Detection of Activating Enzymes for 4-Nitroquinoline 1-Oxide Activation with a Microbial Assay System

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SUMMARY

4-Nitroquinoline 1-oxide (4NQO) damages bacterial genomes but not phage genomes. A reduction product of 4NQO, 4-hydroxyaminoquinoline 1-oxide (4HAQO), damages both the bacterial and phage genomes. These results imply that Salmonella typhimurium is able to convert 4NQO to 4HAQO and that 4HAQO reacts with the genomes. When phage P22 is treated with 4HAQO and assayed on S. typhimurium strains lysogenic for P221, a rapid inactivation of P22 and a greatly increased frequency of recombination between P22 and the prophage P221 are observed. With the use of this phage assay system, experiments were designed to search for activating enzymes which convert 4NQO to 4HAQO or a reactive intermediate that inactivates bacteriophage. When phage P22 was incubated with 0.1 mM 4NQO, 0.1 mM NADH, and bacterial protein fractions obtained by diethylaminoethyl cellulose column chromatography, it was found that only one protein peak was able to cause phage inactivation and recombination; this same peak was the only one with bacterial diaphorase and 4NQO-reducing activity. Similarly, experiments with rat liver cytosol fractions showed that there are two peaks that activate 4NQO, and these also corresponded to diaphorase activity. These results suggest that diaphorase is an activating enzyme for 4NQO.

INTRODUCTION

4NQO, a potent carcinogen, inactivates bacteria but does not inactivate bacteriophage (10). However, a reduction product of 4NQO, 4HAQO, inactivates both bacteria and bacteriophage, suggesting that 4HAQO interacts directly with DNA. Since 4NQO does not itself interact with phage DNA, it must in all likelihood be converted to 4HAQO by enzymes in bacterial cells (3) before interacting with the bacterial genome.

In this paper, we describe a method we designed for detection of activating enzymes that convert 4NQO to 4HAQO or a reactive intermediate that inactivates bacteriophage in the presence of enzyme fractions, NADH, and 4NQO. This method may be generally applicable in the search for activating enzymes that convert potential carcinogens to proximal carcinogens.

MATERIALS AND METHODS

Bacterial Strains

Salmonella typhimurium strains Q1, LT-2 (St for short) and their her+ and recA mutants (10) were used. Their lysogenic strains for phage P221b (11) were also used for the prophage induction and the phage recombination experiments. For assay of prophage induction, a strain resistant to phage P22 and streptomycin, St/22 Smr+, was used. All bacterial strains were cultured by aeration in nutrient broth.

Bacteriophages

The clear plaque-forming mutants (c2) of Salmonella bacteriophage P22 were used for phage inactivation and recombination. P221bc+ was used in the preparation of the lysogenic strains (11). Phage P22 has a short tail with hexagonal base plate and 6 spikes, whereas phage P221 has a long, flexible tail. Although they are serologically unrelated, they have a partial genetic homology for the entire region containing c1, c2, c3, g, and h21 markers (7—9).

Media

Regular nutrient broth consisting of 8 g of Difco (Detroit, Mich.) nutrient broth and 5 g of sodium chloride per liter of distilled water was used to make phage lysate and bacterial aeration culture. For phage plating, we used hard agar containing 23 g of Difco nutrient agar (Difco) and 5 g of sodium chloride; we overlayed soft nutrient agar containing 7.5 g of Difco bacto-agar (Difco), 5 g of sodium chloride and 8 g of Difco nutrient broth (Difco) per liter. Phosphate-buffered NaCl solution contained M/15 phosphate in 0.1 M NaCl at pH 7.0.

Reagents

4NQO (Iwai Chemical Co., Tokyo, Japan) was kindly sent by Dr. Y. Tagashira. 4HAQO was kindly supplied by Dr. Y. Kawazoe (National Cancer Center Research Institute, Tokyo, Japan).
Inactivation of Bacteria by 4HAQO

Bacteria (about 10^7 cells/ml) were treated with 4HAQO in phosphate-buffered NaCl solution. Samples were withdrawn at various intervals, diluted, and assayed for inactivation of bacteria by means of colony-forming ability.

Prophage Induction of P221b Lysogen

A log-phase culture of lysogenic cells (10^7 to 10^8 cells/ml) was treated with 4HAQO in phosphate-buffered NaCl solution and diluted 100- to 1000-fold in fresh nutrient broth. After 30 min incubation at 37°, samples were plated on St/22 Smr with soft agar containing dihydrostreptomycin (200 µg/ml for rec^+ (P221b) and 25 µg/ml for hcr^- (P221b) and rec^- (P221b); streptomycin kills lysogenic cells on the plates, but does not inhibit phage production of cells previously induced (4, 5, 10). Therefore, only cells that are induced by 4HAQO give P221b infectious centers on St/22 Smr.

Fractionation of Bacterial Protein

Bacterial cells were cultured in nutrient broth and collected by centrifugation. Packed cells were homogenized with 4 volumes of phosphate-buffered NaCl solution, pH 7.0, and 0.2-mm diameter glass beads by a VirTis homogenizer. The homogenate was centrifuged at 74,000 X g for 90 min. The supernatant was fractionated with (NH_4)_2SO_4 and the precipitate, between 50 and 75% saturation, was dissolved and dialyzed against water. After centrifugation, the dialyzed extract was placed on a DEAE-cellulose column (1.3 x 20 cm) buffered with 0.01 M phosphate buffer, pH 6.4. Elution was carried out with gradient of 0.01 to 0.3 M phosphate buffer, pH 6.4 (1).

Fractionation of Rat Liver Protein

Rat liver was homogenized with 9 volumes of phosphate-buffered NaCl solution in a Teflon-glass homogenizer and the homogenate was centrifuged at 74,000 X g for 90 min. After centrifugation, the procedure used was the same as the fractionation method for bacterial protein.

Inactivation of Bacteriophage by Incubation with 4NQO in the Presence of NADH and Enzyme

Bacteriophage P22c_2 (about 2 x 10^7 particles/ml) was incubated with 0.1 mM 4NQO, 0.1 mM NADH and an appropriate amount of enzyme in phosphate-buffered NaCl solution, pH 7.0, at 30°. The samples were withdrawn at various times, diluted, and assayed on strains lysogenic for P221b. After 8 to 10 hr of incubation at 37°, the number of surviving plaques and c^+ recombinants (8) of phage P22 were scored.

Assay of Enzyme Activities

Diaphorase Activity. For assay of diaphorase activity, we followed the method of Ernster et al. (1). Dichlorophenolindophenol was used as hydrogen acceptor. The reaction media contained 0.04 mM dichlorophenolindophenol, 0.1 mM NADH, and an appropriate amount of enzyme in M/15 phosphate-buffered NaCl solution, pH 7.0. Activity was assayed by the change of absorbance at 600 mµ at 30°.

4NQO Reducing Activity. Activity was assayed by the method of Hashimoto et al. (2). The components of the reaction media were 0.1 mM 4NQO, 0.1 mM NADH, and an appropriate amount of enzyme in M/15 phosphate-buffered NaCl, pH 7.0. The reaction was measured by the decrease of NADH at A_340 at 30°.

RESULTS

Inactivation of Bacteria by 4HAQO. As reported previously 4NQO inactivates bacteria and repairs deficient bacterial strains. Mutants hcr^- and recA^- of Salmonella typhimurium are far more sensitive to 4NQO than the wild-type cell (10). However, bacteriophage P22, when treated in vitro, is insensitive to 4NQO, whereas it is rapidly inactivated by 4HAQO (10) (see Table 1). These observations suggest that 4NQO is converted to 4HAQO by bacterial enzyme(s) and that

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<td><strong>Effect of 4NQO and its derivative on bacteria and bacteriophage</strong></td>
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^a+, inactivation; −, no effect.
Search for Activating Enzyme by Microbial System

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4HAQO and that 4HAQO does react with bacterial genome, because 4NQO does not interact with DNA and phage genome (10).

Search for Activating Enzyme in Bacterial Cells. For determination of whether bacterial cells contain an enzyme for conversion of 4NQO to 4HAQO, fractions from bacterial homogenates were separated by DEAE-cellulose and were incubated with 4NQO, NADH, and bacteriophage P22c2. As

the latter compound inactivates the bacterial genome. Therefore, we studied the effect of 4HAQO on bacterial cells. As shown in Chart 1, P221b lysogens of repair-deficient mutants hcf and recA were drastically inactivated, whereas the wild-type strain was inactivated only slowly. Incubation for about 20 min showed that hcf and recA mutant cells were inactivated down to several hundred-fold, whereas only 20% of the wild-type cells were inactivated. Moreover, tremendous prophage induction was observed from hcf and wild-type lysogens. After incubation for 15 min the prophage induction of hcf lysogens reached a maximal induction, whereas the wild type reached a maximal induction at about 50 min.

Since the repair-deficient mutants were extremely sensitive to 4HAQO, 4HAQO damages genomes, and the damaged genome is repairable by wild-type enzymes. Greatly increased prophage induction of these strains also indicates the damage of bacterial genome.

These findings are practically the same as the results for 4NQO treatment reported previously (10). Thus, these results support the hypothesis that bacteria can convert 4NQO to

Chart 2. Search for activating enzyme of Salmonella typhimurium by fractionation with DEAE-cellulose column. a, protein fraction pattern at A280 (•—•) and phage inactivation activity (X—X); b, diaphorase activity (•—•) and 4NQO reducing activity (X—X).

Chart 3. Inactivation kinetics of phage P22c2 by 4NQO in the presence of NADH and the peak fraction of enzyme and recombination between damaged P22c2 and prophage P221bc+. The concentration of agents used were 0.1 mM 4NQO and 0.1 mM NADH. •, survivors; o, c+ recombinants.

Chart 4. Inactivation of phage P22c2 by 4HAQO and recombination between 4HAQO-damaged P22c2 and prophage P221bc+. 4HAQO, 1 mM, was used for this experiment. •, survivors; o, c+ plaques of recombinant.
Chart 5. Fractionation of rat liver enzyme with DEAE-cellulose column. a, protein fraction pattern at A_{280} (•—•) and phage inactivation (X--X); b, diaphorase activity (•—•) and 4NQO reducing activity (X--X).

shown in Chart 2a, one sharp peak for phage inactivation was found. Since 4HAQO formation is a reductive reaction, and Sugimura et al. (6) showed that rat liver diaphorase can convert 4NQO to 4HAQO, we examined each fraction for 4NQO reduction activity and for diaphorase activity. Peaks for 4NQO reducing activity and diaphorase activity shown in Chart 2b corresponded to the peak for the phage inactivation shown in Chart 2a.

For demonstrating that the inactivation of phage is due to damage of the phage genome, the protein fraction at the peak of phage inactivation was mixed with 4NQO, NADH, and phage P22c2. At various incubation intervals, samples were withdrawn and plated on P221 lysogens of various bacterial mutants. As shown in Chart 3, inactivation of phage P22 and a great increase of c+ recombinants were observed. This result is similar to the effect of 4HAQO on phage P22 as shown in Chart 4. In Chart 3, however, a short lag period for both inactivation and recombination was observed. This suggests that the formation and accumulation of 4HAQO were required for the inactivation of phage. Therefore, it is concluded that the bacterial diaphorase is able to convert 4NQO to 4HAQO and that 4HAQO reacts with genomes.

Activating Enzyme in Rat Liver. For a further test of the feasibility of detecting activating enzymes for 4NQO, this method was applied to rat liver cytosol. Chart 5a shows that rat liver contains 2 protein fractions with phage inactivation activity, the peaks of which also correspond with peaks of 4NQO reducing activity and diaphorase activity shown in Chart 5b. Evidently, liver contains 2 diaphorase isozymes separable by DEAE-cellulose chromatography.

DISCUSSION

The data presented in this paper show that a metabolic intermediate of 4NQO activated by diaphorase interacts with bacteriophage genomes to cause inactivation and recombination. Sugimura et al. (6) reported that mammalian diaphorase can convert 4NQO to 4HAQO and assumes this is the form in which this potent carcinogen interacts with genomes. In vitro treatment of phage showed that 4 HAQO does indeed interact with genomes and, together with the microbial assay method described in this paper, strongly points to diaphorase as an activating enzyme for 4NQO. This microbial assay system should provide an approach to rapid detection of activating enzymes for various other chemical carcinogens and should be a useful tool for understanding activation mechanisms and identification of various proximal carcinogens.

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