Role of Tryptophan and Cysteine in the Binding of Gene 32 Protein from Phage T4 to Single-Stranded DNA. Modification of Crucial Residues by Oxidation with Selective Free-Radical Anions†

José Ramon Casas-Finet, Jean-Jacques Toulmé,* Christian Cazenave, and René Santus

ABSTRACT: Cysteinyl and tryptophyl residues of a single-stranded binding protein, the gene 32 protein (gp 32) of phage T4, can be selectively oxidized at pH 6.2 by $I_2^{-}\cdot$, $Br_2^{-}\cdot$, and $SCN_2^{-}\cdot$ radical anions produced by steady-state γ-radiolysis. Gene 32 protein was found to be very sensitive to radical attack, and thus low irradiation doses were required for inhibition of its ability to bind to nucleic acids. The $I_2^{-}\cdot$ radicals react with two cysteines susceptible to 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) attack. They also oxidize one cysteine which is inaccessible to the reaction with DTNB. The cysteine oxidation produces a partial inhibition of the biological activity of gp 32. When $I_2^{-}\cdot$ radicals react with gp 32 denatured DNA complexes, only one exposed SH group and one buried SH group are available for the oxidation. At low doses with $Br_2^{-}\cdot$ or $SCN_2^{-}\cdot$ as oxidizing species, two tryptophyl residues are readily oxidized in addition to three SH groups, leading to a complete inhibition of the DNA-binding properties of gp 32. In the complex formed with denatured DNA, these two tryptophans are fully protected while one exposed cysteine residue and one buried cysteine residue are oxidized by $SCN_2^{-}\cdot$. It is concluded that the two exposed tryptophans are essential for the activity of gp 32 most probably by stabilizing the complex via stacking interactions with the bases of single-stranded DNA.

As indicated in Toulmé et al. (1984), the gene 32 protein (gp 32)[1] plays an important role as a helix-destabilizing protein; it is involved in several steps of phage T4 DNA metabolism. Aromatic amino acids are known to form stacked complexes with nucleic acids (Montenay-Garestier & Hélène, 1968, 1971). These stacking interactions provide oligopeptides with a specificity for binding to single-stranded nucleic acids (Brun et al., 1975; Mayer et al., 1979). Consequently, it may be thought that some of the aromatic amino acids (eight Tyr and five Trp) contained in gp 32 may interact with the DNA bases and stabilize the association between the protein and the DNA. The contribution of tryptophyl residues in this binding has already been suggested by several methods (Hélène et al., 1976; Toulmé & Hélène, 1980; Toulmé et al., 1984; Le Doan et al., 1984).

Some time ago, Adams and co-workers showed that the radiolysis technique could be used as a tool to identify key residues present in the active site or capable of maintaining the three-dimensional structure of enzymes. This technique involves the specific reaction of a few residues (Cys, Trp, His, Met, and Tyr) with inorganic radical anions such as $Br_2^{-}\cdot$, $SCN_2^{-}\cdot$, $I_2^{-}\cdot$ formed during γ-radiolysis of an aqueous solution of the corresponding salt under suitable experimental conditions (Adams et al., 1972; Adams, 1972). We used this technique to modify cysteinyl and tryptophyl residues of gp 32 either free or bound to heat-denatured DNA.

Materials and Methods

Materials

Details regarding the gene 32 protein preparation and purification have been given in Toulmé et al. (1984). Fluorescent etheno-DNA (ε-DNA) was prepared by incubating heat-denatured DNA with chloroacetaldehyde at 37 °C for 48 h (Kohwi-Shigematsu et al., 1978). All other chemicals of the purest commercially available grade were used as received.

Methods

Spectrophotometry. Absorbance, circular dichroism, and fluorescence measurements have already been described (Toulmé et al., 1984).

γ-Irradiations were performed with a ^{60}Co γ source (Institut du Radium, Paris) at a dose of 82 Gy min^{-1}. Samples (1 or 0.2 mL) containing 3.5 μM gp 32 and 10 mM KBr (or KSCN or KI) in 1 mM phosphate buffer, pH 6.2, in septum-capped vials, were saturated with N_2O prior to irradiation by maintaining a stream of N_2O just above the meniscus for 15 min. This procedure avoided protein denaturation. Up to four samples could be irradiated at the same time.

Analysis of Tryptophan and Cysteine Content. The tryptophan content of irradiated or unirradiated gp 32 was measured by using a fluorometric method (Sasaki et al., 1975). However, this method consistently leads to an underestimate of the number of tryptophyl residues (4.3 instead of 5) per gp 32 molecule (Toulmé et al., 1984). Cysteinyl residues were determined by using Ellman's method (1959). Upon reaction with SH groups, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) forms the yellow 2-nitro-5-thiobenzoate anion having a molar extinction coefficient of 13 600 M^{-1} cm^{-1} at 412 nm. The reactivity of the free SH groups in the protein depends on steric factors. Consequently, the change in the 412-nm absorbance as a function of time after addition of DTNB makes it possible to distinguish SH groups of various accessibility in the protein. The Ellman reaction was performed at pH 8.0 in a 10 mM phosphate buffer containing 1 mM EDTA and 1 mM DTNB with or without 8 M urea.

† From the Laboratoire de Biophysique, INSERM U.201 and ERA 951 du CNRS (Photobiologie Moléculaire), Muséum National d'Histoire Naturelle, 75005 Paris, France (J.R.C.-F., J.-J.T., and C.C.), and the Laboratoire de Physico-Chimie de l'Adaptation Biologique, ERA 951 du CNRS (Photobiologie Moléculaire), Muséum National d'Histoire Naturelle, 75231 Paris Cedex 05, France (R.S.). *Received August 2, 1983.* This work has been supported by INSERM (Grants ATP 77-79-109 and CRL 79-403-32), by DGRST (Grant 81 E 12 26), and by grants from the Ligue Nationale contre le Cancer and the Fondation pour la Recherche Médicale.

[1] Abbreviations: gp 32, gene 32 protein; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CD, circular dichroism; Trp, tryptophan; Tyr, tyrosine; Cys, cysteine; ε-DNA, DNA modified with chloroacetaldehyde; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; poly(HgU), poly(5-mercuriuridylic acid).

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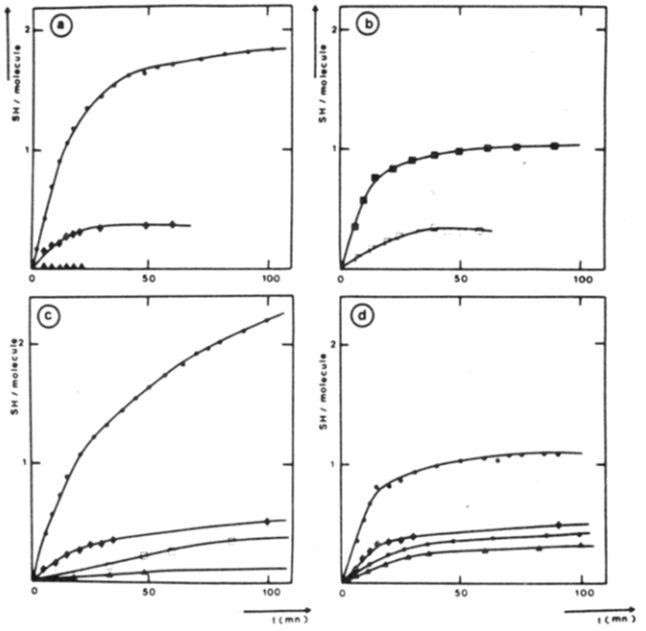


FIGURE 1: (a) Titration of cysteinyl residues of gp 32 by Ellman's method after reaction with $I_2^{-}\cdot$ radical anions formed upon γ-radiolysis of N_2O-saturated buffered aqueous solutions (pH 6.2, 1 mM phosphate buffer) containing 3.5 μM gp 32 and 10 mM KI: (●) no irradiation; (♦) after γ-irradiation (dose 42 Gy); (▲) after γ-irradiation (dose 84 Gy). (b) Same as (a) except the solution contained 75 μM denatured DNA: (■) before γ-irradiation; (□) after γ-irradiation (42 Gy). (c) Same as (a) except KI is replaced by KSCN: (●) before irradiation; (♦) 42 Gy; (□) 84 Gy; (▲) 380 Gy. (d) Same as (c) except the solution contained 75 μM denatured DNA: (●) before γ-irradiation; (♦) 42 Gy; (○) 84 Gy; (▲) 380 Gy.

Results and Discussion

Role of Cysteine Residues in the Association of gp 32 with Denatured DNA. The titration of the accessible thiols by DTNB in native gp 32 shows that there is a very reactive (and thus very accessible) SH group (Figure 1). A second one can be titrated on a longer time scale. It should be noted that two SH groups are found accessible both by Ellman's method and by reaction with *N*-ethylmaleimide (Le Doan et al., 1984). When the protein is bound to denatured DNA, only one SH group is accessible. Furthermore, the titration performed on the denatured protein leads to four cysteines per molecule. The primary structure shows that three of them are found in a region which is probably involved in the binding to DNA (Williams et al., 1980). It is therefore possible that some of these residues are involved in the stabilization of the gp 32–single-stranded DNA complex. Since $I_2^{-}\cdot$ and $O_2^{-}\cdot$ react very selectively with SH groups (Adams et al., 1972; Armstrong & Buchanan, 1978), we have looked for the consequence of the attack of these radicals on the gp 32 binding affinity.

(*A*) *Reaction of $I_2^{-}\cdot$ Radical Anions with gp 32 in N_2O-Saturated Solutions.* Formation of the oxidizing $I_2^{-}\cdot$ radical anions in N_2O-saturated solutions of gp 32 results from the following reaction sequence:

$$H_2O \xrightarrow{\gamma\text{-radiolysis}} \underbrace{e^-_{aq} + OH\cdot + H_2O_2, H\cdot, H_3O^+, \ldots}_{\text{major species}} \quad (1a)$$

$$N_2O + e^-_{aq} \xrightarrow{H^+} N_2 + OH\cdot \quad (1b)$$

$$OH\cdot + I^- \rightarrow I\cdot + OH^- \quad (1c)$$

$$I\cdot + I^- \rightleftarrows I_2^{-}\cdot \quad (1d)$$

$I_2^{-}\cdot$ radical anions are thus produced with a radiolytic yield $G = 6.1$ when the iodide concentration is 10 mM.

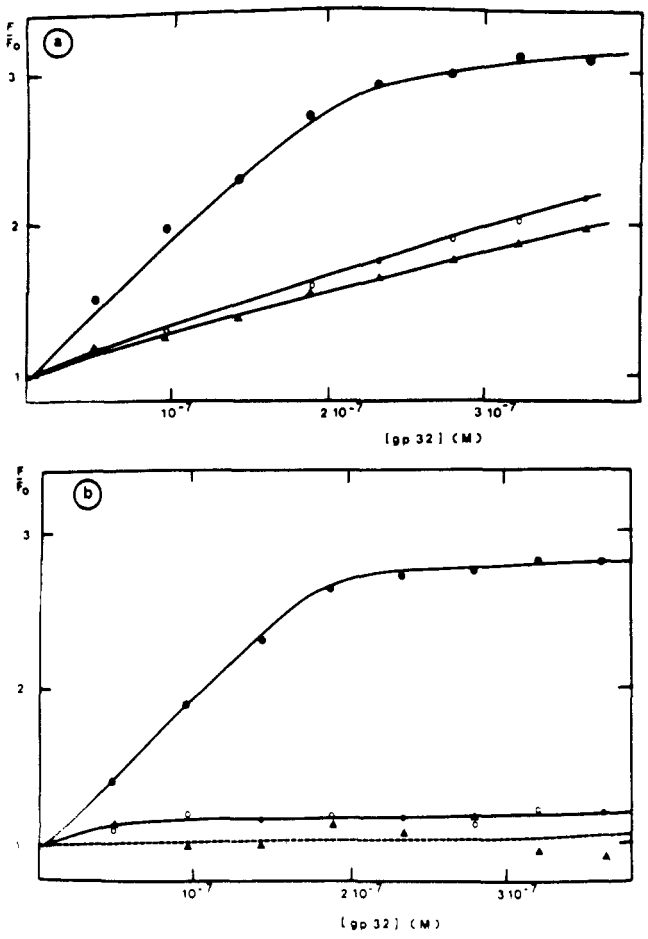
Table I: Number of Cysteinyl Residues Measured by Ellman's Method in the Presence or Absence of 8 M Urea Using $I_2^{-}\cdot$ or $SCN_2^{-}\cdot$ as the Oxidizing Species[a]

conditions	no. of reactive cysteines per protein molecule: unirradiated sample	no. of reactive cysteines per protein molecule: after γ-irradiation at 42 Gy
gp 32 (IK) (urea)	3.9	1.1
gp 32 (IK)	2	0.4
gp 32 + DNA (IK)	1	0.3
gp 32 (KSCN) (urea)	4.1	0.4
gp 32 + DNA (KSCN) (urea)	3.8	1.6
gp 32 (KSCN)	2.5	0.5
gp 32 + DNA (KSCN)	1	0.5

[a] The protein is either free (gp 32) or bound to heat-denatured DNA (gp 32 + DNA). The dose (42 Gy) has been chosen in order to double the slope (partial inhibition) of the F/F_0 vs. DNA concentration plot (see Figure 3) upon oxidation with $I_2^{-}\cdot$ (IK) and to totally inhibit the protein (see Figure 2b) upon reaction with $SCN_2^{-}\cdot$ (KSCN). All solutions were N_2O saturated before irradiation. The error in the determination of the number of cysteinyl residues can be estimated to be ±0.5 because of the low protein concentration (0.8 μM) used for Ellman's reaction.

As can be seen from Figure 1a, the two exposed cysteines of gp 32 that can be titrated with DTNB in the presence of I^- ions are rapidly destroyed upon reaction with $I_2^{-}\cdot$. Results reported in Table I suggest, by comparing the SH loss measured in the presence to that in the absence of 8 M urea, that in addition one "buried" SH is also oxidized. The initial G value for the SH loss is 2.4. Tryptophyl residues can also react with $I_2^{-}\cdot$ radicals (Adams et al., 1972), although the bimolecular reaction rate constant for tryptophan oxidation is 2 orders of magnitude smaller than with cysteine. In the present study, analysis of tryptophyl residues after irradiation shows no tryptophan loss. However, the fluorescence of gp 32 is decreased by 20% after irradiation with a dose corresponding to the loss of two exposed and one buried cysteinyl residues. This result suggests that a conformational change occurs upon oxidation of cysteinyl residues accessible to $I_2^{-}\cdot$ attack provided one can exclude the iodination of the SH group after reaction with $I_2^{-}\cdot$ and possible subsequent reactions (Bonifacic & Asmus, 1980). As a matter of fact, the presence of cysteinyl residues in the vicinity of tryptophyl residues evidenced by heavy atom effects in the mercurated protein (Le Doan et al., 1984) makes it possible that an iodinated SH group may quench the gp 32 fluorescence via a heavy atom effect due to the iodine atom. We will see below that this hypothesis is rather unlikely.

(*B*) *Effect of the $I_2^{-}\cdot$ Radical Attack on the Binding Capacity of gp 32.* As shown in the previous papers (Toulmé et al., 1984; Le Doan et al., 1984), the DNA-binding activity of gp 32 can be determined by measuring the fluorescence of tryptophyl residues, which is quenched upon binding to single-stranded DNA. The gp 32 binding capacity can also be probed by using a modified DNA (ϵ-DNA) in which adenine bases have been made fluorescent upon $NH_2(6)$ and N(1) bridging with an etheno group (Kohwi-Shigematsu et al., 1978). The binding of gp 32 to ϵ-DNA results in an increased ϵ-DNA fluorescence due to bases unstacking. From fluorescence titration curves, ϵ-DNA has the same binding properties (i.e., the same apparent binding constant and size of the binding site) as denatured DNA with respect to the association with gp 32. As shown in Figure 2a, the modification of cysteinyl residues alters the association of gp 32 with ϵ-DNA.



GURE 2: (a) Fluorescence enhancement ratio (F/F_0) at 4 °C of 1 μM ϵ-DNA upon addition of gp 32 in a pH 7.6 10 mM phosphate buffer containing 0.1 M NaCl. The ϵ-DNA fluorescence emission was recorded at 425 nm upon excitation at 310 nm: (●) unirradiated gp 32; (○) γ-irradiated gp 32 in the presence of 10 mM KI (dose 42 Gy); (▲) γ-irradiated gp 32 in the presence of 10 mM KI (dose 84 Gy). (b) Same as (a) except the γ-irradiation was performed in the presence of 10 mM KSCN. All irradiated solutions were N_2O saturated.

Therefore, the use of ϵ-DNA provides an alternative method for measuring the affinity of gp 32 for nucleic acids.

The gradual decrease in the binding capacity as a function of the dose (data not shown) suggests that the fourth SH group is oxidized at a higher dose, and this reaction further destabilizes gp 32. This is confirmed by the titration of SH groups by DTNB [in denaturing conditions (8 M urea)] performed on a sample irradiated with 450 Gy. It cannot be excluded, of course, that some SH oxidation by I_2 occurs on a long-time scale because of the following reaction in γ-irradiated iodide solutions:

$$2I_2^{-}\cdot \rightleftarrows 2I^- + I_2 \qquad (2)$$

Reaction 2 may thus explain part of the subsequent loss of SH groups by oxidation with iodine after long irradiation times (data not shown). In addition, it must be noted that the SH titration could not be performed immediately after γ-irradiation because of the ^{60}Co γ-source location, this fact favoring oxidation by iodine. This reaction has also been suggested to account for the inactivation of lactate dehydrogenase (Buchanan & Armstrong, 1976).

The primary structure is unchanged by γ-radiolysis; i.e., no smaller gp 32 fragments are formed upon oxidation of the cysteine residues as shown by electrophoresis on a polyacrylamide–SDS gel. As reported, modification of the two exposed cysteinyl residues does not lead to any change in the

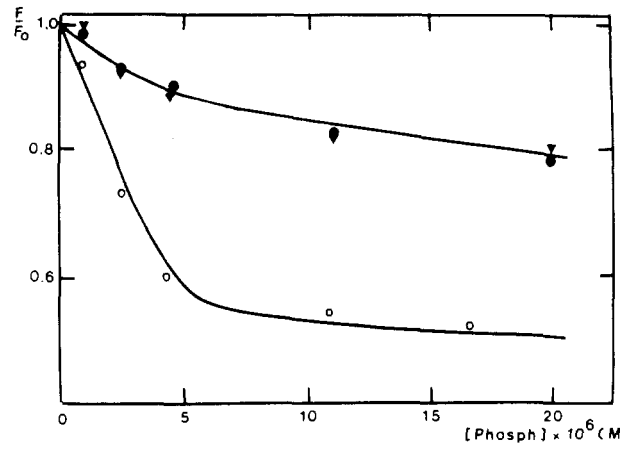


FIGURE 3: Fluorometric titration at 4 °C of 0.5 μM gp 32 by denatured DNA. The protein was irradiated with (○) 0, (●) 31, and (▼) 540 Gy in air-saturated solutions containing 5×10^{-2} M KI and 1 μM gp 32. The fluorescence was excited at 282 nm and read at 340 nm.

CD spectrum (Le Doan et al., 1984). However, as suggested by the decrease in the fluorescence of gp 32, a local conformational change in the vicinity of the attacked cysteinyl residues cannot be excluded. Moreover, longer irradiation times leading to the modification of more cysteinyl residues induce a change of the CD spectrum. Consequently, the present data suggest that the oxidation of buried cysteines leads to structural changes. These residues could be involved in some structure crucial for maintaining the activity of the gp 32 molecule.

(C) Reaction of $I_2^{-}\cdot$ with gp 32 in Air-Saturated Solutions. In air-saturated solutions, hydrated electrons (e^-_{aq}) and H· atoms formed by water radiolysis are rapidly scavenged by O_2, yielding the superoxide radical anion $O_2^{-}\cdot$. This radical does not react with gp 32 as shown by the absence of SH loss during irradiation with large γ doses (600 Gy) of an air-saturated solution of gp 32 (1 μM) containing 10 mM potassium formate which converts OH· radicals into $O_2^{-}\cdot$ radicals according to reaction 3 (Draganic & Draganic, 1971):

$$HCOO^- + OH\cdot \rightarrow CO_2^{-}\cdot + H_2O \qquad (3a)$$

$$CO_2^{-}\cdot + O_2 \rightarrow O_2^{-}\cdot + CO_2 \qquad (3b)$$

In addition, no loss of the DNA-binding capacity of gp 32 can be observed under the same irradiation conditions. This result indirectly suggests that hydrogen peroxide formed upon γ-radiolysis does not significantly alter the protein.

The absence of reactivity of $O_2^{-}\cdot$ with the protein is in keeping with the low reaction rate constant of $O_2^{-}\cdot$ with cysteine (Armstrong & Buchanan, 1978). Consequently, large irradiation doses are required for appreciable oxidation of the SH groups by $O_2^{-}\cdot$. However, γ-irradiation of air-saturated solutions of 1 μM gp 32 in the presence of I^- rapidly and totally inhibits the binding capacity of gp 32 to denatured DNA (Figure 3). Under these conditions, there are only two radical species formed in the solution, namely, $I_2^{-}\cdot$ and $O_2^{-}\cdot$. These observations must be compared to those obtained under N_2O saturation where SH oxidation by $I_2^{-}\cdot$ radical ions produced in a higher yield [$G(I_2^{-}\cdot) = 6.1$ (compared to 3.1 in air-saturated solutions] leads to a progressive loss of activity. It is likely that in aerated solutions the thiyl radical formed upon γ-radiolysis is readily oxidized, leading to sulfone or sulfine derivatives (Barton & Packer, 1970) whose interaction with neighboring residues "amplifies" the destabilization of gp 32 as already observed in the absence of oxygen. This hypothesis is consistent with the buried Cys residue integrity being es-

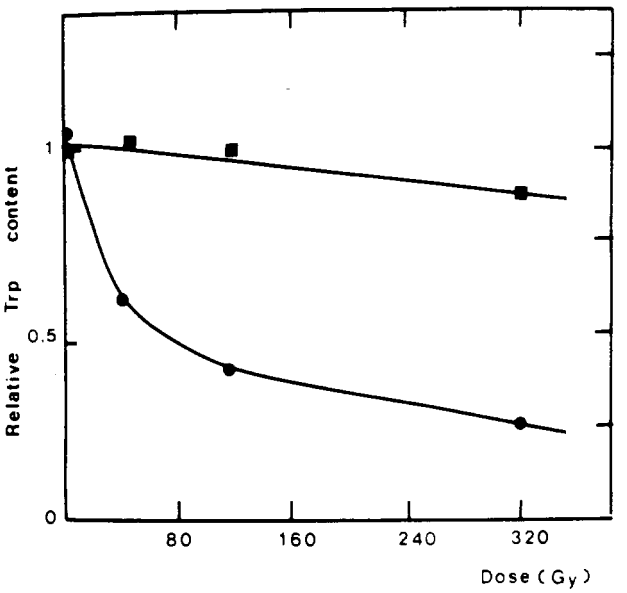


FIGURE 4: Variation of the relative content of intact Trp residues per protein molecule of gp 32 free (●) or bound to 70 μM denatured DNA (■) as a result of $SCN_2^{-}\cdot$ attack. Solutions were saturated with N_2O before γ-radiolysis and contained 10 mM KSCN, 3.5 μM gp 32, and 1 mM phosphate buffer (pH 6.2).

sential for maintaining the gp 32 structure. In addition, the rapid reaction of oxygen with the thiyl radical supports the view that iodination of the SH group does not compete with oxidation as emphasized above.

(D) Reaction of $I_2^{-}\cdot$ Radical Anions with the gp 32–Denatured DNA Complex. γ-Radiolysis of 3.5 μM gp 32 in N_2O-saturated pH 6.2 phosphate buffer (1 mM) containing 10 mM $I_2^{-}\cdot$ and 70 μM denatured DNA does not modify the fluorescence of gp 32. Under these conditions, 99% of the gp 32 molecules are bound to DNA. Consequently, one may suggest that the reactivity of the fluorescent Trp residues toward $I_2^{-}\cdot$ oxidation is not increased as a result of the association.

In agreement with the titration of only one exposed SH group in gp 32 complexed to denatured DNA, the rate of cysteine loss is halved upon reaction of the complex with $I_2^{-}\cdot$ radical anions as compared to that with free gp 32 (see Figure 1 and Table I). It must be noted that $I_2^{-}\cdot$ radicals do not react with DNA (Adams, 1972).

Role of Tryptophyl Residues in the Association of gp 32 with Denatured DNA. In addition to the four cysteinyl residues, gp 32 possesses five tryptophan, eight tyrosine, and four histidine residues which can be oxidized by $X_2^{-}\cdot$ radical anions ($X^- = Br^-, SCN^-$) formed in the radiolysed solution by the reactions given in eq 1 (Williams et al., 1980). The selectivity of the $X_2^{-}\cdot$ attack can be further improved by adjusting the pH conditions. Thus, in our experimental conditions (pH 6.2), the $SCN_2^{-}\cdot$ and $Br_2^{-}\cdot$ radical anions should almost exclusively react with Trp and Cys residues (Adams et al., 1972).

(A) Reaction of $Br_2^{-}\cdot$ and $SCN_2^{-}\cdot$ with gp 32. The reaction of $Br_2^{-}\cdot$ or $SCN_2^{-}\cdot$ with 1 μM gp 32 in N_2O-saturated solutions leads to a rapid loss of two tryptophyl residues as measured by the Sasaki method (1975). Further irradiation provokes the modification of one more tryptophyl residue (Figure 4). This destruction of tryptophan is accompanied by a decrease in the fluorescence of gp 32. Under denaturing conditions (8 M urea), the fluorescence loss is nearly independent of the radical anion used as the oxidizing species (Figure 5a). We assume that denaturation with 8 M urea leads to the same coil structure of gp 32 whatever the γ-ray dose delivered to the solution. The oxidation of gp 32 by $X_2^{-}\cdot$ radicals is accompanied by the formation of a fluorescent radioproduct (Figure 5b) which absorbs and emits at longer wavelengths than tryptophan. As indicated above, under our experimental conditions, $X_2^{-}\cdot$ radical anions also react with SH groups. As a consequence, the oxidation of three cysteines parallels the tryptophan loss (Figure 1 and Table I). The loss of tryptophyl residues and the formation of the radioproduct make it difficult to correctly monitor the activity of gp 32 by measuring the fluorescence quenching of tryptophan residues upon DNA addition. However, as already shown above, the affinity of gp 32 for polynucleotides can be conveniently checked by using the fluorescent ε-DNA. As shown in Figure 2b, the destruction of two tryptophyl and three cysteinyl residues is sufficient to inhibit the binding while it is only partially affected by the oxidation of the cysteinyl residues. This result agrees with filter binding assays. It was previously shown that gp 32 whose tryptophyl residues have been oxidized by $SCN_2^{-}\cdot$ radicals had a reduced ability to retain DNA on nitrocellulose filters as compared to native gp 32 (Toulmé, 1984). It can thus be inferred that these tryptophyl residues play a critical role in

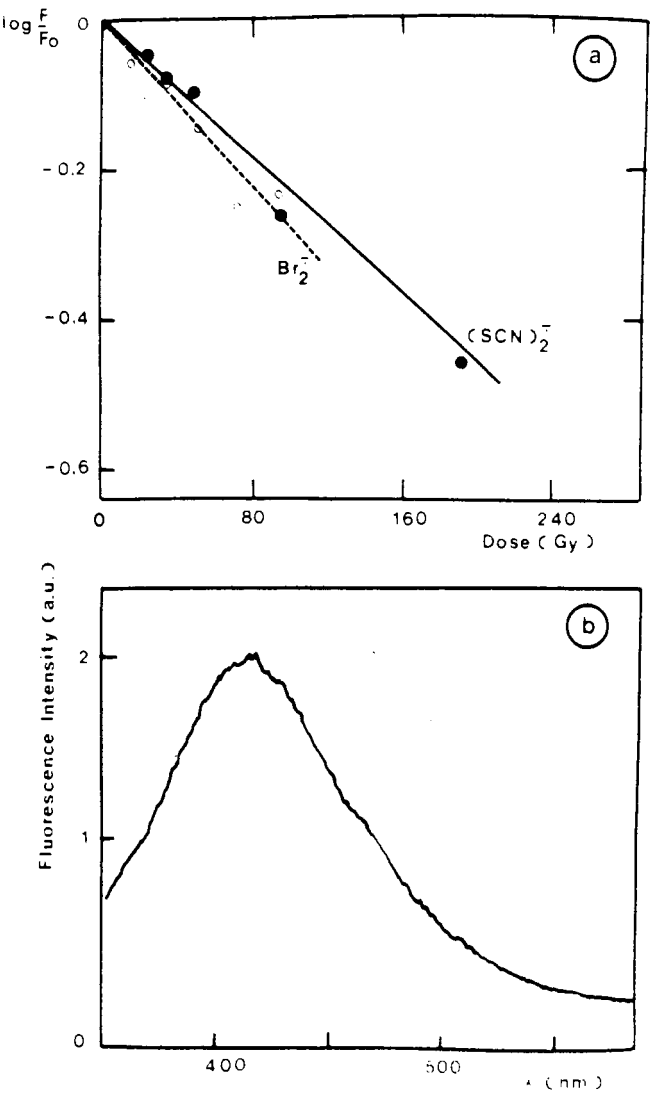


FIGURE 5: (a) Relative fluorescence loss (F/F_0) of gp 32 as a function of the irradiation dose in the presence of 10 mM Br^- (○) or 10 mM SCN^- (●). The concentration of gp 32 used for the fluorescence measurement was 0.5 μM. The protein was denatured with 8 M urea in a pH 7.0 phosphate buffer at 20 °C. The fluorescence was excited at 282 nm and measured at 355 nm. (b) Fluorescence emission spectrum of the radioproduct formed upon reaction of the Trp residues of gp 32 with $SCN_2^{-}\cdot$ in pH 7.0, 10 mM phosphate buffer. The excitation was set at 315 nm. The gp 32 solution (pH 6.2) has been irradiated with 480 Gy and contained 10 mM KSCN.

complex formation with heat-denatured DNA. Therefore, with respect to results obtained upon oxidation of cysteinyl residues by $I_2^{-}\cdot$, it may be concluded that both residues are crucial for gp 32–ϵ-DNA association.

It should be noted that the present data indicate that the reacting cysteinyl and tryptophyl residues have practically the same reactivity with respect to $SCN_2^{-}\cdot$. This is somewhat surprising when compared to their reactivity as free amino acids. Thus, at pH 6.2, the reaction rate constant for the oxidation of tryptophan by $SCN_2^{-}\cdot$ is an order of magnitude higher than that observed with cysteine (Adams, 1972). The observed results suggest that these residues are located in a cluster of basic residues whose electrostatic attraction increases the local concentration of $X_2^{-}\cdot$ radical anions. The effect of the gp 32 conformation on the selectivity of the $X_2^{-}\cdot$ radical attack is further illustrated by pulse radiolysis data obtained with native and denatured gp 32 reacting with $Br_2^{-}\cdot$. Thus, in the native protein, about half of the $Br_2^{-}\cdot$ radical anions react with Trp and Tyr residues, the rest reacting with His and Cys residues. On the other hand, the $Br_2^{-}\cdot$ radicals react almost exclusively with Trp and Tyr residues in the denatured protein or at high ionic strength (1 M KBr), which induces a reversible conformational change of gp 32 (Casas-Finet et al., 1984).

(B) Reactions of $SCN_2^{-}\cdot$ with the gp 32–Denatured DNA Complexes. The reactivity of $X_2^{-}\cdot$ radical anions toward gp 32 bound to DNA has been studied by using N_2O-saturated solutions containing 3.5 μM gp 32, 70 μM denatured DNA, and 10^{-2} M KBr or KSCN at pH 6.2 (1 mM phosphate buffer). Under these conditions, tryptophan oxidation by $X_2^{-}\cdot$ radical anions is notably reduced, with only one residue being oxidized with a slow rate analogous to the one observed with the slow-reacting residue in the absence of DNA (Figure 4). We also note the formation of the same radioproduct as that obtained with the free protein (Figure 5b). Dissociation of the irradiated complex induces an increase in the radioproduct fluorescence, suggesting its involvement in some interaction with either DNA or adjacent protein molecules bound to the same DNA strand. One accessible SH group and one buried SH group are still oxidized as shown from data in Figure 1 and Table I. Furthermore, the irradiated complex can be dissociated as demonstrated by the increase in the fluorescence intensity of gp 32 upon addition of 1 M NaCl. This result indicates that no covalent binding of the protein to the DNA occurs upon oxidation of the SH group and that the irradiated complex still has some binding capacity. Therefore, it can be concluded that the two cysteines oxidized are not essential for binding although their oxidation may explain some loss of affinity of gp 32 for DNA. It may be suggested that the Trp and Cys protected from $SCN_2^{-}\cdot$ attack by association with the DNA play a role in the stabilization of the complex.

The protection of two tryptophyl residues and one cysteinyl residue against the radical attack brought about by the association of the protein to the DNA may be due to two effects: first, a repulsive electrostatic interaction between the negatively charged $X_2^{-}\cdot$ radical and the negatively charged phosphate backbone of DNA; second, the steric hindrance exerted by the DNA on the accessibility of these tryptophyl and cysteinyl residues. The fact that these residues are protected from $SCN_2^{-}\cdot$ attack suggests that they are in close contact with the DNA. In support of this hypothesis, it has been shown that one cysteine was in the vicinity of the polynucleotide in the gp 32–polynucleotide complex and was close to a tryptophyl residue (Le Doan et al., 1984). These observations complement those obtained with the free protein where we showed that integrity of the exposed Cys and Trp residues was necessary to achieve strong binding to the DNA.

Conclusions

The oxidation of cysteinyl and tryptophyl residues by $X_2^{-}\cdot$ radical anions shows that the integrity of several of these residues is crucial for the activity of gp 32. Our study suggests that at least one buried cysteinyl residue could maintain some crucial structure of the protein. One cysteinyl residue is protected from the radical attack upon complexation with DNA, suggesting that it could be located in the binding site of gp 32. This idea is supported by the fact that binding of gp 32 to poly(HgU) results in the formation of protein–polynucleotide adducts (Le Doan et al., 1984). Binding of gp 32 to nucleic acids involves electrostatic interactions between basic amino acids and phosphate groups (Kowalczykowski et al., 1981). This also accounts for the unexpected modification of cysteinyl residues by $SCN_2^{-}\cdot$ radicals in the free gp 32 because the presence of basic amino acids in the vicinity of cysteine would increase the oxidation reaction rate constant and, therefore, the oxidation efficiency.

Similarly, the protection of two tryptophyl residues of gp 32 upon binding to DNA is in favor of their location in regions involved in binding. However, due to the cooperative mode of binding of gp 32 to heat-denatured DNA, we cannot exclude that protection could be due to interactions with adjacent protein molecules. Pulse radiolysis experiments also suggest that one tryptophyl residue is in the vicinity of nucleic acid bases (Casas-Finet et al., 1984). The heavy atom effect observed with gp 32–poly(5-mercuriuracil) complexes strongly supports the idea that at least one tryptophyl residue is located in the binding site of the protein (Le Doan et al., 1984).

The decrease in the affinity of DNA for gp 32 when two tryptophyl and three cysteinyl residues have been modified suggests that these residues could play a role in the binding process. The fact that gp 32 whose only cysteinyl residues have been oxidized is still able to bind to DNA (although with a reduced affinity) and the results obtained with gp 32 UV irradiated in the presence of trichloroethanol (Toulmé et al., 1984) point to the involvement of tryptophyl residue(s) in some crucial interaction with nucleic acid constituents. The type of interaction in which the indole ring could be involved is not yet elucidated. Model studies have shown that tryptophan derivatives can form stacked complexes with nucleic acid bases [Montenay-Garestier & Hélène, 1968, 1971; Brun et al., 1975; see Hélène & Maurizot (1981) for a review]. Such interactions in gp 32–nucleic acid complexes account for several results. The binding of gp 32 to polynucleotides results in a quenching of the protein fluorescence. Fluorescence quenching of a partially proteolyzed gp 32 upon binding to nucleic acids is identical with that of native gp 32 even though binding cooperativity is lost. Therefore, fluorescence quenching is not due to protein–protein interactions (Spicer et al., 1979). Stacking is the main mechanism which contributes to fluorescence quenching of tryptophan in protein–nucleic acid complexes (Hélène et al., 1982). The formation of stacked complexes requires an increase in the base–base distance. Electron micrographs of gp 32–fd DNA complexes have shown a lengthening of the DNA chain from 3.4 to 5.3 Å per base (Delius et al., 1972; Coleman & Oakley, 1980). It was also demonstrated that pyrimidine dimers are cleaved upon UV irradiation of UV-damaged DNA–gp 32 (Hélène et al., 1976). Stacking interactions between tryptophan and nucleic acid bases can also account for this reaction (Toulmé et al., 1974).

Stacking interactions between nucleic acid bases and aromatic amino acids (tyrosine and phenylalanine) have been demonstrated in the case of another single-strand binding

protein, the gene 5 protein from phage fd (Coleman & Oakley, 1980). The primary sequence of gp 32 shows that the region between positions 72 and 144 contains three tryptophyl and seven tyrosyl residues (Williams et al., 1981). This domain probably contains the DNA-binding site required for DNA replication and recombination (Breschkin & Mosig, 1977). In addition to the results presented here and in the accompanying papers (Toulmé et al., 1984; Le Doan et al., 1984), Anderson & Coleman (1975) have shown that tyrosyl residues could also be involved in some interactions with DNA. Therefore, stacking interactions between aromatic amino acids and nucleic acid bases could be a general mechanism for the recognition of single-stranded nucleic acids by single-strand binding proteins.

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Registry No. Trp, 73-22-3; Cys, 52-90-4; I_2^-, 12190-71-5; Br_2^-, 12595-70-9; SCN_2^-, 34504-17-1.

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