# Properties of Crystalline Reduced Nicotinamide Adenine Dinucleotide Phosphate-adrenodoxin Reductase from Bovine Adrenocortical Mitochondria. II. Essential Histidyl and Cysteinyl Residues at the NADPH Binding Site of NADPH-adrenodoxin Reductase†

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ABSTRACT: The binding site of NADPH in NADPH-adrenodoxin reductase was examined using crystalline enzyme from bovine adrenocortical mitochondria by studies on the effects of photooxidation and chemical modifications of amino acid residues in the reductase. (1) Photooxidation decreased the enzymatic activity of NADPH-adrenodoxin reductase. Photooxidation of the reductase was prevented by $NADP^+$, adrenodoxin, or reduced glutathione, but not $NAD^+$. Photoinactivation caused loss of a histidyl residue, but not of tyrosyl, tryptophanyl, cysteinyl, or methionyl residues of the reductase. It did not affect the circular dichroism spectrum of the reductase appreciably. (2) NADPH-adrenodoxin reductase activity was inhibited by diethyl pyrocarbonate and the inhibition was partially reversed by addition of hydroxylamine. The inhibition was prevented by $NADP^+$, but not $NAD^+$. (3) NADPH-adrenodoxin reductase activity was inhibited by 5,5′-dithiobis(2-nitrobenzoate) and the inhibition was reversed by reduced glutathione. It was also protected by $NADP^+$, but not $NAD^+$. The results indicate that a histidyl residue and a cysteinyl residue of NADPH-adrenodoxin reductase are essential for the binding of NADPH by the reductase.

NADPH[1]-adrenodoxin reductase is a component of the monooxygenase system for steroid hydroxylation in adrenocortical mitochondria (Nakamura et al., 1966; Omura et al., 1966). To elucidate the mechanism of the monooxygenase system and of steroid hydroxylation, the interactions between NADPH-adrenodoxin reductase and its substrate and substrate derivatives were investigated. The interaction between NADPH-adrenodoxin reductase and adrenodoxin and the interaction between flavin and the aporeductase have been reported previously (Hiwatashi et al., 1976).

This paper describes studies on the NADPH binding site of NADPH-adrenodoxin reductase which examined the effects of photooxidation and of chemical modification of amino acid residues on enzyme activity and the protective effects of various compounds.

## Materials and Methods

*Materials.* Crystalline NADPH-adrenodoxin reductase was prepared from bovine adrenocortical mitochondria as reported previously (Hiwatashi et al., 1976). The specific activities of adrenodoxin-linked NADPH-cytochrome *c* reductase and NADPH-ferricyanide reductase were 8.8 and 20.0 $\mu$mol $min^{-1}$ $mg^{-1}$ protein, respectively, at 25 °C. Adrenodoxin was crystallized from bovine and pig adrenocortical mitochondria (Ichikawa, 1971; Suhara et al., 1972; Estabrook et al., 1973) and ferredoxin from spinach leaves (Tagawa and Arnon, 1962). Crystalline catalase was prepared from bovine liver (Shirakawa, 1953) and superoxide dismutase from bovine erythrocytes (McCord and Fridovich, 1969).

*Chemicals.* NADPH, $NADP^+$, $NAD^+$, and cytochrome *c* (type III) were purchased from Sigma Chemical Co. Sulfhydryl reagents and diethyl pyrocarbonate were obtained from Nakarai Chemical Co. FAD was obtained from Nakarai Chemical Co. and purified by the method of Massey and Swoboda (1963). All other reagents were of the highest purity available commercially.

*Assays of Enzymatic Activities.* The enzymatic activities of NADPH-adrenodoxin reductase were measured at 25 °C as reported previously (Hiwatashi et al., 1976), unless otherwise stated.

*Measurements of Optical Absorption Spectra.* Optical absorption spectra were measured in a Cary Model 17 spectrophotometer with a thermostatically controlled cell holder and a cuvette of 1-cm light path. A neodymium glass filter was used as a standard of wavelength.

*Measurements of Circular Dichroism Spectra.* The circular dichroism spectra of NADPH-adrenodoxin reductase solutions before and after photooxidation were measured at a sensitivity of $s = 1 \times 10^{-5}$/mm, using a sample cuvette of 1-cm light path.

*Electrophoresis.* Electrophoresis was carried out as reported previously (Hiwatashi et al., 1976).

*Analytical Procedures.* The molar extinction coefficients of NADPH and NADH were taken as $6.22 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 340 nm and those of $NADP^+$ and $NAD^+$ as $18.0 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 260 nm (Okunuki and Nozaki, 1956). Protein concentration was determined by the biuret reaction (Gornall et al., 1949) and by the method of Lowry et al. (1951), using bovine serum albumin as a standard. The concentration of NADPH-adrenodoxin reductase was determined by the molar extinction coefficient of $11.3 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 450 nm (Hiwatashi et al., 1976). The concentrations of adrenodoxin and ferredoxin were estimated by their molar extinction coefficients of $9.8 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 414 nm (Ichikawa, 1971) and $9.68 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 423 nm (Tagawa and Arnon, 1968), respectively. The molar extinction coefficient of reduced minus oxidized cytochrome *c* was taken as $18.5 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 550 nm (Margoliash, 1954) and that of oxidized FAD as $11.3 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 450 nm (Yagi and

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[1] Abbreviations used: NAD, nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide.

Matsuoka, 1956). The concentration of *p*-chloromercuribenzoate was determined spectrophotometrically, assuming that the molar extinction coefficient was $16.9 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 232 nm at pH 7.0 (Boyer, 1954). The sulfhydryl content of proteins was determined using a molar extinction coefficient of $7.6 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 250 nm at pH 7.0 (Boyer, 1954). The purity of diethyl pyrocarbonate was determined by the method of Ovadi et al. (1967). The molar extinction coefficient of the diethyl pyrocarbonate complex with histidine or histidyl residues of proteins was taken as $3.2 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 240 nm between pH 6.0 and 8.0 at 25 °C.

*Photooxidation Procedure.* Test solutions in a 20-ml beaker under air were illuminated with a tungsten 100-W spot light lamp at the distance of 8 cm above the surface. The test solutions were stirred gently with a magnetic stirrer during illumination. A solution of 0.2 M cupric sulfate, of 15 mm depth, was used as a screen to exclude infrared radiations in the light. Photochemical reactions were carried out at 0 °C. Change in temperature during illumination was prevented by circulation of cold water. The light beam intensity of the illumination was $1.1 \times 10^3$ erg $mm^{-2}$ $s^{-1}$, determined with a dc breaker amplifier (Beckman Model-14) (Kondo and Jagger, 1966).

*Photooxidation of NADPH-adrenodoxin Reductase.* A solution of 2 mg of NADPH-adrenodoxin reductase and 0.25 mg of methylene blue in 5 ml of 10 mM potassium phosphate buffer, pH 7.9, was illuminated, and samples were taken at intervals for determinations of enzymatic activity, amino acid composition, and the circular dichroism spectrum and for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Results were compared with those for control samples of a solution kept in the dark under air. After illumination, the methylene blue was removed by dialysis for 10 h at 2 °C in the dark. When the enzyme was illuminated in the presence of adrenodoxin, the adrenodoxin was removed from the sample before amino acid analysis. This was achieved by applying the sample to a DEAE-cellulose column (1 × 8 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.4, washing the column with 100 ml of ice-cold solution of the same buffer, and then eluting the yellow band of reductase with 50 mM potassium phosphate buffer, pH 7.4.

*Amino Acid Analysis.* For amino acid analysis, a solution of 0.5 mg of the untreated or photoinactivated NADPH-adrenodoxin reductase was frozen and then rapidly lyophilized under anaerobic conditions. The residue was dissolved in 1 ml of 6 N HCl in the absence or presence of 2% thioglycolic acid (Matsubara and Sasaki, 1969) and hydrolyzed at 110 °C for 24 h in an evacuated, sealed ampule. Then the solution was evaporated to dryness in a desiccator, and the residue was dissolved in 1.5 ml of 0.2 M sodium citrate-HCl buffer, pH 2.2, and 0.5 ml of the solution was applied to a Hitachi KLA-3B amino acid analyzer. The content of tryptophanyl residues of the reductase was also estimated by the method of Beaven and Holiday (1952) as reported previously (Hiwatashi et al., 1976).

*Content of Sulfhydryl Groups in NADPH-adrenodoxin Reductase.* The number of sulfhydryl groups in NADPH-adrenodoxin reductase was determined by the methods of Ellman (1959) and Boyer (1954) as reported previously (Hiwatashi et al., 1976).

*Content of Histidyl Residues in NADPH-adrenodoxin Reductase.* Diethyl pyrocarbonate was dissolved in cold ethanol and its concentration in fresh solution was determined spectrophotometrically. The reductase solution was incubated with 0.5 mM diethyl pyrocarbonate for 20 min at 25 °C. Modification of histidyl residues was followed by measuring the difference between the absorbance at 240 nm of the modified sample in the sample cell and that of an unmodified sample treated with cold ethanol, in the reference cell. A molar extinction coefficient of $3.2 \times 10^3$ $M^{-1}$ $cm^{-1}$ at 240 nm for free histidine was used to determine the content of histidyl residues of the reductase.

*Sugar Analysis.* The sugar composition of NADPH-adrenodoxin reductase was determined as described previously (Hiwatashi et al., 1976).

## Results

*Effects of the Concentrations of NADPH-adrenodoxin Reductase and Methylene Blue on its Photooxidation.* Reductase solutions at concentrations of 0.05 to 2.50 mg/ml were illuminated for 10 min in the presence of 50 μg/ml of methylene blue. When the residual activities of the solutions after illumination were measured, photoinactivation of the reductase increased with decreases in its concentration to a constant rate at concentrations of less than 1.25 mg of protein per ml under the experimental conditions used.

NADPH-adrenodoxin reductase solution containing 400 μg of protein per ml was illuminated in the presence of various concentrations (0–100 μg/ml) of methylene blue for 10 min. The concentration of methylene blue was rate limiting up to a concentration of 25 μg/ml. Based on these results, 400 μg of reductase per ml and 50 μg of methylene blue per ml were used in subsequent experiments.

*Effect of pH on the Photooxidation of NADPH-adrenodoxin Reductase in the Presence of Methylene Blue.* NADPH-adrenodoxin reductase was dissolved in 10 mM potassium phosphate buffer of pH 5.8 to 8.0. The solutions were kept at 0 °C for 10 min and then illuminated as described in the Methods and Materials. The pH dependency of photooxidation of the reductase with methylene blue gave a sigmoidal curve. The optimum pH of the photooxidation of the NADPH-adrenodoxin reductase in the presence of methylene blue was 7.9. This curve for photooxidation of the reductase with methylene blue at different pH values was similar to those obtained in experiments on the photooxidation of other enzymes; the optimum pH values of photooxidation were alkaline (Weil et al., 1952; Ichikawa and Yamano, 1963).

*Kinetics of the Photooxidation of NADPH-adrenodoxin Reductase.* Decrease in the NADPH-cytochrome *c* reductase activity of NADPH-adrenodoxin reductase during photooxidation followed first-order kinetics for the first 20 min with an apparent rate of $8.1 \times 10^{-2}$ $min^{-1}$ (Figure 1). But on longer illumination, the rate of photoinactivation of the reductase gradually decreased. A control solution kept in the dark showed no loss of reductase activity. The apparent first-order rate constant of photooxidation of histidyl residues of the reductase was $1.9 \times 10^{-2}$ $min^{-1}$. The photooxidations of amino acids and proteins with methylene blue have been investigated in detail by Weil et al. (1952, 1953) and Weil and Seibles (1955), and their results showed that histidyl, tryptophanyl, tyrosyl, and cysteinyl residues of proteins were very susceptible to photooxidation. However, as shown in Figure 1, no change in the contents of tryptophanyl, tyrosyl, or cysteinyl residues in NADPH-adrenodoxin reductase was observed, although specific decomposition of the histidyl residue of the reductase was observed on photooxidation for 20 min.

*Effects of Photooxidation on the Amino Acid, Flavin, and Sugar Compositions of NADPH-adrenodoxin Reductase.* The amino acid and sugar compositions and FAD content of NADPH-adrenodoxin reductase were measured before and after photooxidation. The contents of tryptophanyl and cysteinyl residues of the reductase were determined spectropho-

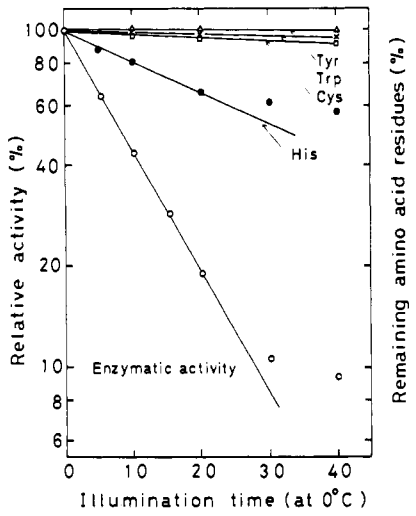


FIGURE 1: Inactivation of NADPH-adrenodoxin reductase and loss of constituent amino acid residues with time during illumination. A solution of 2 mg of NADPH-adrenodoxin reductase in 5 ml of 10 mM potassium phosphate buffer, pH 7.9, was illuminated in the presence of 50 $\mu$g/ml methylene blue. Samples were taken at intervals for determinations of reductase activity and amino acid composition.

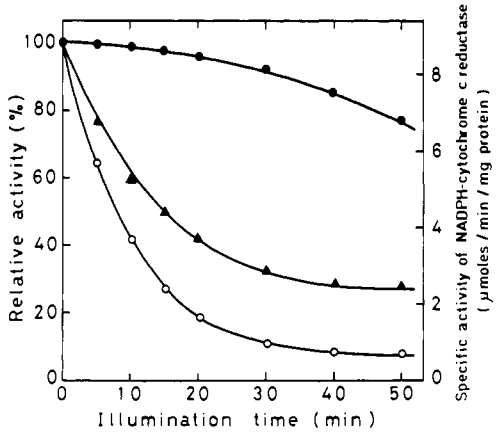


FIGURE 2: Effects of $NAD^+$ and $NADP^+$ on the photooxidation of NADPH-adrenodoxin reductase. NADPH-adrenodoxin reductase was dissolved in 10 mM potassium phosphate buffer, pH 7.9. The solution was preincubated with or without 50 $\mu$M $NAD^+$ or $NADP^+$ for 10 min at 0 °C, and then illuminated in the presence of 50 $\mu$g/ml methylene blue. (○) No addition; (▲) with 50 $\mu$M $NAD^+$; (●) with 50 $\mu$M $NADP^+$.

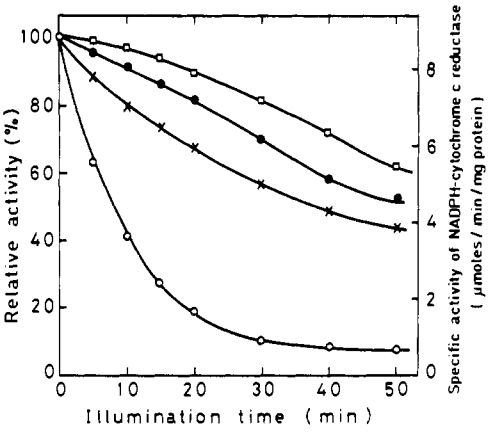


FIGURE 3: Effect of adrenodoxin on the photooxidation of NADPH-adrenodoxin reductase. NADPH-adrenodoxin reductase in 10 mM potassium phosphate buffer, pH 7.9, with or without 8 $\mu$M adrenodoxin or ferredoxin was kept for 10 min at 0 °C and then illuminated in the presence of 50 $\mu$g/ml methylene blue. (○) No addition; (□) with 8 $\mu$M bovine adrenodoxin; (●) with 8 $\mu$M pig adrenodoxin; (X) with 8 $\mu$M spinach ferredoxin.

tometrically. During photooxidation, the content of histidyl residues decreased to 68% after illumination for 20 min, but the contents of sulfhydryl groups, cysteinyl residues, and other amino acid residues did not change. In addition, the sugar compositions and FAD content of the reductase were not affected by the experimental illumination, which was sufficient to inactivate 80% of the reductase activity.

*Effects of $NADP^+$ and Adrenodoxin during Photooxidation of the NADPH-adrenodoxin Reductase.* Figure 2 shows the effects of $NAD^+$ and $NADP^+$ on the photooxidation of NADPH-adrenodoxin reductase. Without addition, the activity of adrenodoxin-linked NADPH-cytochrome *c* reductase decreased to 20% of the initial activity during photooxidation for 20 min at 0 °C. In the presence of 50 $\mu$M $NADP^+$, the activity only decreased to 92% of the original activity. In the presence of 50 $\mu$M $NAD^+$, decrease in activity was similar to that without additions. Thus $NADP^+$ protected the reductase from photooxidation, but $NAD^+$ did not. Similar results were obtained using ferricyanide as an electron acceptor instead of adrenodoxin and cytochrome *c*.

The photoinactivation curves of NADPH-adrenodoxin reductase and the complex with adrenodoxin are shown in Figure 3. The complex of the reductase with adrenodoxin lost only 13% of its NADPH-cytochrome *c* reductase activity during photooxidation for 20 min. Thus the activity is protected considerably against photooxidation by formation of a complex with adrenodoxin. Pig adrenodoxin and spinach ferredoxin as well as bovine adrenodoxin protected the reductase against photoinactivation.

*Circular Dichroism Spectra of Photooxidized NADPH-adrenodoxin Reductase.* Figure 4 shows the circular dichroism spectra of NADPH-adrenodoxin reductase before and after the photoinactivation. No significant change in the spectrum of the reductase in the visible or ultraviolet region during photooxidation was detected. These results do not exclude the possibility of a slight conformational change of the reductase during photooxidation which does not affect the circular dichroism spectrum.

*Effect of Diethyl Pyrocarbonate on NADPH-adrenodoxin Reductase.* Figure 5 shows that the activity of NADPH-adrenodoxin reductase was inhibited by rather low concentrations of diethyl pyrocarbonate in 0.1 M potassium phosphate buffer, pH 7.4 at 25 °C. The difference spectrum in the ultraviolet region of diethyl pyrocarbonate-treated reductase minus untreated reductase showed the characteristic difference spectrum of carboethoxyhistidine only, with a sharp maximum between 238 and 243 nm and no significant peak between 250 and 300 nm. The content of histidyl residues of the modified reductase is shown in Figure 6 as a function of the enzymatic activity. Extrapolation of the reductase activity to zero shows the extent of carboethoxylation of histidyl residues per mole of the reductase. There is a stoichiometric relation between loss of activity and modification of one histidyl residue of the reductase until less than 40% of the initial activity remains. The time courses of inactivation of the reductase with 80 $\mu$M diethyl pyrocarbonate, at pH 7.4 and 25 °C, with and without $NADP^+$ or $NAD^+$, are shown in Figure 7. Without additions and in the presence of 50 $\mu$M $NAD^+$, the activity of NADPH-adrenodoxin reductase decreased to 45% of the initial value on incubation with 80 $\mu$M diethyl pyrocarbonate at 25

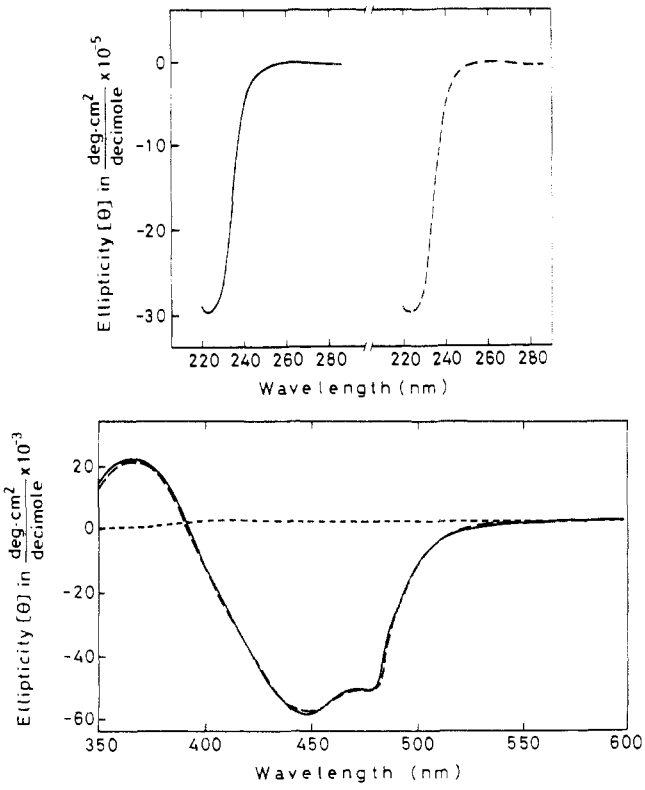


FIGURE 4: Circular dichroism spectra of NADPH-adrenodoxin reductase before and after photooxidation. A solution of 400 $\mu$g/ml of NADPH-adrenodoxin reductase in 10 mM potassium phosphate buffer, pH 7.9, was illuminated for 20 min in the presence of 50 $\mu$g/ml methylene blue. Then the methylene blue was removed by dialysis for 10 h at 2 °C in the dark. Measurements were performed at a sensitivity of $s = 1 \times 10^{-5}$/mm, using a sample cuvette with a light path of 20.0 mm for the visible region and of 10.0 mm for the ultraviolet region. The concentration of NADPH-adrenodoxin reductase was 5 $\mu$M for the visible region and 1 $\mu$M for the ultraviolet region. (—) Before photooxidation; (— — —) after photooxidation; (- - -) baseline.

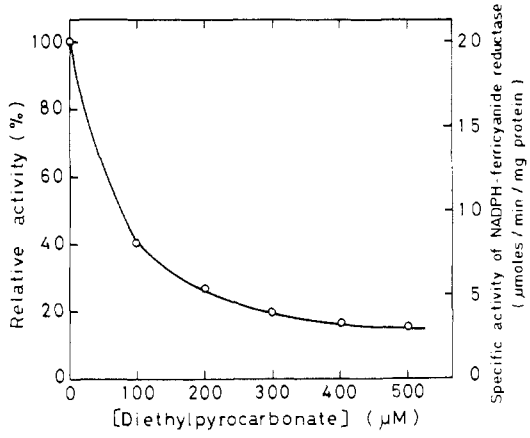


FIGURE 5: Inhibition of NADPH-adrenodoxin reductase activity by diethyl pyrocarbonate. The reaction mixture contained in a final volume of 1.0 ml, 400 $\mu$g of NADPH-adrenodoxin reductase, potassium phosphate buffer, pH 7.4 (100 $\mu$mol), and diethyl pyrocarbonate (0 to 500 nmol as indicated). After incubation for 30 min at 25 °C, samples (0.01 ml) of the mixture were diluted with 2.0 ml of the standard mixture for NADPH-ferricyanide reductase assay.

°C for 20 min. However, in the presence of 50 $\mu$M $NADP^+$, only 15% of the original activity was lost.

*Reversal of Inhibition by Diethyl Pyrocarbonate of NADPH-adrenodoxin Reductase.* When the activity of

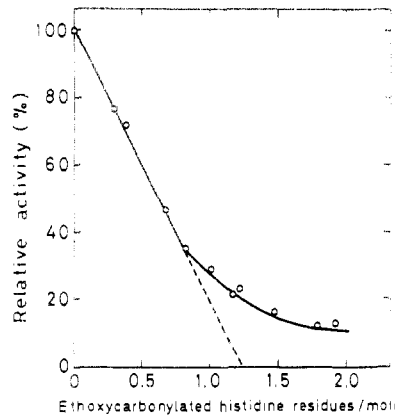


FIGURE 6: Relationship between NADPH-adrenodoxin reductase activity and the number of histidyl residues modified by treatment with various concentrations of diethyl pyrocarbonate. The reaction mixture contained in a final volume of 1.0 ml: 400 $\mu$g of NADPH-adrenodoxin reductase, potassium phosphate buffer, pH 7.4 (100 $\mu$mol), and diethyl pyrocarbonate (0 to 800 nmol). After incubation for 30 min at 25 °C, NADPH-ferricyanide reductase activity and the histidine content of the reductase were determined.

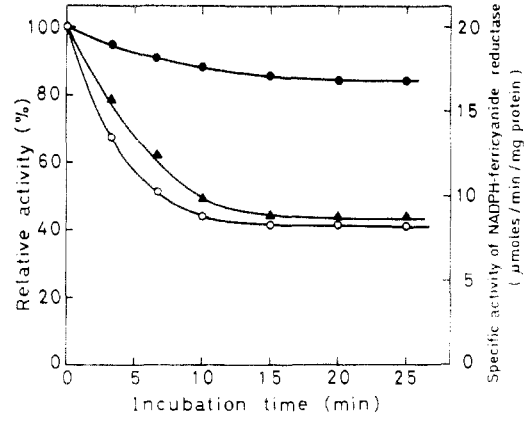


FIGURE 7: Time courses of inactivation of NADPH-adrenodoxin reductase on treatment with diethyl pyrocarbonate with and without $NAD^+$ or $NADP^+$. Solutions of 400 $\mu$g/ml of NADPH-adrenodoxin reductase in 0.1 M potassium phosphate buffer, pH 7.4, with and without 50 $\mu$M $NAD^+$ or $NADP^+$ were incubated for 10 min at 25 °C. Then 80 $\mu$M diethyl pyrocarbonate was added and the mixtures were incubated at 25 °C for the indicated times. (○) No addition; (▲) with 50 $\mu$M $NAD^+$; (●) with 50 $\mu$M $NADP^+$.

NADPH-adrenodoxin reductase had been inhibited 80% by addition of 0.5 mM diethyl pyrocarbonate, 60% of the initial activity could be restored by dilution of the mixture with 3 volumes of 0.1 M potassium phosphate buffer, pH 7.4 at 0 °C, containing 1 M hydroxylamine, incubation for 2 h at 25 °C, and then dialysis for 12 h at 2 °C in the dark to remove the hydroxylamine. No activity could be restored by dialysis in the absence of hydroxylamine.

*Effects of Sulfhydryl Reagents on NADPH-adrenodoxin Reductase Activity.* NADPH-adrenodoxin reductase activity was inhibited by various sulfhydryl reagents such as 5,5′-dithiobis(2-nitrobenzoate), *p*-chloromercuribenzoate, *N*-ethylmaleimide, mercury chloride, mersalyl acid, and iodoacetate. Results on the effects of these compounds are summarized in Table I. 5,5′-Dithiobis(2-nitrobenzoate), *p*-chloromercuribenzoate, mercury chloride, and mersalyl acid were strongly inhibitory. However, *N*-ethylmaleimide and iodoacetate were only weakly inhibitory. After inhibition by *p*-chloromercuribenzoate, 80% of the original activity could be restored by

TABLE I: Effects of Sulfhydryl Reagents on NADPH-adrenodoxin Reductase Activity.

| Sulfhydryl Reagent | $K_i$[a] (M) | Restoration[b] (%) |
|---|---|---|
| Mersalyl acid | $4 \times 10^{-7}$ | 75 |
| Mercury chloride | $5 \times 10^{-7}$ | 78 |
| *p*-Chloromercuribenzoate | $2 \times 10^{-6}$ | 80 |
| 5,5′-Dithiobis(2-nitrobenzoate) | $1 \times 10^{-5}$ | 62 |
| *N*-Ethylmaleimide | $2 \times 10^{-3}$ | 24 |
| Iodoacetate | $5 \times 10^{-3}$ | 15 |

[a] NADPH-adrenodoxin reductase ($1 \times 10^{-7}$ M) was incubated with various sulfhydryl reagents for 30 min in 0.1 M potassium phosphate buffer, pH 7.4, at 25 °C, before addition of the reductase to the reaction mixture. The NADPH-ferricyanide reductase activity of NADPH-adrenodoxin reductase was assayed in the presence of the sulfhydryl reagents. The column labeled $K_i$ shows the concentration of sulfhydryl reagent giving 50% inhibition. [b] After inhibition by $8 \times 10^{-6}$ M sulfhydryl reagent, the reductase was incubated with $1 \times 10^{-3}$ M reduced glutathione for 60 min in 0.1 M potassium phosphate buffer, pH 7.4, at 25 °C. The experiments were done three times and this table shows the average values.

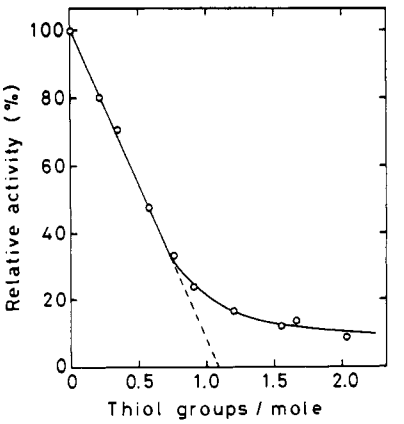


FIGURE 9: Relationship between NADPH-adrenodoxin reductase activity and the number of cysteinyl residues modified by treatment with various concentrations of 5,5′-dithiobis(2-nitrobenzoate). The reaction mixture contained in a final volume of 1.0 ml: 400 μg of NADPH-adrenodoxin reductase, potassium phosphate buffer, pH 7.4 (100 μmol), and 5,5′-dithiobis(2-nitrobenzoate) (0 to 150 nmol). After incubation for 30 min at 25 °C, NADPH-ferricyanide reductase activity and the cysteine content of the reductase were determined.

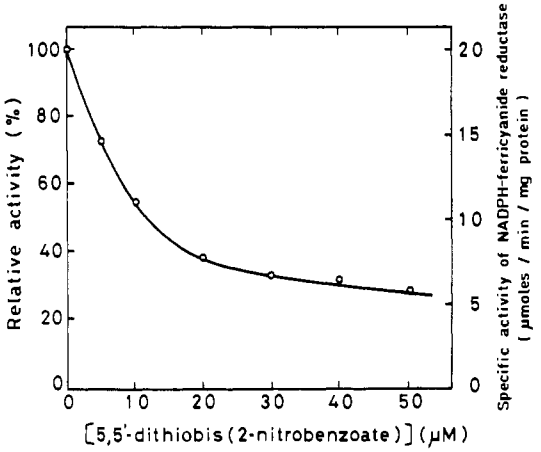


FIGURE 8: Inhibition of NADPH-adrenodoxin reductase activity by 5,5′-dithiobis(2-nitrobenzoate). The reaction mixture contained in a final volume of 1.0 ml: 400 μg of NADPH-adrenodoxin reductase, potassium phosphate buffer, pH 7.4 (100 μmol), and 5,5′-dithiobis(2-nitrobenzoate) (0 to 50 nmol as indicated). After incubation for 30 min at 25 °C, samples (0.01 ml) of the mixture were diluted with 2.0 ml of the standard mixture for NADPH-ferricyanide reductase assay.

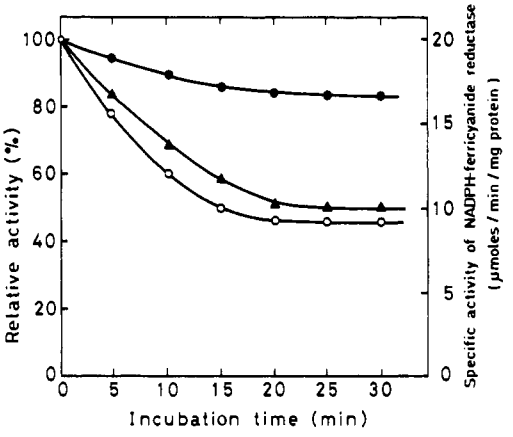


FIGURE 10: Time courses of inactivation of NADPH-adrenodoxin reductase on treatment with 5,5′-dithiobis(2-nitrobenzoate) with and without $NAD^+$ or $NADP^+$. Solutions of 400 μg/ml of NADPH-adrenodoxin reductase in 0.1 M potassium phosphate buffer, pH 7.4, with and without 50 μM $NAD^+$ or $NADP^+$ were incubated for 10 min at 25 °C; then 10 μM 5,5′-dithiobis(2-nitrobenzoate) was added and the mixtures were incubated at 25 °C for the indicated times. (○) No addition; (▲) with 50 μM $NAD^+$; (●) with 50 μM $NADP^+$.

addition of reduced glutathione.

Figure 8 shows that NADPH-adrenodoxin reductase was readily inactivated by 5,5′-dithiobis(2-nitrobenzoate). The optical absorption spectrum of the reductase after treatment with 0.1 mM 5,5′-dithiobis(2-nitrobenzoate) showed increased absorbance at 412 nm. Figure 9 shows the relation between modification of cysteinyl residues and loss of reductase activity. Extrapolation of the reductase activity to zero indicates that one cysteinyl residue was modified per mole of the reductase. There was a stoichiometric relation between decrease in reductase activity and modification of cysteinyl residues until about 35% of the initial activity remained.

Figure 10 shows the time courses of inhibition of the reductase activity with 10 μM 5,5′-dithiobis(2-nitrobenzoate), with and without 50 μM $NADP^+$ or $NAD^+$. Without additions or with $NAD^+$, the activity of NADPH-ferricyanide reductase was inhibited about 55% by 10 μM 5,5′-dithiobis(2-nitrobenzoate). However, in the presence of 50 μM $NADP^+$, the inactivation of NADPH-ferricyanide reductase was only 18%.

The number of cysteinyl residues per mole of reductase was found to be six in the presence of 8 M guanidine hydrochloride as reported previously (Hiwatashi et al., 1976). Thus two cysteinyl residues of the reductase were sensitive to 5,5′-dithiobis(2-nitrobenzoate), and so appeared to be present on the surface of the reductase whereas the other four cysteinyl residues were buried inside the molecule. The same result was obtained using *p*-chloromercuribenzoate instead of 5,5′-dithiobis(2-nitrobenzoate).

Table II shows the binding of 5,5′-dithiobis(2-nitrobenzoate) to NADPH-adrenodoxin reductase and the modification of that binding by prior treatment of the reductase with diethyl pyrocarbonate. The binding of sulfhydryl reagent to the reductase was inhibited by previous modification of the reductase with diethyl pyrocarbonate. This suggests that a sulfhydryl group of the reductase is present near the histidyl residue of

TABLE II: The Binding of 5,5′-Dithiobis(2-nitrobenzoate) to NADPH-adrenodoxin Reductase and the Modification of that Binding by Prior Treatment of the Reductase with Diethyl Pyrocarbonate.

| | $\Delta OD_{412}$[a] |
|---|---|
| NADPH-adrenodoxin reductase | 0.063 |
| NADPH-adrenodoxin reductase + 5,5′-dithibis(2-nitrobenzoate) | 0.314 |
| NADPH-adrenodoxin reductase + diethyl pyrocarbonate + 5,5′-dithiobis(2-nitrobenzoate) | 0.110 |

[a] Reductase solution (0.5 mg of protein/ml in 0.1 M potassium phosphate buffer, pH 7.4) with and without previous treatment with 0.5 mM diethyl pyrocarbonate for 30 min at 25 °C was incubated in the presence of 0.5 mM 5,5′-dithiobis(2-nitrobenzoate) for 30 min at 25 °C and then the absorbance at 412 nm was measured spectrophotometrically. The experiments were done three times and this table shows the average values.

NADPH-adrenodoxin reductase and that the sulfhydryl group and histidyl residue are essential for NADPH-adrenodoxin reductase activity.

## Discussion

In this work we found that photooxidation of a histidyl residue and chemical modifications of a histidyl residue and sulfhydryl group of NADPH-adrenodoxin reductase were closely related to inactivation of the reductase. Photooxidation did not affect the contents of other amino acid residues in the reductase. We also found that the reductase activity was inhibited reversibly by diethyl pyrocarbonate. $NADP^+$ protected the reductase against photoinactivation and inhibition by diethyl pyrocarbonate. $NAD^+$ had no protective effect. This means that only bound pyridine nucleotide has a specific protective effect for $NADP^+$, because the $K_m$ of NADPH-adrenodoxin reductase for $NADP^+$ is much smaller than that for $NAD^+$ (Chu and Kimura, 1973). These results support the idea that a histidyl residue of the reductase is necessary for its binding to NADPH.

The NADPH-adrenodoxin reductase has nine histidyl residues per mole. Figure 4 shows that photooxidation affected three to four of these histidyl residues and that its effect was nonspecific, affecting parts of all nine residues. Assuming that the apparent $pK_a$ is defined by the pH value at which the half-maximum rate of photoinactivation of the reductase is observed, the apparent $pK_a$ value of 6.6 of photoinactivation of the reductase corresponds to the $pK$ of the histidyl residue in the reductase and reflects the normal ionization of an imidazole moiety. NADPH-adrenodoxin reductase was also inactivated by diethyl pyrocarbonate and this inactivation was reversed by hydroxylamine. This shows that a histidyl residue of the reductase is essential for its activity. Similar results were obtained with sulfhydryl reagents. It has been reported that a histidyl residue of several oxidases (D-amino acid oxidase (Ichikawa and Yamano, 1963; Thomé-Beau et al., 1971), L-amino acid oxidase (Thomé-Beau et al., 1971), and monoamine oxidase (Hiramatsu et al., 1975), is essential for the activities of these enzymes.

The above results suggest that a histidyl residue and a sulfhydryl group of NADPH-adrenodoxin reductase may have catalytic roles in removal of a proton from the substrate of the reductase. However, before concluding that a histidyl residue and sulfhydryl group of the reductase are obligatory for the binding of NADPH, the following possibilities must be considered: (1) The structure of NADPH-adrenodoxin reductase may undergo a conformational change on photooxidation of a histidyl residue or chemical modification with diethyl pyrocarbonate or 5,5′-dithiobis(2-nitrobenzoate), and this may hinder the binding of NADPH and so cause loss of reductase activity. (2) Photooxidation may cause irreversible polymerization of the reductase, like that of D-amino acid oxidase (Tu and McCormick, 1973). (3) On photooxidation or chemical modifications, flavin adenine dinucleotide may be released from the holoreductase. (4) Diethyl pyrocarbonate may modify another amino acid residue of the reductase besides the histidyl residue. Possibility 1 can be excluded from the results of circular dichroism measurements of the reductase. Possibility 2 was investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It was found that both the untreated and photooxidized preparations gave a single band of reductase and the molecular weights of the two were both 52 000. Possibility 3 can be excluded, because the activity of the photooxidized reductase was not to be restored by incubation with 0.05 $\mu$M FAD at 25 °C for 30 min. Diethyl pyrocarbonate might modify the serinyl, tyrosyl, and cysteinyl residues of the reductase (Vallee and Riordan, 1969). Therefore, the effects of diisopropyl phosphofluoridate (which specifically combines with serinyl residues), 2,3-butanedione (which specifically affects arginyl residues), and pyridoxal 5′-phosphate (which attacks amino groups specifically) were also examined. It was found that the activity of NADPH-adrenodoxin reductase was not inhibited by any of these reagents. Thus possibility 4 can be excluded. Thus it is concluded that a histidyl residue and a sulfhydryl group of the reductase are essential for its binding to NADPH.

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# Enzymatic Determination of Nonrandom Incorporation of 5-Bromodeoxyuridine in Rat DNA†

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ABSTRACT: Secondary cultures of normal rat embryo cells were synchronized by a double thymidine block and pulsed with $10^{-7}$ M 5-[$^3$H]bromodeoxyuridine (BrdUrd) or $10^{-7}$ M [$^3$H]thymidine during an entire S phase (7.5 h). To examine the pattern of [$^3$H]BrdUrd incorporation in rat genomes as compared to [$^3$H]thymidine, DNA was immediately extracted and purified at the completion of the S phase. CsCl density gradient centrifugation revealed that substitution for thymine by bromouracil was less than 7%. Single-strand specific nucleases obtained from *Aspergillus oryzae* and *Neurospora crassa* were allowed to react with native and partially depurinated (24–29%) [$^3$H]thymidine- and [$^3$H]BrdUrd-labeled rat DNA samples, and the products were assayed by hydroxylapatite column chromatography. Approximately 4–6% of the native, nondepurinated rat DNA was hydrolyzed by both nucleases. However, 24–28% of the partially depurinated, [$^3$H]thymidine-labeled rat DNA was hydrolyzed by both enzymes as determined by loss of mass as well as radioactivity. Whereas comparable levels of depurinated, [$^3$H]BrdUrd-labeled DNA were physically hydrolyzed by both nucleases, nearly 65% of the radioactivity was not recovered. Native, as well as depurinated, enzyme-treated DNA samples were sequentially and preparatively reassociated into highly repetitive, middle repetitive, and nonrepetitive nucleotide sequence components. The absolute and relative specific activities of each subfraction of native [$^3$H]thymidine-labeled DNA were comparable. [$^3$H]BrdUrd was differentially concentrated in the middle repetitive sequences as compared to other reiteration frequency types. When depurinated, nuclease-treated DNA samples were similarly fractionated, [$^3$H]thymine moieties were uniformly distributed throughout all sequences. However, a differential loss of [$^3$H]BrdUrd moieties was detected predominantly from the middle repetitive nucleotide fraction. Melting profiles of the renatured DNA samples were characteristic of each respective DNA subfraction regardless of isotopic precursor. These results suggest that [$^3$H]BrdUrd may be differentially incorporated into A + T rich clusters of rat DNA, especially in the moderately repeated chromosomal elements.

Halogenated pyrimidine analogues have been widely used in the study of differential gene expression and regulation in eukaryotic cells. In particular, the thymidine analogue 5-bromodeoxyuridine (BrdUrd)[1] has been extensively utilized in many experimental biological systems to determine molecular mechanisms and schedules for phenotypic and biochemical differentiation (Coleman et al., 1970; Rutter et al., 1973; Turkington et al., 1971; Walther et al., 1974). Moreover, BrdUrd and 5-iododeoxyuridine have been shown to be potent activators of RNA and DNA tumor virus-specific expression from selected animal cells (Hampar et al., 1974; Lowy et al., 1971; Schwartz et al., 1974b; Verwoerd and Sarma, 1973). In order for most of these events to occur, it is necessary that the thymidine analogues be incorporated into the chromosomal DNA of the respective cells (Levitt and Dorfman, 1973; Rutter et al., 1973; Teich et al., 1973). A search for a molecular mechanism for pyrimidine analogue induced alterations of

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[1] Abbreviations used are: BrdUrd, bromodeoxyuridine; PBS, phosphate buffered saline; SSC, standard saline-citrate.