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Formation of the Red Neotetrazolium Formazan by Reduced Nicotinamide Adenine Dinucleotide Phosphate-Cytochrome c Reductase in the Presence of Triton X-100[1]

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Neotetrazolium (NT) was reduced to purple formazan (P-NTF) by hepatic microsomes in the presence of NADPH-generating system but to red formazan (R-NTF) if Triton X-100 (none-ionic detergent) was added to the reaction mixture. The visible spectrum and thin-layer chromatogram of R-NTF were different from those of P-NTF.

As a result of purification of the enzyme in microsomes which could form R-NTF in the presence of Triton X-100, this enzyme was found to be NADPH-cytochrome c reductase. Effect of some compounds on formation activity of both formazans was examined and EDTA, ascorbic acid, Cu^{2+}, and Mn^{2+} affected both activities different. It was presumed that the reduction of NT by NADPH-cytochrome c reductase in the presence or absence of Triton X-100 would be different.

Since it has been reported that NADPH-cytochrome c reductase could produce superoxide anion, either purple or red formazan might be formed by the reductive action of superoxide anion and the effect of superoxide dismutase purified from bovine erythrocytes was examined. It was found that the formation activity of red formazan was strongly inhibited by superoxide dismutase and NT could be also reduced to red in xanthine oxidase system which could produce superoxide anion. From these results, it was clarified that the R-NTF would be formed by the reductive action of superoxide anion.

It is well known that neotetrazolium (NT) is reduced to purple formazan (P-NTF) by sodium hydrosulfite, and similarly by mitochondria with succinate.[3a–d] Williams and Kamin[4] have reported that purified reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase (NADPH-cytochrome c reductase), which was solubilized with lipase and highly purified from hog liver microsomes, could also form P-NTF in the presence of NADPH. On the other hand, Okui, *et al.*[3d] have suggested that two tetrazolium rings of NT was stepwisely reduced by mitochondria in the presence of succinate because NT was converted to a red formazan (R-NTF) in a short incubation time and gradually changed to P-NTF with further incubation.

In our laboratory, it was observed that a R-NTF was formed by microsomes in the presence of NADPH and Triton X-100. The present report describes an enzyme which participates in the formation of R-NTF in the presence of Triton X-100 and difference in the formation mechanism between R- and P-NTF.

Materials and Methods

1. Commercial Enzymes, Substrates, and Reagents——Xanthine oxidase from milk was a product of Boehringer Mannheim GmbH (Denmark). Lipase from hog pancreas (Grade II) was a product of Sigma Chemical Company (USA). 2,6-Dichlorophenol indophenol, hypoxanthine, and neotetrazolium chloride

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3) *a*) H. Kamin, R.H. Gibbs, and A.D. Merritt, *Federation Proc.*, **16**, 202 (1957); *b*) A.L. Smith and R.L. Lester, *Federation Proc.*, **19**, 34 (1960); *c*) R.L. Lester and A.L. Smith, *Biochim. Biophys. Acta*, **47**, 475 (1961); *d*) S. Okui, Y. Suzuki, K. Momose, and A. Ogano, *J. Biochem.* (Tokyo), **5**, 500 (1963).

4) C.H. Williams, Jr. and H. Kamin, *J. Biol. Chem.*, **237**, 587 (1962).

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were the products of Wako Pure Chemical Industries, Tokyo. But neotetrazolium chloride was purified with silica column chromatography by the method of Okui, *et al.*[3d] Silica thin-layer plates and Triton X-100 were the products of Tokyo Kasei Kogyo and Nakarai Chemicals, respectively.

2. **Preparation of NADPH-Cytochrome c Reductase from Rat Liver Microsomes**——Purification of NADPH-cytochrome c reductase from rat liver microsomes is as follows: Male rats of Wistar strain were injected intraperitoneally with phenobarbital (80 mg/kg of body weight, once a day) for 4 days and 112 g of their liver was homogenized with 1000 ml of 0.14 M KCl. The homogenate was centrifuged at $9000 \times g$ for 20 min. The supernatant fluid was centrifuged at $105000 \times g$ for 60 min. The obtained microsomal pellets were washed 3 times by homogenization with 0.14M KCl and centrifugation ($105000 \times g$, 60 min). Washed microsomes were suspended in 0.01M Tris buffer (pH 7.5) (Step I). This suspension was incubated aerobically with 1 mg of lipase per 100 mg of microsomal protein at 37° for 40 min. After the treatment of microsomes with lipase, the suspension was centrifuged at $105000 \times g$ for 60 min, the supernatant fluid was collected, and applied on a hydroxylapatite column (3.8×25 cm), which had been equilibrated with 0.01M Tris buffer (pH 7.5), and chromatographed. The enzyme adsorbed on the base was fractionated by elution with a linear gradient of phosphate buffer (pH 7.5). Initially, the mixing flask and reservoir contained 500 ml of 0.005M phosphate buffer (pH 7.5) and 500 ml of 0.3M phosphate buffer (pH 7.5), respectively. The enzyme activity was eluted at about 0.15M phosphate buffer. Eluate from the column (tube Nos. 52—69, total volume 180 ml) was collected (Step III) and concentrated in a collodion bag. The concentrated enzyme solution was diluted to 0.005M phosphate buffer and applied on a diethylaminoethyl (DEAE)-Sephadex column (1.2×24 cm) which was previously equilibrated with 0.005M phosphate buffer (pH 7.5). The column was eluted by the gradient method using 100 ml of 0.005M phosphate buffer (pH 7.5) in the mixing flask and 100 ml of 0.5M NaCl in 0.005M phosphate buffer (pH 7.5) in the reservoir. The enzyme activity was eluted at about 0.4M NaCl (Step IV). Eluate from column (tube Nos. 54—63, total volume 30 ml) was concentrated in a collodion bag.

3. **Preparation of Superoxide Dismutase from Bovine Erythrocytes**——Superoxide dismutase was purified from 1500 ml of bovine erythrocytes by the method of McCord and Fridovich,[5] using DEAE-Sephadex instead of DE-32 in the last step. The yield of protein was 60 mg and 0.43 μg of purified enzyme inhibited 50% of cytochrome c reduction activity of xanthine oxidase.

4. **Measurement of Enzyme Activities**——1) NADPH-Cytochrome c Reductase and 2,6-Dichlorophenol Indophenol Reductase: Reduction activities of 2,6-dichlophenol indophenol (DCIP) and cytochrome c by NADPH-cytochrome c reductase were determined in the same way as described in the previous report.[6]

The activity to form P-NTF (P-NTF activity) by microsomes was determined as follows: 0.2 ml of 1M Tris buffer (pH 7.5), 0.1 ml of 40 mM glucose-6-phosphate, 0.1 ml of 4 units/ml glucose-6-phosphate dehydrogenase, 0.1 ml of 1.2 mM NADP, enzyme, 0.3 ml of 1.0 mM NT, and H_2O in a total volume of 2.0 ml. The reaction mixture was incubated at 37° for 10 min and its absorption at 540 nm was measured after addition of 2.0 ml of the reaction-stopper, which contained 100 ml of 1M formate buffer (pH 3.5), 36 ml of 10% Triton X-100, 50 ml of 40% formaldehyde, and 400 ml of H_2O.

The activity to form R-NTF (R-NTF activity) was assayed by a method described for P-NTF activity except that 0.1 ml of 16% Triton X-100 was added to the reaction mixture and that the color was read at 500 nm. R-NTF activity was gradually increased with a increase of Triton X-100 concentration and reached a maximum at 0.4—0.8% of Triton X-100.

The quantity of P-NTF was calculated from the molecular extinction coefficient which was determined by the addition of a small amount of sodium hydrosulfite crystals to NT. In the case of R-NTF, its molecular extinction coefficient was determined by the enzymic reduction of NT in the presence of Triton X-100. That is, 100 nmoles of NT was incubated with excess microsomes and NADPH-generating system in the presence of Triton X-100 until the absorption at 500 nm reached a maximum. Then, the reaction-stopper was added to the reaction mixture and its absorption was measured at 500 nm.

2) Superoxide Dismutase Activity: The reaction mixture contained 0.2 ml of 0.5M phosphate buffer (pH 7.5), 0.1 ml of 1.0 mM EDTA, 0.1 ml of 0.5 mM hypoxanthine, 0.2 ml of 0.2 mM cytochrome c, 0.1 ml of 100 μg/ml xanthine oxidase, and H_2O in a total volume of 1.0 ml. The reaction was started at room temperature by addition of xanthine oxidase and its absorption at 550 nm was measured at 20 sec intervals. Under these defined conditions, the amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome c by 50% is defined as 1 unit of activity.

3) Formation Activity of R-NTF by Xanthine Oxidase: The reaction mixture contained 0.2 ml of 0.5M phosphate buffer (pH 7.5), 0.2 ml of 2.0 mM hypoxanthine, 0.1 ml of 1.0 mM ethylenediamine tetraacetic acid (EDTA), xanthine oxidase, and 0.3 ml of 1.0 mM NT in a total volume of 2.0 ml. After this mixture was incubated at 37° for 5 min, 2.0 ml of the reaction-stopper was added and its absorption was measured at 500 nm.

5) J.M. McCord and I. Fridovich, *J. Biol. Chem.*, **244**, 6049 (1969).
6) I. Ishiguro, R. Shinohara, A. Ishikura, and J. Naito, *Yakugaku Zasshi*, **94**, 1240 (1974).

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Results

When NT was incubated with microsomes and NADPH-generating system, the reaction mixture changed to purple. However, in the same system containing Triton X-100, it changed to red and different colored NT formazans were obtained. As shown in Fig. 1, P- and R-NTF exhibited absorption maxima at 540 and 500 nm, respectively. Other difference in NT formazans was shown by means of thin-layer chromatography. Both formazans were extracted with ethyl acetate and spotted on a thin-layer plate of silica and developed with acetone-methanol (9:1 by volume). *Rf* value of R-NTF on the chromatogram was 0.13 and that of P-NTF was 0.83. When sodium hydrosulfite solution was sprayed on the chromatogram, color of R-NTF changed to purple. It was speculated from these results that P- and R-NTF were structurally different and that an enzyme reducing NT to red in the presence of Triton X-100 was present in microsomes.

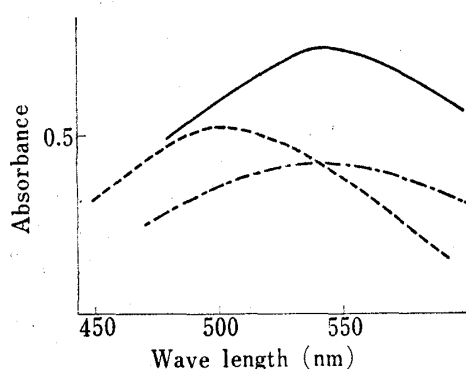

Fig. 1. Visible Absorption Spectra of NT Formazans formed in Various Conditions

(a) After NT (80 nmoles in 2.0 ml) was reduced by a small amount of sodium hydrosulfite crystals, 2.0 ml of the reaction-stopper was added. (b) After NT (300 nmoles) was incubated at 37° for 5 min with microsomes (120 μg of protein) and NADPH-generating system in 2.0 ml, 2.0 ml of the reaction-stopper was added. (c) To the same reaction mixture as (b), 0.1 ml of 16% Triton X-100 was added and incubated.

——— : a, —·—·— : b, ------- : c

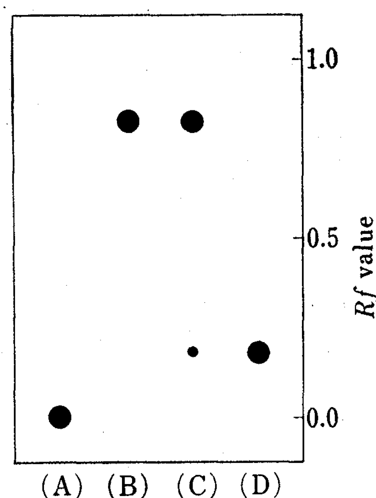

Fig. 2. Silica Thin-Layer Chromatogram of NT Formazans

(A) NT. This spot was detected by spraying of sodium hydrosulfite solution. Spots of (B), (C), and (D) were NT formazans which were extracted with ethyl acetate from a, b, and c in Fig. 1, respectively.

Williams and Kamin[4] had reported that purified NADPH-cytochrome c reductase could reduce various electron acceptors such as cytochrome c, DCIP, NT and $K_3Fe(CN)_6$. In order to examine the possibility that R-NTF is also produced by NADPH-cytochrome c reductase in the presence of Triton X-100, NADPH-cytochrome c reductase was purified from microsomes and its activity was assayed at each step of the purification process using DCIP and NT as a substrate. Furthermore, the ratio of the activities for the two substrate was estimated. The purification method is described in the "Materials and Methods" and the result is shown in Table I. NADPH-cytochrome c reductase was purified about 250 fold. The reduction activity of purified enzyme for cytochrome c was 9.9 μmole/min/mg protein at room temperature (20°). Its reduction activity for $K_3Fe(CN)_6$ determined by the method of Williams and Kamin[4] was 15.5 μmole/min/mg protein. This enzyme could also reduce NT in the absence of Triton X-100, but P-NTF activity could not be determined exactly. Because it had been reported that a plot of P-NTF activity versus amount of solubilized with lipase were nonlinear[4] and in addition, it was observed that a plot of P-NTF activity versus incubation time was also nonlinear.

The ratio of reductive activity of DCIP to that of NT in the presence of TritonX-100 was almost constant and was in the range of 3.1 to 4.1. These results indicated that the enzyme which could reduce NT to R-NTF in the presence of Triton X-100 was identical with

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Table I. Purification of NADPH-Cytochrome c Reductase

Step	Protein (mg)	R-NTF activity: Activity (units/mg)	Total activity (units)	Purity	DCIP reduction activity: Activity (units/mg)	Total activity (units)	Purity	DCIP/NT
I	2700.0	0.029	77.8	1.0	0.091	245.8	1.0	3.14
II	994.7	0.077	76.7	2.7	0.315	313.7	3.5	4.09
III	35.0	1.146	40.2	39.5	3.796	133.0	41.7	3.31
IV	0.83	7.180	6.0	247.6	24.275	20.2	266.8	3.38

One unit is 1.0 μmole of reduced product/min mg. DCIP/NT shows the ratio of activity for DCIP and NT.

NADPH-cytochrome c reductase.

Thus, it was clarified that NADPH-cytochrome c reductase reduces NT to a P-NTF in the absence of Triton X-100, but to R-NTF in the presence of Triton X-100. It was considered that there are some differences in the formation process of R- and P-NTF in the presence or absence of Triton X-100. To investigate these differences, effect of various substances on P- and R-NTF activities were examined. CN^-, EDTA, and *o*-phenanthroline as chelators, ascorbic acid, cysteine, and glutathione as reductants, Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, and Ba^{2+} as metal ions were used. In these substances, CN^-, EDTA, cysteine, ascorbic acid, Cu^{2+}, and Mn^{2+} strongly activated or inhibited NADPH-cytochrome c reductase. As shown in Table II, both activities were equally activated by CN^-, but R-NTF activity was activated more than P-NTF activity by cysteine. In addition, EDTA remarkably activated R-NTF activity at a very low concentration but it did not affect the P-NTF activity. Especially, Cu^{2+} inhibited about 80% of the activity at such a low concentration as 10^{-7}M. From these results, it might be presumed that formation mechanisms of P- and R-NTF by NADPH-

Table II. Effects of Various Substances on P- and R-NTF Activity

Additive	Amount (M)	P-NTF activity (%)	R-NTF activity (%)
None	—	100.0	100.0
CN^-	10^{-3}	126.0	119.1
	5×10^{-3}	140.0	149.2
	10^{-2}	157.2	156.0
EDTA	5×10^{-6}	100.0	164.6
	10^{-5}	100.0	175.4
	5×10^{-5}	100.0	190.8
Cysteine	5×10^{-5}	112.3	166.3
	10^{-4}	120.3	174.3
	2.5×10^{-4}	127.2	184.0
Ascorbic acid	5×10^{-5}	95.1	86.2
	10^{-4}	91.4	31.3
	2.5×10^{-4}	82.7	6.4
	5×10^{-4}	77.8	0.0
Cu^{2+}	2×10^{-8}	100.0	66.7
	6×10^{-8}	100.0	43.9
	10^{-7}	100.0	27.3
	2×10^{-6}	96.5	0.0
	10^{-5}	84.5	0.0
Mn^{2+}	10^{-7}	91.5	71.5
	2×10^{-7}	87.5	51.1
	5×10^{-7}	84.5	29.0

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cytochrome c reductase would be different from each other.

It has been reported by Perderson and Aust[7] that the purified NADPH-cytochrome c reductase from microsomes catalyzed the NADPH-dependent peroxidation of isolated microsomal lipids. Also, they mentioned that lipid could be peroxidized by superoxide anion which was produced by NADPH-cytochrome c reductase in the presence of NADPH because lipid peroxidation activity was inhibited by superoxide dismutase of bovine erythrocytes.

It has been known that superoxide anion was rich in its reactivity and was responsible to both reductive and oxidative action, such as reduction of cytochrome c or oxidation of anthranilate.[8] Therefore, a reductive reaction by superoxide anion which was produced in enzymic reaction of NADPH-cytochrome c reductase may be detected in the formation of either P- or R-NTF. The effect of superoxide dismutase on P- and R-NTF activities was then examined. As shown in Fig. 3, P-NTF activity was hardly inhibited but R-NTF activity was inhibited by 50% by the addition of 0.5 μg of superoxide dismutase. This result indicates that superoxide anion, which would be produced by NADPH-cytochrome c reductase in its reaction process, participated in the formation of R-NTF.

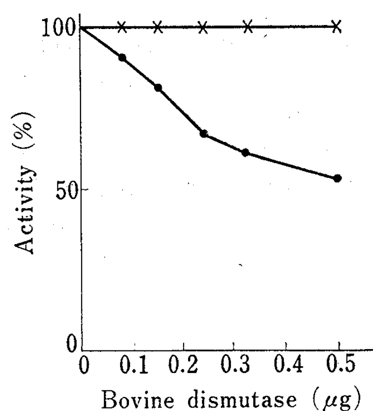

Fig. 3. Effect of Bovine Dismutase on P- and R-NTF Activity

×—×: P-NTF activity, ●—●: R-NTF activity

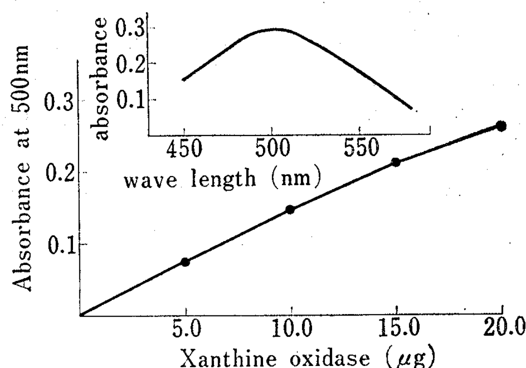

Fig. 4. Formation and Visible Spectrum of R-NTF produced by Xanthine Oxidase in the Presence of Triton X-100

On the other hand, it has been reported that xanthine oxidase-hypoxanthine (or xanthine) system could produce superoxide anion.[9] If R-NTF could be formed by the reductive action of superoxide anion in the presence of Triton X-100, it must be formed in xanthine oxidase-hypoxanthine system in the presence of Triton X-100. As shown in Fig. 4, a red product of NT was obtained in xanthine oxidase system as expected. This red product was spectrophotometrically identical with R-NTF produced in the NADPH-cytochrome c reductase system.

Discussion

NT is usually reduced by NADPH-cytochrome c reductase and NADPH, and changes to purple formazan but, in the presence of Triton X-100, a red formazan was obtained in the same enzyme system. Both formazans were apparently different in respect to visible spectrum and thin-layer chromatography. Since NT has two tetrazolium rings in its structure, two NT formazans could be assumed in one of which, one tetrazolium ring was reduced and the other with two rings reduced. R-NTF would correspond to the product with one tetrazolium ring in two rings of NT was reduced because the red formazan in thin-layer chromatogram

7) T.C. Perderson and S.D. Aust, *Biochem. Biophys. Res. Commun.*, **48**, 789 (1972).
8) P. Prema, S.D. Ravindranath, C.S. Vaidyanathan, and N.A. Rao, *Biochem. Biophys. Res. Commun.*, **49**, 1422 (1972).
9) R.F. Knowles, J.F. Gibson, F.M. Pick, and R.C. Bray, *Biochem. J.*, **111**, 53 (1969).

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on silica changed to purple when sprayed with sodium hydrosulfite solution.

Okui, *et al.*[3d] reported that R-NTF was formed in a short incubation time by mitochondria in the presence of succinate, but if its time was long, R-NTF disappeared gradually and changed to purple formazan. Perhaps, mitochondria-succinate system will be able to reduce R-NTF to purple formazan because tetrazolium derivatives having one tetrazolium ring such as 2,3,5-triphenyl-tetrazolium or 2,5-diphenyl-3-(*p*-diphenyl)-tetrazolium can be reduced in the same system. However, P-NTF could not be detected in microsomes or purified NADPH-cytochrome c reductase system in the presence of Triton X-100 even if the incubation time was long.

On the other hand, action of Triton X-100 added to the reaction system was also an important problem, and its action could be considered as follows: Triton X-100 would affect either the enzyme protein or an oxidation-reduction potential of NT. The latter speculation is more likely since Triton X-100 did not affect the reduction of cytochrome c and DCIP by microsomes or purified NADPH-cytochrome c reductase. This speculation was also confirmed from the fact that NT could not be reduced by ascorbic acid, but was reduced to red if ascorbic acid was added to NT solution in the presence of Triton X-100 (These data were not shown in this paper).

The formation mechanism of R- and P-NTF was assumed to be different because R- and P-NTF activity was affected different by ascorbic acid, Cu^{2+}, and Mn^{2+}, as shown in Table II. In addition, R-NTF activity was strongly inhibited by superoxide dismutase purified from bovine erythrocytes whereas P-NTF activity was not affected.

As shown above, it was clarified that NT was reduced to a red formazan in the presence of Triton X-100 by superoxide anion which was produced by NADPH-cytochrome c reductase.

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