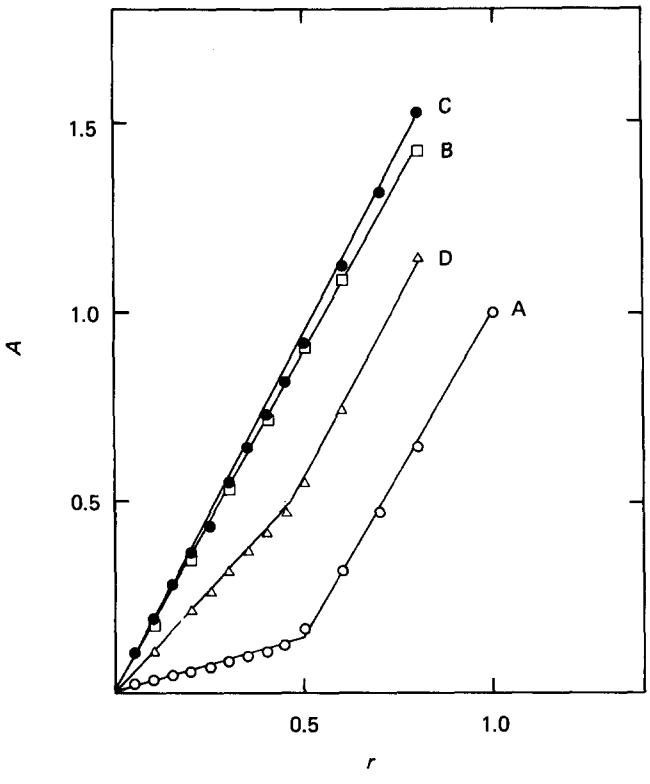

Figure 1 Titration of bovine serum albumin with 2-chloromercuri-4-nitrophenol. Curve A, serum albumin pH 7.1; curve B, iodoacetamide treated serum albumin pH 7.1; curve C, serum albumin pH 9.1; curve D, serum albumin pH 8.0. *A*, the absorbance at 405 nm; *r*, ratio of 2-chloromercuri-4-nitrophenol to albumin. The number of free SH groups in this sample was 0.4

OH group, as already reported[11]. For example we found that coupling with mercaptoethanol and cysteine resulted in a p*K* shift of the phenolic group towards 7.0, both in KCl and $CaCl_2$ containing solutions. A similar p*K* change is to be expected when coupling with albumin occurs. The reaction with albumin was performed at several pH values by titrating a protein solution with a solution of 2-chloromercuri-4-nitrophenol. Some representative results are shown in *Figure 1*. At pH 7.1 (see curve A) the reaction with albumin gives rise to a reasonably sharp equivalence point, corresponding with the amount of free SH groups. This equivalence point is a result of the p*K* change of the phenolic group of 2-chloromercuri-4-nitrophenol when coupled to the albumin SH group. The apparent p*K* of this phenolic group is near 7.9 (see below) whereas in the free form it is 6.6. At pH 7.1 the phenolic group is partly dissociated giving rise to an increase in absorbance. When all free SH groups have reacted, further addition of the reagent results in binding at non-specific sites where it will have a much lower p*K* value causing a much larger contribution to the observed absorbance. In accordance with this phenomenon are the results (*Figure 1*, curve B) representing the reaction with albumin that has no free SH groups, obtained by reaction with iodoacetamide. In this situation no equivalence point can be detected, indicating no reaction with SH groups. Moreover the straight line (curve B) runs parallel to the part of curve A which represents the non-covalent binding. When the coupling is performed at pH 9.1 the equivalence point has disappeared (*Figure 1*, curve C). This pH is well above the p*K* of 2-chloromercuri-4-nitrophenol, whether free or bound. Increasing the pH from 7.1 to 9.1 gives the expected changes, as shown by the results obtained at pH 8.0 (*Figure 1*, curve D).

Similar experiments were performed using 2-chloromercuri-4,6-dinitrophenol. Since, according to McMurray and Trentham[11], the p*K* of this compound is 4.0 we were unable to observe any equivalence points. We assumed that this compound reacted similarly to 2-chloromercuri-4-nitrophenol.

The conclusion so far is that both reagents do react with albumin SH groups. In the studies that follow albumin was allowed to react with an equivalent amount (on SH basis) of the organomercurials. It was assumed that the presence of albumin, with no covalently bound reagent, did not interfere with the reported experiments.

Mercurinitrophenol as reporter group

The question is whether the organomercurials can function as a reporter group for local or other conformational changes, especially the N–B transition. Such a conformational change might cause a perturbation of the p*K* of the nitrophenol, due to changes in the solvent structure around the organomercurial. Therefore the p*K* values were measured spectrophotometrically under various conditions. *Table 1* reports some typical results. We observed a p*K* shift which was similar to that observed after coupling with simple SH-containing compounds (see No. 3 and No. 4). The Hill coefficient is significantly lower than 1. Moreover, in the presence of Ca^{2+} ions the observed p*K* is significantly lower than in the presence of Cl^- only. This effect of Ca^{2+} is probably not a direct effect of Ca^{2+} on the p*K* of the reagent, since Ca^{2+} did not affect the p*K* of simple model compounds. The most simple explanation is that in the first instance Ca^{2+} influences the N–B equilibrium and as a result the p*K* of the attached label. When the SH is blocked with iodoacetamide the 2-chloromercuri-4-nitrophenol becomes bound to other non-specific sites. This results in lower p*K* values. Ca^{2+} now has much less influence (*Table 1*, No. 5 and No. 6).

It is attractive to link the observed lowering of p*K* by Ca^{2+} with the well-known shift to lower pH of the midpoint of the N–B transition[3]. A simple model which might be used to describe the measurements is described below. When the label is bound to the SH group and the protein is totally in the N conformation, the phenolic group of the label has a p*K* given by pK_N, and the dissociation process is described by equation (2).

$$pH = pK_N + \log(\alpha_N/(1-\alpha_N)) \quad (2)$$

When the protein is in the B conformation similarly

$$pH = pK_B + \log(\alpha_B/(1-\alpha_B)) \quad (3)$$

At pH values where a fraction f_B of the protein occurs in the B conformation, the observed absorbance is a function of α_N, α_B, f_B and of the molar absorption coefficients. Such a pH dependent absorbance curve may be analysed as if it were a simple ionization process that could be described by an equation analogous to equations (2) or (3). However the degree of dissociation in this situation is now given by equation (4).

$$F = \alpha_N(1-f_B) + \alpha_B f_B \quad (4)$$

Equation (4) holds if the molar absorption coefficient of the dissociated form of the label is not influenced by the conformational state of the protein. For 2-chloromercuri-4-nitrophenol this assumption cannot be checked because

at the high pH required for such a measurement the protein will be totally converted to the B form. However for the other label used, 2-chloromercuri-4,6-dinitrophenol, this assumption could be confirmed. This means that the pK value obtained for the label when bound to albumin is a complicated function. Equations (2)–(4) were used to perform some model calculation. Values for f_B were taken from Zurawski and Foster[9] and the values of pK_N and pK_B were varied. Apparent pK values (obtained as described before) corresponding to the ones shown in *Table 1* could easily be obtained. However all such curves resulted in titration curves that had Hill coefficients much higher than 1, namely 1.2 to 1.3. The reason for this is the strong cooperativity observed in the f_B *versus* pH measurement.

The albumin-2-chloromercuri-4-nitrophenol product proved to have an induced c.d. signal near its optical band at 405 nm. This spectrum was pH dependent. In *Table 1*, No. 7 and No. 8 refer to these experiments. The confidence intervals here are generally larger due to the smaller number of experimental points. A comparison of No. 3 and No. 7 from *Table 1* leads to the conclusion that pK and n deduced from absorbance measurements or from c.d. are not significantly different in the absence of Ca^{2+}. In the presence of Ca^{2+}, the c.d. measurements give a higher Hill coefficient than does the spectrophotometric method (compare No. 4 and No. 8), whereas pK is lower than in the absence of Ca^{2+} (compare No. 7 and No. 8), as was observed earlier (compare No. 3 and No. 4). The results obtained so far lead to the conclusion that the spectrophotometric and c.d. measurements done on the label 2-chloromercuri-4-nitrophenol are not the same, but very similar.

The situation seems to become more complicated when the induced c.d. signal of a non-covalently bound drug like diazepam is measured as well. Diazepam is known to display a pH-dependent induced c.d. signal, at least when bound to human serum albumin[16]. This c.d. signal also was measured and analysed (No. 9 and No. 10 in *Table 1*). In accordance with results obtained with human serum albumin cooperativity is observed both in the presence of Cl^- and Ca^{2+} (albeit that the confidence interval in the case of Ca^{2+} is rather large). The significant lowering of the pK by Ca^{2+} should also be noted. Normally this type of measurement is used to characterize the N–B transition of albumin. When No. 3 and No. 4 are compared with the corresponding No. 9 and No. 10 it will be clear that different conformational changes are detected by the two labels. The interesting question that then arises is whether or not these changes are part of one and the same major conformation change, or whether they represent different, separate changes. Further experiments using human serum albumin (about which more information is available) are in progress to study these questions in more detail.

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