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BINDING OF A SPIN-LABELLED PHOTOALLERGEN TO HUMAN SERUM ALBUMIN

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The binding site for 3,3',4',5-tetrachlorosalicylanilide (T_4CS), a potent photoallergen, on human serum albumin (HSA) was studied by electron spin resonance spectroscopy using a spin-labelled analogue 3,5-dichlorosalicylamido-4-(2,2,6,6-tetramethylpiperidine 1-oxyl) (DCS-TEMPO) of T_4CS in the absence of ultraviolet irradiation. DCS-TEMPO bound non-covalently ($K = 5.8 \times 10^6$ M^{-1}) to one major binding site on HSA. This binding site could be blocked by the photochemical binding of T_4CS to the protein. Limited tryptic digestion of HSA or chemical modification of its single tryptophan residue with 2-hydroxy-5-nitrobenzyl bromide was found to reduce the binding constant of the T_4CS/DCS-TEMPO-binding site. These observations are in good agreement with earlier conclusions on the nature of the T_4CS-binding site and suggest a location for this site close to the single tryptophan residue of the HSA molecule.

1. Introduction

3,3',4',5-Tetrachlorosalicylanilide (T_4CS) is a highly potent photosensitiser which reacts with human skin in the presence of ultraviolet light (366 nm) to produce photodermatitis [1]. T_4CS has been shown to react photochemically with a number of proteins, including serum albumin, to form covalent photocomplexes [2–4]; its photocomplex with serum albumin has been implicated in the mechanism of the photoallergic response [5]. In previous work, we have demonstrated that human serum albumin (HSA) contains one major site for the photochemical binding of T_4CS [6]. This site is located in residues 182–298 of the HSA sequence [7].

Previous studies have suggested that strong non-covalent binding is a prerequisite for the photochemical reaction of T_4CS with protein [3]. Binding experiments in the absence of ultraviolet light using 3,5-dichlorosalicylamido-4-(2,2,6,6-tetramethyl-piperidine 1-oxyl) (DCS-TEMPO), a spin label analogue of T_4CS, have demonstrated the presence of a single strong binding site for T_4CS on HSA.

In this paper we describe studies of the binding of DCS-TEMPO to enzymically and chemically modified HSA, using the electron spin resonance (ESR) spin labelling technique. (Several excellent reviews on the theory and application of spin labels are available [8,9].)

2. Experimental

HSA (fraction V, fatty acid free), trypsin (bovine pancreas type XI, DPCC treated) and 2-hydroxy-5-nitrobenzyl bromide (HNB) were purchased from Sigma Chemical Co., Poole, U.K.

T_4CS was purchased from Ciba-Geigy Ltd., and recrystallised from isopropanol/ dioxan. The synthesis of DCS-TEMPO is described elsewhere [6].

Monomeric HSA was prepared from the commercial sample by chromatography on Sephadex G-150 [10]. The HSA-T_4CS photocomplex was

prepared using [^{14}C]T_4CS and its stoicheiometry determined as described previously [6].

The tryptic main fragment of HSA was prepared by the method of Sjodin et al. [11] and purified by chromatography on Sephadex G-100 in 0.2 M ammonium formate, pH 2.9.

Modification of the tryptophan residue of HSA by treatment with HNB in 10 M urea, pH 4.5, was carried out using the procedure of Karkhanis et al. [12]. The modified protein had a ratio of HNB/HSA of 1.47. A second sample of HSA was treated similarly, but omitting the HNB, to serve as a control.

ESR spectra were obtained on a Varian E-4 X-band spectrometer using a quartz aqueous sample cell at ambient temperature. Concentrations of DCS-TEMPO solutions were determined relative to a standard solution of 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl by measuring spectra under identical conditions of modulation amplitude, microwave power, etc.

For the binding studies, stock solutions of DCS-TEMPO in methanol were added by microsyringe to 1 ml aliquots of protein in 0.01 M Tris-HCl, pH 7.4, or to 1 ml portions of the same buffer alone as controls. The methanol content of the samples never exceeded 2.5% (v/v).

The addition of protein to a solution of DCS-TEMPO resulted in a reduction in the intensity of the nitroxide ESR signal. This reduction of intensity was accompanied by the appearance of a broad signal of much lower amplitude arising from DCS-TEMPO bound to the protein. Concentrations of free and bound spin probe were calculated from the change in intensity of the high-field line of the ESR spectrum between the protein-containing and control samples. The high-field line is used for these measurements as it is the line least affected in intensity by the presence of the broad (bound) ESR signal.

Protein concentrations were determined using the method of Lowry et al. [13].

All other chemicals used were the best grades available.

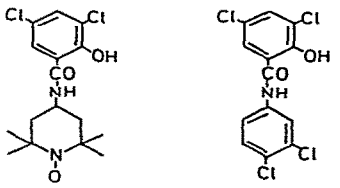
3. Results and discussion

The chemical structures of T_4CS and DCS-TEMPO are shown in fig. 1. When designing a spin label analogue of a chemically or biologically active molecule, it is important to know that the changes made to the molecular structure in incorporating the nitroxide radical do not alter those properties which are of interest. Since *N*-ethyl-3,5-dichlorosalicylamide has been shown to bind and react photochemically with proteins [3,14] in the same way as T_4CS, we can anticipate that DCS-TEMPO also contains sufficient of the original chemical structure to react similarly.

The ESR spectra of DCS-TEMPO alone in aqueous solution and in the presence of HSA are shown in fig. 2. The broad signal arising from the bound spin probe can be seen clearly, together with the three narrow lines from the free spin probe.

A Scatchard plot of the binding of DCS-TEMPO to HSA monomer in the absence of ultraviolet light is shown in fig. 3. Extrapolation of the linear portion of the plot shows that there is a single strong binding site (intercept = 0.90) with a binding constant of 5.8×10^6 M^{-1}. From the tailing off at higher values of r, it appears that there are also a number of additional sites with much lower binding constants.

The binding of DCS-TEMPO to the T_4CS-HSA photocomplex (substitution ratio of T_4CS : HSA = 0.46) is also shown in fig. 3. The covalent binding of T_4CS to about half of the HSA molecules significantly reduces the extent of binding of DCS-TEMPO to the protein, demonstrating that



DCS-TEMPO T_4CS

Fig. 1. Chemical structures of DCS-TEMPO and T_4CS.

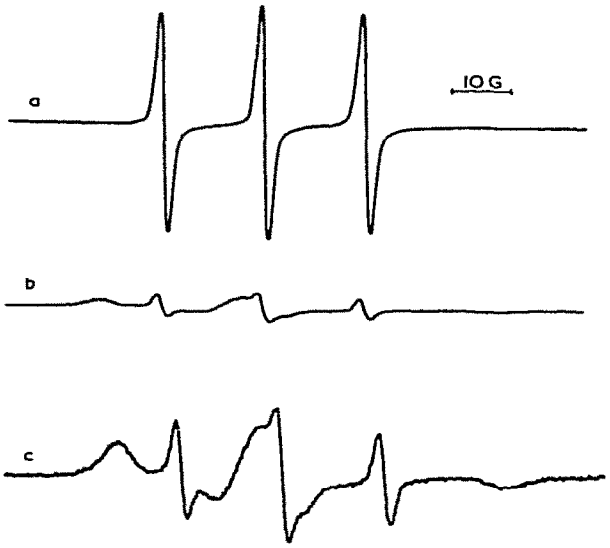


Fig. 2. ESR spectra of DCS-TEMPO (2.5×10^{-5} M in Tris, pH 7.4): (a) alone, (b) + HSA (2.4×10^{-5} M) and (c) as b run with 2× modulation amplitude and 3.5× receiver gain.

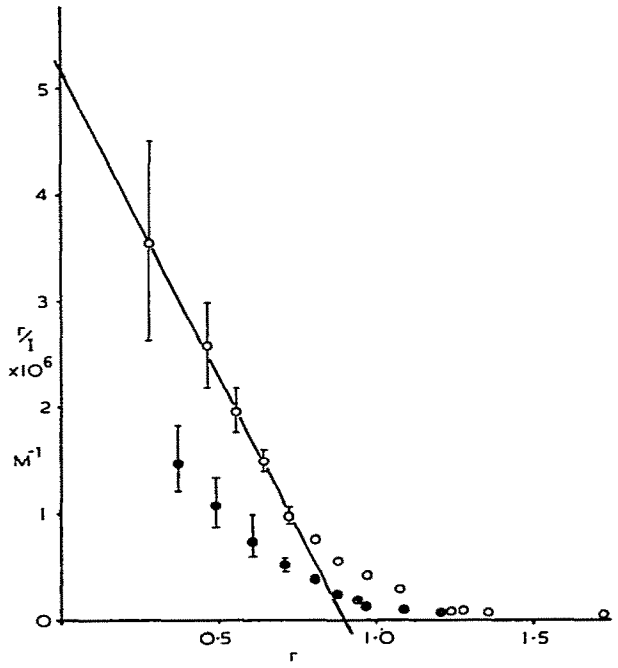


Fig. 3. Scatchard plot of the binding of DCS-TEMPO to HSA monomer (2.4×10^{-5} M) (hollow circles) and to T_4CS-HSA photocomplex (T_4CS : HSA = 0.46) (solid circles). *r* is the number of DCS-TEMPO molecules bound/molecule of HSA and *I* the concentration of unbound DCS-TEMPO at equilibrium.

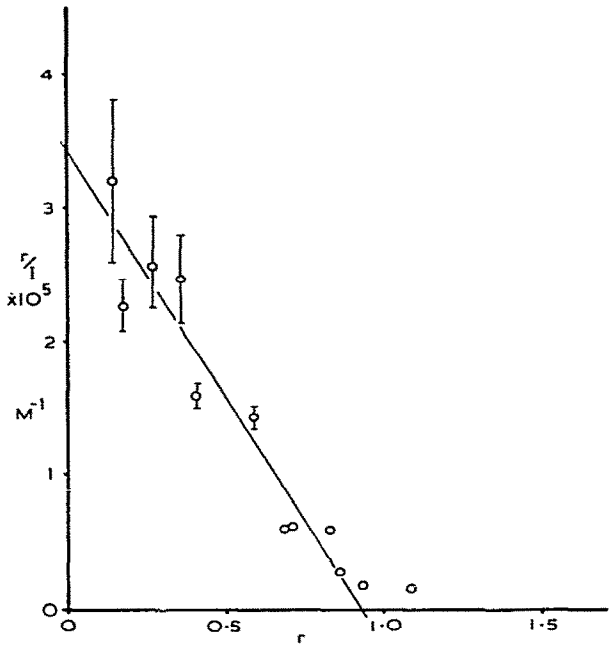


Fig. 4. Scatchard plot of the binding of DCS-TEMPO to the tryptic main fragment of HSA (1.65×10^{-5} M).

the major site of photochemical (covalent) binding of T_4CS and the strong binding site for DCS-TEMPO are indeed the same.

Extensive digestion of HSA with trypsin at pH 8.8 has been shown to yield essentially one main fragment which is resistant to further tryptic digestion [10]. This main fragment has been characterised and shown to consist of residues 182–585 of the HSA molecule.

A Scatchard plot of the binding of DCS-TEMPO to the tryptic main fragment of HSA is shown in fig. 4. Extrapolation of the linear portion of the plot shows that, as in the case of HSA, there is a single binding site (intercept = 0.93) with a binding constant of 3.7×10^{5} M^{-1}. This binding constant is 16-times lower than that

obtained for the intact HSA molecule. Evidently, whilst the major binding site for DCS-TEMPO and T_4CS is still present in the tryptic main fragment, its geometry has been considerably affected by the removal of the N-terminal region of the molecule. This result is consistent with our previous findings that the major binding site for T_4CS on HSA is located in residues 182–298 of the sequence [7]. Extensive breakdown of the HSA primary structure by cyanogen bromide (at the six methionine residues) resulted in the reduction of the binding constant for DCS-TEMPO by more than three orders of magnitude [6].

A Scatchard plot of the binding of DCS-TEMPO to HNB-HSA is shown in fig. 5. Extrapolation of the linear portion of the plot shows that there is a single strong binding site (intercept = 0.97) with a binding constant of $2.4 \times 10^6\ M^{-1}$. This represents a reduction of 60% from the binding constant to HSA; the control sample treated in an identical manner, but omitting the HNB-Br, gave a binding constant of $6.0 \times 10^6\ M^{-1}$.

HNB-Br is a specific reagent for tryptophan residues in proteins and peptides [15]. The fact that chemical modification of the single tryptophan

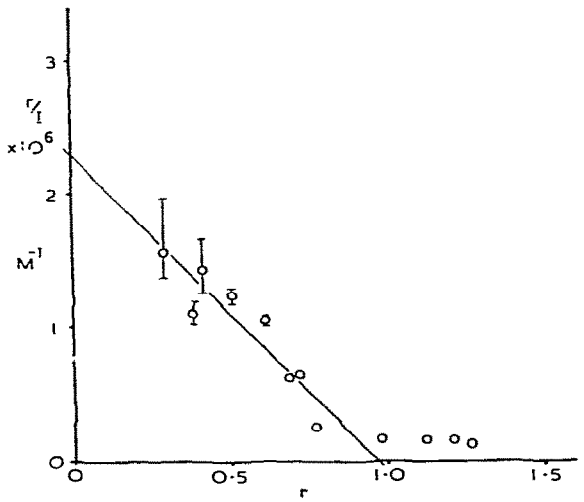


Fig. 5. Scatchard plot of the binding of DCS-TEMPO to HNB-HSA (2.4×10^{-5} M).

(residue 214) of HSA reduces the binding constant for DCS-TEMPO is evidence of the proximity of this tryptophan residue to the T_4CS/DCS-TEMPO binding site. This is in good agreement with our previous work [7], since the tryptophan residue lies between residues 182 and 298 of the HSA sequence.

Further evidence for the proximity of the T_4CS binding site to the tryptophan residue of HSA has been provided by energy-transfer measurements (J. Philp, unpublished data). These experiments indicate that the distance between the tryptophan residue and a T_4CS molecule located at the binding site of HSA is approx. 19 Å.

4. Conclusions

HSA contains a single strong binding site for DCS-TEMPO, a spin label analogue of the photoallergen tetrachlorosalicylanilide (T_4CS). Blocking of this binding site by T_4CS and the subsequent reduction in the binding of DCS-TEMPO. shows that DCS-TEMPO is a good probe for studying the binding properties of T_4CS.

Limited tryptic digestion of HSA or chemical modification of its single tryptophan residue reduces the binding constant of the T_4CS/DCS-TEMPO-binding site. These observations are in good agreement withe earlier conclusions on the nature of the T_4CS-binding site and suggest a location for this site, close to the single tryptophan residue of the HSA molecule.

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