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PHOTOCONTROLLED BINDING OF CYTOCHROME *c* TO IMMOBILIZED SPIROPYRAN

ISAO KARUBE, YOSHIO ISHIMORI, and SHUICHI SUZUKI

Research Laboratory of Resources Utilization
Tokyo Institute of Technology
4259 Nagatsuda-machi, Midori-ku
Yokohama, Japan

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Agarose gel was modified with polyethyleneimine and β-1-[3,3-dimethyl-6′-nitrospiro-(indoline-2,2′-2*H*-benzopyran)] propionic anhydride and the binding of cytochrome *c* was investigated. Cytochrome *c* was not retained on agarose modified only with polyethyleneimine. However, cytochrome *c* interacted with the immobilized spiropyran during illumination, but only a relatively small amount was bound to the immobilized spiropyran in the dark. The maximum difference of cytochrome *c* binding was observed at pH 6.8. Forty-six percent of cytochrome *c* bound on immobilized spiropyran in the column under visible light was released from the gel in the dark. This released cytochrome *c* showed the same spectrum and properties as those of native cytochrome *c*.

INTRODUCTION

The selective isolation and purification of enzymes and other biologically important macromolecules by affinity chromatography exploits the unique biological property of these proteins to bind ligands specifically and reversibly (1). In practice, bound proteins can be isolated by eluting with a large amount of buffer solution including a linear NaCl gradient (2). However, rapid isolation of bound proteins is desirable for the practical application of chromatography.

Substances that undergo reversible color formation under irradiation with light are called photochromic compounds. We have previously found that photosensitive enzymes could be prepared by modifying enzymes with photochromic spiropyran compound (3,4). The polarity of spiropyran drastically changes with light. Therefore, photocontrol of binding and releasing of compounds is possible with light irradiation by using photochromic compounds as a ligand. This new technique may be applied to affinity chromatography in the near future.

Agarose gel was modified with polyethyleneimine and spiropyran. Photocontrolled binding of cytochrome *c* to immobilized spiropyran is examined in this paper.

MATERIALS AND METHODS

Materials

Agarose gel (Sepharose 4B) was purchased from Pharmacia Fine Chemicals. Polyethyleneimine (molecular weight 3000, 6.7 equiv amino groups/mol polyethyleneimine) was obtained from Nihon Shokubai Company. Cytochrome *c* (type III, from horse heart, oxidized form) was obtained from Sigma Chemical Company.

Photochromic Spiran

β-1-[3,3-Dimethyl-6'-nitrospiro-(indoline-2,2'-2*H*-benzopyran)] propionic anhydride was synthesized by the method described previously (4).

Coupling of Polyethyleneimine to Agarose Gel

In a well-ventilated hood, 20 g (wet) of well-washed agarose gel was mixed with 20 ml of water, and 6.0 g of finely divided cyanogen bromide was added at once to the stirred suspension. The pH of the suspension was maintained between 10.5 and 11.5. The reaction was completed after 10 min. The suspension was transferred quickly to a Buchner funnel and washed under suction with cold buffer. Polyethyleneimine, 4.5 g, was added to 20 ml of water containing activated agarose (pH 10) and was allowed to stand for 20 h at room temperature. After reaction, the gel was washed with 500 ml of distilled water. Bound polyethyleneimine was determined indirectly by the method of Okuyama and Satake (5). This treatment resulted in a derivative having 61 mg (2.1×10^{-5} mol) of polyethyleneimine per gram of wet agarose gel.

Coupling of Spiropyran Compound to Modified Agarose Gel

Fifteen grams of the modified agarose in 10 ml of distilled water was added to 40 ml of dioxane solution containing 600 mg of the anhydride. The reaction mixture was allowed to stand at room temperature for 20 h with stirring. After reaction, the suspension was transferred to a Buchner funnel and washed with a large volume of dioxane and distilled water. Bound spiropyran compound was determined indirectly spectrophotometrically (6). This reaction resulted in a derivative having 5.8×10^{-5} mol spiropyran compound/g wet modified agarose gel.

Determination of Cytochrome c

Cytochrome *c* was determined by the method of Lowry et al. (7).

Reaction of Cytochrome c with the Immobilized Spiropyran

Unless otherwise noted, the reaction of cytochrome *c* and the immobilized spiropyran was carried out in test tubes as follows: A reaction mixture of 10 ml of 0.05 M phosphate buffer (pH 6.8) containing 2.0 mg of cytochrome *c* and 200–250 mg (wet) of the immobilized spiropyran was incubated for 1.5 h under visible light (the surface of the test tube was 30,000 lux) at 4°C. Then the gel was separated by filtration and thoroughly washed with 0.05 M phosphate buffer. The gel was suspended in 10 ml of 0.05 M phosphate buffer (pH 6.8) and incubated in the dark for 3 h at 4°C. Then the gel was separated by filtration and thoroughly washed with 0.05 M phosphate buffer. Released cytochrome *c* was determined spectrophotometrically (7).

The reaction of cytochrome *c* and the immobilized spiropyran in the column (0.8 cm × 13.5 cm) was carried out as follows: 1.0 g of the immobilized spiropyran was charged in the column. The column was equilibrated with 0.05 M phosphate buffer (pH 6.8) and irradiated with visible light (the surface of the column was 30,000 lux). Then 2 mg of cytochrome *c* was applied to the column of the immobilized spiropyran under visible light. The column was equilibrated with 0.05 M phosphate buffer and allowed to stand in the dark for 20 h at 4°C. Elution was carried out with 0.05 M phosphate buffer (pH 6.8). Released cytochrome *c* was determined spectrophotometrically.

RESULTS

The spiropyran-modified agarose gel showed reverse photochromism (Fig. 1). It was colored red in the dark (Δ) and bleached with visible light irradiation ($h\nu$). Cytochrome *c* was not retained on agarose gel modified only with polyethyleneimine; however, it interacted with the immobilized spiropyran. Preliminary experiments showed that cytochrome *c* was bound to the immobilized spiropyran under illumination. However, a relatively small amount of cytochrome *c* was bound to the immobilized spiropyran in the dark.

The effect of pH on the binding of cytochrome *c* on the immobilized spiropyran is shown in Fig. 2. The experiments were performed in test tubes. As shown, the amount of cytochrome *c* bound on the immobilized spiropyran under illumination was larger than that in the dark in the pH range from

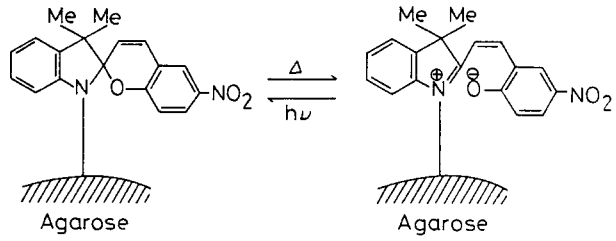

FIG. 1. Photoreversible isomerization of the spiropyran-modified agarose gel.

6 to 7. The maximum difference in cytochrome *c* binding was observed at pH 6.8.

The effect of the phosphate buffer concentration on the binding of cytochrome *c* under visible light and in the dark was examined at pH 6.8. The maximum difference in the cytochrome *c* binding was observed at 0.05 M. Further increase or decrease of the phosphate buffer concentration decreased the difference in cytochrome *c* binding on the immobilized spiropyran.

It was assumed that cytochrome *c* bound on the immobilized spiropyran under visible light was released from the gel in the dark. This assumption was examined by the column system. The elution pattern of cytochrome *c* released in the dark is shown in Fig. 3. This released cytochrome *c* showed the same spectrum as that of native oxidized cytochrome *c* (Fig. 4) and both showed an adsorption maximum at 695 nm, whereas denatured cytochrome

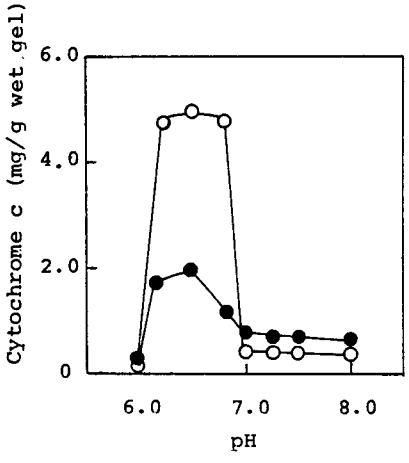

FIG. 2. Effect of pH on the binding of cytochrome *c* on the spiropyran-modified agarose gel. The reaction was carried out under standard conditions at various levels of pH. Phosphate buffer 0.05 M was employed. ○, Under visible light irradiation; ● in the dark.

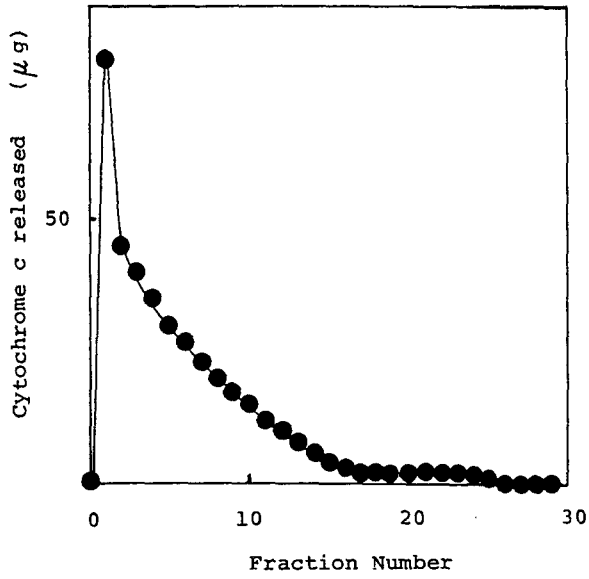

FIG. 3. Elution pattern of cytochrome *c*. Experiments were carried out under standard conditions. ●, Cytochrome *c* released in the dark.

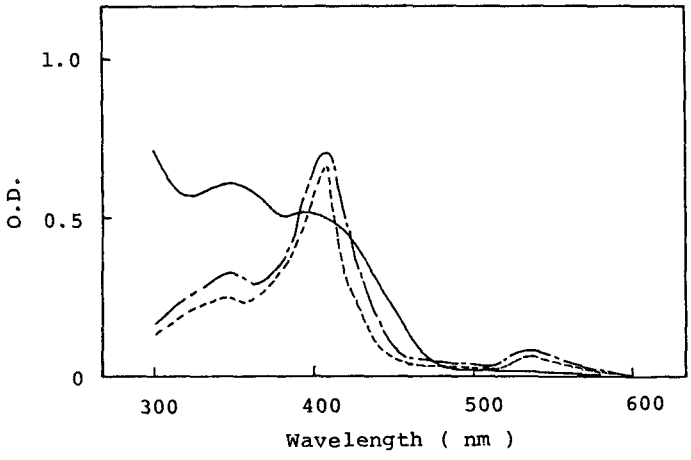

FIG. 4. Spectra of the released cytochrome *c*. Cytochrome *c* solutions, 2 μM, were employed for the experiments. — · — · —, Native cytochrome *c*; – – –, cytochrome *c* released in the dark; —— cytochrome *c* released with 50% ethylene glycol.

c does not show an adsorption maximum at that wavelength (8). Furthermore, released cytochrome *c* shows the adsorption maximum at 550 nm in the presence of a slight excess of sodium dithionate. Total amount of cytochrome *c* bound on the immobilized spiropyran in the column under visible light was 0.95 mg/g wet gel, and the amount of the cytochrome *c* released in the dark was 0.44 mg/g wet gel. Therefore, 46% of cytochrome *c* was released during incubation in the dark. Bound cytochrome *c* in the column was less than the amount of cytochrome *c* bound in test tube experiments (Fig. 2). This may be caused by the insufficient light irradiation (especially in the center region of the column).

The immobilized spiropyran was used repeatedly for experiments (Fig. 5). The experiments were performed as follows: Cytochrome *c* was applied to the column during visible light irradiation and the column was allowed to stand in the dark at 4°C for 20 h. The elution was carried out with phosphate buffer. The amount of cytochrome *c* bound during illumination was initially about 0.9 mg/g wet gel. However, the amount of cytochrome *c* bound under visible light and that released in the dark decreased gradually with increasing use cycles. As shown, cytochrome *c* was not released from the immobilized spiropyran after four cycles. Thereafter, the elution was carried out with 50% (vol/vol) ethylene glycol and 0.87 mg of cytochrome *c* was eluted from the column. The cytochrome *c* of the effluent shows a spectrum different

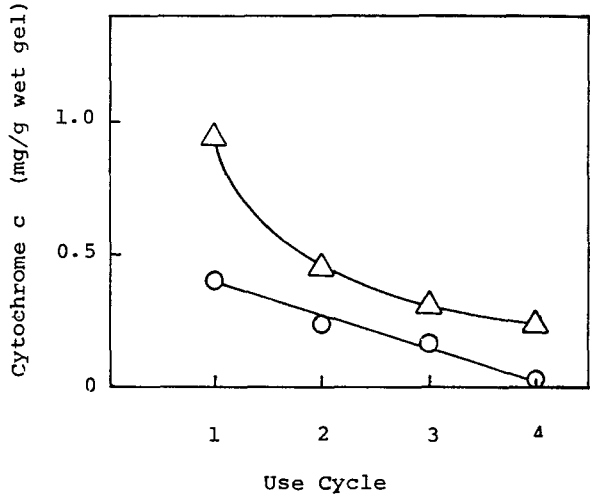

FIG. 5. Reusability of the spiropyran-modified agarose gel. △, cytochrome *c* bound under visible light; ○, cytochrome *c* released in the dark.

from that of native cytochrome *c* (Fig. 4). On the other hand, cytochrome *c* incubated with 50% ethylene glycol for 3 h showed the same spectrum as that of native cytochrome *c*.

DISCUSSION

This report describes photocontrol of the binding and releasing of cytochrome *c* on the immobilized spiropyran. First, cyanogen bromide (9) was employed for the direct coupling of β-1-[3,3-dimethyl-6′-nitrospiro-(indoline-2,2′-2*H*-benzopyran)] propionic anhydride to agarose. However, the amount of the spiropyran compound introduced to agarose gel was small. Thereafter, polyethyleneimine was used to premodify the agarose, and the amount of the spiropyran compound bound to PEI-modified agarose was about 10 times larger than that bound to plain agarose. As previously reported (3), the spiropyran compounds show photochromism and changes in polarity with light irradiation. Colorless spiropyrans (apolar) are converted into colored forms (red, polar) with u.v. light ($h\nu$) irradiation, and then they are converted into colorless forms with visible light ($h\nu'$) irradiation or when they are placed in the dark (Δ). The immobilized spiropyran showed reverse photochromism: Spiropyran compounds show reverse photochromism in organic solvents having higher polarity than ethanol (6). These results are attributed to the stabilization of ground state of the colored form due to the interaction with solvent. Since agarose gel is hydrophilic, the immobilized spiropyran showed the reverse photochromism in a similar manner as enzyme–spiropyran membranes (4). Colorless spiropyran under visible light converts into a colored form (polar) when placed in the dark. The results show that the amount of cytochrome *c* bound on the immobilized spiropyran under visible light is larger than that in the dark (Fig. 2) and that cytochrome *c* bound on the immobilized spiropyran under visible light was released during incubation in the dark (Fig. 3). Since no interaction was observed between agarose gel modified with polyethyleneimine and cytochrome *c*, it can be concluded that cytochrome *c* binds to the immobilized spiropyran moiety.

Since spiropyran derivatives are hydrophobic under visible light, it is assumed that the interaction between cytochrome *c* and the immobilized spiropyran is due to hydrophobic forces. The binding of cytochrome *c* on the immobilized spiropyran is influenced by both pH and concentration of the buffer which affect the conformation of cytochrome *c* and its binding to the immobilized spiropyran. Bound cytochrome *c* is released in the dark (spiropyran derivative has charges). Therefore, cytochrome *c* bound on spiropyran derivatives may be released with the ionic repulsion between

spiropyran compounds and cytochrome *c*. However, the detailed mechanism of the binding and releasing of cytochrome *c* is far from understood.

Cytochrome *c* released in the dark showed the same spectrum and properties as those of native cytochrome *c*. This suggests that no property changes occur in cytochrome *c* during the binding and releasing process.

The amount of the cytochrome *c* bound under visible light and that released in the dark gradually decreased with increasing use cycles (Fig. 5).

However, most of the unreleased cytochrome *c* (97%) was eluted with 50% of ethylene glycol, and the released cytochrome *c* showed a different spectrum from that of native cytochrome *c* (Fig. 4). Therefore, we have only been able to elute it in a denatured form. No difference in the spectrum was observed between native cytochrome *c* and ethylene glycol-treated cytochrome *c*. The possibility of conformational change in the proteins upon binding on the hydrophobic ligand was suggested by Yon and Simmonds (10). Therefore, the conformation of cytochrome *c* may be changed upon binding on the immobilized spiropyran. As reported previously (4), irreversible conformational changes of the spiropyran derivatives occur during light irradiation. Furthermore, the ratio of the irreversibly isomerized spiropyran derivatives increases with increasing use cycles. Therefore, it is possible that cytochrome *c* bound on the irreversibly isomerized spiropyran derivatives is not released in the dark and is released with ethylene glycol. It is known that ethylene glycol decreases the polarity of the medium and tends to weaken hydrophobic binding. Thus, the association of denatured cytochrome *c* and the immobilized spiropyran may also be a hydrophobic one.

Further developmental studies in this laboratory are being directed toward applying the photocontrol of binding and releasing to affinity chromatography.

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