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Control of the thermal reaction of a photochromic spirobenzopyran by the enzyme-like activity of albumins

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Abstract

The thermal ring-opening reaction of a negative photochromic spirobenzopyran was investigated in the presence and absence of albumins. In the presence of the proteins, the formation of the merocyanine form from the spiro form is enhanced markedly by two orders of magnitude. The results for five different albumins indicate the enzyme-like activity of the proteins. The influence of pH, temperature and various ligands was examined in detail for bovine serum albumin (BSA) and human serum albumin (HSA). In particular, it was deduced that warfarin shows reversible purely competitive inhibition for BSA with an inhibitor constant K_i of 1.6×10^{-5} M. The results suggest that the catalytic centre for the reaction with the spirobenzopyran is different for BSA and HSA. Albumin can control the velocity of the thermal reaction of the photochromic system. © 1997 Elsevier Science S.A.

Keywords: Albumin; Enzyme-like activity; Inhibitors; Spirobenzopyran; Thermal ring opening

1. Introduction

Photochromic systems have been investigated extensively, because of their relevance to the photoregulation of various physical and chemical properties of polymers and, in particular, biological systems [1–3]. The light-stimulated control of biocatalytic reactions has been documented in the literature, e.g. the reversible activation or deactivation of enzymes [4–10]. For the photoregulation of bioaffinity reactions, only a few examples are known: the photocontrolled uptake and release of a photochromic hapten by monoclonal antibodies [11], the photoregulated binding of monosaccharides to concanavalin A modified with a thiophene derivative [12] or a spirobenzopyran [13], and the photocontrolled binding and dissociation of agonist molecules to the acetylcholine receptor [14].

For the photocontrol of proteins, the possibility of the thermal reaction of photochromic molecules must be considered in addition to photochemical switching. Moreover, there may be an influence on the photochemical and/or thermal reaction of the photochromic molecule by the protein to be controlled or by other proteins present in the reaction medium. This knowledge is indispensable in order to achieve an effectively photocontrolled system and to regulate both the photochemical and thermal reaction. Recently, we have reported preliminary results which indicate that serum albumin exhibits enzyme-like activity towards a photochromic spirobenzopyran [15]. Willner et al. [16] have briefly demonstrated a similar activity of an antibody against dinitrophenol. However, until now, systematic studies of the influence of proteins on the properties of photochromic molecules have not been performed.

In this paper, a first attempt is made to examine the influence of an important group of proteins, the albumins, on the thermal reaction of a spirobenzopyran, and the possible control of the formation of the mero form is discussed. Albumins were chosen as proteins because of their ability to interact with molecules representing a spectrum of chemically diverse substances. For a deeper understanding of the binding experiments, it is necessary to provide a brief summary of the present knowledge of the binding regions, as discussed by Kragh-Hansen [17] extensively for human serum albumin (HSA) and by Carter and coworkers [18,19] for HSA and horse serum albumin (ESA). Recently, the three-dimensional structures of HSA [19] and ESA [18] were determined by X-ray crystallographic methods revealing a three-domain structure. Each domain consists of two subdomains possessing common structural motifs.

The binding sites and binding regions of albumins, mainly HSA, have been studied previously [17,20,21]. Most research has been performed using competitive binding

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experiments. Kragh-Hansen [17] deduces six plus one binding regions: (1) long fatty acids; (2) indole and benzodiazepine, medium fatty acids, L-tryptophan; (3) bilirubin; (4) Cu^{2+} and Ni^{2+}; (5) haemin; (6) salicylate and various drugs; (7) warfarin. However, it has also been reported that only four regions (long fatty acids, Cu ions, two additional regions) may exist [17]. Region (7) and (2) were confirmed to be domains IIA and IIIA by X-ray [19].

Many ligands have one high affinity binding site, the so-called primary binding site, and may possess various other binding sites with low affinity. This must be considered when carrying out inhibitory experiments with ligand to albumin ratios higher than unity. Until now, a specific examination of the influence of all the binding regions using all the model ligands discussed by Kragh-Hansen [17] has not been performed.

The influence of the enzyme-like activity of five albumins on the thermal ring-opening reaction of 6,8-dinitro-1',3',3'-trimethylspiro[2H-1-benzopyran-2,2'-indoline] (6,8-dinitro-BIPS) was investigated. Because of the possibility that several binding sites may be involved in the catalytic process, albumin was used in excess relative to the spirobenzopyran; the mathematical equations for catalysis as well as for reversible competitive inhibition are described. The results show that the thermal ring-opening reaction of 6,8-dinitro-BIPS is accelerated in the presence of albumins. For HSA, bovine serum albumin (BSA), ESA, dog serum albumin (CSA) and pig serum albumin (PSA), the kinetic constants of the enzyme-like process were determined. For BSA and HSA, the influence of the temperature, pH and various ligands was examined in detail. In particular, experiments using different ligands bound to the protein show that several binding regions are involved in the catalytic reaction. For the ligand warfarin and BSA, the inhibition pattern was deduced; for warfarin and HSA, it was suggested.

Albumins are important for drug transport in serum, making them interesting candidates for the photoregulation of drug release by modification with spirobenzopyrans. However, for an optimized modification of a bioaffinity molecule, it is absolutely necessary to investigate the influence of proteins on the thermal reaction.

2. Experimental details

2.1. Materials and apparatus

6,8-Dinitro-BIPS was synthesized according to the procedure of Koelsch and Workman [22]. The product and its solubility were verified as described previously [15]. All albumins were obtained from Sigma; BSA, HSA and PSA were essentially fatty acid free and essentially globulin free; CSA and ESA were essentially fatty acid free. $CuSO_4 \cdot 5H_2O$ (Aldrich) was 99.999% pure. Palmitic acid sodium salt (Sigma), warfarin (Sigma), salicylic acid sodium salt (Aldrich), bilirubin (Sigma), haemin (Sigma), L-thyroxin (Aldrich) and L-tryptophan (Sigma) were used as obtained. Phosphate-buffered saline solution (pH 7.4) (PBS buffer) was obtained from Sigma and contained 0.01 M phosphate, 0.0027 M KCl and 0.137 M NaCl. Acetonitrile (LAB-Scan) was of high performance liquid chromatography (HPLC) grade. All other solutions were prepared using reagents of analytical grade.

The UV–visible spectra and the change in absorbance with time were measured using a UV–visible spectrometer (Perkin–Elmer Lambda 2). Irradiation to achieve ring closure of 6,8-dinitro-BIPS was performed with a Wacom superhigh pressure Hg lamp (500 W) or with an ordinary 500 W light bulb (Osram) giving the same results. To select the excitation wavelengths above 500 nm, GIF (Nikon) and Y50 (Toshiba) filters were used.

2.2. Sample preparation and measurements

Samples for the measurement of the parameters describing the catalytic reaction and for the pH- and temperature-dependent experiments were prepared as follows. A solution of the mero form of 6,8-dinitro-BIPS in acetonitrile was irradiated with $\lambda > 500$ nm to obtain the spiro form. This solution was added to buffer solution in the presence or absence of the protein, finally containing 2.9% (v/v) of acetonitrile. When measuring the influence of ligands, ligand and albumin were preincubated overnight at 4 °C and for 2 h at room temperature to obtain equilibrated conditions; the spiro form in acetonitrile was then added as described above.

Albumin was used in excess to avoid complications due to multiple reactive sites [23]. The buffer solution was 0.01 M PBS buffer (pH 7.4) (2.9% (v/v) acetonitrile) for the determination of k_{cat}, the temperature-dependent experiments and most ligand experiments. A 0.01 M tris(hydroxymethyl)aminomethane buffer solution (pH 7.4) was used for copper ions as ligand to avoid the formation of a precipitate with phosphate. Due to the low solubility at pH 7.4, 0.01 M phosphate buffer (pH 8) was used in the case of bilirubin, haemin and warfarin as ligands. Solutions of different pH were prepared using KH_2PO_4 and $Na_2HPO_4 \cdot 2H_2O$ as described in Ref. [24].

All measurements were performed in quartz cells having a path length of 5 cm. The cells were kept at a constant temperature of 32 °C for the rate constant measurements, 25 °C for the measurement of the pH dependence and the influence of the ligands and 10–35 °C for the measurement of the temperature dependence. The thermal ring-opening reaction was followed directly by monitoring the appearance of the merocyanine form at 480 nm using the time-drive mode of the spectrophotometer. The light of the spectrophotometer was sufficiently weak such that it did not influence the amount of merocyanine. The reactions were observed during the first 15 min to obtain the initial reaction velocities; pseudo-first-order plots were assumed for all reactions of this study. All results are the mean values of at least three measurements.

2.3. Calculation of kinetic and thermodynamic parameters

According to Scheme 1, the spiro form undergoes a slow ring-opening reaction in the absence of albumin. An apparent first-order rate constant k_{sp} can be expressed as

$$-d[Sp]/dt = k_{sp}[Sp] \quad (1)$$

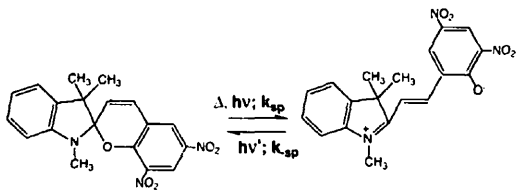
where [Sp] is the concentration of the spiro form. The rate constant k_{-sp} of the thermal reaction of the mero form to the spiro form is negligibly small within the applied time range.

In the presence of albumin, the conversion of the spiro form to the mero form proceeds through the following two pathways: (1) the spontaneous thermal ring-opening reaction; (2) the enzyme-like reaction with albumin where the spiro form is combined, in a first approximation, only to one binding site with high affinity (Scheme 2(a)).

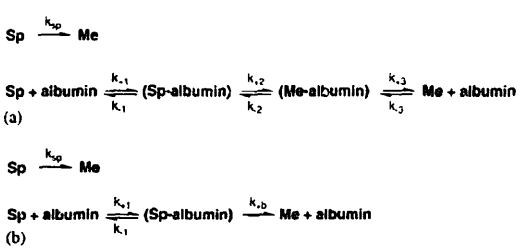
In Scheme 2(a), k_i is the rate constant of the reaction step i; in particular, k_{+1} and k_{-1} represent the rate constants of the formation and dissociation of the complex between the spiro form (Sp) and albumin and k_{+2} is the rate constant of the formation of the merocyanine–albumin complex (Me–albumin). Sp–albumin is the spirobenzopyran–albumin complex, Sp is the free spiro form and Me is the free mero form.

Because the initial velocity measurements at the early stage of the reaction (less than 15% change in the substrate concentration) are used, the product concentration is negligible and therefore the formation of any enzyme complex with the product can be ignored [25,26]. As considered in Refs. [26,27], enzymatic reactions for a single substrate reaction show an identical type of equation no matter how many intermediates are involved; only the physical significance of the constants K_m and k_{cat} will change. Hence the reaction scheme can be simplified as shown in Scheme 2(b).

The following equations can then be derived



Scheme 1.



Scheme 2.

$$-d[Sp]/dt = -k_{-1}[Sp\text{–albumin}] + k_{+1}[Sp][albumin] + k_{sp}[Sp] \quad (2)$$

$$-d[Sp\text{–albumin}]/dt = k_{+1}[Sp][albumin] - (k_{-1} + k_{+b})[Sp\text{–albumin}] \quad (3)$$

The total concentration of the spiro form at a time t in the presence of albumin decreases due to the ring-opening reaction and the reaction with albumin. In terms of the proposed mechanism, the rate is expressed by

$$-d[Sp]_t/dt = -d([Sp] + [Sp\text{–albumin}])/dt = k_{sp}[Sp] + k_{+b}[Sp\text{–albumin}] \quad (4)$$

The observed apparent first-order rate constant k_{obs} is given as

$$-d[Sp]_t/dt = k_{obs}[Sp]_t = k_{obs}([Sp] + [Sp\text{–albumin}]) \quad (5)$$

For all albumin concentrations, a linear relationship between the albumin concentration and the initial velocity was found. Hence the steady state assumption that the concentration of the enzyme–substrate complex is constant under the applied conditions can be made [27,28], and the Michaelis constant K_m can be expressed as follows

$$K_m = (k_{-1} + k_{+b})/k_{+1} = [Sp][albumin]/[Sp\text{–albumin}] \quad (6)$$

k_{+b} is the catalytic constant k_{cat}. The following equation can be derived using Eqs. (4)–(6)

$$(k_{obs} - k_{sp})^{-1} = K_m(k_{cat} - k_{sp})^{-1}[albumin]_0^{-1} + (k_{cat} - k_{sp})^{-1} \quad (7)$$

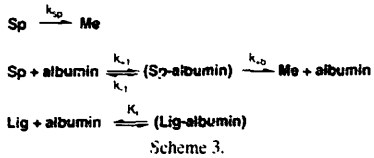
assuming that [albumin] = $[albumin]_0$, where $[albumin]_0$ is the total concentration of albumin, which is justified for albumin in excess.

In the presence of a ligand that acts as a reversible competitive inhibitor, the reaction between albumin and the ligand must be considered as presented in Scheme 3. Here Lig is the ligand and Lig–albumin is the ligand–albumin complex which is a dead end complex. The inhibitor constant K_i is defined as

$$K_i = [albumin][Lig]/[Lig\text{–albumin}] \quad (8)$$

Now the total albumin concentration can be calculated as

$$[albumin]_0 = [albumin] + [Sp\text{–albumin}] + [Lig\text{–albumin}] \quad (9)$$



Scheme 3.

However, since albumin is used in excess compared with the spiro form, the concentration of the Sp–albumin complex will be negligible

$$[\text{albumin}]_0 \approx [\text{albumin}] + [\text{Lig}-\text{albumin}] \quad (10)$$

On combining Eq. (8) and Eq. (10), the following equation is obtained

$$[\text{albumin}] = [\text{albumin}]_0(1+[\text{Lig}]/K_i)^{-1} \quad (11)$$

Eq. (7) must be modified, because the assumption that the free albumin concentration is equal to the total albumin concentration is no longer valid, giving the following

$$(k'_{obs}-k_{sp})^{-1} = K_m(1+[\text{Lig}]/K_i)^{-1}(k_{cat}-k_{sp})^{-1} \times [\text{albumin}]_0^{-1} + (k_{cat}-k_{sp})^{-1} \quad (12)$$

where k'_{obs} is the observed apparent first-order rate constant in the presence of albumin and ligand. Eq. (12) now includes the added total albumin concentration and the free ligand concentration, which can be considered to be equal to the total ligand concentration when using a sufficiently high ligand concentration with respect to added albumin. Hence Eq. (12) is a Lineweaver–Burk-type equation similar to Eq. (7) with the intercept unchanged and the slope increased by a factor $(1+[\text{Lig}]/K_i)^{-1}$, as observed for reversible competitive inhibition in so-called "conventional kinetics" using the substrate in excess.

A plot of the slope a of the primary plot, the Lineweaver–Burk-type plot, against the ligand concentration should be linear with the intercept of the x axis giving the value of $-K_i$

$$a = K_m + K_m K_i^{-1}[\text{Lig}]_0 \quad (13)$$

Eq. (12) can also be rearranged to give a Dixon-type plot which is linear for $(k'_{obs}-k_{sp})^{-1}$ vs. $[\text{Lig}]_0$

$$(k'_{obs}-k_{sp})^{-1} = K_m K_i^{-1}(k_{cat}-k_{sp})^{-1}[\text{albumin}]_0^{-1} \times [\text{Lig}]_0 + (k_{cat}-k_{sp})^{-1}\{K_m[\text{albumin}]_0^{-1}+1\} \quad (14)$$

All the Dixon-type plots for different albumin concentrations should intersect at one point with an x value of $-K_i$.

Hence, when the following characteristics are obtained

- linear Lineweaver–Burk plots with no change in the intercept of the y axis
- linear secondary plot
- linear Dixon plots intersecting at one point with a negative x value and positive y value

an inhibitor can be classified as a reversible purely competitive inhibitor (for nomenclature on enzyme kinetics with excess substrate, see Refs. [25–27]).

k_{sp} can be obtained experimentally in the absence of albumin, k_{obs} in the presence of albumin and k'_{obs} in the presence of albumin and ligand by following the change in absorbance at λ_{max} of the mero form. k_{sp}, k_{obs} and k'_{obs} were determined as first-order rate constants k from absorbance measurements according to Eq. (15)

$$\ln[(A_\infty - A_t)/(A_\infty - A_0)] = -kt \quad (15)$$

where A_0, A_t and A_∞ are the absorbances at time zero (obtained by extrapolation), time t and at the completion of the reaction. The value of A_∞ was obtained from solutions containing the mero form instead of the spiro form. This procedure was checked by keeping solutions of the spiro form until a negligibly small absorbance change was detected. k was then calculated by the linear least-squares method from the slope using at least 60 sets of points.

K_m and k_{cat} can be calculated from the plots resulting from Eq. (7). K_i can be obtained from the secondary plot (Eq. (13)) or the intersection of the Dixon plots.

The temperature dependences of k_{sp} and k_{cat} were analysed using the Arrhenius equation. The activation energy E_a was obtained from the slope. $\Delta H^{\#}$, $\Delta S^{\#}$ and $\Delta G^{\#}$ were calculated in the usual way [26].

According to the literature, the molecular weights of the albumins were 69 000 for HSA [29], 66 200 for BSA [29], 65 700 (calculated) for ESA [18], 65 000 (calculated) for CSA [20] and 65 000 (estimated) for PSA [30]. The concentrations of HSA and BSA were checked using the extinction coefficients $E_{278}^{0.1\%} = 0.531$ (HSA) [23] and $E_{280}^{1\%} = 6.53$ (BSA) [31].

3. Results and discussion

3.1. Determination of the kinetic parameters

Unlike most other spirobenzopyrans, 6,8-dinitro-BIPS is stable in its merocyanine form in which it crystallizes. Hence, when dissolving crystals in an organic solvent, such as acetonitrile, a solution of the mero form is obtained. When irradiated slightly above the absorption maximum of the mero form ($\lambda > 500$ nm), a solution of the colourless spiro form is produced. The ring-opening reaction yielding the mero form proceeds on UV irradiation or thermally (see Scheme 1). The spectra of the mero and spiro forms in PBS buffer solution (pH 7.4) (2.9% acetonitrile (v/v)) are shown in Fig. 1. The absorption maximum ($\lambda_{max} = 480$ nm) can be used to follow the formation of the mero form.

In most organic solvents, 6,8-dinitro-BIPS is reported to be poorly soluble [32]. In addition, the crystals are poorly soluble in aqueous solutions. However, solutions in the region of 9×10^{-5} M in acetonitrile and in the range $(0.5\text{–}3.5)\times10^{-6}$ M in PBS buffer containing 2.9% (v/v) acetonitrile could be obtained without aggregate formation as confirmed by absorption measurements.

When adding albumin to a solution of the spiro form of 6,8-dinitro-BIPS, the thermal ring-opening reaction is enhanced. Fig. 2 shows the initial temporal behaviour of 6,8-dinitro-BIPS in the presence and absence of the protein. Without albumin, the formation of the mero form is slow; in the presence of albumin, the thermal back reaction is enhanced

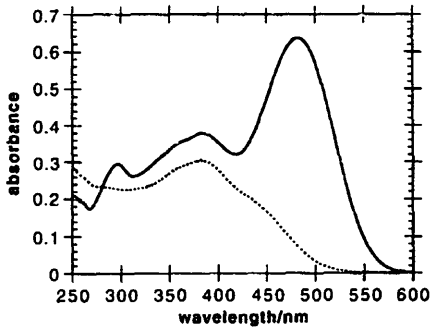


Fig. 1. Spectra of the merocyanine (———) and spirobenzopyran (- - -) forms of 6,8-dinitro-BIPS in 0.01 M PBS buffer (pH 7.4) (2.9% (v/v) acetonitrile); path length, 5 cm.

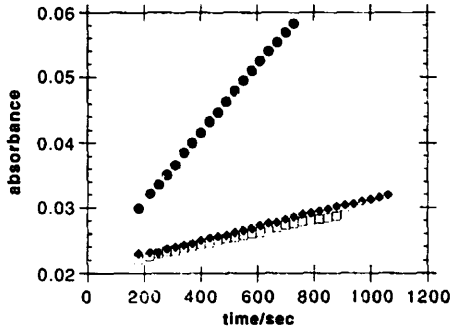


Fig. 2. Initial temporal behaviour of the thermal ring-opening reaction of 6,8-dinitro-BIPS in the absence and presence of proteins in 0.01 M PBS buffer (pH 7.4) (2.9% (v/v) acetonitrile): □, without protein; ●, with HSA; ◆, with avidin; 6,8-dinitro-BIPS, 2.7×10^{-6} M; HSA, 3.0×10^{-6} M; avidin, 1.6×10^{-6} M; monitored at 480 nm; path length, 5 cm; $T=30$ °C.

markedly. In contrast, proteins such as bovine-γ-globulin [15] or avidin show only a negligible influence. This is an indication that the reaction with albumin is specific and not due to a non-specific general process.

An enzyme-like mechanism is postulated. Albumin was used in excess to avoid interactions of the spiro form with low affinity binding sites.

Fig. 3 presents the results for the five albumins as plots according to Eq. (7). k_{sp} was obtained from the thermal ring-opening reaction in the absence of proteins to be $k_{sp}=(3.0\pm0.1)\times10^{-5}\ s^{-1}$ ($n=12$) at $T=32\pm0.5$ °C. k_{cat} and K_m are listed in Table 1 (calculated from the slope and intercept). All albumins show an enzymatic behaviour. The values of K_m are all of the same order of magnitude. However, k_{cat} differs by a factor of six. For CSA, the highest values of k_{cat}/k_{sp} and k_{cat}/K_m are obtained.

It should be noted that, in all cases, the thermal ring-opening reaction is enhanced by two orders of magnitude in the presence of albumins. When comparing these results with those previously published [15], it should be noted that the latter experiments were performed at 23 °C with different lots of albumins.

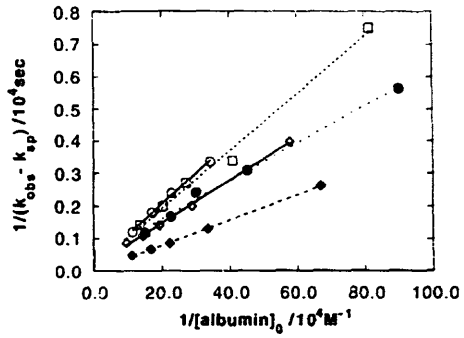


Fig. 3. Plot of $1/(k_{obs}-k_{sp})$ vs. $1/[\text{albumin}]_0$ for the reaction of 6,8-dinitro-BIPS with albumins in 0.01 M PBS buffer (pH 7.4) (2.9% (v/v) acetonitrile): ○, BSA; ●, HSA; □, ESA; ◇, PSA; ◆, CSA.

Table 1
Rate constants and K_m values for the thermal ring-opening reaction of 6,8-dinitro-BIPS in the presence and absence of albumins

Albumin	k_{cat} ($10^{-5}\ s^{-1}$)	K_m (10^{-5} M)	k_{sp} ($10^{-5}\ s^{-1}$)	k_{cat}/k_{sp}
BSA	5.65	5.28	3.04	185
HSA	2.37	1.36	3.00	80
ESA	4.81	4.23	3.47	135
PSA	5.69	3.69	2.92	195
CSA	12.58	4.51	3.04	415

$T=32$ °C; 0.01 M PBS buffer (pH 7.4) (2.9% (v/v) acetonitrile); $T=34$ °C for ESA.

Serum albumins are mainly considered as transport proteins with a unique ability to bind numerous endogenous and exogenous compounds [17,21]. However, in a few cases, the enzymatic or enzyme-like activity of albumin has been reported (for BSA, see Refs. [33–38]; for HSA, see Ref. [39]). Unfortunately, for these examples, no values for k_{cat} or K_m were reported.

6,8-Dinitro-BIPS as a substrate for an anti-dinitrophenol-antibody yielded $k_{cat}/k_{sp}=10^4$ and $K_m=2.3\times10^{-6}$ M [16]; this higher k_{cat} to k_{sp} ratio may be due to the better interaction of the mero form with the antigen binding site of the antibody.

For *p*-nitrophenylacetate (pNPA) and various derivatives, an esterase-like behaviour of BSA and, in particular, HSA has been reported [23,29,40–42]. The results are comparable with those presented here with regard to the experimental conditions and the magnitude of k_{cat} and K_m. However, ESA showed enzymatic activity towards 6,8-dinitro-BIPS, but did not show esterase-like activity towards pNPA [43]. This indicates that the catalytic site for 6,8-dinitro-BIPS is not the same as the catalytic site for pNPA; this is expected because, in the case of pNPA, albumin is acetylated in an initial step [23].

3.2. pH dependence of k_{sp} and k_{cat}

The pH dependence of the ring-opening reaction of the spiro form of 6,8-dinitro-BIPS was determined in the absence

and presence of albumin. The pH range in the presence of the protein was chosen to be pH 5–8, because below pH 4.3 or above pH 8 albumin exists in different isomeric forms with different helical degrees [21]. The results are not directly comparable. For each pH value, data yielding a plot according to Eq. (7) were obtained, and k_{cat} and K_m were calculated. Fig. 4 presents k_{sp} vs. pH; Fig. 5(a) and Fig. 5(b) show k_{cat} and K_m vs. pH for HSA and BSA respectively.

k_{sp} increases slightly with pH between pH 5 and 8; for a pH higher than pH 8, it increases rapidly. Until now, no such data for 6,8-dinitro-BIPS have been reported. The cleavage of the C–O bond results in the transient existence of a cis isomer, which is expected to rearrange itself to the more stable trans isomers [44]. The trans isomers may exist in equilibrium depending on the nature of the particular solvating environment. However, as reported for 6-nitro-BIPS [44], the energy differences between the stable isomers are small, and the absorption spectra are very similar. The apparent spectrum of the mero form is the sum of the spectra of the different isomers. The mero form should not be protonated in the applied pH range, as deduced from the literature on spirobenzopyrans [44–47]. No significant change in the absorption spectrum of the mero form in phosphate buffer (2.9% (v/v) acetonitrile) of different pH could be observed.

Fig. 5(a) and Fig. 5(b) show the pH profiles of k_{cat} and K_m for the reaction of 6,8-dinitro-BIPS with HSA and BSA respectively. For both proteins, a bell-shaped curve for k_{cat} was obtained, which may rise again at pH < 5. As mentioned above, below pH 4.5, a different isomeric form of albumin exists; therefore only the pH range of the so-called N form was examined. From a plot of $\log_{10}(k_{cat})$ vs. pH, $pK_{a1}(HSA) = 6.4$ and $pK_{a1}(BSA) = 6.9$ were obtained. A second pK_{a2} value may occur between 7.5 and 8. These profiles suggest the involvement of at least two amino acids in the catalytic process. One may be an imidazole group having a different microenvironment in the two different proteins. The profile of K_m, which indicates that amino acids are mainly involved in the binding process [26], also shows a dependence on pH. In particular, amino acids with $pK_a > 7.5$ for HSA and $pK_a > 8$ for BSA may be responsible.

In the literature, only a few data are available on the pH profiles of the reaction of albumins with substrates (phenylester derivatives) [23,42,48–52]. In all cases, a single catalytic amino acid group was proposed, similar or different from the group involved in the binding of the substrate. However, the catalytic mechanism of these substrates involves the acetylation of albumin [23].

The results presented here show that, for 6,8-dinitro-BIPS, a different catalytic centre exists for different albumins. It may involve at least two amino acids. Because albumin was used in excess, the pH profiles reflect the catalytic site with the highest affinity. If other low affinity sites exist, different amino acids may be involved in the binding and catalytic process.

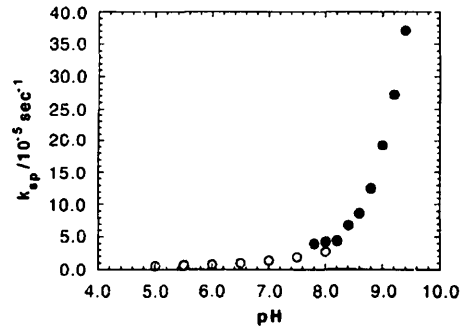


Fig. 4. pH dependence of k_{sp}: ○, 0.07 M phosphate buffer (2.9% (v/v) acetonitrile); ●, 0.1 M borate buffer (2.9% (v/v) acetonitrile).

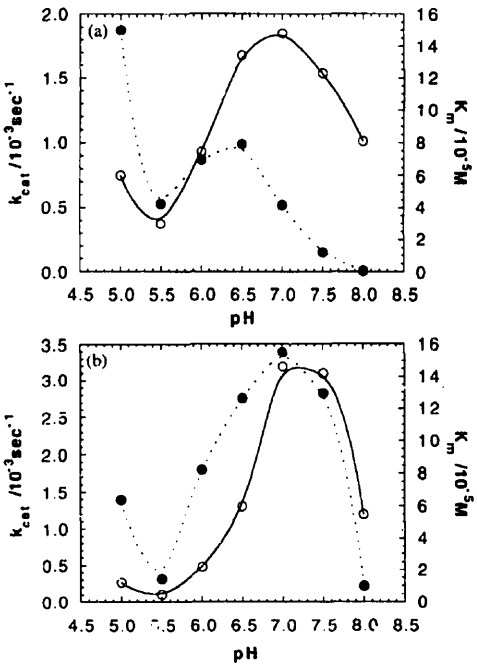


Fig. 5. pH dependence of k_{cat} (○) and K_m (●): (a) HSA; (b) BSA; 0.07 M phosphate buffer (2.9% (v/v) acetonitrile); curves are smooth curve fits for clarity only.

3.3. Temperature dependence

The temperature dependence of the ring-opening reaction of 6,8-dinitro-BIPS was examined between 10 and 35 °C in the absence and presence of HSA and BSA. The corresponding Arrhenius plots are shown in Fig. 6 and the calculated thermodynamic parameters are listed in Table 2. The Arrhenius plots show that the activation energy E_a for the thermal ring-opening reaction in the absence of proteins is higher than that in their presence. E_a for 6,8-dinitro-BIPS in the absence of albumin is about 73 kJ mol^{-1}, which is in good agreement with the data for other spirobenzopyrans [53], but less than the reported value of 96 kJ mol^{-1} for an aqueous solution

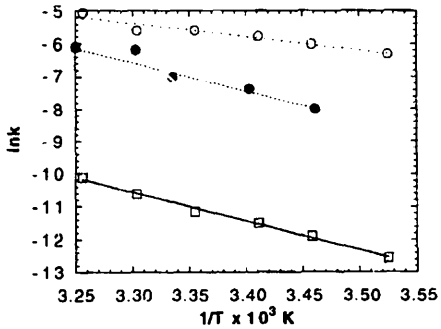


Fig. 6. Arrhenius plot of $\ln(k_{cp})$ vs. $1/T$ and $\ln(k_{cat})$ vs. $1/T$: □, $\ln(k_{cp})$; ○, $\ln(k_{cat})$ for HSA; ●, $\ln(k_{cat})$ for BSA.

Table 2
Thermodynamic parameters of activation for the thermal ring-opening reaction of 6,8-dinitro-BIPS in the absence and presence of albumins

	E_a (kJ mol^{-1})	$\Delta H^{\#}$ (kJ mol^{-1})	$\Delta G^{\#}$ (kJ mol^{-1})	$\Delta S^{\#}$ (J K^{-1})
6,8-Dinitro-BIPS	72.8	70.3	105.7	−119
6,8-Dinitro-BIPS + BSA	33.6	31.6	89.8	−195
6,8-Dinitro-BIPS + HSA	72.1	69.6	90.5	−70

0.01 M PBS buffer (pH 7.4) (2.9% (v/v) acetonitrile).

(pH 5) of BIPS-6-sulphonic acid, which also shows negative photochromism in polar solvents [46].

In the presence of albumin, E_a decreases strongly in the case of BSA and only slightly in the case of HSA. However, the calculated $\Delta G^{\#}$ values are quite similar in the presence of HSA and BSA, but distinctly lower than in the absence of albumin. In the case of HSA, the decrease in entropy ($\Delta\Delta S^{\#} = \Delta S^{\#}$(without albumin) $- \Delta S^{\#}$(with albumin) < 0) can be considered to be the main force for the catalytic process, whereas, for BSA, the decrease in enthalpy ($\Delta\Delta H^{\#} = \Delta H^{\#}$(without albumin) $- \Delta H^{\#}$(with albumin) < 0) is partly compensated by the increase in entropy ($\Delta\Delta S^{\#} > 0$). If the spiro form is compared with the mero form in terms of polarity, it is clear that the mero form is more polar. Hence the transition state complex should also be more polar than the reactant, giving a negative entropy in polar solutions [54], as shown by the negative $\Delta S^{\#}$ values for the ring-opening reaction of 6,8-dinitro-BIPS in the absence or presence of albumin. The even more negative $\Delta S^{\#}$ value in the presence of BSA suggests that the environment of the transition state complex is more oriented than in pure buffer solution causing a restriction of freedom for the ring-opening reaction. For HSA, an opposite effect with regard to entropy is observed; however, a large change in enthalpy occurs. The results suggest that the catalytic process and/or the catalytic centre may be different for the two albumins.

3.4. Influence of ligand binding

To obtain information about the location of the catalytic centre, ligand binding experiments were performed, and the change in the catalytic activity towards 6,8-dinitro-BIPS was determined. The results obtained are presented in Figs. (7)–(9) and summarized in Table 3. In the figures, the ratio of k'_{obs} in the presence of ligand to k_{obs} in the absence of ligand is shown as the ordinate, and the ratio of the initial concen-

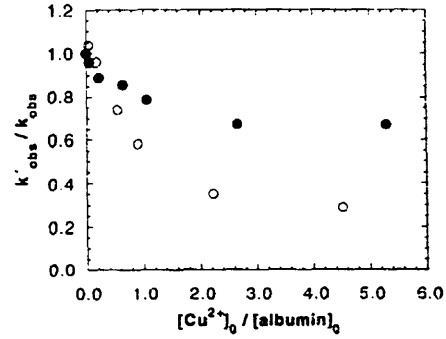


Fig. 7. Effect of Cu^{2+} ions on the reaction of 6,8-dinitro-BIPS with BSA (○) and HSA (●); k'_{obs} is the reaction rate in the presence of ligand; k_{obs} is the reaction rate in the absence of ligand; buffer: 0.01 M tris(hydroxymethyl)aminomethane (pH 7.4) (2.9% (v/v) acetonitrile).

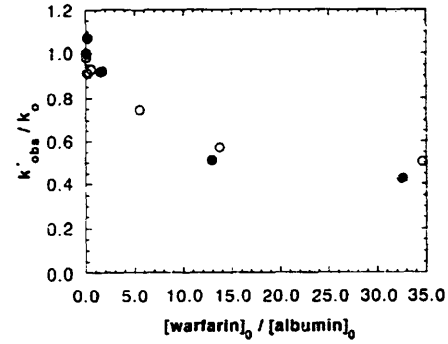


Fig. 8. Effect of warfarin on the reaction of 6,8-dinitro-BIPS with BSA (○) and HSA (●); k'_{obs} is the reaction rate in the presence of ligand; k_{obs} is the reaction rate in the absence of ligand; buffer: 0.01 M phosphate (pH 8) (2.9% (v/v) acetonitrile).

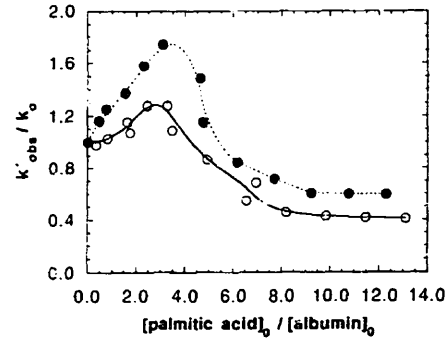


Fig. 9. Effect of palmitate on the reaction of 6,8-dinitro-BIPS with BSA (○) and HSA (●); k'_{obs} is the reaction rate in the presence of ligand; k_{obs} is the reaction rate in the absence of ligand; 0.01 M PBS buffer (pH 7.4) (2.9% (v/v) acetonitrile); curves are smooth curve fits for clarity only.

Table 3
Influence of ligands on the thermal ring-opening reaction of 6,8-dinitro-BIPS in the presence of albumins

Code [a]	Ligand	Albumin	pK_a [b]	$(lig.)_0/(alb.)_0$	$(lig.\text{–}alb.)/(alb.)_0$ [c]	Reduction of k_{obs} (%) $(1-(k'_{obs}/k_{obs})\times 100)$
I	Palmitate	BSA	8.2 + 6.5 [58]	3	1 + 0.7	+ 30
				10	1 + 1	− 60
		HSA	7.8 [21]	3.5	1	+ 65
				10	1	− 50
II	L-Trp	BSA		7		0
		HSA	4.8 [21]	7	0.83	0
II	L-Thyroxin	BSA		1		0
				3		− 10 [d]
		HSA	6.2 [21]	2	1	− 15 [d]
III	Bilirubin [e]	BSA		1		− 55
				2.5		− 65
		HSA	7.7 + 5.7 [21]	1	0.9	− 10
				2.5	1	− 50
IV	Cu^{2+}	BSA	11.1 [59] + 4 [21]	1	1	− 45
				5	1	− 70
		HSA	16.2 + 4 [21]	1	1	− 20
				5	1	− 35
		ESA		5		− 30
		CSA		5		− 20
		PSA		5		− 15
V	Haemin [e]	HSA	8.0 [21]	1.5	0.90	0
VI	Salicylate	BSA		1		+ 10
				20		− 15 [d]
		HSA	5.2 + 4.2 [56]	1	0.55	0
				20	1 + 0.9	− 25 [d]
(VII)	Warfarin [e]	BSA		5		− 25
				30		− 50
		HSA	5.2 + 4.7 [54]	5	0.9	− 25
				30	1 + 0.95	− 55

[a] Binding regions according to Ref. [17].
[b] pK_a values according to the references indicated.
[c] Estimated saturation of the first (+ second) binding site by the ligand before adding 6,8-dinitro-BIPS using the applied concentrations.
[d] Same decrease by applying ligand without albumin.
[e] Buffer: pH 8; others: pH 7.4.
lig., ligand; alb., albumin; lig.–alb., ligand–albumin complex.

tration of ligand to the protein as the abscissa. A total inhibition of the enzymatic reaction would result in the observation of a ratio $k'_{obs} : k_{obs} = k_{sp} : k_{obs} = 0.04$–$0.05$ for the applied conditions, provided that the free ligand has no influence on the thermal ring-opening reaction of 6,8-dinitro-BIPS.

In this work, eight model ligands were examined, covering all of the binding regions discussed in the review of Kragh-Hansen [17]. Table 3 shows clearly that some binding regions are involved in the catalytic process, while others are not, even high ligand concentrations giving no effect. In particular, tryptophan and thyroxin, both considered to share the same binding region [17], as well as haemin and salicylate interact with albumin without interfering with the catalytic ring-opening reaction of 6,8-dinitro-BIPS. Although thyroxin and salicylate seem to reduce k'_{obs} at high ligand concentrations, this is due to the interaction of the free ligand with 6,8-dinitro-BIPS as confirmed by experiments without the protein. Haemin was not checked with BSA, because only primates possess a binding ability of high affinity for this substance [17,21]. Ligands which influence the catalytic activity of albumins towards 6,8-dinitro-BIPS are discussed in more detail below.

3.4.1. Cu^{2+} ions

Cu^{2+} ions reduce the ring-opening reaction of the spiro form of 6,8-dinitro-BIPS markedly when bound to BSA, HSA or ESA. Only a moderate reduction is observed for PSA and CSA (Fig. 7). Cu^{2+} ions themselves, even at high concentrations, have no effect on 6,8-dinitro-BIPS in the absence of protein.

The primary binding of Cu^{2+} or Ni^{2+} occurs at a special and well-defined site [17,21] composed of the first three amino acids of the N-terminal end of the albumins. The amino acids of the first two positions can be exchanged; however, a histidine residue at the third position is essential for binding. CSA and PSA lack histidine at this position and therefore cannot bind copper ions with high affinity. In addition, it has

been reported that Cu^{2+} may bind to the single sulfydryl group and 4–9 Cu ions can be loosely bound ($pK_a = 4$) by albumins [21]. However, these low affinity binding sites are not further specified in the literature. This also means that, although CSA and PSA lack the high affinity binding site, they can bind Cu ions with low affinity to some extent. This is exactly reflected in Table 3.

BSA and HSA show a strong reduction of the catalytic activity towards 6,8-dinitro-BIPS when a 1 : 1 complex between Cu^{2+} and albumin is proposed, as calculated by the pK_a value and the applied concentrations. The activity is reduced further by about 5% (HSA) or 25% (BSA) when the ligand to protein ratio reaches five. A 15% (PSA) or 20% (CSA) reduction is obtained for the same ratio of Cu to albumin (5 : 1). The amount of reduction in activity remains constant up to a ratio of Cu to albumin of 40 : 1. Hence, for CSA and PSA, the secondary binding site influences the catalytic activity, whereas, in HSA and BSA, the primary binding site mainly contributes. Therefore it can be deduced that one catalytic centre is the N-terminal end, in particular, the histidine residue of the third position. This observation is in good agreement with the experiments on the pH dependence of k_{cat}, which were carried out with albumin in excess and therefore reflect the binding site with highest affinity. Allosteric effects may be excluded, because the N-terminal end is very flexible and moves independently of the main molecule [53]. The secondary and/or higher low affinity binding sites are also moderately involved in the catalytic process directly or may give a negative allosteric effect when Cu^{2+} is used in excess or albumin lacks the primary copper binding site.

3.4.2. Bilirubin

The results for bilirubin as ligand differ for HSA and BSA. For low concentration, giving a ligand to protein ratio below or equal to unity, bilirubin has no effect on the catalytic activity of HSA, but decreases the acceleration of the ring-opening reaction of 6,8-dinitro-BIPS by about 60% in the presence of BSA. In the absence of albumin, bilirubin itself has a slight accelerating effect on the reaction. For a ratio of bilirubin to albumin of about 2 : 1, the catalytic activity is reduced by 60% for BSA and 55% for HSA.

Bilirubin has one high affinity binding site ($pK_a = 7.9$ for HSA) and two secondary sites ($pK_a = 5.7$ for HSA) [17,21]. The inhibition experiments suggest that, for HSA, the primary binding site is not involved in the catalytic process. However, binding of bilirubin to the secondary sites shows an influence. For BSA, even the high affinity binding site for bilirubin contributes to the catalytic activity. It should be noted that the experiments examining the influence of bilirubin were performed at pH 8 (different from the experiments discussed above) to avoid the formation of colloids.

3.4.3. Warfarin

The inhibitory effect of warfarin at pH 8 on the thermal ring-opening reaction of 6,8-dinitro-BIPS catalysed by albumin is similar in the presence of HSA and BSA. A 90% saturation of albumin with ligand at the primary binding site reduces the catalytic activity by about 25%. A ratio of warfarin to albumin of 30 : 1 (saturation of the primary binding site; secondary binding site approximately 90% saturated) reduces the catalytic activity by about 50% for BSA and 55% for HSA.

For warfarin, two binding constants for HSA are reported in phosphate buffer: $pK_{a1} = 5.2$–5.7; $pK_{a2} = 3.6$–4.7 (see references and table II in Ref. [55] and Ref. [56]). In this paper, $pK_{a1} = 5.2$ and $pK_{a2} = 4.7$ [56] were used, indicating a rather moderate affinity compared with Cu^{2+} ions or haemin. This means that an initial ratio of ligand to albumin of 1 : 1 will saturate the first binding site only by about 50% as calculated using the applied concentrations. A ratio of 5 : 1 is necessary to achieve about 90% saturation of albumin for the primary binding site. This is reflected in the plots shown in Fig. 8. It can also be suggested that the primary binding site is involved in the catalytic reaction; the secondary site may be involved depending on the "actual" pK_{a2} value.

3.4.4. Palmitate

Fig. 9 shows the influence of the long fatty acid palmitate on the catalytic activity of BSA or HSA towards 6,8-dinitro-BIPS. For both albumins, an acceleration of the thermal ring-opening reaction is observed up to a ratio of palmitate to albumin of 4 : 1 for BSA and 5 : 1 for HSA, increasing the initial rate constant up to 30% for BSA and 70% for HSA. Inhibition then occurs, reducing the initial rate constant k'_{obs} by 60% for BSA and 50% for HSA compared with the rate constant k_{obs} in the absence of palmitate.

BSA is capable of binding as much as seven molecules of palmitate [57]. Peters [21] reports a binding of about six molecules per molecule of albumin. The binding has been characterized as heterogeneous, involving numerous binding sites of varying affinity. The association constants vary from $pK_a = 8$ to $pK_a = 5$ [58]. The first two fatty acids are considered to bind side by side in an antiparallel arrangement [17]. They induce a change in the physical properties of albumin due to conformational changes [21], which may be the reason for an increase in the catalytic activity. These findings are in agreement with preliminary results [15] obtained with commercially available BSA–palmitic acid (4.6 molecules of palmitate per molecule of BSA). It was shown that palmitate has a positive cooperation for the catalytic reaction. This may be the result of conformational changes of the protein, because it is unlikely that fatty acids and 6,8-dinitro-BIPS will share the same binding site due to their large structural differences.

When more than four or five palmitate molecules per molecule of BSA or HSA are bound, further addition of these fatty acid molecules will result in the occupation of weaker binding sites or binding regions. This causes an inhibitory effect of the thermal ring-opening reaction of 6,8-dinitro-BIPS. This may be due to further conformational changes or to interaction with the same binding region to which the

spirobenzopyran interacts. It should be kept in mind that a binding region itself may consist of different binding sites which may overlap with each other [17].

3.5. Determination of the inhibition pattern for warfarin as ligand

After determining which ligands act as inhibitors, it is desirable to establish the type of inhibition and the inhibitor constant K_i. Since Eqs. (12)–(14) are derived for reversible inhibition, tightly bound ligands were not checked. Hence the moderately binding warfarin was the only candidate.

The concentrations of warfarin and albumin (HSA or BSA) were chosen in such a way that the warfarin concentration was not substantially altered and the first binding site was mainly involved. If two binding sites are involved, non-linear plots should be obtained [27].

Fig. 10 shows a representative selection of the Lineweaver–Burk-type plots obtained for BSA as albumin. As predicted for reversible competitive inhibition, the slope for increasing warfarin concentration increases while the intercept remains constant at 609 ± 18 s; the variation coefficient $V_k = 3\%$ ($n = 4$) is therefore within experimental error. For HSA, the intercept is 440 ± 130 s, giving $V_k = 29.5\%$ ($n = 4$). In all cases, linear primary plots were obtained.

The secondary plots according to Eq. (13) for BSA and HSA are shown in Fig. 11. For BSA, the plot is linear, but for HSA the plot is curved slightly upwards. Hence the inhibitor constant was calculated only for BSA to be $K_i = 1.66 \times 10^{-5}$ M.

The Dixon-type plots for BSA according to Eq. (14) are presented in Fig. 12. All plots are linear and the intersection of the plots is at $x = (-1.63 \pm 0.15) \times 10^{-5}$ M and $y = 530 \pm 30$ s. In both cases, V_k is below 10% and therefore within experimental error. K_i is 1.63×10^{-5} M and is in good agreement with the result obtained from the secondary plot.

As a result, warfarin can be considered to be a reversible purely competitive inhibitor for BSA, using 6,8-dinitro-BIPS as substrate, involving a single binding site of warfarin. The calculated inhibitor constant $K_i = 1.6 \times 10^{-5}$ M suggests that the primary binding site of warfarin is involved in the catalytic process. In the case of HSA, reversible competitive binding is probable, but the secondary binding site may be involved to some extent as suggested by the secondary plot.

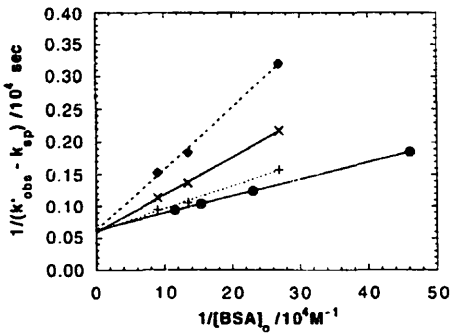


Fig. 10. Plot of $1/(k'_{obs} - k_{sp})$ vs. $1/[\mathrm{BSA}]_0$ for the reaction of 6,8-dinitro-BIPS with BSA in the presence of warfarin in 0.01 M PBS buffer (pH 7.4) (2.9% (v/v) acetonitrile). Concentration of warfarin: ●, 0×10^{-5} M; +, 0.92×10^{-5} M; ×, 2.3×10^{-5} M; ◆, 4.6×10^{-5} M.

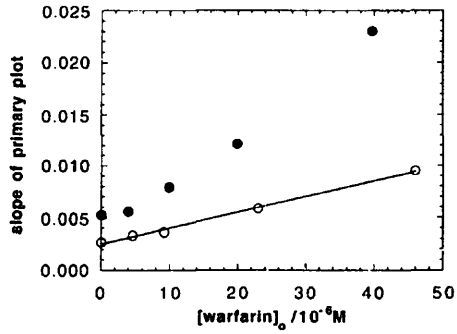


Fig. 11. Secondary plot according to Eq. (13): ○, BSA; ●, HSA.

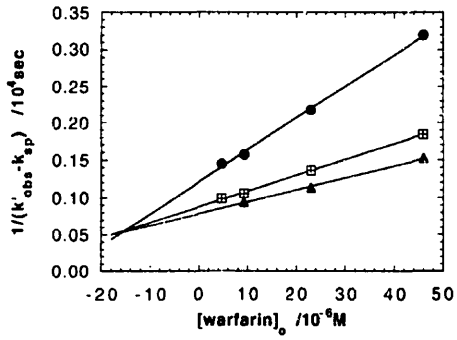


Fig. 12. Plot of $1/(k'_{obs} - k_{sp})$ vs. $[\mathrm{warfarin}]_0$ for the reaction of 6,8-dinitro-BIPS with BSA in the presence of warfarin in 0.01 M PBS buffer (pH 7.4) (2.9% (v/v) acetonitrile). Concentration of albumin: ●, 3.7×10^{-6} M; ⊕, 7.4×10^{-6} M; ▲, 1.1×10^{-5} M.

4. Conclusions

The thermal ring-opening reaction of 6,8-dinitro-BIPS is enhanced markedly by two orders of magnitude in the presence of albumins. The five albumins examined can be considered to show enzyme-like activity. HSA and BSA were checked in more detail, showing differences in the pH and temperature dependence, as well as in the inhibitory effect of the applied model ligands. This suggests that the catalytic centre may be different for the two proteins. It can be deduced that at least two amino acids are involved in the catalytic process. One may be an imidazole group. For BSA and HSA, the Cu^{2+} binding site is one of the catalytic centres. This is consistent with the findings of the pH-dependent experiments which indicate that the imidazole group of histidine is involved in the catalysis. The results of the other ligand binding experiments suggest that the binding regions of warfarin (HSA, BSA) and bilirubin (BSA) may also be catalytic

centres, or the binding of these ligands gives a negative allosteric effect. For palmitate, a positive allosteric effect is obtained for up to 4–5 molecules of fatty acid per molecule of albumin.

For warfarin and BSA, using 6,8-dinitro-BIPS as substrate, the inhibition pattern was determined to be reversible and purely competitive, indicating that it is highly probable that warfarin is competing for the same binding site as 6,8-dinitro-BIPS. The value of the inhibitor constant suggests that the primary site is involved. The HSA and warfarin system also shows reversible competitive inhibition to a great extent, but because of the slightly curved secondary plot other effects may be involved.

The results show clearly that proteins may influence the velocity of the thermal ring-opening reaction of spirobenzopyrans. This may cause an unexpectedly larger amount of one form, and should be considered when preparing systems which are thought to be only switchable by light, in particular when these systems are tested in natural matrices, such as serum, etc., for biomedical purposes. Controlled thermal switching may open up a new field of application of photochromic molecules. A system activated or inactivated by light can then be thermally deactivated or activated, with the velocity of this "back reaction" being controlled by biomolecules.

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