Generation of Superoxide Free Radical by Neocarzinostatin and Its Possible Role in DNA Damage†

Der-Hang Chin and Irving H. Goldberg*

Department of Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: Spectroscopic analysis of the reduction of both nitro blue tetrazolium and ferricytochrome *c* induced by neocarzinostatin shows that superoxide free radical is produced during the spontaneous degradation of the antibiotic. The amount of superoxide free radical produced from neocarzinostatin is not affected by the presence of thiol, although earlier work has shown that DNA damage is stimulated at least 1000-fold by thiol. Transition metals are not involved in this reaction. Although superoxide dismutase inhibits the reduction of nitro blue tetrazolium and cytochrome *c* induced by neocarzinostatin, neither it nor catalase interferes with the action of neocarzinostatin on DNA, whether or not drug has been activated by thiol. The pH profiles for spontaneous base release and alkali-labile base release (a measure of nucleoside 5′-aldehyde formation at a strand break) do not correspond with that for the generation of superoxide free radical from neocarzinostatin. The same holds for supercoiled DNA cutting by neocarzinostatin chromophore in the absence of a thiol, which is an acid-favored reaction. These results indicate that the generation of superoxide free radical by the drug does not correlate with DNA damage activity, whether or not thiol is present. Furthermore, the failure of hydroxyl free-radical scavengers to inhibit drug-induced single-strand breaks in supercoiled DNA in the absence of thiol also indicates that a diffusible hydroxyl free radical is most probably not involved in this reaction.

Neocarzinostatin (NCS),[1] a protein-containing antitumor antibiotic (Ishida et al., 1965), induces base release and DNA strand breaks in a reaction involving molecular oxygen (Kappen & Goldberg, 1978; Burger et al., 1978). Upon activation of the drug by a sulfhydryl in an aerobic environment, NCS is able to produce a DNA strand break with a nucleoside 5′-aldehyde at its 5′ end (Kappen et al., 1982, 1983). The DNA cleavage reaction is stimulated at least 1000-fold by added thiol (Beerman et al., 1977; Kappen & Goldberg, 1978). Since the nonprotein chromophore of NCS carries the full activity of the drug (Napier et al., 1980), the stoichiometry of uptake of molecular oxygen by DNA-bound NCS chromophore has been determined (Povirk & Goldberg, 1983). These studies have shown that although 1 mol of molecular oxygen is utilized per mole of NCS chromophore under DNA damage conditions, a slow autoxidation of the drug can take place in the absence of the other components. Thus, the exact function of molecular oxygen in these reactions was unclear. From ^{18}O labeling experiments, we have shown recently that the oxygen atom of the nucleoside 5′-aldehyde is derived directly from molecular oxygen rather than from solvent water (Chin et al., 1984). Thus, it appears that an irreversibly activated form of molecular oxygen plays a crucial role in the NCS-induced DNA strand breakage reaction.

The hypothetical form of the activated oxygen intermediate has been proposed by this laboratory to be a peroxy adduct of a carbon-centered DNA free radical (Goldberg et al., 1985). On the other hand, a mechanism implicating a diffusible reactive oxygen species has also been proposed in which the NCS-induced DNA damage is mediated by hydrogen peroxide and by superoxide and hydroxyl free radicals (Sim & Lown, 1978; Lown, 1982, 1983). In this paper, an attempt is made to resolve the conflicting proposals for the role of oxygen in NCS-induced DNA damage. First, we sought to determine whether diffusible oxygen species, in particular superoxide free radical, are formed by NCS and, if so, whether they play a role in the generation of DNA damage.

Materials and Methods

Sonicated calf thymus DNA (7.7 mM nucleotide) was prepared as described (Povirk & Goldberg, 1980) and then dialyzed against 20 mM sodium citrate buffer at pH 4. [*methyl*-^{3}H]Thymidine-labeled λ DNA (specific activity approximately 1.8×10^4 cpm/μg) was prepared essentially by described procedures (Maniatis et al., 1982; Hedgpeth et al., 1972). φX174 RF DNA (95% form I) was obtained from Bethesda Research Laboratories. Tris(hydroxymethyl)-aminomethane (Tris)–phosphate–acetate buffers were mixed from equal molar concentrations of Trizma phosphate (Sigma), Trizma acetate (Sigma), and Trizma base (Sigma). Sodium acetate buffers were adjusted to have the same concentration of sodium cation by adding various amount of 1 M NaCl. Agarose was purchased from Bio-rad Laboratories. Nitro blue tetrazolium chloride monohydrate (NBT) was from Aldrich. Horse heart type III ferricytochrome *c* was from Sigma.

NCS Chromophore. Clinical NCS (holo NCS) (gift of Dr. W. T. Bradner, Bristol Laboratories) was dialyzed against distilled water and lyophilized. The nonprotein chromophore was extracted either with 0.1 N acetic acid in methanol at 0 °C for 2 h or with pure methanol at 0 °C for 2 h after lyo-

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[1] Abbreviations: NCS, neocarzinostatin; holo NCS, neocarzinostatin apoprotein plus nonprotein chromophore; Tris, tris(hydroxymethyl)-aminomethane; NBT, nitro blue tetrazolium chloride monohydrate; AET, *S*-(2-aminoethyl)isothiouronium bromide hydrobromide; DETAPAC, diethylenetriaminepentaacetic acid; SOD, superoxide dismutase; PTTU, 1-phenyl-3-(2-thiazolyl)-2-thiourea; *P*/*D*, molar ratio of DNA nucleotide phosphorus to drug; EDTA, ethylenediaminetetraacetic acid.

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philization with 20 mM sodium citrate, pH 4. The resulting chromophore solution (approximately 0.3 mM) (Povirk et al., 1981) was stored at −70 °C for at least 2 months without detectable degradation.

Spectroscopic Analysis. All spectra were recorded by a Perkin-Elmer 552A UV–vis spectrophotometer at 20 °C. The samples contained NCS chromophore, other components as indicated, and an O_2^{-}·-detecting agent, either NBT or cytochrome *c*. The reaction was initiated by a pH change with a specified buffer. The reduction of NBT was followed for 30 min by recording the increase in absorption at 535 nm due to the production of formazan (Younes et al., 1980). The reduction of ferricytochrome *c* was monitored by recording the increase in absorption at 550 nm resulting from the production of ferrocytochrome *c* (Younes et al., 1980).

Base Release. DNA damage induced by NCS chromophore in the presence of a thiol at various pH values was determined by measuring both spontaneous and alkali-labile thymine release (Kappen & Goldberg, 1983). The latter is a measure of the formation of nucleoside 5′-aldehyde. The NCS chromophore–DNA reaction was initiated by the addition of 62.5 mM Tris–phosphate–acetate buffer (at the desired pH) to a mixture of 109 μM calf thymus DNA, 43 μM [*methyl*-^{3}H]-thymidine-labeled λ DNA, 0.4 mM *S*-(2-aminoethyl)isothiouronium bromide hydrobromide (AET), 3 mM diethylenetriaminepentaacetic acid (DETAPAC), 15.2 μM NCS chromophore (pH 4), 11.8% v/v methanol, and 3.28 mM sodium citrate (all concentrations specified are final ones). A 10-μL aliquot of each sample was added to 0.3 mL of water for the determination of total radioactivity in the sample. The rest of the solution was divided into two equal portions. One part was heated at 90 °C for 30 min after an adjustment to pH 13 with 1 M NaOH. After the solution was neutralized with the same amount of 1 N HCl, the treated portion (for the determination of alkali-labile base release), as well as the untreated part of the solution (for the determination of spontaneous base release), was monitored for thymine release by a paper chromatographic method as described (Povirk et al., 1978). The running solvent contained a mixture of 7 parts of ethanol and 3 parts of 1 M sodium acetate, and the running period was approximately 5 h. The released thymine was measured by cutting the paper into 0.5-in.-long sections and vortexing with 0.3 mL of water. The samples sat at room temperature for 15 min to assure complete dissolution; hydrofluor, a scintillation solution from National Diagnostics, was added, and the radioactivity of each segment was measured by a Searle Delta 300 liquid scintillation counter.

Agarose Gel Electrophoresis. The extent of DNA strand damage induced by NCS in the absence of a thiol was monitored by following the conversion of supercoiled DNA (form I) to its relaxed circular form (form II). Individual experiments were done with both holo NCS and NCS chromophore. The chemical reaction was initiated by a pH change upon addition of a buffer solution into a mixture of φX174 RF DNA solution that contained final concentrations of 3 mM DETAPAC and either 60 μM φX174 RF DNA with 15.2 μM NCS chromophore or 54.5 μM φX174 RF DNA with 14 μM NCS or NCS chromophore. Reaction mixtures (total volume 20 μL) were incubated at 25 °C for 30 min for samples containing NCS chromophore and 37 °C for 2 h for samples containing holo NCS. Each sample was then added to 5 μL of a gel loading solution that consisted of 50% w/v glycerol, 0.25% bromphenol blue, and 25 mM EDTA, pH 8. Individual samples were loaded immediately into a 1% w/v agarose horizontal slab gel (23 cm × 12 cm × 0.6 cm) that contained 1 μg/mL ethidium bromide, 40 mM Tris-HCl at pH 8, 5 mM sodium acetate, and 1 mM EDTA. Following electrophoresis at constant voltage (120 V for a period of approximately 5 h), the gels were visualized with 254-nm UV light and photographed with a red filter on Polaroid type 57 film.

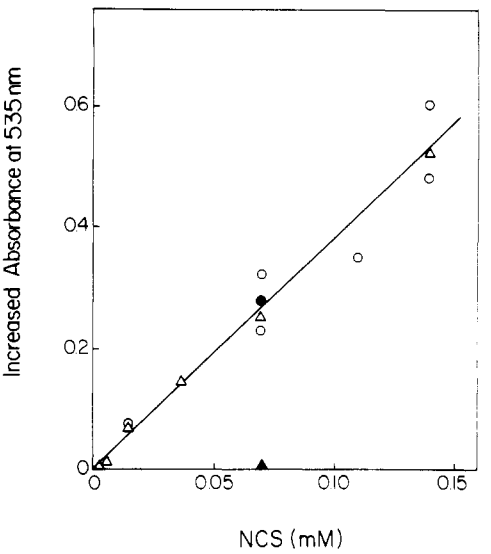

FIGURE 1: Reduction of NBT to formazan by NCS chromophore. The reaction was initiated by a pH change with the addition of 62.5 mM Tris-HCl at pH 8 into a solution which contained NCS chromophore and the following: (1) 3 mM DETAPAC–0.5 mM NBT (all concentrations specified are final concentrations) (△); (2) same as (1) with an additional 0.5 mM AET (○); (3) same as (2) with an additional 200 μg/mL SOD (▲); (4) same as (2) with an additional 400 μg/mL catalase (●).

RESULTS

Generation of Superoxide Free Radical by NCS in the Absence of Thiol. To directly examine whether superoxide free radical is generated from NCS, the chemical reduction of two widely used superoxide detection reagents, NBT and cytochrome *c*, was followed. However, in addition to their superior reactivity toward superoxide free radical, NBT and cytochrome *c* are also susceptible to direct chemical reduction by a number of other reductants (McCord et al., 1977). Hence, it is important to determine which chemical source initiates the reduction reaction and what the nature of the reduction is. Experimental results from spectroscopic analysis of the reduction of NBT at pH 8 induced by NCS are shown in Figure 1. Similar results were obtained when the reduction of ferricytochrome *c* was followed (data not shown). Because of the linear relationship between NCS concentration and the reduction of NBT and cytochrome *c*, NCS can be considered as the major chemical source in the system for the reductive reactions. Since NCS chromophore spontaneously degrades in aqueous solution as the pH is increased above 5 (Kappen & Goldberg, 1980), the reduction of NBT and cytochrome *c* presumably is produced in conjunction with the NCS degradation process. A pH profile of the reduction of NBT by NCS (Figure 2) clearly illustrates that the reduction does not occur near pH 4, where both holo NCS and NCS chromophore are stable. Additional evidence relating NCS degradation and NBT reduction is the finding that holo NCS does not cause reduction of NBT at pH 7.2 (Figure 2). Holo NCS is known to be much more resistant to degradation than the unprotected NCS chromophore and is relatively stable at this pH level at 20 °C (Kappen & Goldberg, 1980). In contrast to the lack of effect of catalase on the reduction reaction (Figure 1), addition of superoxide dismutase (SOD) inhibits

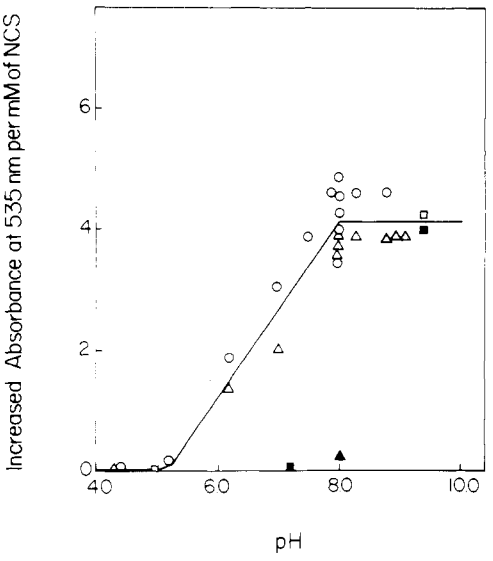

FIGURE 2: Reduction of NBT by NCS at different pH values. The reaction was initiated by a pH change with an addition of 62.5 mM Tris–phosphate–acetate buffer into a solution which consisted of the following: (1) NCS chromophore, 3 mM DETAPAC, and 0.5 mM NBT (all concentrations specified are final concentrations) (△); (2) same as (1) with an additional 0.5 mM AET (○); (3) same as (2) with an additional 200 μg/mL SOD (▲); (4) holo NCS and 0.5 mM NBT (■); (5) same as (4) with an additional 3 mM DETAPAC (□).

NBT reduction by more than 95% (Figures 1 and 2). Similar results were obtained in the case of cytochrome *c* reduction (data not shown). This effect of SOD indicates that the reduction is due to the formation of superoxide free radical rather than to a direct chemical reaction with the NCS. In summary, in the absence of added thiol, superoxide free radical appears to be produced during the spontaneous degradation of NCS as revealed by the following characteristics of the reduction of NBT and cytochrome *c*: (1) linear relationship with the concentration of NCS at pH 8; (2) nonreactivity at pH 4 for both holo NCS and NCS chromophore; (3) nonreactivity at pH 7.2 for holo NCS but not for NCS chromophore; and (4) inhibition by SOD.

The pH profile in Figure 2 exhibits an essentially linear relationship in the range pH 5–8 for the reduction of NBT by NCS. It thus appears likely that the generation of superoxide from NCS is a hydroxide anion stimulated reaction. Above pH 8, the production of superoxide free radical reaches a plateau, indicating that the amount of hydroxide anion is sufficient to drive all NCS molecules into superoxide production.

Under the conditions used in our experiments, NBT and cytochrome *c* coprecipitate with DNA, making it not possible to obtain accurate measurements of superoxide free-radical formation by DNA–drug complexes.

Since a trace amount of transition metals is known to be implicated in the formation of superoxide free radical in various reaction systems, the effect of DETAPAC, a metal chelating agent whose chelate possesses no additional coordination bonds, has also been examined in the reduction of NBT by NCS. Figure 2 shows that DETAPAC has essentially no effect on the production of superoxide free radical from NCS. Presumably, transition metals are not involved in this reaction.

Generation of Superoxide Free Radical by NCS in the Presence of Thiol. It is known that superoxide free radical can be produced during the autoxidation of thiols (Misra, 1974). Thiols that have been commonly used to activate NCS in the DNA strand scission reaction, namely, 10 mM 2-mercaptoethanol, 0.5 mM AET, 5 mM L-cysteine, 5 mM cysteamine, 5 mM glutathione, and 1 mM DL-dithiothreitol, were tested by NBT reduction for the generation of superoxide free radical under the same conditions used with NCS. Except for AET, the other thiols produced significant amounts of formazan compared to that with NCS alone. The low background in the case of AET is possibly due to the low concentration required for activation of NCS. For this reason, AET was chosen to examine the effect of an added thiol on the production of superoxide free radical from NCS chromophore. To minimize the already small background due to the autoxidation of AET, both reference cell and sample cell contained the same quantity of AET. The effect of added AET on the reduction of NBT by NCS is illustrated in Figures 1 and 2. Similar results were obtained on the reduction of cytochrome *c*. In general, the amount of superoxide free radical produced from NCS is not affected by addition of a thiol. NCS is known to be inactivated irreversibly by thiols at pHs above 5 (Kappen & Goldberg, 1978), and its reaction product with a thiol is also known to be different from its reaction product without a thiol (Povirk & Goldberg, 1983). The fact that an added thiol does not perturb the system generating superoxide from NCS implies that the inactivation of NCS by thiol and the generation of superoxide free radical from NCS by hydroxide anion are two unrelated processes that may occur at two different reactive sites of NCS. However, it should be noted that the addition of AET to NCS sometimes enhances slightly the reduction of NBT, and the effect increases with time. Therefore, most spectroscopic data collected in this paper were obtained within the first 30 min of the reaction.

NCS Chromophore-Induced DNA Damage in the Presence of Thiol. To examine whether the superoxide free radical generated from NCS is actually involved in the DNA scission reaction, the activity of NCS at different pH values was measured in the presence and absence of thiol. In the situation where NCS is activated by thiol, DNA damage was measured by thymine base release. pH profiles of total thymine release, alkali-labile thymine release, and spontaneous thymine release are illustrated in Figure 3. The calculation of the percentages of thymine released is based upon the total number of thymine molecules in the DNA. Alkali-labile base release, a measure of strand breaks having a nucleoside 5′-aldehyde at the 5′ end (Kappen & Goldberg, 1983), exceeds spontaneous base release at pHs above 7.0. The difference in the pH patterns between spontaneous base release and alkali-labile base release indicates that they result from different chemical reactions. To examine whether either of the two processes involves the generation of superoxide by NCS, one may compare their pH patterns with that in Figure 2. It is clear that neither the spontaneous base release nor the alkali-labile base release pH profile corresponds with that for the generation of superoxide free radical from NCS. The same holds for total thymine release which represents 73% of the total DNA damage (Kappen & Goldberg, 1983). These results strongly indicate that the generation of superoxide free radical from the drug does not correlate with the drug activity, as measured by two parameters of DNA damage.

Since not all NCS molecules participate in DNA damage (Povirk & Goldberg, 1983), but may also be involved in side reactions, the percentage of NCS chromophore molecules utilized in the DNA damage reaction at various pHs can be calculated from the data in base release experiments. The calculation is based on kinetic results showing that (1) each NCS chromophore molecule is used only once during the DNA

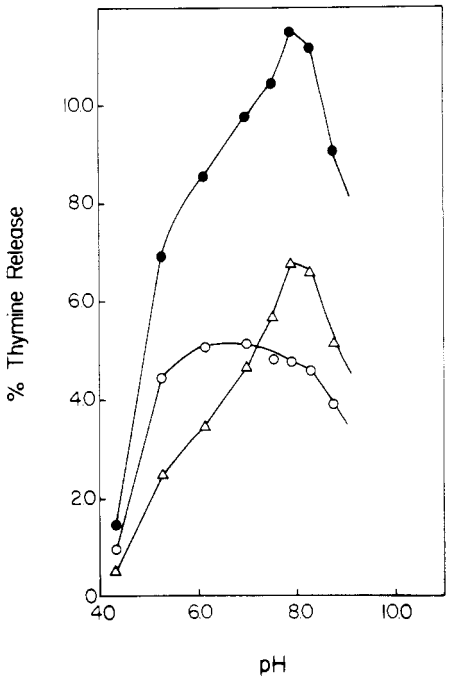

FIGURE 3: Effect of pH on the DNA damage activity of NCS chromophore in the presence of thiol. The activity was measured by thymine base release by paper chromatography. Standard incubation in 62.5 mM Tris–phosphate–acetate buffer at different pH values was carried out at room temperature for 20 min. The final drug level was 15.2 μM. Total concentration of DNA (calf thymus and [*methyl*-^{3}H]thymidine-labeled λ DNA) was 152 μM. 0.4 mM AET was used to activate the drug. Spontaneous thymine release (○), alkali-labile thymine release (△), and total thymine release (●) were calculated on the basis of the percentage of total thymine in DNA.

Table I: Utilization of NCS Chromophore for DNA Damage and for the Generation of Superoxide Free Radical at Various pHs[a]

pH	% of NCS chromophore utilized for particular DNA damage: spontaneous base release	alkali-dependent base release	total base release	% of NCS chromophore utilized for generation of $O_2^{\cdot-}$
4.3	3.3	1.7	5.0	0.0
5.3	15.3	8.4	23.7	3.0
6.2	17.3	11.9	29.2	38.7
7.0	17.5	15.9	33.4	64.7
7.5	16.5	19.4	35.9	82.4
7.9	16.2	23.3	39.5	94.1
8.3	15.7	22.5	38.2	100
8.8	13.4	17.6	31.0	100

[a] The values were calculated from data in Figures 2 and 3. Percentage of NCS chromophore utilized for particular DNA damage was calculated according to the percentage of total thymine in the DNA released. Percentage of NCS chromophore utilized for generation of $O_2^{\cdot-}$ was calculated on the basis of the reduction of NBT by NCS and was normalized to 100% where NBT reduced by NCS reaches its plateau.

scission reaction and (2) each thymine molecule released requires only one NCS chromophore (Povirk & Goldberg, 1983). The results are presented in Table I. The percentage of NCS chromophore utilized in the generation of superoxide free radical at different pH values was also calculated on the basis of a normalized value of 100% at pH 8. Since NCS chromophore-induced reductions of both NBT (Figure 2) and cytochrome *c* (data not shown) reach their plateau at the same pH level (pH 8), and the ratio of the reduction at a certain pH value to that at pH 8 is approximately the same for both assays, the ratio of the extent of reduction at a particular pH to its plateau represents the percentage of NCS chromophore

Table II: Effect of SOD and Catalase on NCS Chromophore-Induced DNA Damage in a Thiol-Activated Reaction[a]

reagent	concn of reagent (μg/mL)	concn of CH_3OH (% v/v)	spontaneous thymine release (%)	alkali-labile thymine release (%)	total thymine release (%)
SOD	0	20	2.87	3.07	5.94
	50	20	2.59	3.39	5.98
SOD	0	2.7	1.98	2.93	4.91
	50	2.7	2.31	3.69	6.00
SOD	0	2.7	1.87	3.36	5.23
	471	2.7	2.60	4.28	6.88
catalase	0	2.7	1.92	2.89	4.81
	2.65	2.7	3.42	3.49	6.91
catalase	0	20	3.04	4.53	7.57
	2.65	20	3.37	5.09	8.46

[a] The standard incubation contained 114 μM [*methyl*-^{3}H]thymidine-labeled λ DNA, 9.5 μM NCS chromophore, 3 mM DETAPAC, and 10 mM 2-mercaptoethanol. DNA damage was initiated by a pH change with 62.5 mM Tris-HCl at pH 8 and was carried out at room temperature for 20 min. All concentrations specified are final ones. Drug activity was measured as the percentage of total thymine base release.

utilized in superoxide production. When these values (Table I) are compared with the values of drug utilization for spontaneous base release, alkali-labile base release, and total base release at the same pH levels, obvious differences in pH patterns are noted. Nevertheless, if superoxide free radical were responsible for the drug-induced DNA damage, one might not expect it to be fully utilized for the DNA reaction, since superoxide free radical is an active species and other side reactions could take place and compete with the reaction toward DNA. Therefore, one might consider that the lack of correlation between the drug activity and the generation of superoxide free radical is due to the competitive side reactions of superoxide. Hence, it is significant to note that at pH 5.3 only 3% of NCS chromophore molecules was used for the generation of superoxide free radicals, while 23.7% of NCS chromophore was used for DNA damage. Since more NCS chromophore molecules were used in DNA damage than that in producing superoxide, it is not possible to assign the lack of correlation to the competitive side reactions.

To obtain direct proof that superoxide is not involved in the DNA scission reaction induced by NCS, the effect of free-radical scavengers on drug activity has been examined. Neither SOD nor catalase inhibits the thiol-activated NCS chromophore–DNA reaction. As shown in Table II, they, in fact, enhance slightly the drug activities whether they are expressed as alkali-labile thymine release or as total thymine release. It is thus clear that the thiol-activated NCS chromophore–DNA reaction is not mediated by a diffusible superoxide free radical and the generation of diffusible superoxide free radical from NCS is not responsible for the drug activity in the presence of a thiol.

NCS Chromophore-Induced DNA Damage in the Absence of Thiol. Although thiol stimulates NCS-induced DNA damage at least 1000-fold, NCS at high levels in the absence of an added thiol is still able to produce a small number of strand breaks (Beerman et al., 1977; Kappen & Goldberg, 1978; Sim & Lown, 1978). To determine whether superoxide free radical plays a role in the formation of single-strand breaks under non-thiol conditions, where overall DNA damage is small, it was necessary to use a more sensitive assay than base release. Accordingly, we followed the drug-induced conversion of supercoiled (form I) DNA to its singly nicked species, circular duplex (form II) DNA, by agarose gel electrophoresis. As shown in Figure 4, φX174 RF DNA was cleaved by a high

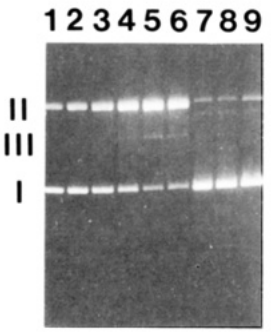

FIGURE 4: Agarose gel electrophoretic pH profile of ethidium bromide stained ϕX174 RF DNA after treatment with NCS chromophore in the absence of thiol. The standard solution mixture contained 15.2 μM DNA, 60 μM NCS chromophore, 3 mM DETAPAC, 17.5% methanol, 1 mM sodium citrate, and 62.5 mM Tris–phosphate–acetate buffer. Lane 1, pH 9.0; lane 2, pH 8.0; lane 3, pH 7.2; lane 4, pH 6.4; lane 5, pH 5.4; lane 6, pH 4.4; lane 7, untreated DNA at pH 9.0; lane 8, untreated DNA at pH 7.2; lane 9, untreated DNA at pH 4.4.

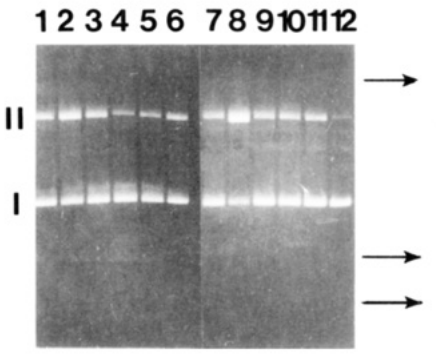

FIGURE 5: Effect of free-radical scavengers on NCS-mediated supercoiled DNA strand scission in the absence of thiol. The standard solution mixture contained 54.5 μM ϕX174 RF DNA, 14 μM NCS chromophore, 3 mM DETAPAC, 0.92 mM sodium citrate, 4.6% methanol, and 50 mM potassium phosphate buffer at pH 7.2. Lane 1, without a free-radical scavenger reagent; lane 2, added 50 μg/mL SOD; lane 3, 50 μg/mL catalase; lane 4, 40 mM sodium benzoate; lane 5, 40 mM sodium chloride; lane 6, 5 mM histidine; lane 7, 100 mM mannitol; lane 8, 40 mM thiourea; lane 9, 0.5 mM PTTU; lane 10, 40 mM sodium acetate lane 11, 40 mM Tris–acetate; lane 12, without NCS chromophore.

level of NCS chromophore after incubation at 25 °C for 30 min in the absence of thiol to generate mainly form II and small amounts of form III (linear duplex) DNA. Inactivation of the drug either by previous treatment at an alkaline pH or by its complexation by NCS apoprotein inhibits the DNA cleavage reaction completely (data not shown). The production of form II DNA increases as the pH decreases. Although linear duplex DNA, produced by a single double-strand break, could hardly be detected at higher pHs, it appeared in reactions at lower pH values. It is clear that the non-thiol-activated NCS–DNA reaction is an acid-favored reaction. As noted earlier, the generation of superoxide free radical from NCS is not favorable at low pH values. In fact, the pH profile of DNA cutting by NCS chromophore in the absence of a thiol is just opposite that for the production of superoxide free radical by NCS (shown in Figure 2). This is also true for holo NCS on going from pH 6 to pH 4.

The presence of DETAPAC did not influence the DNA damage reaction by either holo NCS or the isolated chromophore (Figure 4 and unpublished data). Therefore, the major cause of DNA damage in the absence of thiol by NCS chromophore or holo NCS cannot be explained by the action of superoxide free radical, a reaction presumably requiring transition-metal complexes (Brawn & Fridovich, 1981). This is most clearly seen in the reaction at pH 4.4. Figure 2 clearly shows that there was no detectable superoxide free radical generated at pH 4.4 from NCS chromophore, whereas a comparatively large amount of DNA damage was produced at this pH by NCS chromophore (Figure 4) and holo NCS (data not shown).

One possible explanation for the non-thiol-type of NCS–DNA reaction being favored under acidic conditions concerns the stability of NCS chromophore. NCS chromophore is known to be more stable in acidic solution. The degradation rate of NCS chromophore increases with the increase in pH value, perhaps making the NCS–DNA reaction less competitive with the degradation process. Since NCS chromophore is more stable in an acidic solution at a higher content of methanol, one would expect that DNA cleavage by NCS chromophore would increase with the methanol content, at least up to a point. Agarose gel analysis revealed this to be true (data not shown).

Figure 5 shows the most direct evidence to rule out the possibility of the involvement of superoxide in the DNA cleavage reaction. The addition of SOD not only is unable to inhibit the drug activity but also actually enhances slightly the DNA cutting reaction. Catalase also does not inhibit the drug activity. The same phenomena were also observed in the situation where a thiol was added (Table II). Therefore, both diffusible superoxide free radical and hydrogen peroxide appear unlikely to participate in the strand-break reaction whether or not the drug has been activated by a thiol. Several hydroxyl-radical scavengers, such as histidine, sodium benzoate, mannitol, thiourea, sodium acetate, and 1-phenyl-3-(2-thiazolyl)-2-thiourea (PTTU), have also been tested for inhibition of the non-thiol reaction. As reported earlier (Kappen & Goldberg, 1978) for the thiol-activated reaction, none of them has a significant inhibition effect, except for sodium benzoate. If one compares the inhibition by sodium benzoate with that by the same concentration of NaCl, however, it appears that most of the inhibition by sodium benzoate is due to the effect of the sodium content rather than to its free-radical scavenging function. The stimulatory effect of thiourea is probably due to its rearrangement to a thiol form. Since thiourea, histidine, PTTU, and the like are hydroxyl free-radical-specific scavengers, the lack of inhibitory effect of those reagents is evidence that a diffusible hydroxyl free radical is most probably not involved in the DNA cleavage reaction induced in the non-thiol-type NCS chromophore reaction.

Discussion

Since molecular oxygen is required for base release and DNA strand scission by NCS (Kappen & Goldberg, 1978; Burger et al., 1978; Povirk & Goldberg, 1984), it was reasonable to expect that the active DNA-damaging species might be a diffusible, reduced form of activated oxygen, such as superoxide free radical, hydrogen peroxide, or hydroxyl free radical. Since superoxide free radical itself is a weak DNA-damaging agent, it is generally believed that it exerts its effect via a hydroxyl free radical, generated by a Haber–Weiss type of reaction (Brawn & Fridovich, 1981). Such a mechanism has been implicated in the DNA strand breakage induced under reducing conditions by a number of antitumor antibiotics, many of which are quinoidal in structure (Lown, 1983). Where studied, these reactions have been shown to be inhibitable by SOD, catalase, and by various hydroxyl free-radical scavengers, such as mannitol, isopropyl alcohol, and sodium benzoate (Lown, 1983).

Consistent with earlier results (Povirk & Goldberg, 1983) on the consumption of molecular oxygen by NCS in the ab-

sence of thiol, as well as in its presence, we show here that NCS undergoes autoxidation with the formation of superoxide free radical. Superoxide free-radical formation, however, cannot be involved in the production of DNA damage, whether or not thiol is present. The evidence leading to this conclusion is as follows: (1) the amount of superoxide free radical generated by NCS is not stimulated by thiol, whereas DNA damage activity is greatly stimulated by thiol; (2) the pH profile for the generation of superoxide free radical does not correspond to that for DNA damage, whether or not the drug has been activated by thiol; (3) at low pH, the amount of NCS chromophore used to generate superoxide free radical is not sufficient to account for the DNA damage; and (4) SOD, catalase, and various hydroxyl-radical scavengers do not interfere with the action of NCS on DNA. It is thus clear that under the conditions used in our experiments diffusible superoxide free radical generated from NCS autoxidation is not responsible for a significant fraction of the DNA damage produced. Our data, of course, do not eliminate the possibility that hydroxyl free radical (via $O_2^{-}\cdot$ formation) is generated at the site of bound drug on the DNA and acts locally to produce deoxyribose damage, but for steric reasons is not susceptible to the action of scavengers. It is possible, furthermore, that earlier evidence implicating superoxide free radical in DNA strand breakage in the absence of thiol (Sim & Lown, 1978; Lown, 1982, 1983) involved reactions in which a significant Haber–Weiss-type reaction takes place due to the presence of adventitious transition metals. The conditions we have used appear to be insufficient for hydroxyl-radical formation, since our results are not altered by the presence of the metal chelator DETAPAC, which has been shown by Brawn and Fridovich (1981) to be a potent inhibitor of the Haber–Weiss reaction. Additionally, analysis of the NCS chromophore itself for transition metals has failed to reveal their presence (Napier et al., 1981), although other components in the reaction could be such a source.

Recent studies have clarified the role of molecular oxygen in DNA strand breakage induced by NCS activated by thiol (Charnas & Goldberg, 1984; Kappen & Goldberg, 1985) and are consistent with the above findings and conclusions. Activated NCS chromophore abstracts a hydrogen from C-5′ of deoxyribose in DNA to generate a presumptive carbon-centered radical at C-5′ (Charnas & Goldberg, 1984; Kappen & Goldberg, 1985). ^{18}O Experiments have shown that molecular oxygen adds to the carbon-centered radical intermediate at C-5′ (Chin et al., 1984) to form a presumptive peroxy derivative that eventuates in a strand break with a nucleoside 5′-aldehyde at its 5′ end. The finding that a hydrogen from C-5′ ends up in a nonexchangeable position on the NCS chromophore itself rules out an activated oxygen moiety, whether diffusible or not, as the abstracting species. Furthermore, molecular oxygen is not essential in the formation of nascent DNA damage, a carbon-centered radical at C-5′ of deoxyribose, since C-5′ hydrogen abstraction occurs under anaerobic conditions (Kappen & Goldberg, 1985), and the NCS chromophore forms covalent adducts with the reactive site on the deoxyribose, preferentially in the absence of molecular oxygen (Povirk & Goldberg, 1984, 1985).

A structure for the nonprotein chromophore of NCS has recently been proposed (Hensens et al., 1983; Edo et al., 1985). Although it does not contain a quinone, it is a highly unsaturated structure, containing a highly strained nine-membered ring, that possesses two acetylenic bonds. The mechanism involved in the autoxidation (or thiol activation) of the NCS chromophore remains to be elucidated.

Registry No. $O_2^{-}\cdot$, 11062-77-4; OH^{-}, 3352-57-6; neocarzinostatin, 9014-02-2; neocarzinostatin chromophore, 81604-85-5.

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Hexokinase Receptor Complex in Hepatoma Mitochondria: Evidence from *N,N'*-Dicyclohexylcarbodiimide-Labeling Studies for the Involvement of the Pore-Forming Protein VDAC†

Richard A. Nakashima,‡ Patrick S. Mangan,§ Marco Colombini,§ and Peter L. Pedersen*,‡
Laboratory for Molecular and Cellular Bioenergetics, Department of Biological Chemistry, School of Medicine, The Johns Hopkins University, Baltimore, Maryland 21205, and Laboratories of Cell Biology, Department of Zoology, University of Maryland, College Park, Maryland 20742

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ABSTRACT: In rapidly growing, highly glycolytic hepatoma cells as much as 65% of the total cell hexokinase is bound to the outer mitochondrial membrane [Parry, D. M., & Pedersen, P. L. (1983) *J. Biol. Chem. 258*, 10904–10912]. In this paper, we describe the purification to apparent homogeneity of a mitochondrial pore-forming protein from the highly glycolytic AS-30D rat hepatoma cell line. The purified protein shows a single 35 000-dalton band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, an amino acid composition slightly more hydrophobic than that of the rat liver pore protein (also known as VDAC or mitochondrial porin), and a channel-forming activity of 136 channels min^{-1} (μg of protein)$^{-1}$. In addition to displaying the properties characteristic of VDAC (single-channel conductance, voltage dependence, and preference for anions), we observe that the AS-30D VDAC protein is one of only three mitochondrial proteins that bind [^{14}C]dicyclohexylcarbodiimide (DCCD) at relatively low dosages (2 nmol of DCCD/mg of mitochondrial protein). Significantly, treatment of intact mitochondria isolated from either rat liver or the AS-30D hepatoma with DCCD results in an almost complete inhibition of their ability to binding hexokinase. Fifty percent inhibition of binding occurs at less than 2 nmol of DCCD/mg of mitochondrial protein. In contrast to DCCD, water-soluble carbodiimides are without effect on hexokinase binding. These results suggest that the pore-forming protein of tumor mitochondria forms at least part of the hexokinase receptor complex. In addition, they indicate that a carboxyl residue located within a hydrophobic region of the receptor complex may play a critical role in hexokinase binding.

One of the most consistent phenotypic markers of transformed cell lines is an increase in glucose utilization and lactic acid production rates compared to normal cells from the same tissue of origin (Warburg et al., 1924; Cori & Cori, 1925; Weinhouse, 1966; Burk et al., 1967; Bustamante et al., 1981). The increased rates of glucose catabolism promote rapid tumor growth by providing an alternate source of ATP production, accounting for up to 60% of total cell ATP production in highly glycolytic cancer cells (Aisenberg, 1961; Pedersen, 1978; Nakashima et al., 1984), and by increasing intracellular levels of glucose-6-phosphate, a required precursor in the de novo synthesis of nucleic acids and other essential macromolecules (Weber, 1977). One of the factors that appears to be related to increased glycolytic activity in tumor cells is an increase in total activity and a change in the subcellular distribution of hexokinase (EC 2.7.1.1), which occurs as a predominantly mitochondrially bound enzyme in rapidly growing transformed cell lines (Rose & Warms, 1967; Bustamante & Pedersen, 1977). Binding of hexokinase to the mitochondrial outer membrane has been reported to result in a loss of feedback inhibition by the product glucose 6-phosphate (Kosow & Rose, 1968; Gumaa & McLean, 1969; Wilson, 1968; Bustamante & Pedersen, 1977; Singh et al., 1974) and in a preferred access to mitochondrially generated ATP (Gumaa & McLean, 1969; Gots & Bessman, 1974; Inui & Ishibashi, 1979). Previous studies in our laboratory have shown that the elevated levels of mitochondrially bound hexokinase are essential for the high rates of glycolysis observed in rapidly growing cancer cell lines (Bustamante & Pedersen, 1977; Bustamante et al., 1981).

An outer membrane hexokinase receptor site appears to be of wide distribution in mitochondria, even in those tissues that apparently exhibit little hexokinase binding in vivo (Rose & Warms, 1967; Parry & Pedersen, 1983). Felgner et al. (1979) were the first to isolate a hexokinase binding protein from the outer mitochondrial membrane of rat liver. This integral membrane protein of apparent M_r 31 000–32 000 was shown by Linden et al. (1982a) and Fiek et al. (1982) to be identical with the outer membrane pore-forming protein (also known as VDAC[1] or mitochondrial porin) of rat liver mitochondria.

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* Address correspondence to this author.
‡ The Johns Hopkins University.
§ University of Maryland.

[1] Abbreviations: VDAC, voltage-dependent, anion-selective, channel-forming protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DCCD, *N,N'*-dicyclohexylcarbodiimide; BSA, bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDAC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; HPLC, high-pressure liquid chromatography; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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