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NADH- and NADPH-Dependent Formation of Superoxide Anions by Bovine Heart Submitochondrial Particles and NADH–Ubiquinone Reductase Preparation

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1. Both NADH and NADPH supported the oxidation of adrenaline to adrenochrome in bovine heart submitochondrial particles. The reaction was completely inhibited in the presence of superoxide dismutase, suggesting that superoxide anions (O_2^-) are responsible for the oxidation. The optimal pH of the reaction with NADPH was at pH 7.5, whereas that with NADH was at pH 9.0. The reaction was inhibited by treatment of the preparation with *p*-hydroxymercuribenzoate and stimulated by treatment with rotenone. Antimycin A and cyanide stimulated the reaction to the same extent as rotenone. The NADPH-dependent reaction was inhibited by inorganic salts at high concentrations, whereas the NADH-dependent reaction was stimulated. 2. Production of O_2^- by NADH–ubiquinone reductase preparation (Complex I) with NADH or NADPH as an electron donor was assayed by measuring the formation of adrenochrome or the reduction of acetylated cytochrome *c* which does not react with the respiratory-chain components. *p*-Hydroxymercuribenzoate inhibited the reaction and rotenone stimulated the reaction. The effects of pH and inorganic salts at high concentrations on the NADH- and NADPH-dependent reactions of Complex I were essentially similar to those on the reactions of submitochondrial particles. 3. These findings suggest that a region between a mercurial-sensitive site and the rotenone-sensitive site of the respiratory-chain NADH dehydrogenase is largely responsible for the NADH- and NADPH-dependent O_2^- production by the mitochondrial inner membranes.

The production of superoxide anions (O_2^-) by antimycin-inhibited bovine heart submitochondrial particles with succinate or NADH as an electron donor has been reported (Boveris *et al.*, 1976). Ubiquinone was postulated as the site of the O_2^- generation in the NADH-dependent reaction, from the observations that the H_2O_2 production of NADH–ubiquinone reductase preparation (Complex I) was inhibited by rotenone and was stimulated by supplementation with short-chain ubiquinone homologues (Cadenas *et al.*, 1977). In higher-plant mitochondria, the flavoprotein of the NADH dehydrogenase (EC 1.6.99.3) and ubiquinone–cytochrome *b* region were proposed as the sites of O_2^- production (Rich & Bonner, 1978).

We have previously reported that bovine heart submitochondrial particles catalyse NADPH-dependent lipid peroxidation (autoxidation) in the presence of Fe^{3+} and adenine nucleotides (Takeshige & Minakami, 1975). In the present paper, we have tried to characterize both NADH- and NADPH-dependent O_2^- production by bovine heart submitochondrial particles or Complex I, and to define the possible site of its reaction in the respiratory chain. The reaction with either NADH or NADPH seemed to occur largely at a region between a mercurial-sensitive site and the rotenone-sensitive site of the respiratory-chain NADH dehydrogenase.

Experimental

Preparation of submitochondrial particles and Complex I

Bovine heart mitochondria were prepared from slaughterhouse material by the method of Blair (1967). Submitochondrial particles were prepared by modifying the method of Knowles & Penefsky (1972) as follows. The mitochondria were suspended in 250mM-sucrose/5mM-Tris/HCl buffer, pH 7.8, and sonicated with a Tomy model UR-150P sonicator at an output of 4 A for 1 min at 4°C. A fraction that sedimented between 27000g × 15 min and 77000g × 60 min at 4°C was washed twice with the sucrose/Tris solution by centrifugation at 77000g × 60 min at 4°C. The washed particles were essentially free from superoxide dismutase (EC 1.15.1.1).

Complex I was prepared by the procedure of Hatefi & Rieske (1967).

Preparation of acetylated cytochrome c

Cytochrome *c* was acetylated with acetic anhydride as described previously (Minakami *et al.*, 1958; Kakinuma & Minakami, 1978). Acetic anhydride (170mol/mol of cytochrome *c*) was slowly added to horse heart cytochrome *c* (100mg) dissolved in 10ml of half-saturated sodium acetate solution with continuous stirring at 4°C. After 60min, the solution was dialysed at 4°C for 24h against 4×5 litres of water. The sample was applied to a CM-cellulose column (0.8cm×5cm) equilibrated with 10mM-potassium phosphate buffer, pH7.8, and eluted with the same buffer. The eluate was stored at −20°C. The extent of acetylation was determined by the ninhydrin method (Hirs, 1967) and calculated as described by Azzi *et al.* (1975). About 70% of the lysine residues were acetylated.

Reagents

Superoxide dismutase was purified from ox blood by the method of McCord & Fridovich (1969). Acetylpyridine–adenine dinucleotide, horse heart cytochrome *c* and milk xanthine oxidase were purchased from Boehringer und Söhne, Mannheim, Germany, and adrenaline was from Merck, Darmstadt, Germany. NAD^+, $NADP^+$, NADH and NADPH were obtained from Oriental Yeast Industries, Tokyo, Japan. Antimycin A and rotenone were obtained from Kyowa Fermentation Industries, Tokyo, Japan and Nakarai Chemicals, Kyoto, Japan respectively. Other reagents were of analytical grade.

Analytical procedures

The formation of O_2^- was determined by measuring either the formation of adrenochrome (Misra & Fridovich, 1972; Loschen *et al.*, 1974) or the reduction of acetylated cytochrome *c* (Azzi *et al.*, 1975; Kakinuma & Minakami, 1978). The former method was applied for both submitochondrial particles and Complex I, whereas the latter was used only for Complex I. Adrenochrome formation was assayed as follows. Submitochondrial particles or Complex I were preincubated for 5min at 37°C in a buffered 250mM-sucrose solution. The buffers used were 50mM-sodium Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] for pH6.5–8.25 and 50mM-sodium borate for pH8.25–10. After the addition of freshly dissolved 1nM-adrenaline, the reaction was started by the addition of 1mM-NADH or 1mM-NADPH, unless otherwise stated. Adrenochrome formation was measured at 37°C in dual-wavelength mode ($A_{485}-A_{575}$nm) with a Hitachi 556 spectrophotometer. The absorption coefficient used was 2.96×10^3 litre·mol^{-1}·cm^{-1} (Green *et al.*, 1956).

The reduction of acetylated cytochrome *c* was similarly assayed in a reaction mixture consisting of the buffered 250mM-sucrose and 35μM-acetylated cytochrome *c*. The reaction was initiated by the addition of 1mM-NADH or 1mM-NADPH, and was followed at 37°C in dual-wavelength mode ($A_{550}-A_{540\,nm}$). The absorption coefficient of native cytochrome *c* (19.1×10^3 litre·mol^{-1}·cm^{-1}) was used for the acetylated derivatives, because essentially no changes in the visible spectra were observed after acetylation (Minakami *et al.*, 1958). The NADPH-dependent adrenochrome formation in the rat liver microsomal fraction was assayed by the method of Aust *et al.* (1972) with some modifications: the microsomal fraction was preincubated in 250mM-sucrose/50mM-Tris/HCl buffer, pH8.25, for 5min at 37°C, and after the addition of 1mM-adrenaline, the reaction was started with 0.5mM-NADPH.

The $NAD(P)^+$ transhydrogenase (EC 1.6.1.1) activity was determined by measuring the increase in A_{375} at 30°C in a reaction mixture consisting of 100mM-potassium phosphate buffer, pH6.5, 1mM-acetylpyridine–adenine dinucleotide and 2μM-rotenone in 1.2ml (Kaplan, 1967). The superoxide dismutase activity was determined by measuring the inhibition of the reduction of cytochrome *c* in the xanthine–xanthine oxidase system (McCord & Fridovich, 1969). The amount of superoxide dismutase required to inhibit the rate of the reduction of cytochrome *c* by 50% is defined as 1 unit of activity. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Production of O_2^- by submitochondrial particles

The formation of adrenochrome from adrenaline was observed in bovine heart submitochondrial particles with NADH or NADPH as electron donor. The reaction was completely inhibited by superoxide dismutase, indicating the contribution of O_2^- to the reaction. The activity in whole mitochondria was low and variable, probably because of the presence of endogenous superoxide dismutase. Catalase did not affect the reaction. Because of the difference in optimal pH values, as shown below, the NADH-dependent reaction was assayed at pH9.0 and the NADPH-dependent reaction at pH7.5. The reaction was not linear with time and started with an appreciable lag time, but if we took the apparently linear part as the rate of the reaction, the rate was essentially proportional to the amount of submitochondrial particles used. The rates of adrenochrome formation (nmol/min per mg of protein; means ± S.D., $n = 5$) supported by NADH and NADPH were 10.7 ± 3.5 and 8.2 ± 2.8 respectively, corresponding roughly to 1% of the NADH oxidase activity and 2.5% of the

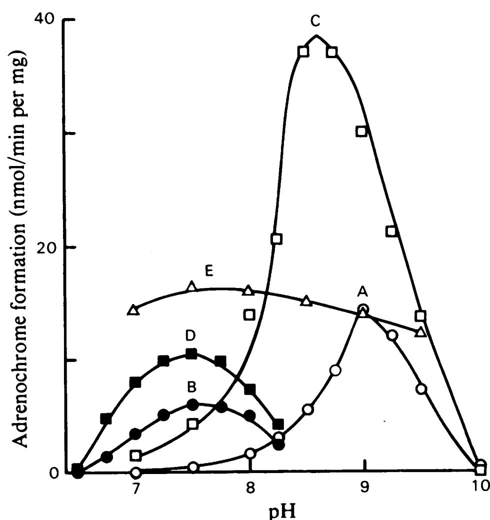

Fig. 1. *Effect of pH on adrenochrome formation by submitochondrial particles preincubated in the presence or absence of rotenone*

Submitochondrial particles (0.5 mg/ml) were preincubated for 5 min at 37°C in a buffered 250 mM-sucrose solution in the presence or absence of 2 μM-rotenone. The buffers used were 50 mM-sodium Hepes buffer for the pH between 6.5 and 8.25, and 50 mM-sodium borate buffer for the pH between 8.25 and 10.0. After the addition of 1 mM-adrenaline, the reaction was started with NAD(P)H. Curves: A (○), without rotenone and with 1 mM-NADH; B (●), without rotenone and with 1 mM-NADPH; C (□), with rotenone and with 1 mM-NADH; D (■), with rotenone and with 1 mM-NADPH; E (△), with rotenone and with 0.2 mM-NADH.

NADPH oxidase activity. The apparent K_m values were 0.33 mM for NADH and 0.31 mM for NADPH.

pH-dependence of the reaction and effects of rotenone

The effects of pH on NADH- and NADPH-dependent O_2^- production are shown in Fig. 1. The pH optimum of the NADH-dependent reaction was at pH 9.0 and that of the NADPH-dependent reaction was at pH 7.5, when the concentrations of nicotinamide coenzymes were 1.0 mM (Curves A and B). When submitochondrial particles were pretreated with 2 μM-rotenone for 5 min at 37°C (rotenone-treated preparation), the O_2^- production supported by either NADH or NADPH was distinctly stimulated. At the same time, the pH optimum of the NADH-dependent reaction was shifted from pH 9.0 to 8.5 (Curve C) in contrast with that of the NADPH-dependent reaction, which was not shifted (Curve D). This shift of the pH optimum of the NADH-dependent reaction to neutral may be explained by an accumulation of a reduced component, which is rapidly oxidized through the respiratory chain at neutral pH when the inhibitor is not present. A similar shift of the pH optimum was observed after treatment with antimycin A or KCN.

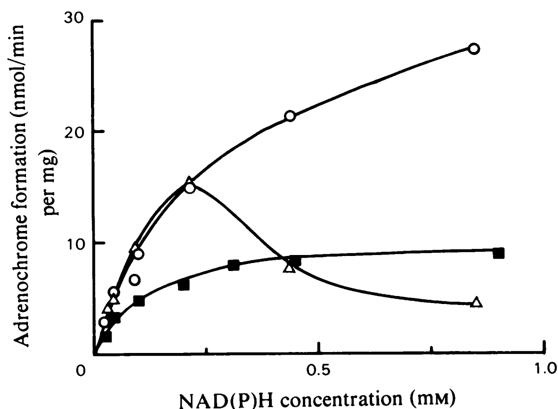

Fig. 2. *Effects of NAD(P)H concentrations on adrenochrome formation by rotenone-treated submitochondrial particles*

Submitochondrial particles (0.5 mg/ml) were treated with 2 μM-rotenone in a buffered 250 mM-sucrose solution at 37°C for 5 min. The subsequent assay conditions were as in Fig. 1. The apparent K_m values of the reactions were 0.19 mM for NADH at both pH 7.5 and 8.5, and 0.11 mM for NADPH at pH 7.5. ○, With NADH at pH 8.5; △, with NADH at pH 7.5; ■, with NADPH at pH 7.5.

The pH-dependence curve of the NADH-dependent reaction was also affected by NADH concentration. When this was decreased to 0.2 mM, the pH curve became flat; the activity at alkaline pH decreased and the activity at neutral pH increased (Fig. 1, Curve E). The situation for the coenzyme-dependence curve of rotenone-treated submitochondrial particles is also shown (Fig. 2). The NADH-dependent activity at pH 7.5 was inhibited by NADH at high concentrations (higher than 0.2 mM). This is in agreement with the observations that NADH–ferricyanide reductase and NADH oxidase activities are strongly inhibited by excess NADH (Minakami *et al.*, 1962; Hatefi & Stempel, 1969). The NADH-dependent activity at pH 8.5 and the NADPH-dependent activity at pH 7.5 was not inhibited by NADH at high concentrations.

Effect of inorganic salts

The NADPH-dependent O_2^- formation was strongly inhibited by high concentrations of KCl, which is similar to the effect observed for NADPH-dependent lipid peroxidation in bovine heart submitochondrial particles (Takeshige & Minakami, 1975). The NADPH-dependent O_2^- generation measured at pH 7.5 with 1 mM-NADPH was inhibited by 100 mM- and 500 mM-KCl to 31 and 2%

Table 1. *Effects of respiratory-chain inhibitors on adrenochrome formation by submitochondrial particles and Complex I*

Submitochondrial particles (0.5 mg/ml) or Complex I (90 µg/ml) were incubated for 5 min at 37°C with inhibitors (concentrations given in parentheses) before the initiation of the reactions.

	Submitochondrial particles				Complex I			
	Activity with 1 mM-NADH at pH 8.5		Activity with 1 mM-NADPH at pH 7.5		Activity with 1 mM-NADH at pH 8.5		Activity with 1 mM-NADPH at pH 7.5	
	(nmol/min per mg of protein)	(%)	(nmol/min per mg of protein)	(%)	(nmol/min per mg of protein)	(%)	(nmol/min per mg of protein)	(%)
Control	4.3	100	7.2	100	24.4	100	18.2	100
+ Ethanol (150 mM)	4.3	100	7.4	102	23.8	98	18.0	100
+ Rotenone (2 µM)	31.2	720	15.3	212	64.4	264	26.4	145
+ Antimycin A (1 µM)	30.7	715	15.2	210	24.5	100	17.9	98
+ KCN (1 mM)	29.2	680	15.5	215	23.5	96	17.8	98
+ *p*-Hydroxymercuribenzoate (1 mM)	2.5	57	3.9	54	12.9	53	8.2	45

of the control activity respectively, whereas, in the presence of the same concentrations of KCl, the NADH-dependent activities measured at pH 9.0 with 1 mM-NADH were stimulated to 103 and 114% respectively, and the NADPH-dependent O_2^- generation by rat liver microsomal fraction was activated to 192 and 220% of the control activity respectively. Other inorganic salts (NaCl, Na_2SO_4 and potassium phosphate buffer) of the same ionic strength showed a similar effect. Inhibition by high concentrations of inorganic salts seems to be a characteristic property of NADPH-dependent reactions of bovine heart submitochondrial particles.

Effects of respiratory-chain inhibitors

O_2^- generation was influenced by various inhibitors of the mitochondrial respiratory chain (Table 1). Submitochondrial particles were preincubated with the inhibitors for 5 min at 37°C. NADH-dependent activities were assayed at pH 8.5, because the effects were most pronounced at this pH. Rotenone stimulated the reaction as described above: about 2-fold with NADPH and 7-fold with NADH. Antimycin A and KCN stimulated the reaction with NADH or with NADPH essentially to the same extent as rotenone. Ethanol, which was used to dissolve rotenone and antimycin A, did not affect the activity. *p*-Hydroxymercuribenzoate inhibited O_2^- generation. These observations imply that the main region of O_2^- generation lies between a mercurial-sensitive site and the rotenone-sensitive site of the respiratory-chain NADH dehydrogenase.

Trypsin digestion of submitochondrial particles

There remains a possibility that the NADPH-dependent reaction is catalysed by a coupled reaction consisting of NAD(P)$^+$ transhydrogenase and the NADH-dependent reaction: NADPH reduces endogenous NAD$^+$ to NADH and the latter is used in NADH-dependent O_2^- formation. This possibility could be ruled out by an experiment in which submitochondrial particles are treated with trypsin, because transhydrogenase is known to be highly sensitive to trypsin digestion (Juntti *et al.*, 1970; Djavadi-Ohaniance & Hatefi, 1975). After treatment of submitochondrial particles (25 mg/ml) in 250 mM-sucrose/100 mM-Tris/HCl buffer, pH 7.0, with trypsin (0.1 mg/mg of protein) at 0°C for 120 min and subsequent addition of soya-bean trypsin inhibitor (0.5 mg/mg of protein), the transhydrogenase activity was completely inactivated, whereas NADPH-dependent O_2^- generation was essentially not affected. Furthermore, the concentration of NAD$^+$ in a reaction mixture consisting of 0.5 mg of submitochondrial particles and 1.0 mM-NADPH is below the limit of detection (less than 130 nM), when measured by a highly sensitive assay method described by Rydström *et al.* (1973).

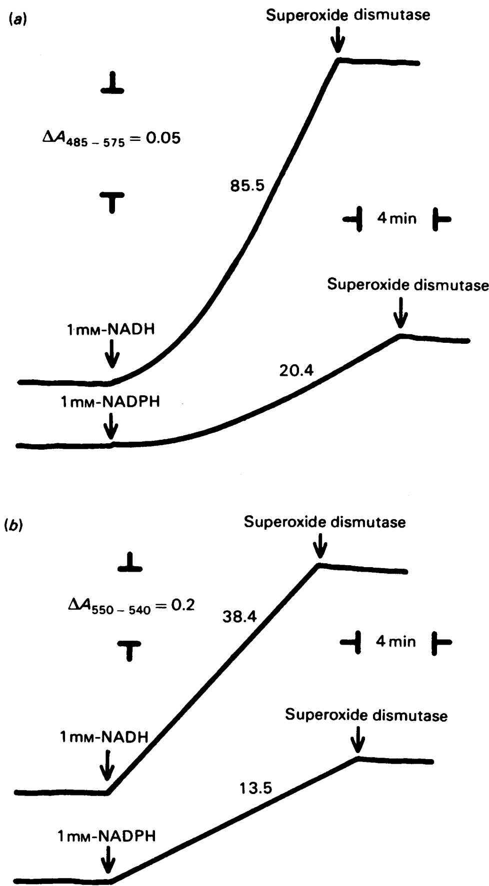

Fig. 3. *Adrenochrome formation and reduction of acetylated cytochrome c by Complex I*

Complex I (90 μg/ml) was preincubated for 5 min at 37°C in a buffered 250 mM-sucrose solution. The buffers used were 50 mM-sodium borate buffer, pH 9.0, for the NADH-dependent reaction and 50 mM-sodium Hepes buffer, pH 7.5, for the NADPH-dependent reaction. Adrenochrome formation was assayed at 37°C by the successive additions of 1 mM-adrenaline and 1 mM-NAD(P)H (*a*), and the reduction of acetylated cytochrome *c* was assayed at 550–540 nm by the successive additions of 35 μM-acetylated cytochrome *c* and 1 mM-NAD(P)H (*b*). Superoxide dismutase (10 μg/ml; 2500 units/mg) was added as indicated. The activities are given on the traces as nmol/min per mg of protein.

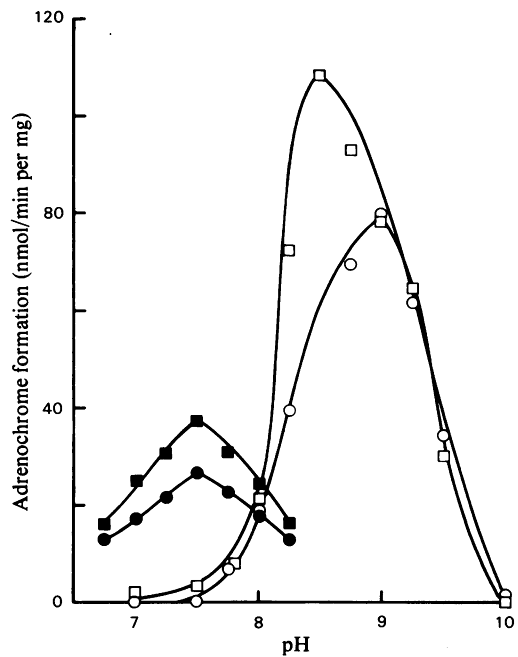

Fig. 4. *Effect of pH on adrenochrome formation by Complex I with or without rotenone pretreatment*

The assay conditions were the same as in Fig. 1, except that Complex I (90 μg/ml) was used. ○, Without rotenone and with 1 mM-NADH; ●, without rotenone and with 1 mM-NADPH; □, with rotenone and with 1 mM-NADH; ■, with rotenone and with 1 mM-NADPH.

Production of O_2^- by NADH–ubiquinone reductase preparation

Complex I generated O_2^- with NADH or NADPH as electron donor. The reaction was assayed by measuring either the formation of adrenochrome or the reduction of acetylated cytochrome *c*. Both the adrenochrome formation and the reduction of acetylated cytochrome *c* were completely inhibited by superoxide dismutase. The time course of adrenochrome formation was not linear, as observed for submitochondrial particles (Fig. 3*a*), whereas that of the reduction of the cytochrome derivative was linear without a lag time (Fig. 3*b*). We used acetylated cytochrome *c*, because the derivative does not interact with respiratory-chain components (Minakami *et al.*, 1958; Azzi *et al.*, 1975; Kakinuma & Minakami, 1978). The reduction of native cytochrome *c* was only slightly sensitive to superoxide dismutase. The rate of adrenochrome formation was higher than the rate of reduction of acetylated cytochrome *c*; the former may be overestimating the O_2^- generation, whereas the latter may be underestimating the generation, because the oxidation of adrenaline by O_2^- is known to be a complex autocatalytic reaction (Misra & Fridovich, 1972) and acetylated cytochrome *c* is susceptible to autoxidation (Minakami *et al.*, 1958).

Properties of the NADH- and NADPH-dependent O_2^--generating activities in Complex I were essentially

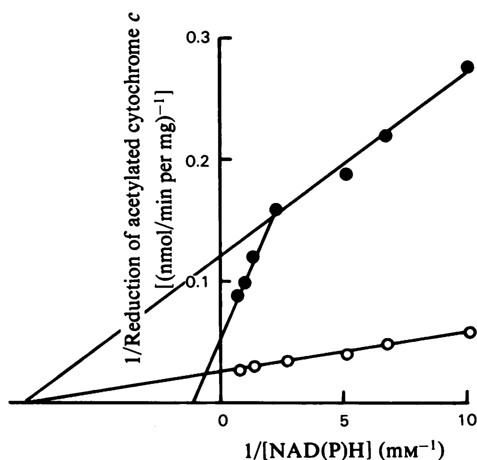

Fig. 5. *Double-reciprocal plots of the reduction of acetylated cytochrome c against NAD(P)H concentrations*
The assay conditions were the same as described for Fig. 3(*b*), except NAD(P)H concentrations. ○, NADH at pH 9.0; ●, NADPH at pH 7.5. The apparent K_m value for NADH was 0.13 mM. The NADPH-dependent reaction has two apparent K_m values of 0.13 and 0.91 mM.

similar to those of submitochondrial particles. The pH optimum for the NADPH-dependent reaction was pH 7.5 and that for the NADH-dependent reaction was pH 9.0, when the concentrations of reduced nicotinamide coenzymes were 1 mM (Fig. 4). *p*-Hydroxymercuribenzoate inhibited and rotenone activated both the NADH- and NADPH-dependent reactions. As expected, antimycin A and KCN were ineffective (Table 1). The NADPH-dependent reaction was inhibited by inorganic salts at high concentrations, whereas the NADH-dependent reaction was slightly stimulated (results not shown).

Kinetic constants of the NADH- and NADPH-dependent O_2^--generating activities of Complex I were determined by using acetylated cytochrome *c*. Double-reciprocal plots of the O_2^--generating activities against NAD(P)H concentrations are shown in Fig. 5. The plot of the NADPH-dependent reaction appeared to be biphasic, whereas that of the NADH-dependent reaction was not. The K_m value for NADH (0.13 mM) apparently agreed with that of the NADH–ferricyanide reductase activity of an NADH dehydrogenase preparation (108 μM; Minakami *et al.*, 1963) or of Complex I (100 μM; Dooijewaard & Slater, 1976). The low K_m value for NADPH (0.13 mM) may correspond to that for NADPH by submitochondrial particles (55 μM; Hatefi, 1973), but we have no explanation for the high K_m value (0.91 mM) of the NADPH reaction.

Discussion

Submitochondrial particles generate O_2^- not only with NADH but also with NADPH as electron donor. NADPH-dependent O_2^- formation by the preparation is not due to the contamination of the microsomal fraction, because the microsomal fraction of bovine heart muscle did not show as much NADPH-dependent O_2^- formation as that of liver. Moreover, the mitochondrial NADPH-dependent activity was affected by inhibitors of the respiratory chain, and the apparent K_m value for NADPH of the reaction in the submitochondrial preparation (about 0.1 mM) was much higher than that of the rat liver microsomal reaction (0.01 mM). The NADPH-dependent reaction could not be ascribed to a coupled reaction of $NAD(P)^+$ transhydrogenase reaction and the NADH-dependent reaction, because the preparation lost transhydrogenase activity as a result of trypsin treatment but retained O_2^- production with NADPH.

Both NADH- and NADPH-dependent reactions seemed to be catalysed by the respiratory-chain NADH dehydrogenase, because (*a*) both NADH- and NADPH-dependent activities were stimulated by rotenone, and (*b*) Complex I (NADH–ubiquinone reductase preparation) catalysed the formation of O_2^- with either NADH or NADPH and showed similar properties to those of submitochondrial particles. Direct oxidation of NADPH by respiratory-chain NADH dehydrogenase has been demonstrated (Hatefi, 1973). Distinct differences in properties, however, were observed between the NADPH- and NADH-dependent reactions. The optimal pH for the former was 7.5, whereas that for the latter was 9.0. The former reaction was strongly inhibited by inorganic salts at high concentrations, whereas the latter was slightly stimulated. The NADH-dependent reaction at pH 7.5 was inhibited by NADH at high concentrations, whereas the NADPH-dependent reaction was not inhibited by NADPH at high concentrations. The differences in interaction of NADH and NADPH with the NADH dehydrogenase might be explained by either the presence of an additional negative phosphate group in NADPH or the possible existence of different NADH and NADPH sites in the dehydrogenase.

The site of O_2^- production in the respiratory chain can be ascribed to a region between a mercurial-sensitive site and the rotenone-sensitive site, because the production was inhibited by *p*-hydroxymercuribenzoate and stimulated by rotenone and antimycin A. The site of the mercurial inhibition is not clear, but a slowly reacting thiol group, which is essential for NADH–ubiquinone reductase activity but not essential for the reduction of flavin by NADH (Minakami *et al.*, 1964; Tyler *et al.*, 1965), might be a candidate. A possible involvement of an iron–

sulphur component of NADH dehydrogenase as the site of O_2 reduction has been discussed by Tyler (1975). The extents of the stimulation by rotenone and antimycin A were essentially the same, which excludes the possibility that ubiquinone is the main site of O_2^- formation. This conclusion is apparently different from the proposal of Cadenas *et al.* (1977) that a reduced form of ubiquinone is responsible for the reduction of O_2 to O_2^-. Their conclusion was based on the observations that the formation of H_2O_2 by Complex I was inhibited by rotenone. We observed stimulation of O_2^- formation, when submitochondrial particles or Complex I was treated with rotenone. This discrepancy might arise from several differences in experimental conditions: Cadenas *et al.* (1977) assayed H_2O_2, added rotenone 5s before the start of the reaction and used a low concentration of NADH (3μM).

Superoxide anions or other active oxygen species derived from them have been shown to threaten the integrity of living cells. One of the actions of superoxide anions or their products on cellular components is the peroxidation of lipids. We have previously reported that bovine heart submitochondrial particles catalyse NADPH-dependent lipid peroxidation (Takeshige & Minakami, 1975). NADH can also support autoxidation of lipid in submitochondrial particles under different conditions (R. Takayanagi, unpublished work).

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