Role of Tryptophyl Residues in the Binding of Gene 32 Protein from Phage T4 to Single-Stranded DNA. Photochemical Modification of Tryptophan by Trichloroethanol†

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ABSTRACT: From previous studies on model peptides and on single-strand binding proteins, aromatic amino acids are expected to play a role in the specific binding of the gene 32 protein from bacteriophage T4 to single-stranded nucleic acids. In this paper, we report the results obtained with gene 32 protein whose tryptophyl residues have been photochemically modified by trichloroethanol. Trichloroethanol is an efficient quencher of the fluorescence emission of indole derivatives and is able to form covalent adducts with tryptophan in its first excited singlet state upon UV irradiation. From UV absorption and fluorescence measurements, it is concluded that irradiation of the gene 32 protein from bacteriophage T4 in the presence of trichloroethanol leads to the formation of only one type of photoproduct. This photochemical reaction results in a complete quenching of the protein fluorescence. However, determination of the tryptophan content of the irradiated protein demonstrates that two tryptophans out of five have not been photochemically modified under conditions where all the fluorescence is abolished. These two residues are protected from photochemical reaction by an intramolecular singlet–singlet energy transfer to the trichloroethanol–tryptophan photoproduct of the other tryptophyl residues. The photochemically modified protein loses its ability to induce hyperchromism of poly(rA), is not able to compete with native protein for binding to either double-stranded or heat-denatured DNA, and does not promote the retention of heat-denatured DNA on nitrocellulose filters. No significant conformational change has been detected by circular dichroism measurements after UV irradiation at low doses, in the presence of trichloroethanol. This suggests that some tryptophyl residue could be involved in crucial interactions with nucleic acid constituents.

Proteins which bind preferentially, and in general cooperatively, to single-stranded polynucleotides and nucleic acids have been isolated from both prokaryotic and eukaryotic cells, bacteriophages (Alberts & Frey, 1970; Reuben & Gefter, 1973; Alberts et al., 1972), viruses and bacteria (Molineux et al., 1974; Van der Vliet & Levine, 1973; Yeh et al., 1976), fungus (Banks & Spanos, 1976), calf thymus (Herricks & Alberts, 1976), and rat liver (Thomas & Patel, 1976; Duguet & De Recondo, 1978). These proteins, termed single-strand binding (SSB)[1] or helix-destabilizing proteins, play an essential role in the cell [see Coleman & Oakley (1980) and Hélène et al. (1982) for reviews].

The protein encoded by gene 32 of bacteriophage T4 (gp 32) is involved in several steps of the viral DNA metabolism: *amber* or *ts* 32 mutants exhibit an abnormal or a stopped lytic cycle (Epstein et al., 1963). gp 32 has been demonstrated to be involved in the multienzymatic complex of replication and to specifically stimulate T4 DNA polymerase (Hibner & Alberts, 1980; Huberman et al., 1971). An active product of gene 32 is also required for phage DNA recombination (Kozinski & Felgenhauer, 1967; Tomizawa et al., 1966), and direct interaction with T4 ligase has been proposed during this process (Mosig & Breschkin, 1975). UV irradiation of T4 *Escherichia coli* infected cells results in an overproduction of gp 32, suggesting that this protein is involved in the repair of UV damages (Baldy, 1970; Wu & Yeh, 1973; Krisch & Van Houwe, 1976). Moreover, it regulates its own synthesis by specific binding to 32 mRNAs (Krisch et al., 1974; Gold et al., 1976; Lemaire et al., 1978).

These properties of gp 32 are mainly due to its ability to bind cooperatively to single-stranded nucleic acids (Albert & Frey, 1970; Jensen & Von Hippel, 1976). This allows it to protect single-stranded regions of DNA from endonucleolytic attack (Curtis & Alberts, 1976) and to destabilize hairpins or A-T-rich regions in native DNA (Jensen et al., 1976; Yaniv et al., 1975). Moreover, the intrinsic association constant (i.e., the binding constant for an isolated gp 32 molecule) is higher for any investigated single-stranded polynucleotides than for double-stranded ones (Newport et al., 1981). Nothing is known yet on the molecular mechanism which is responsible for the specific recognition of single-stranded polynucleotides by gp 32 and other SSB proteins in general.

Aromatic amino acids form stacked complexes with nucleic acid bases either in a rigid medium at 77 K or in concentrated fluid solutions (Montenay-Garestier & Hélène, 1968, 1971; Dimicoli & Hélène, 1971). Using model tripeptides such as Lys-X-Lys (where X stands for tryptophyl or tyrosyl residues), it has been shown that stacking interactions provide these peptides with a specificity of binding to single-stranded polynucleotides as compared to double-stranded ones (Brun et al., 1975; Toulmé & Hélène, 1977; Mayer et al., 1979). Moveover, the peptide Lys-Trp-Lys is able to recognize locally destabilized regions in the vicinity of damages following UV irradiation (Toulmé & Hélène, 1977) or chemical modification of the DNA (Toulmé et al., 1980; Hélène et al., 1982). Stacking interactions could therefore be involved in SSB protein–polynucleotide complexes.

gp 32 contains five tryptophyl and eight tyrosyl residues (Williams et al., 1981). From genetic experiments (Mosig et al., 1977) and results obtained for in vitro binding of proteolytic fragments of the protein to DNA (Hosoda & Moise, 1978), it appears that three tryptophans and six tyrosines are located

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[1] Abbreviations: gp 32, gene 32 protein; SSB proteins, single-strand binding proteins; Lys-Trp-Lys, lysyltryptophyl-α-lysine; NATA, *N*-acetyl-L-tryptophanamide; CD, circular dichroism; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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in the region between residues 30 and 120 which includes the potential nucleic acid binding site of the protein. Chemical modifications of gene 32 protein by tetranitromethane demonstrate that 5 tyrosine residues are protected from nitration when the protein is bound to DNA (Anderson & Coleman, 1975). Evidence has been presented for the presence of at least one tryptophan residue in the vicinity of nucleic acid bases in gp 32–single-stranded polynucleotide complexes (Hélène et al., 1976; Toulmé & Hélène, 1980). Whether this residue is involved in interactions with nucleic acid bases still remains unknown. In this and the following papers [see Le Doan et al. (1984) and Casas-Finet et al. (1984)], we have tried to shed some light on the role that could be played by aromatic residues in the binding of SSB proteins to single-stranded polynucleotides, by photochemical, radiation chemical, and spectroscopic methods.

We report here the results obtained from photochemical modification of gp 32 by trichloroethanol. This compound is an efficient quencher of the indole fluorescence (Eftink & Ghiron, 1976). Moreover, it reacts with indole rings in the first excited singlet state upon UV irradiation (C. Ghiron, personal communication; Privat & Charlier, 1978). The loss of affinity of such a photochemically modified gp 32 for nucleic acids is discussed with respect to the role of tryptophyl residues in its specific binding to single-stranded polynucleotides.

Materials and Methods

Materials. *N*-Acetyl-L-tryptophanamide was obtained from Sigma and trichloroethanol from Aldrich. Calf thymus DNA and proteases (chymotrypsin, trypsin, and Pronase) were purchased from Sigma, and poly(rA) was from Miles. Adenosine 5′-[γ-^{32}P]triphosphate (3000 Ci/mmol) was from Amersham. Heat-denatured DNA was prepared by heating native DNA solutions at 100 °C for 20 min in sealed tubes and then by chilling in ice. ^{32}P-Labeled *E. coli* DNA was prepared by nick translation according to the procedure of Rigby et al. (1977).

gp 32 was prepared from *E. coli* B cells infected with the *am* A292 strain of T4. The procedure used for the preparation of the protein was that previously described by Alberts & Frey (1970) with an additional step on hydroxylapatite for further purification. gp 32 migrates as a single band corresponding to a molecular weight of about 35 000 on a SDS–polyacrylamide gel.

Absorbance, Circular Dichroism, and Fluorescence Measurements. Absorption spectra were recorded on a Cary 15 or on a Uvikon 820 spectrophotometer and circular dichroism spectra on a Jobin Yvon Mark III apparatus. The concentrations of protein and DNA solutions were determined by using molar extinction coefficients of 37 000 and 6500 M^{-1} cm^{-1}, respectively. Fluorescence measurements were performed with a FICA 55 differential spectrofluorometer. Excitation fluctuations were automatically corrected. Unless stated otherwise, experiments were performed in a 10 mM cacodylate buffer (pH 7.6) containing 10 mM sodium chloride and 0.2 mM EDTA.

UV Irradiations. Irradiations were performed with an HBO 250-W mercury lamp. In order to avoid light absorption by chromophores other than tryptophan, the protein solutions were irradiated through a 1-cm cuvette containing toluene whose transmission was 50% at 290 nm and 1% at 282 nm. Irradiation of toluene leads to the formation of photoproducts absorbing at longer wavelengths. Replacement of the toluene filter every 4 min avoided the modification of its transmission properties. Under these conditions, the dose delivered to the sample was 10^{-7} einstein s^{-1}. Irradiations were also performed with monochromatic light in the sample compartment of the Fica spectrofluorometer. The results were quantitatively the same with the two modes of irradiation. As the last procedure required longer times of irradiation, the first method was generally used to prepare photochemically modified samples. UV irradiation was performed either in aerated or in argon-saturated solutions. Similar results were obtained in both conditions.

Analysis of Tryptophan Content. The tryptophan content of native and irradiated gp 32 was determined according to a method derived from that described by Sasaki et al. (1975). Protein samples (0.3 mg/mL in 10 mM cacodylate buffer, pH 7.6) were digested first by Pronase during 8 h at 37 °C and second by trypsin and chymotrypsin overnight at the same temperature. The final concentration of each protease was 0.005% (w/v). The fluorescence of the digested sample was measured in 8 M urea. Increasing amounts of tryptophan were added to the sample, and the fluorescence was measured after each addition. Extrapolation of the linear plot of fluorescence intensity vs. tryptophan concentration to zero fluorescence intensity allows determination of tryptophan content of the hydrolyzed sample.

Nitrocellulose Filter Retention Assay. Sonicated heat-denatured *E. coli* [^{32}P]DNA (0.03 μg) was incubated at room temperature for 10 min either with native or with photochemically modified gp 32 in 500 μL of buffer A (20 mM Tris-HCl, pH 8.0, 130 mM NaCl, 5% glycerol, 2 mM 2-mercaptoethanol, and 1 mM EDTA). The samples were filtered at 1 mL min^{-1} through nitrocellulose filters (prewashed with 5 mL of buffer A containing 1% dimethyl sulfoxide). The filters were then rinsed with 500 μL of buffer A, dried, and counted. Prior to use, the nitrocellulose filters (Schleicher & Schuell, BA 85) were boiled in water for 30 min and soaked overnight in buffer A containing 1% dimethyl sufoxide.

Results and Discussion

Photochemical Modification of gp 32 in the Presence of Trichloroethanol. UV irradiation of *N*-acetyl-L-tryptophanamide (NATA) in the presence of trichloroethanol results in the formation of a photoproduct whose absorption spectrum is shifted toward longer wavelengths as compared to NATA itself and whose fluorescence spectrum is centered at 500 nm (Privat & Charlier, 1978). As shown in Figures 1 and 2, UV irradiation of gp 32 in 0.1 M trichloroethanol at wavelengths longer than 280 nm (see Materials and Methods) also leads to a modification of both its absorption and its emission spectra. The decrease of the absorbance at 280 nm and of the fluorescence emission at 340 nm is accompanied by a concomitant increase of the absorbance at long ($\lambda > 292$ nm) and short ($\lambda < 267$ nm) wavelengths and of the fluorescence intensity at 460 nm; these results indicate that tryptophyl residues have been photochemically modified. The existence of two isosbestic points at 267 and 292 nm and of an isoemissive point at 420 nm suggests that, under our experimental conditions, UV irradiation of gp 32 gives rise to only one type of photoproduct. The excitation spectra of irradiated protein and NATA (recorded for an emission wavelength of 460 nm) are quite similar and exhibit two bands centered at 310 and 345 nm (Figure 3). This agrees with the results previously reported for wheat germ agglutinin (Privat & Charlier, 1978), but the maxima of the emission spectra of the photoproducts are notably different even in 8 M urea: 465 nm for gp 32 and 495 nm for NATA (Figure 3). As the emission is much more sensitive than absorption, the observed difference could be due to the local environment of the photoproduct in the irradiated protein rather than to a different chemical structure. This is

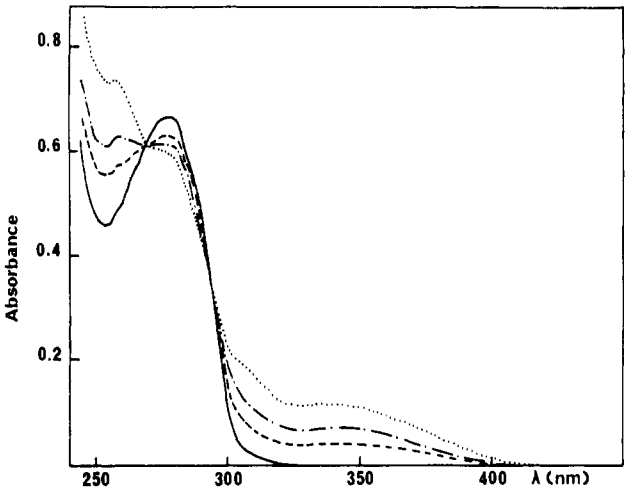

FIGURE 1: Absorption spectra of gp 32 irradiated with a dose of 0 (—), 3×10^{-6} (- - -), 6×10^{-6} (—·—), or 1.8×10^{-5} einstein (···) in 10 mM cacodylate buffer (pH 7.6) containing 0.1 M trichloroethanol (see Materials and Methods for conditions of irradiation).

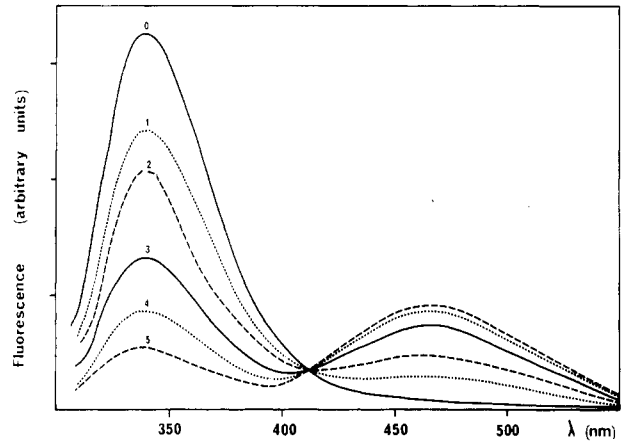

FIGURE 2: Emission spectra of gp 32 irradiated with a dose of 0 (0), 1.5×10^{-6} (1), 3×10^{-6} (2), 7×10^{-6} (3), 1.2×10^{-5} (4), or 2.4×10^{-5} einstein (5) in 10 mM cacodylate buffer (pH 7.6) in the presence of 0.1 M trichloroethanol (excitation wavelength, 280 nm). The spectra were recorded after 100-fold dilution of the irradiated sample in the buffer without trichloroethanol.

supported by the fact that the excitation spectrum remains unchanged when the emission wavelength is set at either 450 or 500 nm.

Analysis of the Photochemically Modified gp 32. Tryptophan contents of native and irradiated proteins were determined according to a fluorometric method described under Materials and Methods (Sasaki et al., 1975). The tryptophan content of the native protein was found to be 4.3 instead of 5 tryptophyl residues given from the gp 32 sequence (Williams et al., 1981). Using tryptophan as a standard in the fluorometric method assumes that the fluorescence quantum yield of the digested protein is equal to that of tryptophan. This assumption could not be valid for gp 32 due to an incomplete digestion by proteases for instance. In this case, a partial quenching of the tryptophan is expected which will result in a systematic underestimate of the tryptophan content of gp 32. This might also be due to an underestimate of the extinction coefficient of the protein.

The number of intact (fluorescent) tryptophyl residues was determined as a function of the irradiation time in the presence of 0.1 M trichloroethanol; as expected, a decrease was observed. The relative decrease of the gp 32 fluorescence induced by UV irradiation ($\Delta F/F_0$) was then plotted vs. the relative residual content of tryptophyl residues ($[Trp]/[Trp]_0$). The

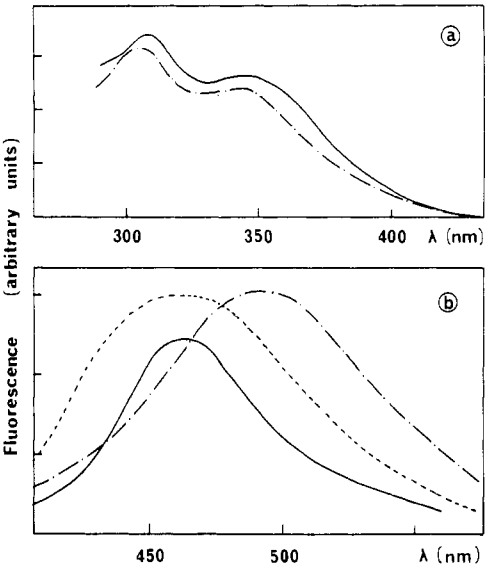

FIGURE 3: (a) Excitation spectra of *N*-acetyltryptophanamide (—·—) and of gp 32 (—) irradiated in the presence of 0.1 M trichloroethanol in 10 mM cacodylate buffer (pH 7.6). Emission wavelength, 460 nm. (The spectra were normalized at 300 nm.) (b) Emission spectra of gp 32 and of *N*-acetyltryptophanamide irradiated in 10 mM cacodylate buffer (pH 7.6) in the presence of 0.1 M trichloroethanol. The gp 32 spectra were recorded either in the cacodylate buffer (—) or in 8 M urea (- - -). The spectrum of NATA is recorded in 8 M urea (—·—) (excitation wavelength, 280 nm). The irradiated samples were diluted as in Figure 2 before the spectra were recorded.

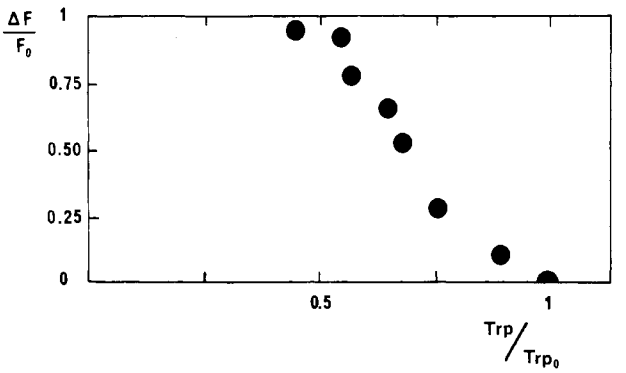

FIGURE 4: Relative decrease of the fluorescence intensity of gp 32 irradiated in the presence of 0.1 M trichloroethanol vs. the relative content of tryptophyl residues per monomer of irradiated protein. The fluorescence intensity was measured at 340 nm (excitation wavelength, 290 nm) after dilution of the samples (see legend to Figure 2).

extent of fluorescence change (ΔF) was obtained from protein samples diluted 100-fold in the cacodylate buffer in order to eliminate the dynamic quenching of tryptophan fluorescence by trichloroethanol (see below). Under such conditions, the relative decrease in fluorescence at 350 nm is entirely due to the photochemical modification of tryptophyl residues.

As shown in Figure 4, the plot of $\Delta F/F_0$ vs. $[Trp]/[Trp]_0$ extrapolates to a relative tryptophan content of about 0.4, i.e., two intact tryptophans for complete loss of the fluorescence ($\Delta F/F_0 = 1$). Thus, irradiation of gp 32 in the presence of trichloroethanol leads to the photochemical modification of three tryptophyl residues. It should be pointed out that the photoreaction of trichloroethanol with tryptophan takes place in the first excited singlet state and that nonfluorescent tryptophyl residues have such a short singlet-state lifetime that they cannot react with trichloroethanol except in the unlikely case where there is a strong binding site of the trichloroethanol in close vicinity to a nonfluorescent tryptophyl residue. The two unmodified tryptophyl residues either are not fluorescent

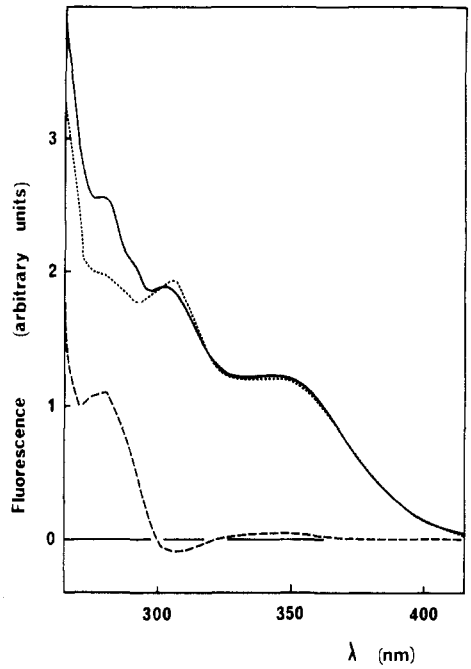

FIGURE 5: Excitation spectra of gp 32 irradiated with a dose of 3×10^{-5} einstein in the presence of 0.1 M trichloroethanol recorded in 6 M urea (···) and in 10 mM cacodylate buffer (—). The difference (×2) between these excitation spectra is also shown (- - -). $\lambda_{em} = 500$ nm.

or are protected from photochemical reaction. The experiments reported below are in favor of the second hypothesis.

Overlap of the emission spectrum of fluorophore D with the absorption of chromophore A can lead to singlet–singlet energy transfer from molecule D (donor) in the excited state to molecule A (acceptor). If such a mechanism occurs, the fluorescence of D will be decreased as compared to conditions in which energy transfer does not take place. The photoproduct resulting from irradiation of tryptophan in the presence of trichloroethanol absorbed at longer wavelengths than unmodified tryptophan, and its absorption exhibits an important overlap with the emission spectrum of the indole ring. This could allow singlet–singlet energy transfer from intact tryptophyl residue(s) to photoproduct(s) in the protein. This mechanism could account for the protection of some tryptophans in the protein. The probability of this process decreases as the sixth power of the distance between the two molecules and depends on their relative orientation (Förster, 1965). A conformational change of the protein will alter both parameters. The fluorescence excitation spectrum of the photochemically modified gp 32 was recorded in 10 mM cacodylate buffer and in 8 M urea with the emission wavelength set at 500 nm. The excitation spectrum of the photoproduct itself was not affected by the presence of urea. Singlet–singlet energy transfer from tryptophan to the tryptophan–trichloroethanol photoproduct should result in an increase of the fluorescence intensity of the photoproduct at 500 nm under excitation between 260 and 300 nm for the sample in nondenaturing conditions as compared with that in urea. As shown in Figure 5 for a sample having lost 85% of its fluorescence, the difference between the excitation spectra of the photochemically modified gp 32 in denaturing and nondenaturing conditions is very similar to that of tryptophan. As direct fluorescence of unmodified tryptophyl residues does not contribute to the 500-nm emission, this difference must be ascribed to energy transfer from intact tryptophan(s) to tryptophan–trichloroethanol photoproduct(s). This process which leads to a rapid deactivation of the excited donor

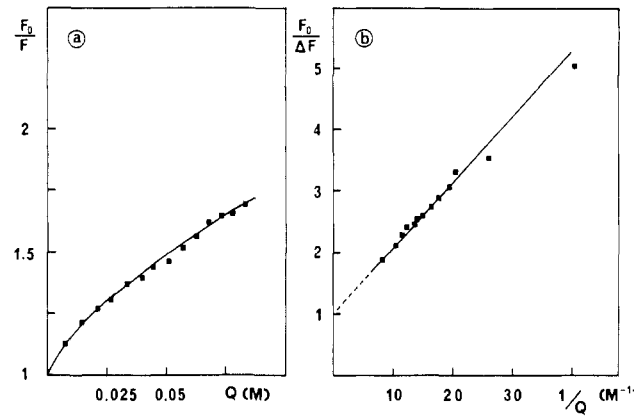

FIGURE 6: Analysis of the fluorescence quenching of gp 32 by trichloroethanol according to the relationships of Stern–Volmer (a) and of Lehrer (b) (see text). The experiments were performed at 4 °C in 10 mM cacodylate buffer (pH 7.6) containing 10 mM NaCl and 0.2 mM EDTA (excitation wavelength, 295 nm; emission wavelength, 350 nm).

tryptophan protects this (these) residue(s) from photoreaction with trichloroethanol. Therefore, at least one of the two intact tryptophyl residues is protected even though it contributes to the fluorescence in the nonirradiated protein.

Quenching of gp 32 Fluorescence by Trichloroethanol. The rate constant for the reaction of tryptophan with trichloroethanol inside a protein depends both on the lifetime of its first excited singlet state and on its accessibility. In the previous section, we have shown that two out of five tryptophyl residues cannot be photochemically modified. These unmodified residues could be partially buried inside the protein structure. The accessibility of gp 32 tryptophyl residues to trichloroethanol molecules was checked by using the quenching properties of this reagent: trichloroethanol is an efficient quencher of both tryptophyl and tyrosyl residues (Eftink et al., 1977). The fluorescence of gp 32 was excited at 295 nm in order to avoid possible complications due to tyrosine excitation. The shape of the fluorescence spectrum of the protein was not modified in the presence of trichloroethanol. The results were analyzed according to the Stern–Volmer relationship:

$$F_0/F = 1 + K_q[Q] \quad (1)$$

or according to the equation derived by Lehrer (1971)

$$\frac{F_0}{F_0 - F} = \frac{1}{f_a K_q[Q]} + \frac{1}{f_a} \quad (2)$$

where F_0 and F are the fluorescence intensities of the protein in the absence and in the presence of quencher, respectively. [Q] is the quencher concentration, K_q is the Stern–Volmer constant, and f_a is the fraction of the fluorescence accessible to quencher molecules. Note that eq 2 assumes that emitters belong to one of the following classes: (i) tryptophyl residues accessible to the quencher with the constant K_q or (ii) tryptophyl residues for which $K_q = 0$ (Lehrer, 1971). In this case, plotting $F_0(F_0 - F)$ vs. $1/[Q]$ leads to a straight line whose intercept and slope are equal to $1/f_a$ and $1/(f_a K_q)$, respectively. As expected from a protein which contains several tryptophyl residues, analysis of the gp 32 fluorescence quenching according to the Stern–Volmer relationship leads to a curved plot (Figure 6). Moreover, analysis according to eq 2 gives a curve which deviates from linearity, indicating that the above assumptions leading to eq 2 (see above) are not valid in the case of gp 32. These results indicate that fluorescent tryptophyl residues are not equally accessible and can be divided into the following two classes: (i) the more exposed residues which

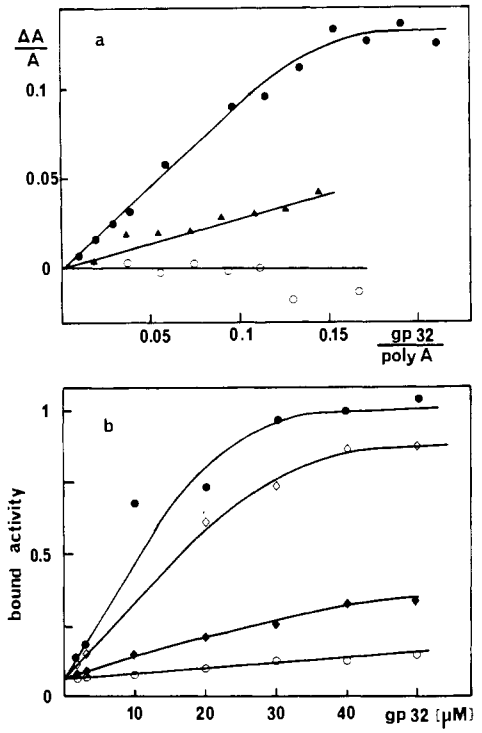

FIGURE 7: (a) Spectrophotometric titration of 1.4×10^{-5} M poly(rA) by native (●) and irradiated gp 32 in the presence of 0.1 M trichloroethanol. The samples were irradiated with a dose of 2.4×10^{-5} (▲) and 3×10^{-5} (○) einstein. The experiments have been performed in 10 mM Tris-HCl buffer, pH 8.0, which contained 100 mM KCl, 1 mM EDTA, and 1 mM 2-mercaptoethanol. Difference absorption spectra between the mixture gp 32 + poly(rA) and the isolated constituents have been recorded after every addition of gp 32. The relative hyperchromism ($\Delta A/A$) measured at 260 nm has been corrected for dilution. (b) Filter retention assays of 0.03 μg of heat-denatured ^{32}P-labeled *E. coli* DNA by native (●) and photochemically modified gp 32 irradiated with a dose of 3×10^{-6} (◇), 1.2×10^{-5} (◆), and 4.2×10^{-5} einstein (○). The samples were filtered as indicated under Materials and Methods.

can be photochemically modified by trichloroethanol and (ii) the partly buried ones which transfer their energy at the singlet level to the photoproducts derived from the exposed tryptophan(s) of class i. It should be noted than even in the presence of 0.1 M trichloroethanol the circular dichroism spectrum of gp 32 remains unchanged, thus indicating that no conformational change of the protein is induced by trichloroethanol.

Binding of Photochemically Modified gp 32 to Nucleic Acids. It was previously shown that at least one tryptophyl residue is located in the binding site of gp 32 and was probably involved in the binding to nucleic acids (Hélène et al., 1976; Jensen & Von Hippel, 1976; Toulmé & Hélène, 1980). Therefore, it was of interest to test whether the photochemically modified protein was still able to bind to single-stranded polynucleotides.

The binding of gp 32 to single-stranded polynucleotides induces a hyperchromism in the absorption spectrum of the polynucleotide (Jensen et al., 1976; Kowalczykowski et al., 1981). With poly(rA), the hyperchromism at 260 nm resulting from gp 32 binding is about 15% of the initial absorbance of the polynucleotide, in 0.1 M salt (Figure 7). Due to the high affinity of gp 32 for poly(rA), the binding stoichiometry can be deduced from the titration curve; the value of 7 ± 1 nucleotides agrees with previous results (Jensen et al., 1976; Hélène et al., 1976). Increasing the ionic strength from 0.1 to 0.3 M KCl leads to a sigmoidal curve when the hyperchromicity is plotted vs. the protein concentration (not shown); this behavior is characteristic of the cooperative mode of

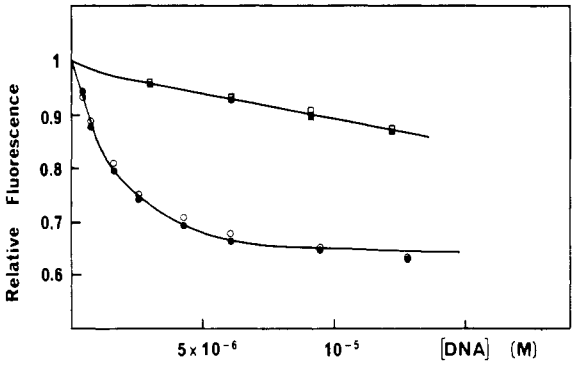

FIGURE 8: Fluorometric titration curves of 2.7×10^{-7} M gp 32 (filled symbols) and of a mixture of 2.7×10^{-7} M gp 32 + 2.7×10^{-7} M irradiated gp 32 (open symbols) by native (□, ■) and heat-denatured DNA (○, ●) in 10 mM cacodylate buffer, pH 7.6. gp 32 was irradiated in the presence of 0.1 M trichloroethanol with a dose of 2.6×10^{-5} einstein. The residual fluorescence of the photochemically modified protein was taken into account.

protein binding. Titration of poly(rA) with photochemically modified protein results in a markedly decreased or totally abolished induced hyperchromism for proteins which have been irradiated with doses of 2.4×10^{-5} and 3×10^{-5} einstein, i.e., whose 2.4 and 2.9 tryptophyl residues have been modified, respectively (Figure 7a). Therefore it appears that gp 32 molecules with photochemically modified tryptophyl residues have lost their ability to bind to single-stranded poly(rA).

We have also investigated the binding of photochemically modified gp 32 to single-stranded DNA using binding assays on nitrocellulose filters (Toulmé, 1983). Native gp 32 is able to promote the retention of DNA: the titration curve of 0.03 μg of heat-denatured *E. coli* DNA by gp 32 shows that a plateau is reached with 0.3 μg of protein (Figure 7b). Under similar conditions, the amount of DNA retained on filters decreased with the extent of protein modification: only 25% of the DNA trapped by native gp 32 remained bound to 0.3 μg of gp 32 which was irradiated with a dose of 1.2×10^{-5} einstein, i.e., having lost 2.1 tryptophyl residues on the average. A sample irradiated with a dose of 4.2×10^{-5} einstein is not able any more to retain DNA on the filters. However, in this last case, a conformational change of the protein was detected (see below). From these results, it appears that photochemical modification of about two tryptophyl residues strongly reduces the affinity of gp 32 for nucleic acids.

Binding of gp 32 to heat-denatured DNA leads to a quenching of about 35% of its fluorescence emission (Figure 8). The intercept of the straight line obtained at low DNA concentration with the limit reached for saturation of the protein allows us to determine the binding stoichiometry. The value of about 6 ± 1 nucleotides per protein molecule calculated from the curve in Figure 8 is in good agreement with the value obtained from absorption experiments (Figure 7a). As the photochemically modified protein has a strongly reduced fluorescence quantum yield, the following competition experiment was carried out: an equimolar mixture of native protein and of molecules in which an average of 2.5 tryptophyl residues have been photochemically modified was titrated by heat-denatured DNA. As the fluorescence emission is essentially due to native protein, an apparent size of the binding site of about 12 nucleotides would be expected if irradiated protein molecules bound to DNA with the same affinity as native ones. As shown in Figure 8, the titration curves of the native protein and of the mixture of native and modified proteins are superimposed. This means that the irradiated molecules do not compete with native ones and therefore have

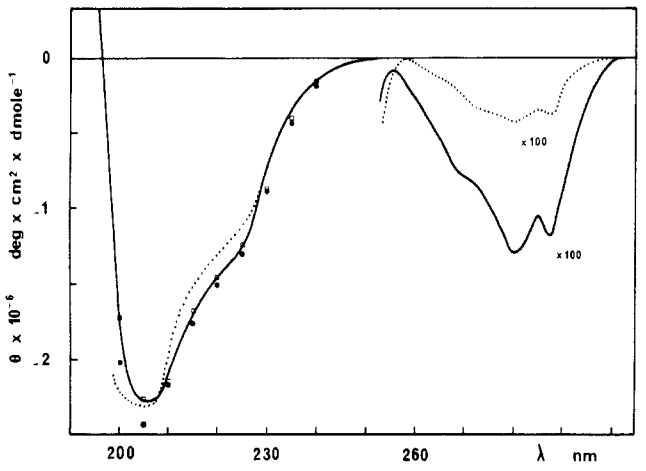

FIGURE 9: Circular dichroism spectra of native gp 32 (—) and of gp 32 which has been irradiated in the presence of 0.1 M trichloroethanol with a dose of 1.5×10^{-5} (□), 2.4×10^{-5} (●), and 3.5×10^{-5} einstein (···).

a strongly reduced affinity for heat-denatured DNA. A similar conclusion was drawn from a competition experiment performed with native DNA (Figure 8).

The structural integrity of the photochemically modified protein was checked by gel electrophoresis and circular dichroism. No chain cleavage was detected by SDS–polyacrylamide gel electrophoresis even with gp 32 irradiated in the presence of 0.1 M trichloroethanol with a dose of 10^{-4} einstein. As expected, the circular dichroism spectra of irradiated samples exhibit an important modification in the aromatic band of the protein (Figure 9). In constrast, no significant change of the peptidic band was detected for irradiated samples which have lost less than 2.3 tryptophyl residues on the average, i.e., irradiated with a dose lower than 2.4×10^{-5} einstein under our conditions (Figure 9). A small change was observed for a sample irradiated with a dose of 4.2×10^{-5} einstein. Analysis of this spectrum according to the method of Chen et al. (1974) gave 19% α-helix and 20% β-structure for this irradiated sample. The corresponding values for native gp 32 are 20% and 22%, respectively. Moreover, it must be pointed out that the position of the fluorescence emission maximum was not shifted during the photochemical reaction. This wavelength is characteristic of the tryptophyl environment and can shift from 330 to 360 nm when the indole ring is in a hydrophobic or a polar medium, respectively. Therefore, UV irradiation in the presence of trichloroethanol does not markedly modify the gp 32 conformation.

Conclusions

The results reported above show that UV irradiation of gp 32 in the presence of trichloroethanol results in the modification of three out of the five tryptophyl residues of the protein. Our results demonstrate that unmodified residues are protected from photochemical modification by singlet–singlet energy transfer to tryptophan–trichloroethanol photoproducts. This did not allow us to determine the number of fluorescent tryptophyl residues in gp 32. However, energy transfer from nonmodified tryptophans to photoproduct(s) involves residues with a long enough lifetime; otherwise they would be non radiatively deactivated before transferring their excitation energy (except in the unlikely case where intact and modified residues are in close contact). The three out of five tryptophyl residues which can be photochemically modified must have a long enough lifetime in the first excited singlet state to encounter a trichloroethanol molecule and react with it. Therefore, it is very likely that the three modified and the unmodified residue(s) which transfer to the photoproduct are long-lived fluorescent residues. At most, one tryptophyl residue could be nonfluorescent.

Our experiments demonstrate that photochemical modification of gp 32 in the presence of trichloroethanol leads to an inactive protein for binding to nucleic acids and polynucleotides. Photodestruction of two out of the five tryptophyl residues yields a protein which is neither able to induce hyperchromism of poly(rA) nor able to retain heat-denatured DNA on nitrocellulose filters. This points to the involvement of a tryptophyl ring in the binding of gp 32 to DNA even though one cannot exclude local conformational change (which would not result in a modified CD spectrum) as being responsible for the loss of binding capacity upon tryptophan modification. Previous results obtained with a fluorescent polynucleotide, poly(1,N^6-ethenoadenylic acid), allowed us to conclude that one tryptophyl residue must be located in close vicinity to nucleic bases in gp 32–polynucleotide complexes (Toulmé & Hélène, 1980). The results obtained with radiation chemical experiments (Casas-Finet et al., 1984) and with mercury-substituted polyuracil (Le Doan et al., 1984) suggest that this crucial tryptophyl residue could be involved in stacking interactions with nucleic acid bases as suggested by studies with model peptides (Toulmé & Hélène, 1977; Hélène et al., 1979).

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Registry No. NATA, 2382-79-8; tryptophan, 73-22-3; trichloroethanol, 115-20-8.

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