BIOSYNTHESIS OF NANAOMYCIN

III. NANAOMYCIN A FORMATION FROM NANAOMYCIN D BY NANAOMYCIN D REDUCTASE *VIA* A HYDROQUINONE

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Nanaomycin D reductase which is involved in the biosynthesis of the antifungal antibiotic nanaomycin catalyzes the formation of nanaomycin A from nanaomycin D in the presence of NADH under anaerobic conditions. On the other hand, under aerobic conditions NADH is consumed and nanaomycin A formation is markedly reduced. These findings suggest that nanaomycin A synthesis is not due to the direct reduction of the 5-membered lactone ring of nanaomycin D. Reduction of various quinones by the enzyme was examined. It was found that nanaomycin A is converted to its hydroquinone derivative in the presence of NADH under anaerobic conditions, whereas NADH consumption alone is observed under aerobic conditions. When *p*-benzoquinone, 1,4-naphthoquinone or menadione is used instead of nanaomycin D, NADH is also consumed. These results indicate that: (1) these compounds act as electron acceptors, (2) O_2 functions as final electron acceptor under aerobic conditions, and (3) nanaomycin D reductase is, in fact, an NADH dehydrogenase (quinone).

Changes in the UV-absorption spectrum of a reaction mixture containing nanaomycin D and NADH indicate that a hydroquinone derivative is formed as an intermediate during nanaomycin A formation. Similar results were obtained when nanaomycin D is reduced chemically with $NaBH_4$ or Zn powder. It was concluded that nanaomycin D is converted to a hydroquinone derivative and that nanaomycin A is then formed nonenzymatically through intramolecular electron transfer.

The nanaomycins (NNMs) are antifungal and antimycoplasmal antibiotics which possess benzoisochromane-quinone skeletons. We have reported that five components, NNM-A, B, C, D and E, are synthesized by *Streptomyces rosa* var. *notoensis* OS-3966,[1~6] and that the carbon skeleton of the antibiotics is derived from eight acetate units.[3] Studies of the biosynthetic relationship of the NNMs using a bioconversion method with cerulenin, a specific inhibitor of fatty acid and polyketide biosyntheses,[7] have revealed that the biosynthetic sequence is: NNM-D→NNM-A→NNM-E→NNM-B.[8]

In an earlier paper,[9] we reported the purification and properties of NNM-D reductase which catalyzes the conversion of NNM-D to NNM-A. The purified enzyme, a flavoenzyme possessing a molecular weight of 68,000, catalyzes the formation of NNM-A from NNM-D in the presence of NADH under anaerobic conditions. By contrast, under aerobic conditions NNM-A synthesis is greatly reduced, whereas NADH consumption is rather enhanced, suggesting that an intermediate step is involved in the reaction sequence. We now provide evidence showing that NNM-D reductase catalyzes the conversion of NNM-D to a hydroquinone which is then converted rapidly to NNM-A nonenzymatically under anaerobic conditions.

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Abbreviation used: NNM, nanaomycin.

Materials and Methods

NNM-D Reductase

An electrophoretically homogeneous enzyme preparation purified from crude extracts of *S. rosa* var. *notoensis* as described in a previous paper[9] was used throughout this investigation.

Chemicals

NNM-A was prepared as described by ŌMURA *et al.*[1,2] and NNM-E and 4*a*-*epi*-NNM-B were made by the method of KASAI *et al.*[8] NNM-D was prepared from NNM-A as reported in an earlier paper.[9] Other chemicals were obtained from commercial sources.

Assay Methods for NNM-A Formation and NADH Consumption by NNM-D Reductase under Aerobic or Anaerobic Conditions

A reaction mixture (final volume, 100 μl) containing 0.2 mM NNM-D, 1.0 mM NADH, 0.1 M phosphate buffer (pH 5.5) and 2 μg (as protein) of NNM-D reductase was incubated at 37°C under aerobic or anaerobic conditions. NNM-A formation was assayed as described previously[9] after addition of 10 μl of 3 N HCl to the reaction mixture. For NADH consumption, absorbance at 340 nm was measured with a Hitachi Model 100-40 Spectrophotometer following additions of 900 μl of 0.1 N NaOH to the reaction mixture.

Assay Method for NADH Dehydrogenase Activity of NNM-D Reductase under Aerobic Conditions

A reaction mixture (total volume, 1.5 ml) containing 0.2 mM electron acceptor (NNM or another quinone), 0.2 mM NADH, 0.1 M phosphate buffer (pH 5.5), and 10 μg (as protein) of NNM-D reductase was incubated at 37°C in an unsealed cuvette of a Shimadzu Double-beam Spectrophotometer (UV-210A) and the decrease in absorbance at 340 nm was monitored.

UV-Absorption Spectrum of the Reaction Mixture during Incubation under Anaerobic Conditions

A reaction mixture (total volume, 2.0 ml) containing 0.1 mM NNM-D or NNM-A, 0.1 mM NADH, 0.1 M phosphate buffer (pH 5.5), and 10 μg (as protein) of NNM-D reductase was incubated at 37°C in an anaerobic Thunberg cell placed in a Hitachi Scanning Spectrophotometer. The UV-visible absorption spectrum was recorded at the times indicated; the region from 600 nm to 300 nm was scanned in a 30 s interval.

NNM-A Formation from NNM-D in the Presence of $NaBH_4$ or Zn Powder

To a solution of NNM-D (10 mg) in CH_3COOH (2 ml) Zn powder (5 mg) was added and mixed vigorously. The mixture was extracted with ethyl acetate (5 ml) after addition of water (2 ml) and the extract was washed with water, evaporated, and then subjected to preparative thin-layer chromatography on silica gel 60 F_{254} (Merck, 0.25 mm thickness, 20×20 cm) using $CHCl_3$ - CH_3OH (10: 1, v/v) as solvent. Under UV light (254 nm) two separate zones of the product (Rf 0.38) and NNM-D (Rf 0.74) respectively could be seen. The slower running substance was scraped from silica gel plate and extracted with $CHCl_3$ - CH_3OH (5: 1, v/v). The filtered extract was evaporated to dryness to give an orange powder (3. 7 mg), which was identified as NNM-A by silica gel thin-layer chromatography and by UV and IR spectroscopy.

To a solution of NNM-D (10 mg) in $CHCl_3$ - CH_3OH (1: 1, v/v, 1 ml) $NaBH_4$ (5 mg) was added and mixed vigorously. $CHCl_3$ (10 ml) and 0.1 N HCl (5 ml) were then added with vigorous mixing. The $CHCl_3$ layer was evaporated to dryness to give an orange powder (4.6 mg), which was identified as NNM-A as described above.

Results

NNM-A Formation from NNM-D and NADH Consumption by NNM-D Reductase under Aerobic and Anaerobic Conditions

As shown in Fig. 1, the conversion of NNM-D to NNM-A with a purified preparation of NNM-D reductase under anaerobic conditions is accompanied by the consumption of NADH. The amount of

Fig. 1. NADH consumption and NNM-A formation by NNM-D reductase under aerobic (○) and anaerobic (●) conditions.

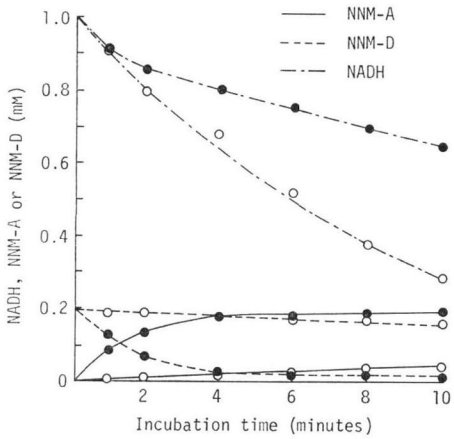


Table 1. The ability of various compounds to serve as substrate (electron acceptor) of NNM-D reductase under aerobic conditions.

Compound	NADH consumed nmole/minute	Relative activity (%)
None	0	0
NNM-D	94.6	100
NNM-A	18.8	20
NNM-E	10.7	11
4*a*-*epi*-NNM-B	4.3	5
Menadione	30.0	32
Ubiquinone-10	0	0
p-Benzoquinone	86.0	91
1,4-Naphthoquinone	91.6	97

NADH consumed was somewhat larger than that of NNM-D consumption because NADH was also consumed in the presence of NNM-A and NNM-D reductase as described below. On the other hand, under aerobic conditions NNM-A formation was markedly reduced, NADH was much consumed and NNM-D consumption was also deminished (Fig. 1). These findings indicate that a reduction product derived from NNM-D was reoxidized in the presence of O_2 to NNM-D, suggesting that NNM-A formation from NNM-D under anaerobic conditions is not due to the direct reduction of the 5 membered lactone ring of NNM-D. This conclusion is based on the finding that NNM-A is not readily oxidized to NNM-D.

Effect of NNMs and Several Quinones on NADH Dehydrogenase Activity of NNM-D Reductase

When NNM-D, other NNMs or a number of quinones were examined as substrates in the reaction, NNM-D reductase activity was assayed by NADH consumption under aerobic conditions. As shown in Table 1, NNM-D proved to be the best electron acceptor; however, other compounds such as *p*-benzoquinone, 1,4-naphthoquinone and, to a lesser extent, menadione also were good electron acceptors. By contrast, ubiquinone, which is an important electron acceptor in the bacterial respiratory chain,[10] was not reduced at all. NNM-A and NNM-E also served as substrates exhibiting 20 and 10 percent, respectively, the activity observed with in NNM-D. These results imply that NNM-D reductase is, in fact, an NADH dehydrogenase (quinone) and that, under aerobic conditions, a quinone is reduced by the enzyme to a semiquinone or hydroquinone which is then converted to the starting compound by autooxidation as described by IYANAGI and YAMAZAKI.[11,12]

Formation of Hydroquinones from NNM-A and NNM-D under Anaerobic Conditions

As described above, when NNM-D was incubated with NNM-D reductase and NADH under anaerobic conditions, NNM-A was formed; by contrast, formation of the product was markedly reduced under aerobic conditions. Aerobically various quinones served as electron acceptors, but the reduction product of a given reaction was not detected because of reoxidation to the starting compound. To identify the reduction product, changes in the UV-absorption spectrum of a reaction mixture were ex-

Fig. 2. Change of UV spectrum of the reaction mixture containing NNM-A, NADH, and NNM-D reductase during incubation at pH 5.5 under anaerobic conditions.

1, without enzyme: 2, 5 minutes; 3, 12 minutes; 4, 60 minutes; 5, after the Thunberg cell incubated for 60 minutes under anaerobic conditions was opened and mixed thoroughly.

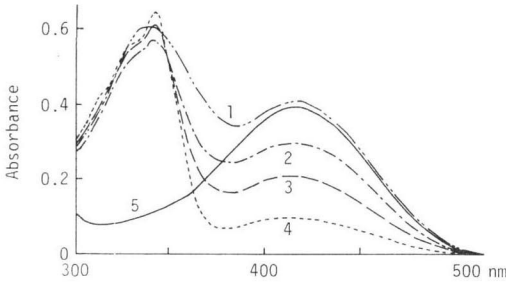
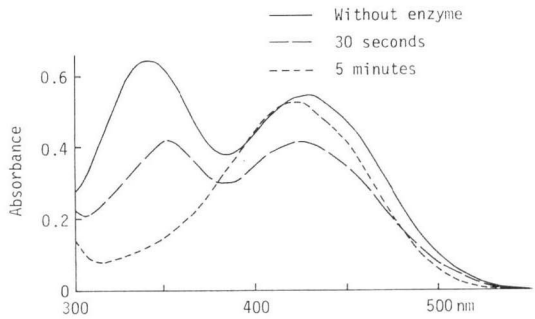


Fig. 3. Change of UV spectrum of the reaction mixture containing NNM-D, NADH, and NNM-D reductase during incubation at pH 5.5 under anaerobic conditions.



amined when NNM-A or NNM-D was incubated with NNM-D reductase and NADH under anaerobic conditions. As shown in Fig. 2, the absorption of NNM-A at 423 nm and of NADH at 340 nm decreased during the 60 minutes incubation; by contrast, there was an increased absorption at 344 nm and 330 nm (shoulder). These results are consistent with the formation of a hydroquinone derivative. When the UV-cell was opened after 60 minutes and the reaction mixture was thoroughly mixed, the absorption spectrum obtained was characteristic of that observed with NNM-A.

When NNM-D instead of NNM-A was used as substrate, a complex series of changes in the UV-absorption spectrum of the reaction mixture were observed. The absorption of NADH and NNM-D at 340 nm and 426 nm, respectively, declined, and a new absorption peak appeared at 352 nm. The novel peak, considered to be due to the putative hydroquinone, appeared after 30-second incubation (Fig. 3). After a further 5-minute incubation the absorption at 352 nm disappeared with the resultant appearance of an absorption spectrum with a maximum at 423 nm characteristic of NNM-A.

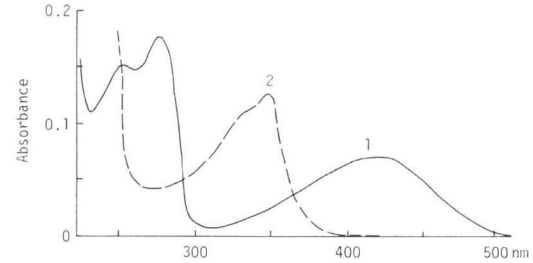
The above results indicate that NNM-A and NNM-D are reduced to their hydroquinone derivatives by NNM-D reductase in the presence of NADH, and that the hydroquinone of NNM-D is readily converted to NNM-A under anaerobic conditions. It seems that NNM-A formation from the hydroquinone occurs *via* a nonenzymatic reaction (s). By contrast, under aerobic conditions, the hydroquinones formed from NNM-A or NNM-D are readily re-oxidized to NNM-A or NNM-D, respectively.

Chemical Reduction of NNM-A and NNM-D

To examine whether the hydroquinone of NNM-D is converted to NNM-A nonenzymatically, the reduction of NNM-A and NNM-D with $NaBH_4$ or Zn powder was carried out. When a solution of NNM-A was treated with $NaBH_4$, a spectrum (with a maximum at 344 nm and a shoulder at 330 nm) similar to that observed in the reduction of NNM-A with NNM-D reductase was seen as shown in Fig. 4. When

Fig. 4. UV spectra of NNM-A and reduced NNM-A (hydroquinone).

1, 15 μM NNM-A in 50 mM tris-HCl (pH 7.5); 2, after incubation of the solution for 5 minutes in the presence of 5 mM $NaBH_4$.



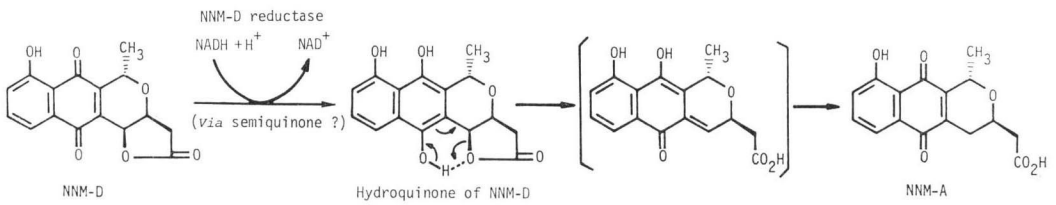
NNM-D was treated with $NaBH_4$ or Zn powder, a similar spectrum was observed. When the reaction mixture was extracted with ethyl acetate and the extract was subjected to preparative thin-layer chromatography on silica gel as described in Materials and Methods, NNM-A was obtained. This finding suggests that the hydroquinone derived from NNM-D was readily converted to NNM-A by a nonenzymic process (es). Under aerobic conditions, the hydroquinone is reoxidized because the oxidation reaction is faster than the conversion to NNM-A.

Discussion

NNM-D reductase isolated from the NNM producer *S. rosa* var. *notoensis* catalyzes the conversion of NNM-D to NNM-A.[9] The enzyme was found to be able to reduce NNM-A, NNM-E, *p*-benzoquinone, 1,4-naphthoquinone, and menadione (vitamin K_3) as well as NNM-D in the presence of NADH. This finding indicates that the enzyme is an NADH dehydrogenase (quinone) [NADH: (quinone-acceptor) oxidoreductase (EC 1.6.99.5)]. The enzyme is a soluble flavoprotein possessing FAD as prosthetic group, requires NADH as electron donor exclusively, and has an optimum pH of 5.5.[9] Ubiquinone which generally functions as an important electron acceptor in the bacterial respiratory chain[10] does not appear to serve as electron acceptor in this reaction whereas menadione is quite active in this respect.

The reaction mechanism of NNM-A formation from NNM-D is proposed to take place as follows: when NNM-D is used as electron acceptor under anaerobic conditions, it is reduced to a hydroquinone derivative by the enzyme (*via* semiquinone ?) and then the hydroquinone is nonenzymatically converted to NNM-A through intramolecular electron transfer as shown in Fig. 5. On the other hand, under aerobic conditions, because O_2 acts as final electron acceptor, the hydroquinone or semiquinone is reoxidized almost completely to NNM-D and NNM-A formation is greatly diminished. In addition, IYANAGI and YAMAZAKI[12] have reported the formation of a semiquinone through one-electron reduction of quinones by microbial flavin enzymes. Further experimentation is necessary to clarify whether the primary product in the reduction by the enzyme is a semiquinone or hydroquinone.

Fig. 5. Possible reaction mechanism from NNM-D to NNM-A by NNM-D reductase.



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