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# A fluorescence study of human serum albumin binding sites modification by hypochlorite

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ABSTRACT

A study has been made on the properties of human serum albumin (HSA) binding sites and how they are modified by pre-oxidation of the protein with hypochlorite. The oxidation extent was assessed from changes in the protein intrinsic fluorescence and production of carbonyl groups. HSA retains its solute binding capacity even after exposure to relatively large amounts of hypochlorite (up to 40 oxidant molecules per protein). From an analysis of the binding isotherms of dansyl sarcosine (DS) and dansyl-1-sulfonamide (DNSA) to native and hypochlorite treated albumin it is concluded that pre-oxidation of the protein reduces the number of active sites without affecting the binding capacity of the remaining binding sites. From DS and DNSA fluorescence anisotropy, Laurdan anisotropy and generalized polarization measurements, it is concluded that both Sites I and II in the native protein provide very rigid environments to the bound probes. These characteristics of the sites remain even after extensive treatment with hypochlorite. This stubbornness of HSA could allow the protein to maintain its function along its *in vivo* lifetime.



## 1. Introduction

Reactive oxygen species (ROS) are present in biological fluids and tissues, both in normal conditions and, particularly, in oxidative stress scenerios, where their rate of production is increased and/or removal rate decreases, leading to an increase in their steady state concentrations. Most biomolecules (lipids, proteins, DNA) are the target of ROS and reactive nitrogen species, suffer from oxidative transformations that can modify their structure and capacity of function. Regarding proteins, ROS can alter their primary structure, lead to fragmentation or oligomerization, and modify their capacity of function. This last aspect has been extensively studied in several enzymes [1–3], but considerably less information is available in toxins [4], channel forming membrane proteins [5,6] or plasma soluble transport protein, albumin [7–10].

Human serum albumin (HSA) is the most abundant protein in blood plasma. During the lifetime (ca. 27 days [11]), it is continuously exposed to different oxidants. This process could be of importance since it has been proposed that albumin could play, besides its transport function, a role as antioxidant [12] in blood due to its free sulfhydryl group at Cys34 [8]. Several studies have been devoted to describe the changes elicited in the protein by their exposure to biologically relevant oxidants [7], but only a few have analyzed how oxidation of the protein modifies its binding capacity and, in particular, how the characteristics and binding capacity of its main binding sites are affected [8]. In this work, we address this point employing hypochlorite as oxidant.

Hypochlorite, produced in stimulated polymorphonuclear leukocytes readily reacts with proteins [9,13], changing the protein primary structure both by direct interaction with the oxidant and secondary processes mediated by the initially formed chloramines. Regarding albumin, it has been shown that titration of HSA with NaOCl affects firstly the sulfhydryl and afterwards the amino groups [9]. In this work, we titrated HSA with hypochlorite and estimated the degree of protein oxidation by the loss of tryptophan (Trp) and the formation of carbonyl groups. Simultaneously, we measured the binding capacity of albumin towards dansylsarcosine, DS (with affinity towards site II) and dansyl-1-sulfonamide, DNSA (with affinity towards site I) [14]. Changes in the characteristics of the binding sites were evaluated from the dansyl derivatives fluorescence anisotropy and Prodan generalized polarization [15].

## 2. Experimental

Human serum albumin (HSA, Sigma; fatty acid free), 6-propionyl-2-(N,N-dimethyl)aminonaphthalene, prodan (Molecular Probes), dansylsarcosine (piperidinium salt), (DS) and dansyl-1-sulfonamide (DNSA), (Sigma) and 2,4-dinitrophenylhydrazine (DNPH; Fluka) were used as received.

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Fluorescence measurements were carried out on an Aminco Bowman spectrofluorometer. Excitation of HSA, which contains a single tryptophan residue, was carried out at 295 nm in order to avoid energy transfer from tyrosine to tryptophan [16,17]. The tryptophan fluorescence emission was registered at 340 nm. After hypochlorite addition, the loss of tryptophan groups was assumed to be equal to the loss of the protein intrinsic fluorescence intensity. DS and DNSA were excited at 329 nm and the fluorescences registered at 476 nm. Prodan was excited at 340 nm and the fluorescence measured at 444 nm. Generalized polarization (GP) of prodan was determined according to the procedure described by Parasassi et al [15].

Steady-state anisotropy measurements were performed by using a Glan-Thomson polarizer. Fluorescence anisotropy values, $r$, were obtained by using the expression $r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH})$, where $I_{VV}$ and $I_{VH}$ are the vertically and horizontally polarized components of the emission after excitation by vertically polarized light, and $G$ is the sensitivity factor of the detection system.

Albumin oxidation was performed as follows: adequate aliquots of a concentrated NaOCl solution (0.1 M) were added to 2 mL of a HSA solution (83 M) in such a way as to obtain the desired [NaOCl]/[HSA] mol:mol ratio; the reaction mixture was maintained under continuous stirring. NaOCl is almost completely consumed in few minutes in the presence of amino acids or proteins [18–21]. In spite of this, measurements were performed after 60 min incubation in the dark at room temperature in order to standardize secondary damage due to the decomposition of the initially formed chloramines [18,22]. However, most of the protein modification was directly elicited by NaOCl since experiments carried out with a shorter incubation time after its addition (5 min) gave similar results.

Carbonyl content in oxidized HSA was quantified according to the method described in the literature [23]. In this method, carbonyl group concentrations were determined by their conversion to the corresponding hydrazones by mixing 0.3 mL of the oxidized protein solution and 1.2 mL of DNPH (0.2% w/v). After incubation for 1 h at room temperature, the protein was precipitated with trichloroacetic acid (final concentration 20% v/v) and incubated in an ice bath for 30 min. Subsequently, the solution was subjected to centrifugation at 11,000g for 3 min, and the supernatant was discarded. The precipitated protein was washed three times with 10 mL of an ethanol ethyl:acatete mixture (1:1 v/v) and afterwards dissolved in 1 mL of 6 M guanidine–HCl solution (pH 2.3). The carbonyl content was calculated from the absorbance at 370 nm using a molar absorption coefficient of 22,000 $M^{-1}$ $m^{-1}$ [24].

All measurements were carried out at room temperature in 10 mM sodium phosphate buffer, pH 7.3.

## 3. Results and discussion

Addition of NaOCl to HSA solution (83 μM, pH 7.3) leads to a progressive modification of the protein. Results obtained for Trp residues bleaching and carbonyl group formations are given in Table 1.

**Table 1**
Production of carbonyl groups and Trp consumption elicited by NaOCl addition to HSA solution (Values are given in mol/mol basis).

| [NaOCl]/[HSA] | [Carbonyl]/[HSA] | [Carbonyl]/[NaOCl] | Δ[Trp]/[HSA][b] | Δ[Trp]/[NaOCl] |
|---|---|---|---|---|
| 0 | 0.011 ($1.66 \times 10^{-6}$)[a] | – | 0 | – |
| 10 | 0.61 ($9.24 \times 10^{-5}$)[a] | 0.06 | 0.47 | 0.047 |
| 40 | 1.17 ($1.77 \times 10^{-4}$)[a] | 0.03 | 0.96 | 0.024 |

[a] Values expressed in nmol carbonyls /mg HSA.
[b] Equated to the fraction of fluorescence intensity loss following hypochlorite addition.

The data given in this table are compatible with a progressive oxidation of the protein elicited by hypochlorite addition. However, the stoichiometric coefficients of the measured oxidation processes are relatively low. This is compatible with previous reports that indicate that the main targets of hypochlorite reactions are sulfur containing amino acids and nitrogen bearing side chains [9,21].

### 3.1. Effect of NaOCl on albumin binding capacity

Addition of albumin to a DNSA or DS solution leads to a progressive increase in fluorescence intensity, directly related to an enhanced fluorescence yield from the protein-bound chromophore. Data obtained in untreated albumin and in albumin pre-treated with hypochlorite are shown in Figs. 1 and 2. Fig. 3 shows similar data, obtained employing prodan as fluorescent probe.

The data collected in Figs. 1–3 show that, even when the protein was reacted with 40 molecules of NaOCl, it retained its capacity to bind the three tested ligands. The results were fitted into a sigmoidal function in order to obtain the maximum intensity (from the plateau) and the concentration of HSA needed to bind 50% of the ligand from the protein concentration at which $F_{1/2} = 0.5F_{plateau}$. $F_{plateau}$ values were determined by the maximum fluorescence intensity of the solutes ligands bound to the modified and native HSA, while $F_{1/2}$ values are a measure of the binding affinity of the active sites. Duplicated measurements and fitting of the data into a sigmoidal function for the native protein are included in Fig. 1. It is observed that the chosen function adequately reproduces the experimental determinations. The same considerations apply to data obtained employing two other fluorescent probes, shown in Figs. 2 and 3. Table 2 shows data obtained from this figures.

It is noticeable that

(i) A plateau is reached with the native and treated proteins, indicative of complete ligand binding. Differences in fluorescence intensities can be related to changes in the characteristics of the environment of the bound ligands resulting from

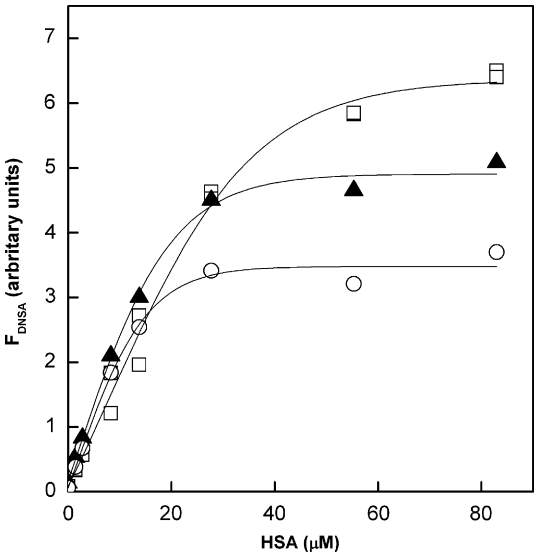


**Fig. 1.** Increase in fluorescence intensity (excitation at 329; emission registered at 476 nm) of a DNSA solution (8.3 μM) elicited by albumin addition. (□) native HSA; (▲) HSA pre-treated with NaOCl at ([NaOCl]/[albumin] = 10, mol/mol); (○) HSA pre-treated with NaOCl at ([NaOCl]/[HSA] = 40, mol/mol). Duplicate measurements for the native protein are shown. Experimental data were fitted into a sigmoidal function.

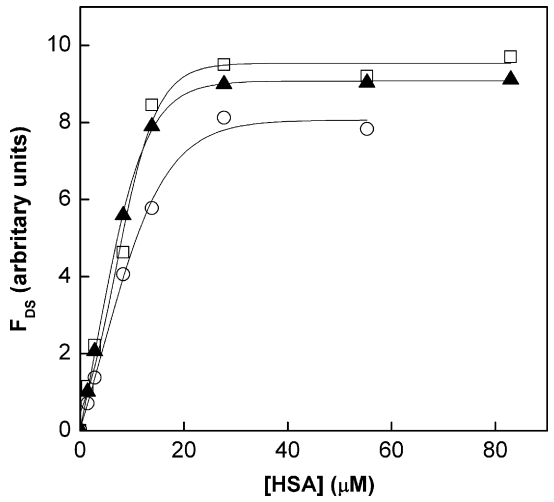


**Fig. 2.** Increase in fluorescence intensity (excitation at 329 nm; emission registered at 476 nm) of a DS solution (8.3 μM) elicited by HSA addition. (□) native HSA; (▲) HSA pre-treated with NaOCl at ([NaOCl]/[HSA] = 10, mol/mol); (○) albumin pre-treated with NaOCl at ([NaOCl]/[HSA] = 40, mol/mol). Experimental data were fitted into a sigmoidal function.

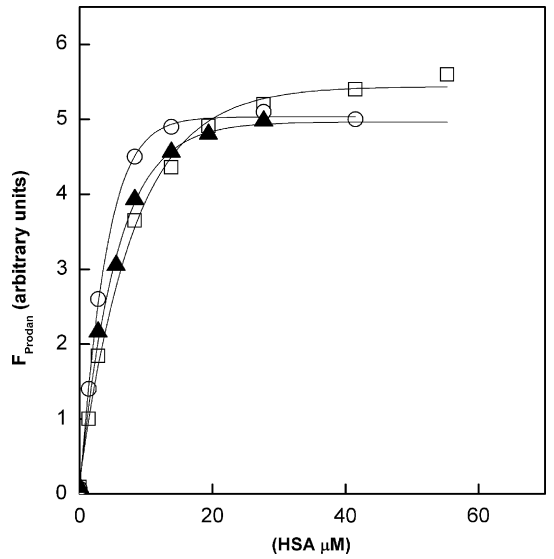


**Fig. 3.** Increase in fluorescence intensity (excitation at 340 nm; emission registered at 444 nm) of a Prodan solution (8.3 μM) elicited by albumin addition. (□) native HSA; (▲) HSA pre-treated with NaOCl at ([NaOCl]/ [HSA] = 10, mol/mol); (○) HSA pre-treated with NaOCl at ([NaOCl]/[HSA] = 40, mol/mol). Experimental data were fitted into a sigmoidal function.

**Table 2**
HSA concentrations needed to bind 50% of the indicated ligand*.

| Protein/ligand | DS (μM) | DNSA (μM) | Prodan (μM) |
|---|---|---|---|
| Native | 7.4 (1.00) | 13 (1.00) | 3.7 (1.00) |
| [NaOCl/[HSA] = 10 | 8.3 (0.95 ± 0.08) | 11 (0.80 ± 0.04) | 3.1 (0.92 ± 0.05) |
| [NaOCl]/[HSA] = 40 | 6.7 (0.84 ± 0.05) | 8.3 (0.54 ± 0.03) | 3.3 (0.91 ± 0.05) |

* Values in parentheses correspond to relative fluorescence intensities at higher HSA concentrations (oxidized/native). Values and errors were calculated from plateaus derived by fitting the experimental data into a sigmoidal function.

changes in the protein's tertiary structure [18,25,26]. The data given in the figures show that DNSA is the probe whose fluorescence was most affected when bound to the pre-oxidized protein.

(ii) The concentrations to reach $F_{1/2}$ values are little affected by the protein pre-exposure to NaOCl. Furthermore, the relative affinity of the remaining sites depends upon the probe employed. In fact, while for DS and prodan remained almost unaltered, binding sites in the oxidized protein showed an increased affinity for DNSA.

Points (i) and (ii) would suggest that even after extensive oxidation, the binding capacity of the remaining sites is similar or even higher than in the native protein.

The data collected in Figs. 1–3 do not give any indication regarding the number of binding sites per protein, and how this number is modified by NaOCl-induced oxidation. In order to evaluate the effect of NaOCl on the number of binding sites per HSA, we performed a titration of a fixed amount of protein with increasing concentrations of the dansyl derivatives. Typical results are given in Figs. 4 and 5 for DS and DNSA, respectively. Plateaus are reached when all the sites are occupied by the probes. In order to evaluate the maximum fluorescence intensity, the data were fitted to a sigmoidal function. The fittings were very good, allowing accurate evaluations of plateau values (examples are shown in Figs. 4 and 5). Duplicate experiments provided plateau values with differences lesser than 10%.

Since the relative fluorescence from each site has been calculated from the data given in Figs. 1 and 2 (see Table 2), a comparison of the intensities measured in the plateaus of Figs. 4 and 5 can be employed to estimate the relative number of binding sites in the modified and native proteins.

In Figs. 1–3, the plateau is reached when all probes are bound to the protein. This allows an estimation of the fluorescence intensity ($F_{\text{bound}}$) associated with a bound fluorophore:

$$F_{\text{bound}} = F_{\text{plateau}}/[\text{fluorophore}] \quad (1)$$

In plots as those shown in Figs. 4 and 5, the plateaus ($F_{\text{plateau at saturation}}$) are reached when the protein is saturated. In this situation

$$F_{\text{plateau at saturation}} = n[\text{protein}]F_{\text{bound}} \quad (2)$$

where $n$ is the average number of bound probes per protein (at saturation). From these equations it can be obtained that

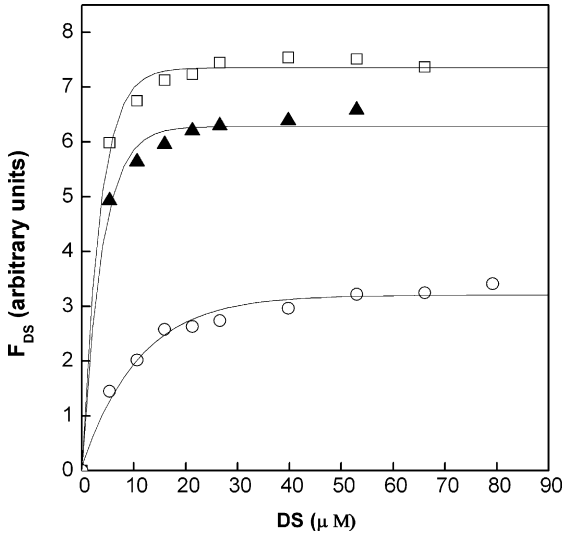


**Fig. 4.** Titration of a HSA solution (1 M) with DS. Excitation wavelength: 329 nm. (□) Native HSA; (▲) HSA pre-treated with NaOCl at ([NaOCl]/ [HSA] = 10, mol/mol); (○) HSA pre-treated with NaOCl at ([NaOCl]/[HSA] = 40, mol/mol). Duplicate measurements for the native protein are shown. Experimental data were fitted into a sigmoidal function.

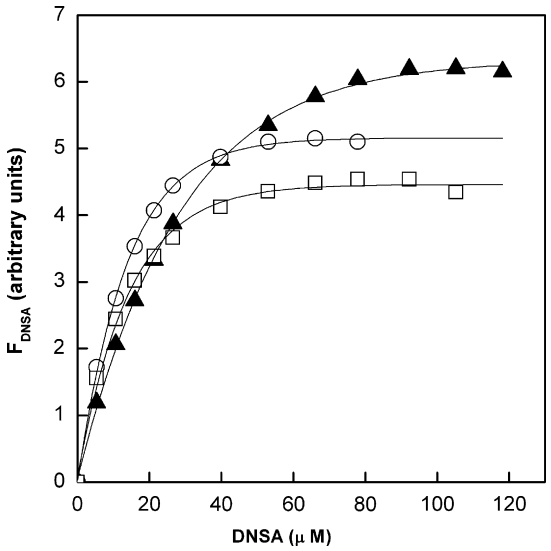


**Fig. 5.** Titration of a HSA solution (1 M) with DNSA. Excitation wavelength: 329 nm. (□) Native HSA; (▲) HSA pre-treated with NaOCl at ([NaOCl]/ [HSA] = 10, mol/mol); (○) HSA pre-treated with NaOCl at ([NaOCl]/[HSA] = 40, mol/mol). Duplicate measurements for the native protein are shown. Experimental data were fitted into a sigmoidal function.

$$n = F_{\text{plateau at saturation}}/(F_{\text{bound}}[\text{protein}]) \quad (3)$$

The fraction of sites remaining after the protein oxidation was taken as the ratio $n/n^{\circ}$, where $n^{\circ}$ is the number of sites in the untreated protein. This type of calculation was not carried out for prodan since, for this probe, a plateau was not clearly reached in a plot like that shown in Figs. 4 and 5. The data obtained are shown in Table 3. These data show that NaOCl treatment reduces the number of active sites. However, the remaining sites have a substrate affinity equal or even larger than those present in the native protein (Table 2). Furthermore, it is noticeable that, even after 40 NaOCl molecules have reacted per HSA, a large fraction of the sites remains active. If it is considered that nitrogen bearing residues, such as lysine and arginine, are the main targets for hypochlorite, the present results are compatible with previous data showing that, even after extensive modification (10 arginines or 56 lysines and 5 tyrosines), the protein retains its conformation and, depending on the solute, its binding capacity [10].

The changes in fluorescence intensity of DS and DNSA associated with the protein oxidation could be due to damage elicited by the added oxidant and/or secondary reactions, such as those promoted by chloramines. These reactions could affect the protein and/or the added fluorescent probe. Furthermore, the oxidative modification of the protein could be partially reverted by added reductants. In order to have some insight in the occurrence of these reactions and their reversibility, we performed two types of experiments:

(i) we added the dansyl derivatives 5 min or 1 h after the hypochlorite; and,

(ii) we added 1 mM cysteine, a compound able to remove chloramines [28,29], prior to dansyl derivatives addition.

No differences were observed in the dansyl fluorescence by one 1 h incubation of the sample prior to their addition (experiment i), suggesting that partial destruction of the chloramines does not change the capacity of the oxidized protein to bind dansyl derivatives or the fluorescent behavior of the bound probes. On the other hand, a significant decrease (ca. 40%) in diminution of the probes fluorescence elicited by hypochlorite (40:1) was observed in presence of cysteine (experiment ii). Taken together, these results would indicate that cysteine addition can partially repair damaged sites. Nevertheless, a limited contribution of fast secondary reactions of chloramines, affecting the protein or the probes, cannot be completely disregarded [30].

### *3.2. Effect of NaOCl pre-treatment on the characteristics of the binding sites as sensed by bound probes*

The characteristics of DS binding site (site II in sub-domain IIIA) and DNSA binding site (site I in sub-domain IIA) can be inferred from the fluorescence anisotropy of the bound probe's fluorescence. The measurements were carried out at relatively high protein concentrations and large protein/probe ratios in order to minimize the amount of unbound probe. Furthermore, since the fluorescence yield of the unbound probe is almost negligible, the small fraction of ligand remaining unbound to the protein did not affect anisotropy values.

The data of Table 4 show a noticeable rigidization of the probe environment associated with its binding to HSA. This was particularly evident for DNSA, whose anisotropy value was very close to that expected from a rigid moiety. On the other hand, smaller *r* values were obtained for DS, suggesting a lower microviscosity and order of the surroundings of this probe (see *r* value in the untreated HSA). The data also show a small but noticeable decrease in *r* values of DS molecules bound to hyprochlorite treated HSA. This would indicate a greater mobility of the probe bound to the oxidized protein. This effect was not observed in DNSA, whose r values remain very high even after addition of large amounts of the oxidant.

In order to further assess the characteristics of the site I and how they are modified by the protein oxidation, we analyzed the characteristics of bound prodan, a probe that specifically bound to the same site where DNSA binds [14,31]. The surroundings of the bound probe can be assessed from the anisotropy and GP values. The data obtained are shown in Table 5.

Taking into account the intrinsic anisotropy (highest value determined in a vitrified matrix) of prodan, $r_0 = 0.336$ [32], the *r* values given in Table 5 point to a rigid environment of the probe incorporated to site I, a condition that was not relaxed by HSA extensive oxidation (see also $r_{\text{DNSA}}$ values in Table 4),. Even more, prodan data show a small but systematic increase in anisotropy with NaOCl addition. Similarly, GP data indicate that solvent relaxation around bound prodan molecules is highly restricted, and that this restriction is systematically increased when HSA is exposed to the oxidant.

**Table 3**
Fraction of sites remaining after NaOCl treatment*.

| Treatment | DS binding sites | DNSA binding sites |
|---|---|---|
| None | 1.00 | 1.00 |
| NaOCl/HSA = 10 | 0.85 ± 0.08 | 0.91 ± 0.10 |
| NaOCl/HSA = 40 | 0.45 ± 0.05 | 0.66 ± 0.05 |

* Number of binding sites calculated with Eq. (2) employing plateau values from Figs. 1, 2, 4 and 5 obtained by fitting the data into a sigmoidal function. Errors were estimated by the errors propagation method [27].

**Table 4**
Fluorescence anisotropy (r) of DS and DNSA adsorbed in native and hypochlorite treated HSA.

| [NaOCl]/[HSA] | $r_{\text{DS}}$ | $r_{\text{DNSA}}$ |
|---|---|---|
| Buffer | 0.006 ± 0.004 0.016[a] | 0.005 ± 0.003 |
| Untreated | 0,262 ± 0.001 | 0.320 ± 0.002 |
| 10/1 | 0.251 ± 0.001 | 0.329 ± 0.020 |
| 40/1 | 0.243 ± 0.001 | 0.323 ± 0.003 |

[a] Data from Ref. [32].

**Table 5**
Generalized polarization (GP) and anisotropy (*r*) of prodan molecules bound to HSA.

| [NaOCl]/[HSA] | GP | *r* |
|---|---|---|
| 0 | 0.249 | 0.301 ± 0.001 |
| 3/1 | 0.312 | 0.304 ± 0.005 |
| 5/1 | 0.318 | 0.312 ± 0.004 |
| 10/1 | 0.322 | 0.316 ± 0.003 |
| 40/1 | 0.324 | 0.317 ± 0.002 |
| Buffer | | 0.033 |

## 4. Conclusions

Addition of NaOCl led to an extensive oxidation of amino acids, as evidenced by the loss of Trp fluorescence and formation of carbonyl groups. However, even when several NaOCl molecules have reacted per protein, this retained its capacity to bind ligands associated with both type I and type II binding sites. Furthermore, the properties of probes bound to these sites remained almost unmodified, suggesting only minor changes in the characteristics of the binding domains. The average number of oxidative modifications in each molecule during the protein *in vivo* lifetime is difficult to asses. In spite of this, these observations could be of relevance regarding its capacity to fulfill its transport role even after suffering a progressive oxidation along its lifetime.

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