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THE PRESENCE OF ESSENTIAL CARBOXYL GROUP FOR BINDING OF CYTOCHROME *c* IN RAT HEPATIC NADPH-CYTOCHROME P-450 REDUCTASE BY THE REACTION WITH 1-ETHYL-3-(3-DIMETHYLAMINOPROPYL)-CARBODIIMIDE

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NADPH-cytochrome P-450 reductase (EC 1.6.2.4) purified from rat hepatic microsomal fraction was inactivated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), a specific agent for modification of carboxyl groups in a protein. The inactivation exhibited pseudo-first order kinetics with a reaction order approximately one and a second-order-rate constant of 0.60 M^{-1} min^{-1} in a high ionic strength buffer and 0.08 M^{-1} min^{-1} in a low ionic strength buffer.

By treatment of NADPH-cytochrome P-450 reductase with EDC, the pI value changed to 6.5 from 5.0 for the native enzyme, and the reductase activity for cytochrome *c*, proteinic substrate, was strongly inactivated. When an inorganic substrate, $K_3Fe(CN)_6$, was used for assay of the enzyme activity, however, no significant inactivation by EDC was observed. The rate of inactivation by EDC was markedly but not completely decreased by NADPH. Also, the inactivation was completely prevented by cytochrome *c*, but not by $K_3Fe(CN)_6$ or NADH. The sulfhydryl-blocked enzyme prepared by treatment with 5,5′-dithio-bis(2-nitrobenzoic acid), which had no activity, completely recovered its activity in the presence of dithiothreitol. When the sulfhydryl-blocked enzyme was modified by EDC, the enzyme in which the carboxyl group alone was modified was isolated, and its activity was 35% of the contol after treatment with dithiothreitol.

In addition, another carboxyl reagent, *N*-ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward reagent K), decreased cytochrome *c* reductase activity of NADPH-cytochrome P-450 reductase. These results suggest that the carboxyl group of NADPH-cytochrome P-450 reductase from rat liver is located at or near active-site and plays a role in binding of cytochrome *c*.

KEY WORDS: Cytochrome P-450 reductase, cytochrome *c*, carboxyl group, substrate-binding site, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, chemical modification.

ABBREVIATIONS: EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; DCPIP, 2,6-dichlorophenolindophenol; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); Woodward reagent K, *N*-ethyl-5- phenylisoxazolium-3′-sulfonate.

ENZYMES: NADPH-cytochrome P-450 reductase, NADPH : ferricytochrome oxidoreductase (EC 1.6.2.4), ferredoxin-$NADP^+$ oxidoreductase, ferredoxin : $NADP^+$ oxidoreductase (EC 1.18.1.2), lactate dehydrogenase, L-lactate : ferricytochrome *c* oxidoreductase (EC 1.1.2.3), estradiol 17β-dehydrogenase, estradiol-17β : NAD^+ 17β-oxidoreductase (EC 1.1.1.62), NAD^+-isocitrate dehydrogenase, *threo*-Ds isocitrate : NAD^+ oxidoreductase (decarboxylating) (EC 1.1.1.41), and 3α(20β)-hydroxysteroid dehydrogenase, 3α(20β)-hydroxysteroid : NAD(P) oxidoreductase (EC 1.1.1.50).

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INTRODUCTION

The cytochrome P-450-linked mixed function oxygenase system in the microsomal fraction of hepatocytes is composed of two protein components, NADPH-cytochrome P-450 reductase and cytochrome P-450. NADPH-cytochrome P-450 reductase catalyzes the transfer of electrons from NADPH to the heme iron of cytochrome P-450 as well as to a number of other electron acceptors, such as cytochrome *c*, DCPIP and $K_3Fe(CN)_6$[1]. NADPH-cytochrome P-450 reductase contains the residues of arginine[2], cysteine[3–6] and histidine[7] located at or near the cofactor-binding site, and a tyrosine residue in the vicinity of the FMN-binding domain which constitutes the active center of the enzyme[8]. In addition to these residues, a number of NAD(P)-dependent enzymes is known to have the residues of aspartic acid or glutamic acid which are essential for the enzyme function, and they have been implicated in cofactor binding[9–11]. The present study was undertaken to examine a possible involvement of the carboxyl group in the functioning of NADPH-cytochrome P-450 reductase using EDC and Woodward reagent K as selective reagents for chemical modification of the aspartic acid or glutamic acid residues.

MATERIALS AND METHODS

Materials

NADPH-cytochrome P-450 reductase in rat hepatic microsomes was purified after trypsin digestion[1] which yielded a fragment of the holoenzyme[12]. Horse heart cytochrome *c* (type III), bovine pancreatic trypsin (type III), protoporphyrin and hemin were obtained from Sigma (St. Louis, MO). EDC and dithiothreitol were purchased from Nakarai Chem. (Kyoto, Japan). DCPIP, DTNB and $K_3Fe(CN)_6$ were purchased from Wako Chem. (Tokyo, Japan). Woodward reagent K was obtained from Aldrich (Milwaukee, WI). NADH and NADPH were purchased from Boehringer (Mannheim, F.R.G.). Ampholyte (40% solution, pH 3.5–10) was obtained from LKB Instruments (Bromma, Sweden).

Assays

The activity of cytochrome *c* reductase of NADPH-cytochrome P-450 reductase was measured at 25°C in 2 ml of 0.1 mM phosphate buffer (pH 7.5) containing 20%(v/v) glycerol, 100 μM NADPH and 20 μM cytochrome *c*. The enzymatic production of the reduced form of cytochrome *c* during the incubation was monitored by absorption at 550 nm for 200 s using a spectrophotometer (Union Giken, SM-401, Osaka, Japan). Enzymatic reduction of DCPIP and $K_3Fe(CN)_6$ in the presence of NADPH was measured by the same procedure as described by Omura and Takesue[1]. Concentrations of DCPIP and $K_3Fe(CN)_6$ for the assay were 40 and 200 μM, respectively. The reductase activities were calculated from the initial rate of reduction of the electron acceptors. One unit is defined as the amount of enzyme activity that caused a change in absorbance of 0.001 at 550 nm under the assay conditions. Each datum indicated the mean of duplicated determinations. The inactivation rate was expressed as a percentage obtained as follows;

$$\% \text{ Inactivation} = \frac{\text{Velocity at 0 h} - \text{Velocity at 2 h}}{\text{Velocity at 0 h}} \times 100.$$

The pseudo-first order rate constant (k_1) of inactivation was estimated from the slope of a plot of log enzyme activity (%) *vs.* time. The reaction order (n) with respect to reagent was determined from the slope of a plot according to the equation:

$$\log k_1 = \log k_2 + n \log [R],$$

where [R] represents reagent concentration. The second order rate constant (k_2) of inactivation was determined from the slope of a linear plot of k_1 *vs.* [R][13]. All slope values were determined by least-squares regression analysis. Protein concentrations were determined by method of Bradford[14] using bovine plasma gamma globulin as the standard.

Modification of NADPH-Cytochrome P-450 Reductase with EDC

EDC was dissolved in 5 mM KH_2PO_4–20% (v/v) glycerol–2 M NaCl (pH 6.5) to give a 100 mM solution before use. NADPH-cytochrome P-450 reductase was mixed with EDC in the phosphate buffer at 25°C. Activity of the modified enzyme was measured at various times as indicated in Results.

Differential Modifications of Carboxyl and Sulfhydryl Groups of the NADPH-Cytochrome P-450 Reductase

The NADPH-cytochrome P-450 reductase was differentially treated with DTNB as the first, EDC as the second and finally with dithiothreitol as the third modification. For the first modification, the enzyme (35 pmol) was incubated in 600 μl of the phosphate buffer containing 0.1 mM DTNB for 60 min at 25°C to block the sulfhydryl groups. An aliquot (90 μl) was sampled after incubation, and cytochrome *c* reductase activity of the modified enzyme was measured. Then, as the second modification, EDC at the final concentration of 1 mM was added to the remaining enzyme solution (510 μl) to modify carboxyl group(s). The enzyme activity in 90 μl of the solution was assayed after 90 min. As the third modification, dithiothreitol (2 mM, as the final concentration) was added to the remaining solution (420 μl) to regenerate free sulfhydryl group from the modified one, and the mixture was incubated for 30 min. Without the NADPH-cytochrome P-450 reductase, cytochrome *c* was partially reduced in the presence of 2 mM dithiothreitol. The velocity for the third modification was calculated by subtraction of the non-enzymatic reduction.

Isoelectric Focusing

Isoelectric focusing was carried out in a 110 ml column. A discontinuous density gradient from 0.42% Ampholytes (pH 3.5–10) in 0% sucrose solution to 1.25% Ampholytes in 47% sucrose solution in the column was prepared by the method of Vesterberg and Svensson[15]. For determination of the isoelectric point of the NADPH-cytochrome P-450 reductase modified with EDC, the native enzyme (14.1 nmoles) was incubated with 50 mM EDC in 4 ml of 25 mM KH_2PO_4–10% (v/v) glycerol–2 M NaCl (pH 6.5) at 25°C. The modified enzyme preparation (87% inactivation) which was obtained after the incubation was dialyzed against 3 l of 25 mM KH_2PO_4 (pH 7.4) at 4°C for removal of an excess reagent. The dialyzed solution of modified enzyme was added to the middle fractions in the column. After the isoelectric focusing was run for 48 h at constant voltage (300V) at 2°C every 70 drops of the eluate were collected

in fractions. Fractions were examined for pH, protein concentration, and cytochrome *c* reductase activity.

RESULTS

Time-, Concentration- and Ionic Strength-dependent Inactivation of NADPH-Cytochrome P-450 Reductase by EDC

Incubation of NADPH-cytochrome P-450 reductase with EDC in 5 mM KH_2PO_4–20% (v/v) glycerol-2 M NaCl (pH 6.5) resulted in a progressive loss of cytochrome *c* reductase activity. As shown in Figure 1A, the inactivation rate of the enzyme by EDC was dependent on concentration of the reagent and on the incubation time. When the enzyme was mixed with 10 mM EDC for 2 h, 18% of the control activity remained. The inactivation followed pseudo-first order kinetics, as indicated by typical semi-log plots of enzyme activity *vs.* time (Figure 1A). The second-order

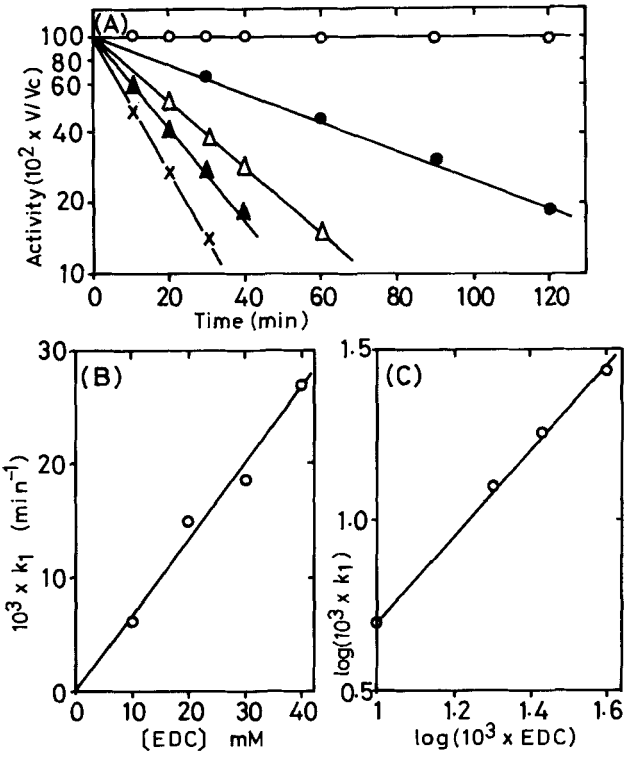

FIGURE 1 Inactivation of NADPH-cytochrome P-450 reductase by EDC. (A) NADPH-cytochrome P-450 reductase (35 pmol) was treated with 0 (○), 10 (●), 20(△) 30(▲) and 40 (×) mM EDC in 60 μl of 5 mM KH_2PO_4–20% (v/v) glycerol–2 M NaCl buffer (pH 6.5) at 25°C. Aliquots (9 μl each) of the reaction mixture were sampled at the indicated times and cytochrome *c* reductase activities of the modified cytochrome P-450 reductase were assayed. V and Vc were the enzyme activities of experimental and control. (B) Plot of k_1 obtained at various concentration of EDC against EDC concentrations. (C) Apparent order with respect to reagent concentration for the reaction between the cytochrome P-450 reductase and EDC. The observed pseudo-first order rate constant (k_1) values were calculated from the data of Figure 1(A).

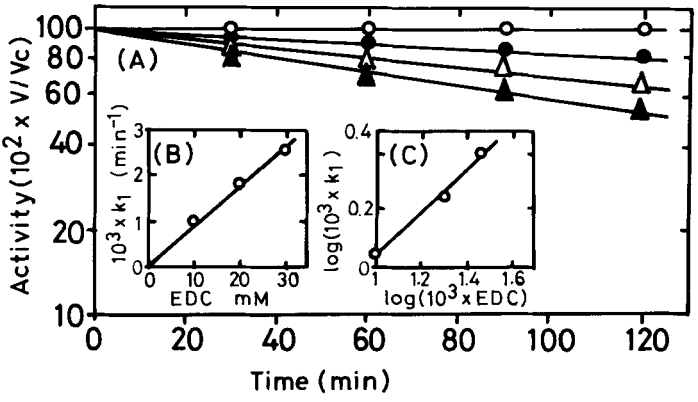

FIGURE 2 Inactivation of NADPH-cytochrome P-450 reductase by EDC. (A) NADPH-cytochrome P-450 reductase (35 pmol) was treated with 0 (○), 10 (●), 20(△) and 30(▲) mM EDC in 60 μl of 5 mM KH_2PO_4–20% (v/v) glycerol buffer (pH 6.5) at 25°C. Aliquots (9 μl each) of the reaction mixture were sampled at the indicated times and cytochrome *c* reductase activities of the modified enzyme were assayed. V and Vc were the enzyme activities of experimental and control. *Inset-(B)* shows the plot of k_1 obtained at various concentration of EDC against EDC concentrations. *Inset-(C)*, Linear double-log plots of pseudo-first order rate constant *vs.* EDC concentration.

rate constant (k_2) was obtained from the slope of the linear plots of pseudo-first order rate constant (k_1) *vs.* reagent concentration (Figure 1B) and was 0.60 M^{-1} min^{-1}. Double log plots of k_1 as a function of reagent concentration (Figure 1C) were also linear, yielding a slope of about 1.1 and a reaction order of one with respect to reagent for the activities studied. In the phosphate buffer without NaCl, the inactivation progressed more slowly, and in a time-dependent manner. Figure 2A shows that 80% of the activity of the control was obtained after 2 h incubation with 10 mM EDC in the buffer (pH 6.5) with the lower ionic strength. The value of the second-order rate constant for the inactivation in the phosphate buffer without NaCl was calculated as 0.08 M^{-1} min^{-1} from Figure 2B. A reaction order of about 0.8 with respect to the inhibitor was calculated from the slope shown in Figure 2C, according to the method of Levy *et al.*[16]

Difference in Inactivation Rate by EDC of NADPH-Cytochrome P-450 Reductase for Cytochrome c, DCPIP and $K_3Fe(CN)_6$ as Substrate

After NADPH-cytochrome P-450 reductase (35 pmol) was incubated with 10 mM EDC in 60 μl of 5 mM KH_2PO_4–20% (v/v) glycerol–2 M NaCl (pH 6.5) at 25°C, the enzyme activity was assayed using cytochrome *c*, DCPIP, and $K_3Fe(CN)_6$ as substrate. The inactivation of cytochrome *c* reductase activity progressed faster than that of reductase activity for DCPIP and $K_3Fe(CN)_6$ (Figure 3). When the enzyme activity was assayed in the presence of $K_3Fe(CN)_6$, about 90% of the control activity remained after incubation for 1 h. with EDC, and no further inactivation was observed.

Change in Isoelectric Point of NADPH-Cytochrome P-450 Reductase by Treatment with EDC

From the analysis of cytochrome *c* reductase activity of NADPH-cytochrome P-450

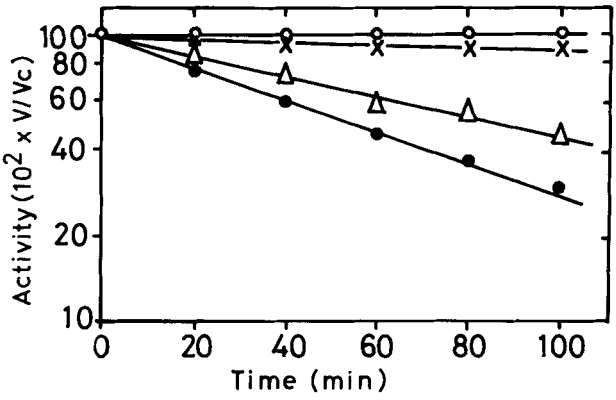

FIGURE 3 Different reducing activities of $K_3Fe(CN)_6$, DCPIP and cytochrome *c* by NADPH-cytochrome P-450 reductase modified with EDC. NADPH-cytochrome P-450 reductase (35 pmol) was incubated with 10 mM EDC in 60 μl of 5 mM KH_2PO_4–20% (v/v) glycerol–2 M NaCl (pH 6.5) at 25°C. Aliquots (9 μl each) of the reaction mixture were sampled at the indicated times and the enzyme activities were assayed using $K_3Fe(CN)_6$ (×), DCPIP (△) and cytochrome *c* (●) as substrate. A control (○) was carried out by the same procedure without EDC. Enzyme activities are expressed as % of the control values, where Vc is the control enzyme activity and V is the enzyme activity at the indicated time.

reductase in each fraction separated by the isoelectric focusing, the enzyme activity was found as a single peak in Fraction 45 corresponding to the protein peak (Figure 4). The isoelectric point of the native enzyme was determined as 5.0 (n = 2). NADPH-cytochrome P-450 reductase (14.1 nmol) was treated with 50 mM EDC in the phosphate buffer at 25°C, and 87% inactivated enzyme was obtained. The isoelectric point for the modified NADPH-cytochrome P-450 reductase was obtained by the measurement of protein content in each fraction, because of diminished activity of the modified enzyme. The modified enzyme preparation showed the existence of the two different protein fractions. Their isoelectric points were determined as 6.5 and 5.0. As the protein with pI 6.5 increased during progress of the reaction with EDC, this fraction was regarded as the modified form of the NADPH-cytochrome P-450 reductase. The protein with pI 5.0 had a weak cytochrome *c* reductase activity and this was even weaker for the more highly inactivated enzyme preparation. Therefore, the protein (pI 5.0) was suggested as the unmodified enzyme with the same isoelectric point as that of the native preparation.

Effect of substrates against Inactivation by EDC of NADPH-Cytochrome P-450 Reductase

The reductase activity of cytochrome *c* by NADPH-cytochrome P-450 reductase was spontaneously decreased to 73–80% of the original level at 25°C for 2 h, as shown in Table I. By addition of cytochrome *c* (0.25 mM, as the final concentration) to the enzyme preparation, the spontaneous inactivation was completely prevented. The inactivation of NADPH-cytochrome P-450 reductase by 10 mM EDC was also completely protected by addition of 0.25 mM cytochrome *c*. Figure 5 shows a relationship between concentration of cytochrome *c* and remaining enzyme activity during the modification by EDC. The inactivation rate of NADPH-cytochrome P-450 reductase by 10 mM EDC was significantly reduced by addition of a lower concentration than

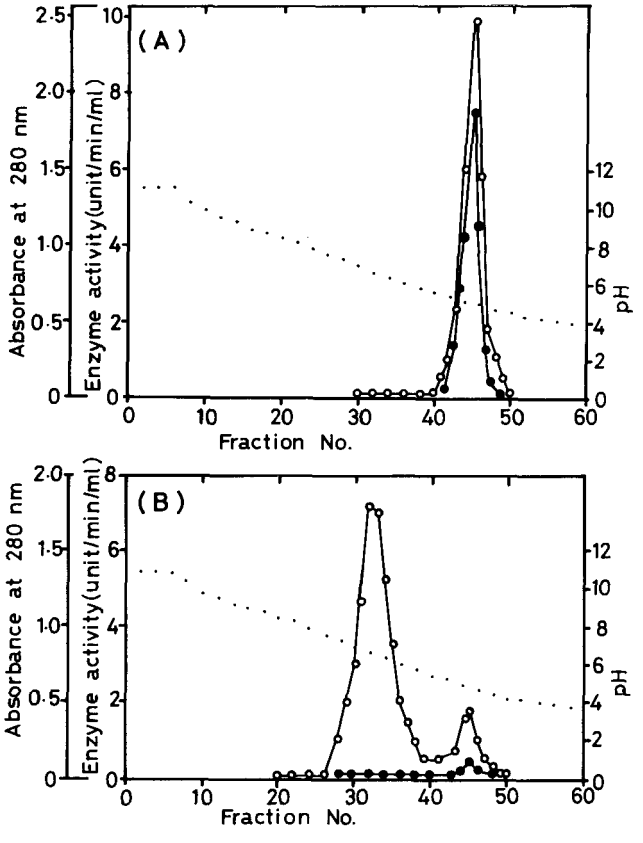

FIGURE 4 Isoelectric focusing of native (A) and modified (B) NADPH-cytochrome P-450 reductases. Details, see in Materials and Methods. Fractions were examined for pH (·), absorbance at 280 nm (○) and cytochrome *c* reductase activity (unit/min/ml of fraction) (●).

0.1 mM cytochrome *c*. However, no protective effect of DCPIP and $K_3Fe(CN)_6$ against the spontaneous inactivation was found. DCPIP showed less protective effect against the inactivation of NADPH-cytochrome P-450 reductase by 10 mM EDC, while $K_3Fe(CN)_6$ had no significant effect.

Effect of Protoporphyrin and Hemin against Inactivation of NADPH-Cytochrome P-450 Reductase by EDC

Since cytochrome *c* was completely protective against the inactivation by EDC of NADPH-cytochrome P-450 reductase, the effect of hemes without a protein moiety, such as protoporphyrin and hemin, was examined. After the hemes were added to the mixture of both the enzyme and EDC in the dark, the inactivation rate by EDC in the presence of the hemes was almost of the same order as that for the control which contained only the enzyme and EDC, as shown in Table II. However, NADPH-cytochrome P-450 reductase activity was markedly inactivated by 2.5 μM protoporphyrin or hemin even in the absence of EDC.

TABLE I

Effect of substrates against inactivation of NADPH-cytochrome P-450 reductase by EDC. NADPH-cytochrome P-450 reductase (35 pmol) was mixed with 10 mM EDC, 0.25 mM cytochrome *c*, 3.3 mM DCPIP or 0.16 mM $K_3Fe(CN)_6$ in 60 μl of 5 mM KH_2PO_4–20% (v/v) glycerol-2 M NaCl (pH 6.5), and its activity in an aliquot (9 μl) was immediately assayed. Then, the remaining enzyme solution (51 μl) was incubated at 25°C for 2 h. Aliquots (9 μl each) were sampled during the incubation, and cytochrome *c* reductase activities of NADPH-cytochrome P-450 reductase were assayed for 200 s

Addition to enzyme	Velocity[a]		% Inactivation
	0 h	2 h	
Buffer	25.2	20.1	20
10 mM EDC	24.3	3.3	86
0.25 mM Cytochrome *c*	27.0	28.8	−6
10 mM EDC + 0.25 mM Cytochrome *c*	29.1	28.8	1
Buffer	24.1	17.6	27
10 mM EDC	23.7	2.3	88
3.3 mM DCPIP	37.2	21.6	42
10 mM EDC + 3.3 mM DCPIP	36.1	12.6	65
0.16 mM $K_3Fe(CN)_6$	22.5	17.1	24
10 mM EDC + 0.16 mM $K_3Fe(CN)_6$	24.4	2.7	89

[a]Unit/min. Details, see Materials and Methods.

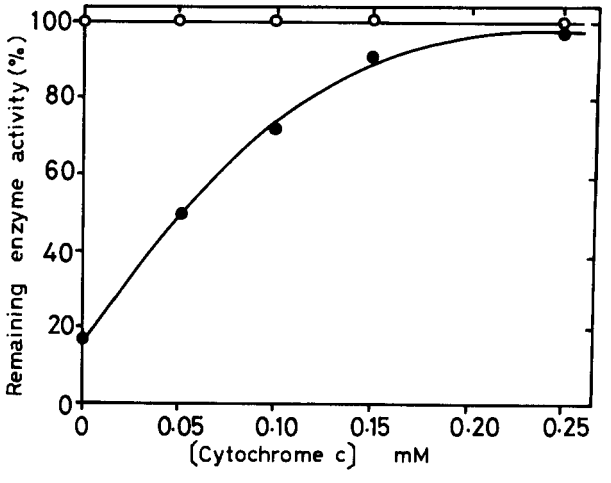

FIGURE 5 Concentration-dependent protection of cytochrome *c* against inactivation of NADPH-cytochrome P-450 reductase by EDC. NADPH-cytochrome P-450 reductase (35 pmol) was mixed with 0–0.25 mM cytochrome *c* in the presence (●) or absence (○) of 10 mM EDC in 60 μl of 5 mM KH_2PO_4–20% (v/v) glycerol-2 M NaCl (pH 6.5) at 25°C. Aliquots (9 μl each) of the reaction mixture were sampled after 2 h and cytochrome *c* reductase activities of NADPH-cytochrome P-450 reductase modified by EDC were assayed. Remaining enzyme activity was expressed as % of the control value in the absence of EDC.

TABLE II
Effect of protoporpyrin and hemin against inactivation of NADPH-cytochrome P-450 reductase by EDC. Experimental procedures were the same as described in Table I.

Addition to enzyme	Velocity[a]		% Inactivation
	0 h	2 h	
Buffer	24.1	17.6	27
10 mM EDC	23.7	2.8	88
2.5 μM Protoporphyrin	24.4	2.0	92
10 mM EDC + 2.5 μM Protoporphyrin	20.4	0	100
2.5 μM Hemin	24.4	1.0	94
10 mM EDC + 2.5 μM Hemin	25.2	0.3	99

[a]Unit/min.

Effect of Cofactors against Inactivation of NADPH-Cytochrome P-450 Reductase by EDC

The inactivation rate of NADPH-cytochrome P-450 reductase by 10 mM EDC was decreased in the presence of 10 mM NADPH. When NADH was added to the mixture of both the enzyme and EDC, however, no preventive change in the inactivation was observed as shown in Table III. NADPH also showed a protective effect against the spontaneous inactivation in the absence of EDC. As shown in Figure 6, when NADPH-cytochrome P-450 reductase was mixed with 10 mM EDC in the absence of cofactor, the enzyme activity was decreased to 15% of the control level after 2 h. By addition of variable concentrations (1–15 mM) of NADPH to the mixture prepared for the modification, the remaining enzyme activities during the process of the modification were linearly increased to 70%. However, no further protective effect was observed by increasing the concentration of NADPH beyond 15 mM. In contrast, in the presence of 25 mM NADH with EDC, the enzyme activity after 2 h was found to be 21% of the control activity.

TABLE III
Effect of cofactors against inactivation of NADPH-cytochrome P-450 reductase by EDC. Experimental procedures were the same as described in Table I.

Addition to enzyme	Velocity[a]		% Inactivation
	0 h	2 h	
Buffer	24.6	17.1	30
10 mM EDC	24.9	3.9	84
10 mM NADH	23.1	12.3	47
10 mM EDC + 10 mM NADH	26.7	4.5	83
10 mM NADPH	24.0	21.9	9
10 mM EDC + 10 mM NADPH	24.6	14.3	36

[a]Unit/min.

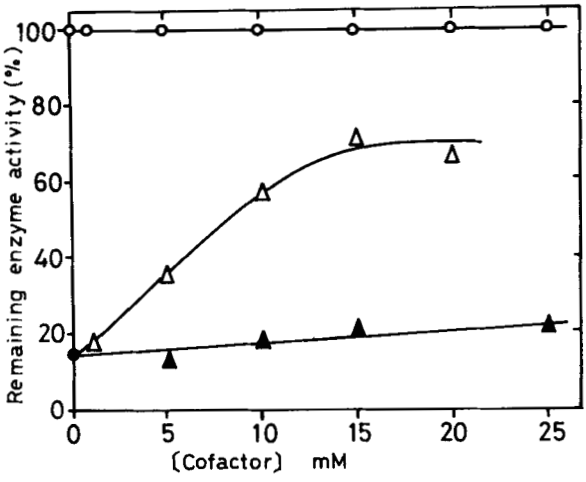

FIGURE 6 Concentration-dependent protection of cofactors against inactivation of NADPH-cytochrome P-450 reductase (35 pmol) was mixed with NADPH (△) or NADH (▲) in the presence of 10 mM EDC in 60 μl of 5 mM KH_2PO_4–20% (v/v) glycerol-2 M NaCl (pH 6.5) at 25°C. The control was carried out in the same manner without 10 mM EDC (○) or with EDC alone in the absence of cofactor (●). Aliquots (9 μl each) of the reaction mixture were sampled after 2 h and cytochrome *c* reductase activities of NADPH-cytochrome P-450 reductase modified with EDC were assayed. Each remaining enzyme activity was expressed as % of the control value in the absence of EDC.

Chemical Modification of Carboxyl and Sulfhydryl Groups of NADPH-Cytochrome P-450 Reductase

To clarify the mode of modification of the carboxyl group in NADPH-cytochrome P-450 reductase, we blocked the sulfhydryl group of the enzyme with 0.1 mM DTNB before the treatment with EDC. As shown in Table IV, the enzyme was almost inactivated by treatment with 0.1 mM DTNB, but the decreased activity recovered to the initial level by further addition of 2 mM dithiothreitol (Experiment III). The enzyme activity was also completely lost by treatment of the native enzyme with 1 mM

TABLE IV

Differential modification of carboxyl and sulfhydryl groups of NADPH-cytochrome P-450 reductase. Experiments I and III were the control for Experiments II and IV, respectively. Modification was carried out as described in Materials and Methods.

Exp.	1st Modification	Velocity[a]	(%)	2nd Modification	Velocity[a]	(%)	3rd Modification	Velocity[a]	(%)
I	0 mM DTNB	5.2	(100)	0 mM EDC	2.8	(54)	2 mM dithiothreitol	5.6	(108)
II	0 mM DTNB	5.2	(100)	1 mM EDC	0	(0)	2 mM dithiothreitol	2.2	(42)
III	0.1 mM DTNB	0.4	(8)	0 mM EDC	0.2	(4)	2 mM dithiothreitol	4.9	(94)
IV	0.1 mM DTNB	0.4	(8)	1 mM EDC	0.2	(4)	2 mM dithiothreitol	1.8	(35)

[a] Unit/min.

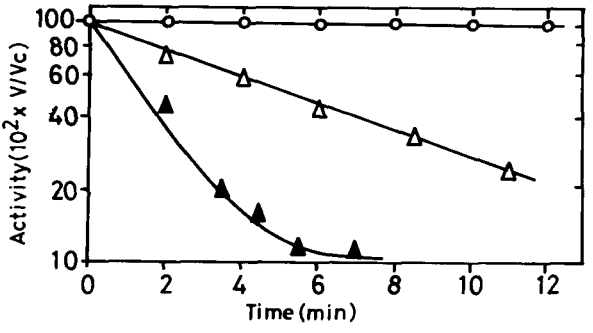

FIGURE 7 Time- and ionic strength-dependent inactivation of NADPH-cytochrome P-450 reductase by Woodward reagent K. NADPH-Cytochrome P-450 reductase (70 pmol) was incubated with 2 mM Woodward reagent K in 60 μl of 5 mM KH_2PO_4–20% (v/v) glycerol (pH 6.5) in the presence (▲) or absence (△) of 2 M NaCl. Aliquots (9 μl each) of the reaction mixture were sampled at the indicated times and enzyme activities were assayed using cytochrome *c* as a substrate. The control value (○) was obtained by the same procedure without Woodward reagent K. Enzyme activities are expressed as % of the control values, where Vc is the control enzyme activity and V is the enzyme activity at the indicated time.

EDC, and the enzyme activity was partially recovered by further addition of 2 mM dithiothreitol to EDC-treated preparation (Experiment II). When NADPH-cytochrome P-450 reductase (35 pmol) was incubated in 60 μl of 5 mM KH_2PO_4–20% (v/v) glycerol–2 M NaCl (pH 7.4) containing 6 μl ethanol at 25°C for 150 min, the activity was reduced to 54% of the initial level (Experiment I). The decreased activity obtained from the second modification of Experiment I recovered fully by addition of dithiothreitol. The DTNB- and EDC-treated enzyme that had very low activity was then further treated with dithiothreitol. The preparation obtained from the 3rd modification of Experiment IV had 35% of the initial activity, suggesting that there were carboxyl groups in the active-site of the NADPH-cytochrome P-450 reductase.

Effects of Woodward Reagent K on NADPH-Cytochrome P-450 Reductase

The role of the carboxyl groups on NADPH-cytochrome P-450 reductase was further studied using Woodward reagent K as a carboxyl-modifying reagent. Figure 7 shows that Woodward reagent K inactivated the cytochrome *c* reductase activity of NADPH-cytochrome P-450 reductase in time- and ionic strength-dependent manners, similar to that by EDC. But inactivation by Woodward reagent K of cytochrome *c* reductase activity of NADPH-cytochrome P-450 reductase was faster than by EDC.

DISCUSSION

NADPH-cytochrome P-450 reductase has been solubilized from the microsomal membrane of rat liver by trypsin digestion[1] or the use of detergent[17]. For this study, we solubilized the holoenzyme by the former method and purified it. Rat hepatic NADPH-cytochrome P-450 reductase purified from the trypsin-treated preparation had 35 residues of aspartic acid and 43 residues of glutamic acid per enzyme mole-

cule[18]. However, no function of the carboxyl groups of those residues has yet been reported. EDC inactivated the cytochrome *c* reductase activity of NADPH-cytochrome P-450 reductase in time- and concentration-dependent manners. The mechanism for the modification of carboxyl groups by EDC probably involved formation of an *O*-acylisourea intermediate which was then rearranged to a stable *N*-acylurea group as described by Perfetti *et al.*[19] By the third modification of Experiment IV (Table IV), we isolated the enzyme which had been modified on the carboxyl groups alone. The inactivation was significantly enhanced in phosphate buffer with a high ionic strength. The results suggest that the carboxyl groups essential for the enzyme activity are buried in the enzyme molecule. But no inactivation was observed, when an inorganic substrate, $K_3Fe(CN)_6$ was used for assay of the activity of NADPH-cytochrome P-450 reductase modified with EDC. From these results, it is suggested that the carboxyl groups of NADPH-cytochrome P-450 reductase do not participate directly in the catalytic function.

In ferredoxin-$NADP^+$ oxidoreductase which is a FAD-containing enzyme related to photosynthetic electron transport from water to $NADP^+$ in chloroplasts, there is a single essential carboxyl group in the nucleotide-binding site of the enzyme[20]. Lactate dehydrogenase had also residues of aspartic and glutamic acids within its cofactor-binding site. The aspartic acid is hydrogen-bonded to the C-2′ hydroxy group of the adenosine ribose in NAD^+ and the glutamic acid is available to cancel the positive charge of nicotinamide Nl of the oxidized form of the cofactor[9]. Recently, we identified the carboxyl groups of the residues of aspartic or glutamic acids at the cofactor-binding site of NAD^+-dependent estradiol 17β-dehydrogenase by chemical modification with EDC[11]. Therefore, the carboxyl group in those enzymes would be considered to bind the cofactor at their cofactor-binding sites, as a primary step of the reactions. The mechanism of the reduction of oxidized NADPH-cytochrome P-450 reductase (FAD-FMN) by NADPH has been reported by Iyanagi *et al.*[21] and Oprian and Coon[22]. In the present experiment, the essential carboxyl group of NADPH-cytochrome P-450 reductase was identified outside its cofactor-binding domain, because incomplete protection against inactivation by EDC was observed by addition of the cofactors.

On the other hand, an essential carboxyl group was found within the substrate-binding site of NAD^+-dependent isocitrate dehydrogenase[23], and NADH-dependent 3α(20β)-hydroxysteroid dehydrogenase[24]. Adrenodoxin, one of the components for cytochrome P-450-linked mixed function oxygenase system in adrenal mitochondrial fraction, was inactivated by modification with EDC[25,26]. The major carboxyl groups modified by EDC were found to be at Glu-74, Asp-79 and -96 in adrenodoxin, which were located in a sequence containing a high negative charge density[25]. The carboxyl groups in adrenodoxin were cross-linked to the amino group of lysine residue in cytochrome *c* in the presence of EDC. In NADPH-cytochrome P-450 reductase, the carboxyl group would participate in binding proteinic substrate, cytochrome *c*, at its substrate-binding site, because of the complete protection against the inactivation even by a very low concentration of cytochrome *c*. But, no participation of the carboxyl group in binding of inorganic substrate, $K_3Fe(CN)_6$, at the substrate-binding site was suggested. We also studied the protective effect of hemes against inactivation of NADPH-cytochrome P-450 reductase by EDC, but the activity of cytochrome *c* reductase was markedly inhibited by a low concentration of protoporphyrin and hemin in the absence of EDC. From those results, it is not yet clear whether the hemes are protective against the inactivation or not. While the isoelectric

point of the native NADPH-cytochrome P-450 reductase was pH 5.0, the pI of the enzyme modified with EDC was 6.5. As a functional role of the carboxyl group in the NADPH-cytochrome P-450 reductase, this group with a negative charge is suggested to be electrostatically bound to cytochrome *c*, a positive charged protein (pI = 9.4)[27] at its substrate-binding site. When the enzyme was modified by EDC, the negative charge due to the carboxyl group would be neutralized, and accordingly the electrostatic binding of the enzyme to cytochrome *c* would be disturbed.

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