CHARGE–TRANSFER INTERACTION INVOLVING SULFHYDRYL GROUPS OF BOVINE SERUM ALBUMIN

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1. Introduction

SH-groups in proteins play an important role in many biochemical and bioenergetic processes. In previous studies, the formation of complexes was shown between the dye 3′·6′-dichlorofluoran ion (DCF) and various SH-compounds including SH-proteins such as egg albumin and bovine serum albumin (BSA) [1–3]. These complexes were formed in the nonphysiological pH range above pH 11. The formation of a similar complex with BSA at neutral pH has been sought and found under unexpected conditions. This communication describes the formation of this new protein complex and its unique characteristics as well as the nature of the dye–protein interactions in comparison with other complexes including nonprotein SH-compounds.

2. Materials and methods

Crystalized BSA (Armour, glutathione, cysteine, thioglycolic acid, mercaptoethylamine (all from Calibiochem.), homocysteine (Nutritional Biochem.), mercaptopropionic acid (Eastman) and *p*-mercuriphenyl sulfonate (Sigma) were used as obtained. DCF was prepared as described previously [2].

Absorption spectra were taken using a Cary 14 spectrophotometer. An Aminco–Keirs spectrophosphorimeter was employed to measure fluorescence and phosphorescence spectra. Solutions containing 50% glycerol at liquid nitrogen temperature were used for the determination of phosphorescence spectra. Electron spin resonance spectra were determined on a Varian E-9 EPR spectrometer. A light source for illumination was a 1 kW-tungsten projection lamp with a Corning filter CS4–94.

3. Results and discussion

As compared with the absorption maximum of DCF at 453 nm, the absorption maxima of DCF complexes with a variety of SH-compounds ranged from 490 to 520 nm, depending upon the structure of SH-compounds. The new BSA complex exhibited a further red-shift to 545 nm at pH 6.5, while it absorbed at 520 nm in alkaline solution (Table 1). Table 1 includes the absorption maxima of complexes with three SH-compounds which were not reported before: mercaptopropionic acid, homocycteine and mercaptoethylamine. Homocysteine was in between mercaptopropionic acid and mercaptoethylamine, which gave rise to the same absorption maxima as thioglycolic acid and cysteine, respectively. The fact that different SH-compounds interacting with the same electron acceptor DCF form complexes which can absorb at different wavelengths indicates that the SH-group as an electron donor is capable of forming charge–transfer complexes. Table 1 also shows that charge–transfer absorptions at different wavelengths yield distinct charge–transfer fluorescences and phosphorescences, further supporting the charge–transfer nature of these complexes involving SH-groups. The thioglycolic acid and cysteine complex fluoresced at 530 and 535 nm and phosphoresced at 615 and 620 nm, respectively.

When 10^{-4} DCF was added to 0.5% BSA in 0.1 M phosphate buffer (pH 6.5), a slight shift of the absorption maximum from 453 to 457 nm was accompanied by a reduction of the absorbance to almost half of

Table 1
Absorption, fluorescence and phosphorescence maxima (nm) of charge–transfer complexes between SH-compounds and DCF

SH-compound	Absorption	Fluorescence	Phosphorescence	References
Mercaptoethanol	490	515	600	[3]
Dimercaptopropanol	490, 520	515	600, 650	[3]
Thioglycolic acid	467, 493	530	615	[1] for absorption
Mercaptopropionic acid	467, 493			
Homocysteine	470, 497			
Mercaptoethylamine	505			
Cysteine	505	535	620	[1] for absorption
Sodium hydrosulfide*	520	–	650	[2]
Glutathione	520	–	650	[2]
Egg albumin	520			[1]
Bovine serum albumin (pH>11)	520	–	650	[2]
Bovine serum albumin (pH 6.5)	545			

* Sodium sulfide forms a complex absorbing at 580 nm [4].

that of DCF alone. This indicates an immediate binding of the dye to the protein. This change was followed by a very slow formation of the complex absorbing at 545 nm (the 545 nm complex), showing a lag time even to several weeks at room temperature. It was therefore suspected that some aging process or bacterial growth in protein solution played a role in this transformation. The experiment done under sterile showed no formation, although the sterilized protein solution retained its ability to form the 545 nm complex under nonsterile conditions. The formation of this complex under conditions of prolonged incubation during which bacterial contamination apparently occurred was interpreted as a probable exposure of SH-groups from the reduction of disulfide bonds by bacterial metabolism. As expected, the addition of SH-compounds such as glutathione to the fresh BSA-DCF mixture greatly accelerated the formation of the 545 nm complex, indicating the participation of SH-groups generated from the reduction of disulfide bonds. SH-compounds by themselves did not form this complex at this pH.

This protein complex underwent bleaching and regeneration with different treatments:

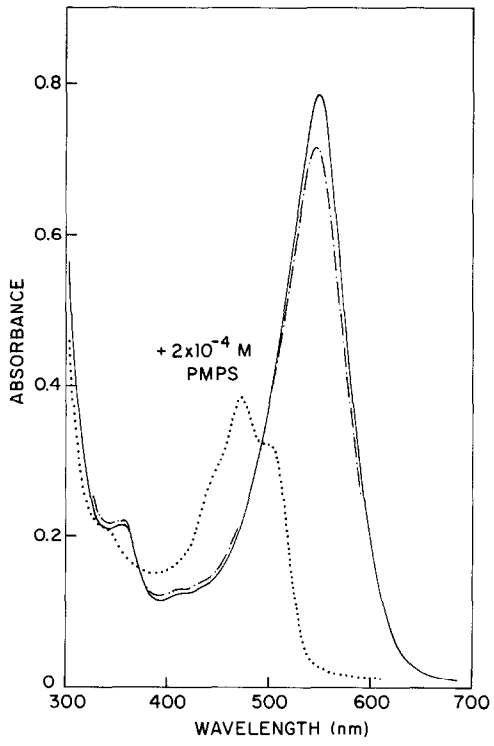

Fig.1. Absorption spectrum of the 545 nm complex (10^{-4} M DCF and 0.5% BSA) (——), its bleaching with *p*-mercuriphenyl sulfonate (PMPS) (2×10^{-4} M) (– – – – –) and subsequent regeneration with excess glutathione (— · —).

3.1. *SH-reagents*

Upon addition of the organic mercurial, *p*-mercuriphenyl sulfonate, the absorption spectrum with a peak

at 545 nm disappeared immediately, leaving a smaller absorption spectrum at 472 nm which was similar to the dye immediately bound to the protein but shifted somewhat to a longer wavelength (fig.1). Subsequent addition of excess glutathione instantly regenerated the original absorption spectrum. This bleaching and regeneration indicate that SH-groups are responsible for the absorption at 545 nm,

3.2. *Oxygen*

The complex exhibited a unique sensitivity to oxygen. It was noted that when the 545 nm complex was formed, the top of the solution exposed to air remained unchanged in color. When mixed with air, the solution underwent bleaching gradually over about one hour in the dark. The resulting spectrum at 470 nm was similar to that obtained with mercurial. Upon standing for several hours, the original absorption at 545 nm reappeared again except at the top of the solution. This phenomenon suggests that by mixing with air oxygen perturbs the charge–transfer interaction involving the SH-group of the protein, while by standing oxygen becomes again inaccessible to the site of the interaction. These processes may be related to the finding by Weber and co-workers [5,6] that the protein matrix provides a steric barrier to the free diffusion of oxygen toward dyes bound to BSA. It should be noted that shaking up the solution in the absence of air did not cause any spectral changes.

After the bleaching by mixing with air, the regeneration was greatly accelerated and completed mostly within 10 min by adding SH-compounds (10^{-3} M) such as mercaptoethanol, cysteine and glutathione. The rate of this regeneration was still slower than that shown by the removal of mercurials by SH-compounds. Although the bleaching and regeneration by mixing and standing in air were repeated many times, this sensitivity to oxygen was lost in the presence of excess SH-compounds, which apparently protected the SH-group of the protein involved in the charge–transfer interaction.

3.3. *Thermo- and photosensitivity*

Another unique property of this complex is its lability to mild temperature decrease. Lowering the temperature to about 4°C overnight bleached the absorption at 545 nm again to about 470 nm. This thermochromic change was reversed slowly when the temperature was returned to room temperature. The bleaching of the complex by exposure to light also occurred and produced a free radical of the dye. This is shown in fig.2 by the appearance of a single ESR spectrum having a width of 10 Gauss. This signal is the same as that observed previously in the protein complex absorbing at 520 nm in alkaline solution [2]. Differing from a short lifetime (about 3 min) of the bleached state in the latter complex, the bleached state of the former was much more stable, lasting for several hours. This long-lived radical state is apparently similar to the case of the cysteine or thioglycolic acid complex in which the bleached state lasts for one hour or two [1].

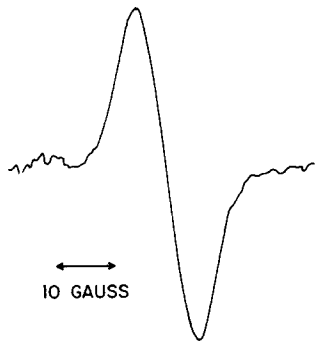

Fig.2. Photo-induced ESR spectrum of the 545 nm complex (3.5×10^{-4} M DCF and 1% BSA).

The protein BSA has a molecular weight of about 67 000 and exists as a single polypeptide chain of approximately 588 amino acids [7]. It contains approximately 0.7 SH-group and 17 disulfide bonds per molecule. The departure of the SH-group from unity is possibly due to the formation of some mixed disulfide with either a cysteinyl or glutathionyl moiety [8]. A low reactivity of the SH-group to SH-reagents suggests that it may be masked in some way. The aging process involves intramolecular SH-catalyzed disulfide–disulfide exchange as well as the formation of dimers and denatured forms [9–11]. SH-compounds slowly reduce the disulfide bonds when BSA is in the native state, but more rapidly after denaturation [12–15]. The finding that incubation of the DCF–BSA mixture with glutathione can markedly accelerate the formation of the 545 nm complex indicates the production of new SH-groups favorable for interacting with the dye

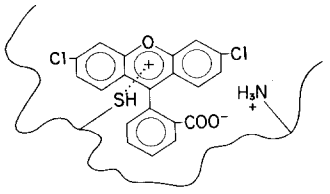

Fig.3. Interacting sites of DCF with BSA in the 545 nm complex.

which is already bound to a group other than SH-groups in the protein.

As shown in fig.3, DCF contains both a negative charge (carboxylate) and a positive charge (oxonium). The resonance structure of DCF may allow the positive charge to be nonlocalized in a central ring structure. The protein BSA possesses an exceptional ability to bind organic anions [16–18], but does not usually bind organic cations of comparable size [19]. The carboxylate group of DCF is probably bound to a cationic group (lysine or arginine residue [20]) of BSA, initially forming an electrostatic complex. The steric configuration or close proximity of the cationic portion of DCF (electron acceptor) and a specific SH-group (electron donor) of BSA seems to be critical in forming the charge–transfer interaction between them. Thus, the region of the BSA molecule involved in the binding of DCF in the 545 nm complex may be considered to have two interacting sites.

SH-groups generated from the reduction of disulfide bonds in the protein can be reoxidized by oxygen [15] which initially removes an electron from the SH-groups. Oxygen may perturb the interaction of the protein SH-group with the dye by involving itself in charge transfer with the SH-group. Reoxidation of this SH-group to disulfide may be inhibited by the bound dye. Excess SH-compounds appear to prevent the effect of oxygen, allowing the dye to interact with the specific SH-group. Thus, the bleaching by SH-reagent or oxygen (probably the thermosensitivity also) can be attributed to inhibition of the charge transfer interaction, whereas the photobleaching is based on the promotion of an electron transfer from the SH-group to the dye resulting in a photoreduced dye radical. Attempts will be made to study further the interaction of completely reduced BSA with the dye as well as the effect of reoxidation by oxygen.

The findings reported here reveal specific interactions between the dye molecule and the SH-protein at neutral pH. Charge–transfer interactions involving SH-groups of proteins could also be significant in some biologically important chromoproteins. The visual pigments respond to SH-reagent under certain conditions, suggesting the possible involvement of SH-groups in their spectral integrity [21–23]. It is also known that biliproteins, photosynthetic accessary pigments (phycocyanin and phycoerythrin) [24–26] and photomorphogenetic pigments (phytochrome P_{FR} and P_R) [27,28] possess the visible absorption bands which are also affected by SH-reagents.

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