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Interaction of a reactive dye with serum albumins and with aminoacids: the dye as a chiral label

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Abstract

HSA and BSA, human and bovine serum albumins, react with a reactive dye, C.I. Reactive Blue 29 (levafix brillant blue EB) giving adducts containing two dye molecules per protein molecule. While cysteine was able to react with the dye, the cysteine(s) present in the proteins was not. The dye-protein adducts reveal strong circular dichroism in the 300–720 nm spectral region where only absorption bands of the achiral dye molecule are active. The intensity of these bands depends on the conformation of the protein: particularly the 530–720 nm band. On tuning the denaturation degree by means of SDS addition and removal, the intensity of this band varies between zero and a maximum when the protein is in fully denaturated and native conditions, respectively. This behavior evidences a chiral label function of the dye that can be useful in studying processes regarding proteins.

Keywords: Reactive Blue 29 dye; Serum albumin; Absorptivity; Circular dichroism; Esr

1. Introduction

Human (HSA) and bovine (BSA) as well as the other serum albumins, are prone to bind effectively many small organic molecules. For instance they are able to complex and transport fatty acids and also many xenobiotics as drugs and dyes [1]. Some applications of these interactions are found in chiral chromatography [2] and, conversely, in the affinity chromatography where a dye covalently bound to a stationary phase is able to interact more or less effectively with different proteins resulting, for example, into an efficient method to purify albumins [3]. C.I. Reactive Blue 29 1 [4] is a synthetic reactive dye structurally similar to C.I. Reactive Blue 2 (Cibacron Blue F3GA), a substrate biomimetic of many enzymes [5].

The question arises if the above *reactive* dye exhibits reactivity also towards albumins. In addition, this reactivity could result in interesting spectroscopic effects. In fact, as complexation of the achiral molecules of a reactive dye [6] or methyl orange [7], by HSA or BSA was shown to give rise to strong complexes revealed by intense

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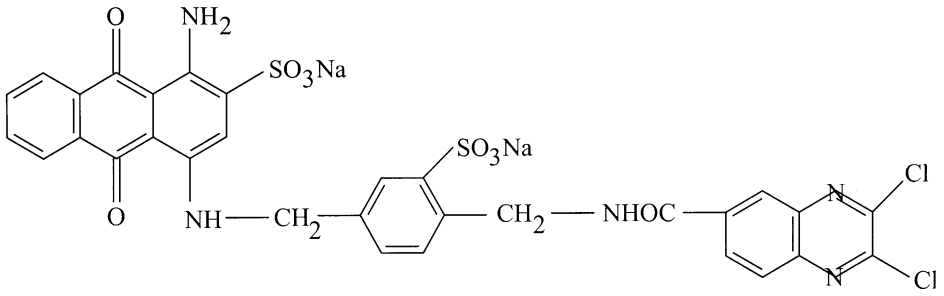

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circular dichroism (CD) in the near-UV/vis absorption spectral region of the dye, 300–730 nm, it is expected that the formation of covalent bond(s) between the reactive dye and these proteins also will result in intense CD. The induced CD should be dependent on the chirality of the protein or, at least, on the chiral structure of a significant part of the protein molecule. Thus, the reactive dyes could be considered, and used, as a chiral label. The CD induced in the low-energy electronic transitions, $\lambda > 300$ nm, of the bound, originally achiral, dye molecule could be measured much more easily than the CD of the peptide backbone transitions at $\lambda < 240$ nm and it could be used to reveal the conformational state of the secondary structure of the protein. As will be shown in the following, these expectations are justified.

2. Results and discussion

We have recently shown that C.I. Reactive Blue 29 interacts with serum albumins in aqueous solution leading to the complexation of up to nine molecules of the dye in three triply degenerate sites, in HSA, and up to 12 units of the dye in two distinct sites, in BSA, respectively [6]. The site complexation constants have been determined through a fitting approach of CD data registered at widely different [protein]/[dye] ratios of freshly prepared solutions.

Fig. 1 shows that the CD spectrum of a freshly prepared solution , buffered to pH = 7, containing 6.2×10^{-5} M dye, and 3.2×10^{-5}M HSA, registered at room temperature, in the 300–700 nm interval, is the spectrum expected in correspondence to the above concentrations on the basis of the known

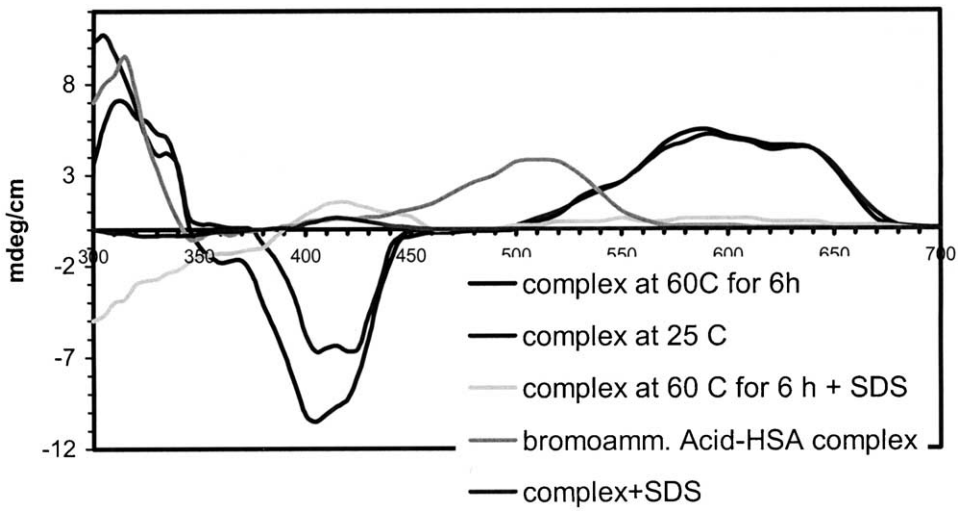
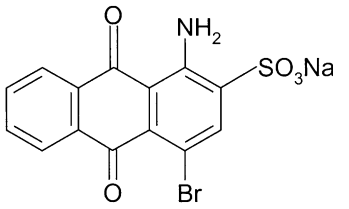


Fig. 1. CD spectra for buffer solutions of 3.2×10^{-5} M HSA and 6.2×10^{-5} M dye in various conditions.

site constants and site-CDs [6]. A positive band is exhibited from 300 up to 340 nm, while an intense negative band is apparent in the 370–450 nm range; at $\lambda > 450$ nm a positive CD spectrum occurs, with maxima at 600 and 640 nm. After 6 hours warming the reaction mixture at 60 °C, and then recovering the room temperature (RT), the spectrum changes are clear and stable. Since these effects could be attributed to protein denaturation, blank experiments were carried out in which an HSA solution, buffered to pH = 7, was kept at 60 °C for several hours. After reverting to RT, no change in the CD spectrum occurred in the 200–300 nm region up to 10 h warming, showing no thermal denaturation of pure HSA.

Spectral changes of the dye–HSA solution similar to those that occurred in 1 h at 60 °C, took place also at RT but needed approximately 48 h.

Once these changes are produced by the warming, or keeping the initial *complex* at RT for enough time, the occurred modifications appear irreversible. We can therefore reasonably attribute this new CD path to the formation of covalent bond(s) between dye and protein. To ascertain the above hypothesis, we proceeded to the addition of a large excess (8 mg to 3 ml of solution) of SDS (sodium dodecyl sulfate), an efficient denaturant [8], both to the dye–protein initial *complex,* and to the *adduct*, that is the product of the above described thermal treatment. We found that SDS addition to both samples results in dramatic, even if different, changes of dichroic activity (Fig. 1). It is worth noting that while SDS annihilates the CD of the *complex,* in the case of the *adduct* we observe simply a *different*, low-intensity, CD. Then we submitted the warmed mixture, added with the denaturant, to an ultrafiltration (UF) experiment with the aim of eliminating SDS; the solution, 3 ml, was diluted with buffer up to 10 ml, then ultrafiltered up to ca.1.5 ml and again washed three times in the same way. After the UFs, and recovery with the buffer to the starting volume, a CD spectrum is found, similar to that measured before SDS addition. The same procedure, applied to the dye–HSA complex (the non-warmed sample), ended in a quasi-zero CD at $\lambda > 300$ nm as the dye was washed out by the UFs. Therefore, a reaction very likely occurred between the dye and protein in the *complex*, leading to covalent bonds formation stable to protein denaturation (*adduct*). On passing, the effects of the above reaction on the CD spectra are clearly shown in Fig. 1. Here, while the longer wavelength CD band is almost coincident for to HSA–**1** adduct and the HSA–**1** complex, the bands in the other spectral regions are different. In the same figure the CD spectrum of the 2:1 complex between bromoaminic acid **2** and HSA is reported. **2** Can be considered a model for the antraquinone chromophore: then the 510 nm band of **2** is, in our opinion, that connected to the 590 nm band in the dye complex.



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We obtained further confirmation that a reaction occurred, and found quantitative data regarding its stoichiometry on looking at the absorption spectra (Fig. 2).

Here, a 6.8×10^{-5} M dye and 3.1×10^{-5} M HSA mixture displays some minor absorbance changes in the 300–700 nm region when the reaction of the complex is carried out at 60 °C for 6 h. Moreover, an evaluation of free- and bonded-dye concentrations can be made on the basis of the following experiment: the heated solution (5 ml) was dried under nitrogen stream, then extracted twofold with MeOH (9 ml two times, 6 + 3 ml), obtaining the dissolution of the free dye, while the protein remained as a precipitate. Each time the mixture was centrifuged, and the surnatant separated. The spectrum of the overall MeOH solution (Fig. 2) showed that only a small part of the dye has been extracted (ca. 10%), with respect to the spectrum of the warmed complex, after the volume was adjusted to the one of starting mixture. The residual, insoluble in MeOH, was dried under nitrogen stream and added with buffer solution until the initial volume was again obtained and, finally, the absorption spectrum registered. This last spectrum (Fig. 2) shows that 90% of the dye has

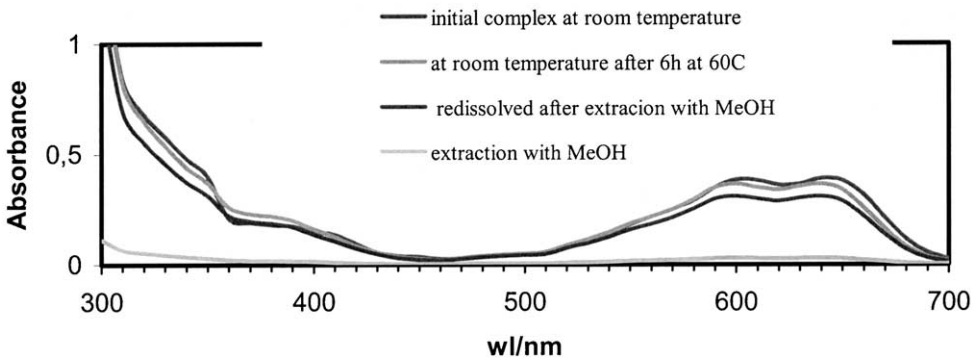


Fig. 2. Absorbance spectra of 3.1×10^{-5} M HSA and 6.8×10^{-5} M dye in buffer.

not been extracted by MeOH, i.e. it was covalently bonded to the protein. The sum of the absorbances of the free dye in MeOH and of the dye bonded to HSA reproduces within experimental errors the spectra measured for the initial mixture and the warmed solution.

To measure the maximum quantity of the dye that could be covalently bonded to the protein, we carried out another, similar, experiment using [dye]/[HSA] = 5, [HSA] = 3×10^{-5} M. Three samples (3 ml each) of 5:1 **1**–HSA mixture were warmed at 60 °C for 70 min, 2 h, and 7 h, respectively. The absorption spectra of these samples were measured at RT: they showed very small differences with respect to an identical sample kept at 4 °C. All samples, i.e. the three warmed and the one at 4 °C, were dried under N_2 stream, and extracted with MeOH (6 ml, then 4 ml three times). Then the four final methanol solutions were reduced to 3 ml volume by evaporation and the absorption spectra registered. The proteic residues were dissolved with 3 ml buffer. The absorption spectra of these solutions indicated that 0.02, 1.51, 1.8, and 2.3 mol of dye/mole of HSA were present in the aqueous solutions. These results were confirmed by the absorbance of previous methanol solutions regarding the free dye.

Then, to minimize the free-dye content in the aqueous solutions, a sequence of three threatments was carried out using an overall quantity of 20 ml buffer, reducing first to 1/25 (on the basis of the measured volumes of permeates and retentates) the content of free low molecular-weight compounds in each sample. In the successive step, final samples were adjusted to the original 3 ml volume. From absorbance measurements the 6-h warmed sample contained 2.0 mol of dye per HSA mol. Third, in order to eliminate any possible free-dye surviving to previous purification we submitted the protein dye fraction to a denaturation/separation process. Thus, a sequence of 3 UFs, in which each time the retentate (1 ml) was diluted with 9 ml SDS solution (82 mg/30 ml water), was performed on the 7 h×60 °C sample. The absorption band remained unchanged. This was taken as a conclusive evidence that the maximum number of dye molecules bond to each HSA was two.

Besides HSA, BSA (bovine serum albumin) has also been proved to be able to complex dye extensively [6]. In this case, six dye units are accommodated in each of two distinct equivalent complexation sites. We therefore added dye to a buffered (0.1 M NaH_2PO_4 , pH = 7) BSA (3×10^{-5} M) solution obtaining a solution 12×10^{-5} M in the dye, and registered the CD spectrum in the 300–700 nm interval, where the spectrum expected for a 4/1 = [dye]/[BSA] mixture was found (Fig. 3).

We note that the resultant CD is almost *antipodic* with respect to that shown in Fig. 1 by the 2:1 **1**–HSA mixture in the same spectral region and, as far as the absorption intensity is concerned we note that *on a dye molar concentration basis* the 600 nm band of the initial complex is ca. 1/4 of the correspondent HSA dye band, while the bands centered at 410 and 325 nm are of similar intensity. Even in this case, leaving the solution at room temperature for enough time, or warming the mixture, significant changes take place in the CD

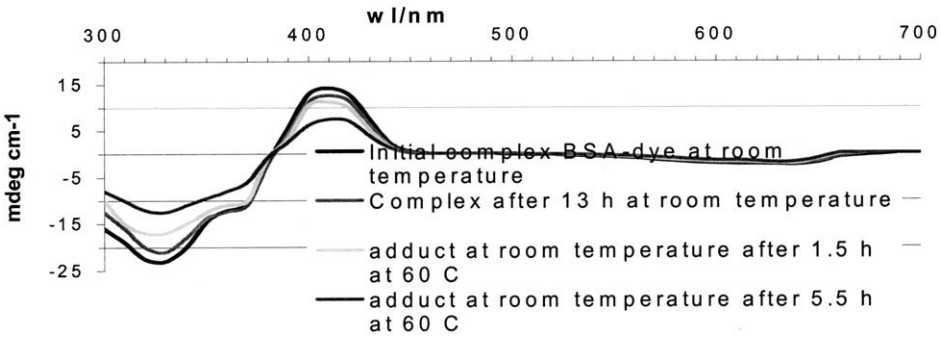


Fig. 3. BSA (3×10^{-5} M) and 1.2×10^{-4} M dye buffer solutions in various conditions.

spectra, consisting in a progressive decrease of the elongation in the 300–450 nm wavelengths interval. For example, some 11% decreasing of the CD bands was observed after 12 h at RT while a 38% decrease accompanied a 1.5 h warming at 60 °C. The change of CD slowed down, after 6 h of further warming, at an overall 50% decrease of the initial value in the violet-UV spectral region while in the yellow–red band marginal variations of the CD intensity were observed. It is to be remembered that, after warming, we found marginal variations of the CD in this spectral region also in the HSA–**1** case. Moreover, the two UV CD-bands of the dye in the two proteins undergo a different fate on warming: a well detectable increment in HSA, and, on the contrary, a partial depletion of the signal in BSA. That the most pronounced differences take place in the UV is not surprising, since these two bands are attributable to the benzoquinoline section of **1**, the reactive moiety of the dye hence the nearest to the protein. On the contrary, the anthraquinone transition in the visible is by far less sensitive to reactive events concerning the dye. The above complicated behavior of the dye shows its ability to distinguish the two proteins that are very like each other, also from the structural point of view.

Minimal variations of the absorbance were found in the case of BSA–dye solutions so that also here the absorbance of the dye is approximately independent of its binding to the protein. As in the case of the dye–HSA adduct the absorbance measurements were used to establish the stoichiometry of the reaction of the dye with BSA. Using the methods previously described for the dye–HSA case we determined the maximum number of molecules of dye bound to BSA, that is two.

2.1. Perturbations

In order to appreciate the sensibility of the CD of the dye–HSA *adduct* to perturbations of the system we proceed to the addition of SDS to the *adduct* on the basis of one SDS molecule/peptide unit. The CD spectra before and after the SDS addition are shown in Fig. 4.

The CD *after* the SDS addition is clearly amenable to the asymmetric perturbations on the chromophores of the dye due to the l-aminoacids to which the two dye molecules are directly bonded. Hence, the strong difference of the CD of the adduct *before* SDS with respect to the CD *after* addition essentially represents the chiral perturbations on the electronic transitions of the dye chromophores attributable to the protein moiety in conformation near native conditions. Finally, on submitting the dye–HSA adduct + SDS mixture to a washing process with buffer through consecutive UFs, the "native" dye–HSA CD spectrum is progressively recovered. In Fig. 4 two intermediate steps in the recovery of the native structure of the protein in the adduct are reported together with the CDs corresponding to the extreme conformational situations. Also reported is the CD spectrum of the adduct obtained through 2-h warming at 70 °C of a 2:1 mixture of dye and HSA: notice that the CD is intermediate between the first and the second UF of the adduct.

Interestingly, addition of oleic acid to the "native" *adduct* slightly alter its CD, while addi-

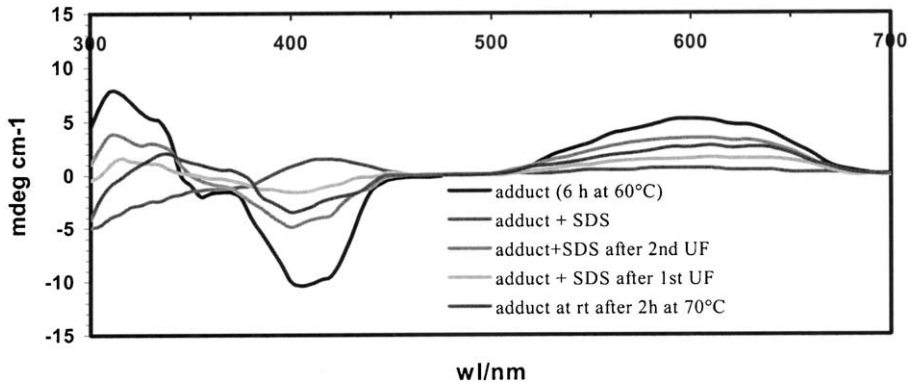


Fig. 4. HSA (3.2×10^{-5} M) and 6.2×10^{-5} M dye buffer solutions in native and non-native situations.

tion of free oleic acid to the *complex* induces very sensible alterations probably connected to a redistribution of the dye molecules between the binding sites due to the change of the relative value of the binding constants when oleic acid is added [6]. We note that moderate addition of oleic acid to HSA (up to 4 molecules per HSA molecule) does not affect the secondary structure of the protein, the tertiary structure only being affected.

2.2. Kinetics

We resorted to two different ways to measure the kinetics of the protein–dye reaction at fixed temperatures. In the first, the spectroscopic-one, we followed the time evolution of CD intensity. In Fig. 5 the 410 nm CD measured at 50 °C is reported. In the second, we extracted with MeOH the free, non-reacted dye, from the reaction mixture at 10, 30, 60 and 120 min warming at 50 °C. Thus, we could evaluate both the free- and reacted-dye concentrations from the absorbance of the methanol extracts and of the protein residual redissolved in buffer, respectively.

The measured CD obeys the simple kinetic law

$$\delta\varepsilon(t) = \delta\varepsilon_1 + (\delta\varepsilon_0 - \delta\varepsilon_1)\mathrm{e}^{-kt} \quad (1)$$

where $\delta\varepsilon_0$, $\delta\varepsilon_1$ and $\delta\varepsilon$?are the CD elongation of the complexes, that of the adducts and that of the reacting mixture at time t, respectively.

The increment of the dye bonded to protein, as above described, follows the same law (Fig. 6), so that we obtain the results of Table 1. The last row

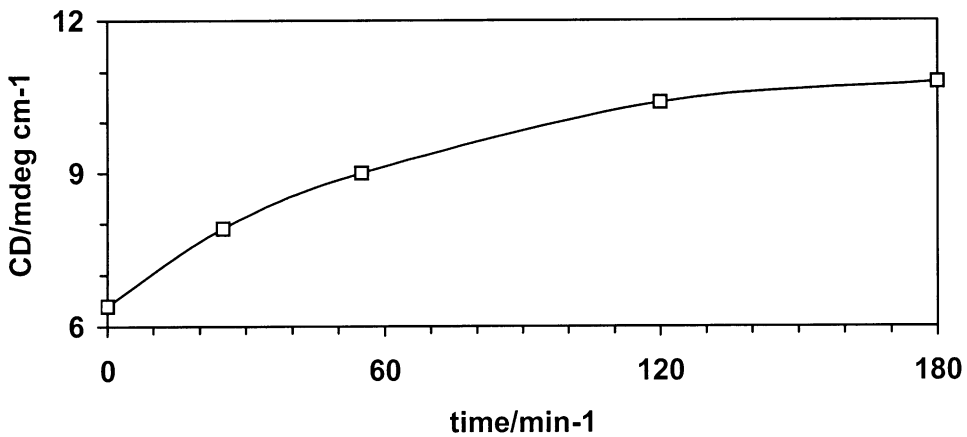


Fig. 5. Dye–HSA reaction followed through CD signal variation at 410 nm (50 °C).

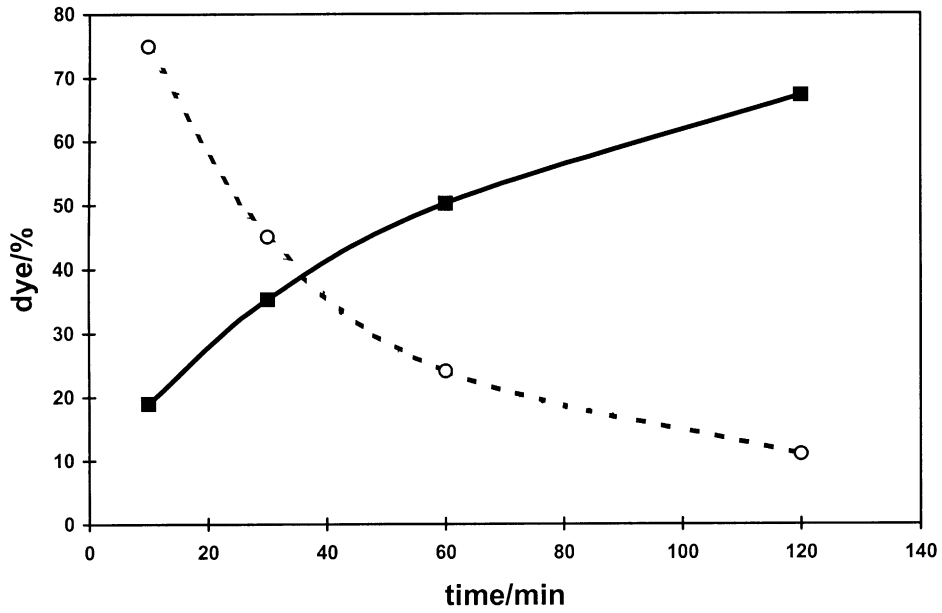


Fig. 6. Reaction between 6.2×10^{-5}M dye and 3.2×10^{-5}M HSA at 60 °C followed via extraction with methanol of unreacted dye (dashed) and with increasing formation of covalent **1**–HSA adduct in water (full line).

Table 1
First-order kinetic constants for the reaction of serum albumins and single aminoacids with the dye in buffer

t (°C)	Technique	k_1/min^{-1}
50	CD, HSA/**1** = 2	1.1×10^{-2}
60	CD, HSA/**1** = 2	1.7×10^{-2}
60	Extraction meoh	1.8×10^{-2}
60	Water re-add.	1.7×10^{-2}
60	CD,BSA/**1** = 4	$8{,}2\times10^{-3}$

of Table 1 reports the kinetic outcome of the reaction between the dye and BSA, that follows the same kinetic order, while the value of the kinetic constant is similar (nearly 50% lesser) to those found for HSA. Looking at the results in Table 1, it is worth noting the reproducibility of the kinetic constant.

2.3. Binding

2.3.1. Reaction of the dye with fragment B of HSA

In order to cast some light on the binding site of the dye in HSA, we firstly verified that **1** does not bind to aminoacids pertaining to the so-called fragment B of HSA, obtained with BrCN scissor, this fragment includes the 1–130 aminoacids [9]. As a matter of fact, no CD signal at $\lambda > 320$ nm was registered nor upon adding dye (7.6×10^{-5}) to a 2×10^{-5}M fragment B buffered solution, nor leaving the above mixture at 60 °C for 6 h. After dialysis of the warmed mixture no absorption at $\lambda > 320$ nm was detected. The final CD spectrum overlapped that of the original fragment B also at $\lambda < 300$ nm. These results seemingly indicate that fragment B of the HSA protein is not responsible for the reaction of the dye with HSA.

2.3.2. Reactions of the dye with aminoacids

Furthermore, we studied the reactions of single aminoacids with the dye, in conditions similar to those previously reported for the HSA and BSA reactions. The reaction mixtures were left to stand at 70 °C for 12 h, at 20:1–40:1 aminoacid:dye molar ratios, using 5×10^{-5}M dye solutions in buffer (NaH_2PO_4 0.1 M). The resulting solutions were examined by CD spectroscopy and the spectra compared with those of the initial, room temperature, dye-aminoacid solutions. The ratio-

nale was the hypothesis that reaction between dye and an aminoacid should result into a new adduct endowed with chirality, therefore showing some CD signal in the absorption region of the dye. A CD signal was reasonably expected as previous works [6,7] showed the strong sensitivity of the achiral dye molecule to a chiral environment even if the absence of a CD signal in the visible and in the near-UV spectral region does not completely exclude the intervention of a reaction between the dye and the aminoacid. The CD of the fresh mixtures, involving glycine, alanine, valine, leucine, isoleucine, phenylalanine, proline, 4-hydroxyproline, hydroxylisine, arginine, lysine, histidine, aspartic acid, glutamic acid, tyrosine, *N*-acetyltyrosine-ethyl ester, cysteine, *N*-acetylcysteine, cystine, tryptophan, serine, threonine, and methionine resulted zero, in the above absorption range at the very beginning. In only two cases, cysteine and *N*-acetylcysteine, non-zero CD resulted after the thermal treatment. In particular, cysteine reacts and the measured CD signal develops following a first order kinetic law, with $k_1 = 1.8\times10^{-2}$ ($\pm0.03\times10^{-2}$) min^{-1} at 70 °C. The stoichiometry involved in the reaction of the dye with cysteine was then investigated and we found that one molecule of dye binds three cysteines [10].

The kinetic order, and the value of the kinetic constant similar to that found in the protein–1 reaction suggest that cysteine could be responsible for the reactivity of HSA towards the dye.

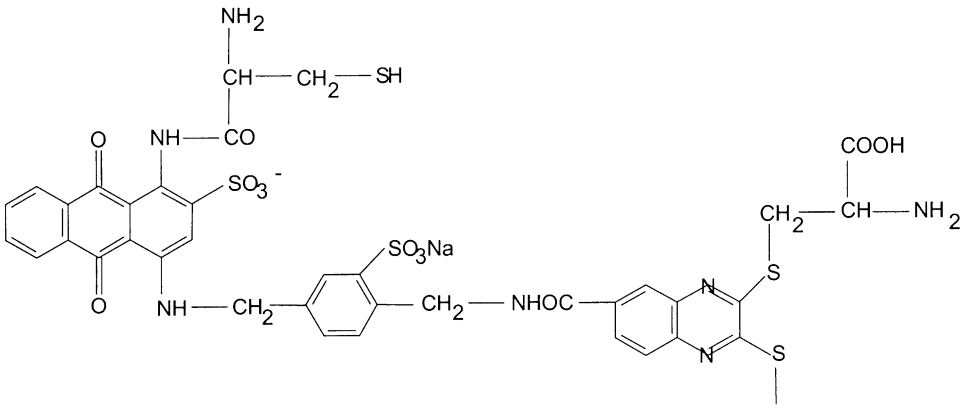
2.3.3. Spin labeling the dye–HSA adduct

Once a reaction has been observed between HSA and the dye, and a similar process detected between the dye and cysteine, but not other aminoacids, the conclusion that the cysteine is responsible for the reactivity of the protein with the reactive dye 1 should be taken into consideration. To ascertain this possibility, we underwent the dye–HSA adduct to the following experiments.

The Ellmann test [11] in the present application failed as the blank experiment, that in this case consists of a solution of pure albumin at the appropriate concentration, did not react with DNTB.

An aliquot of the purified HSA–dye adduct in water was added with aqueous HgCl2. Immediately a blue precipitate, quantitatively involving the protein, appeared. This was considered as evidence that Hg^{++} had reacted with a *free* sulfidryl group present in the protein (the cysteine 34 of HSA).

A "decisive" experiment was finally undertaken: that is we proceed to label with the 3-MAL stable radical both pure HSA [12], and the dye–protein adduct, in order to compare the intensity and the shape of their ESR spectra. Any difference should be due to the different labeling of HSA (only the -SH is reactive) and dye–HSA adduct. In Fig. 7 the results of this approach are reported: no appreciable difference occurs in the spectra. Then we concluded that cysteine-34 is not responsible for the reaction of the HSA with the dye.



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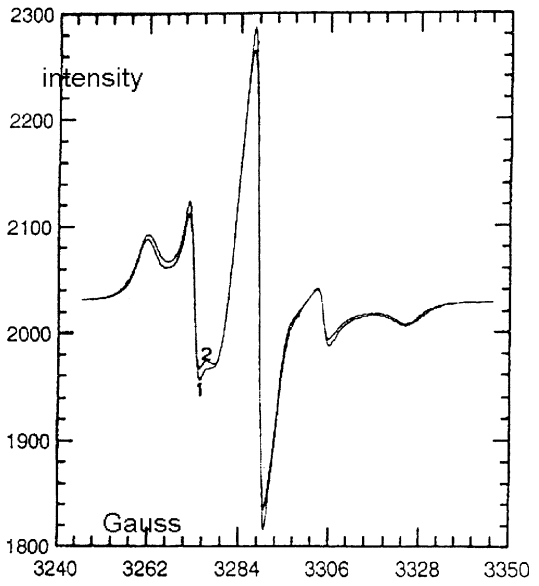

Fig. 7. EPR spectra of HSA (1) and dye–HSA adduct (2) both labeled with 3-MAL.

3. Conclusions

A reaction between the host HSA and the dye ligand has been found to occur. Time evolution of the CD spectra, determination of the reacted and un-reacted dye resulted in a first-order kinetics, probably attributable to an "intramolecular"-reaction of the dye–HSA complex initially formed.

A reaction, similar in order and in the kinetic constant, has been found between cysteine and dye while several other aminoacids, both basic and acid, have been found not able to react with the dye. The **1**–cysteine adduct has been shown to consist of three cysteine units covalently bonded to the dye. An investigation carried out with the 3-maleimido proxyl, specific spin label for the -SH group, has proved that the cysteine of the albumin is not involved in the reaction of the protein with the dye. In conclusion, reactivity with **1** of albumins is certain but the chemical methodology used to clear the binding points failed.

However, an interesting reactive behavior of HSA with the dye is apparent. Thus, some aminoacid *in the protein* reacts with the dye, while the same aminoacid does not react in free form. On the contrary, while *free* cysteine gives rise to a covalent adduct with **1**, the cysteine in the protein does not. The same conclusions are drawn also for the BSA case.

It is to be noted that information on the conformational state of the protein moiety in the HSA–dye adduct have been actually obtained by means of the CD induced in the absorption bands of the dye moiety. In particular the near-UV and visible spectral region bands reveal an intense and easily measurable CD when the protein is in native conditions, as the Kuhn's dissymmetry factor is relatively high for these bands and favorable to CD measurements. The advantages over the usual CD measurements in the deep UV, necessary to gain information on the protein conformation are clear. The intensity of the near-UV and vis CD is directly dependent on the denaturation level of the protein. In particular, the CD band in the red part of the spectrum seems the most indicative of the average level of denaturation of the protein. Terms such as "denaturation level" cannot be defined in an univocal and general fashion even if they are of common use. With albumins it seems possible to give to "denaturation" a meaning in connection to the percentage of the actual CD, in the 600 nm band, with respect to that of the native protein dye adduct. Clearly, it is difficult to assess whether the property of then dye of informing via CD on the conformational state of the protein will be present also with other proteins to which is bound.

4. Experimental section

Many experimental details are not here reported since they are directly included in the results section, where their presence is essential for the description of the actual experiments.

4.1. Materials

Human serum albumin essentially fatty acid and globulin free (HSA) was purchased from Sigma, stored at 4 °C and used without further purification. The CI RB 29 MW taken to calculate its concentrations was 66300. (Levafix Brilliant Blue EB) (Bayer) was purified through precipitation by addition of diethyl ether to a methanol solution of

1. Fragment B of HSA was obtained according to a reported procedure [9].

Aminoacids, sodium dodecyl sulfate (SDS), and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma.

4.2. Spectrophotometric measuremts

CD spectra were registered on a JASCO J40S spectropolarimeter, with a 2 nm bandwidth; The average concentration of the protein was taken as low as 3×10^{-5} M to escape significant dimerization processes.

Absorption spectra were registered on a Cary 219 spectrophotometer. The original **1**–HSA mixture was thermostatted at the temperature and for the time selected, and a CD spectrum was registered in the 300–450 nm range after returning at ambient temperature. After a reasonable time, a new CD spectrum was registered, together with the Absorbance of the solution. Then an aliquot of this solution was dried using a nitrogen stream. The solid adduct was then extracted with methanol (three times, up to total 15 ml volume). The volume was then reduced to 2 ml , and then the UV–vis absorption spectrum registered. The solid residue of the methanolic extraction, was dissolved in 2 ml of water, to register again the CD spectrum. This last solution, at any rate, has been added with few milligrams of SDS, and a new CD has been taken, and absorbance, too. An UF was then carried out, the 2 ml of water volume readjusted, and final CD and absorbance spectra taken. The molar absorptivity of the dye at 600 nm was applied (6800 M^{-1} cm^{-1}).

The standard deviations of kinetic constants were always around 1–5%. The EPR spin labeling was carried out as follows: A 3.7×10^{-4} M HSA–**1** adduct buffered solution (10 ml) was added with 3-maleimido-proxyl free radical (Aldrich) (5.1 mg, 2.1×10^{-3} M). The same labeling, with identical concentrations, was carried out with a parallel solution containing pure HSA, of course. After a couple of days during the which these solutions were left to react at 4 °C, they were ultrafiltrated through a Spectrum C 20 K membrane in a 10-ml Spectra-por cell. 5 consecutive UFs, from 10 to 1 ml, were carried out, re-adding fresh buffer each time. At the last ultrafiltration process, the solution was reduced to the minimum possible volume, and then EPR measurements were brought about on a EPR Varian E112 instrument.

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