CIS-TRANS ISOMERIZATION OF NITROFURAN DERIVATIVES BY XANTHINE OXIDASE

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SUMMARY. Enzymatic cis-trans isomerization of nitrofuran derivatives was demonstrated with milk xanthine oxidase. 3-(5-Nitro-2-furyl)-2-(2-furyl)-acrylamide (AF-2) and 3-(5-nitro-2-furyl)-2-(5-bromo-2-furyl)acrylamide (NFBFA) were mainly converted from the cis to the trans form by this enzyme supplemented with an electron donor. This enzymatic reaction was further characterized with respect to its cofactor requirements. Finally, a new cis-trans isomerization mechanism, which is based on transfer of a single electron by a nitroreductase such as xanthine oxidase to a nitrofuran derivative to give the anion free radical, was proposed.

A few enzymes, capable of catalyzing cis-trans isomerization about carbon-carbon double bonds of maleic acid (1) and its derivatives (2-4) and retinene (5), have been known in mammalia and bacteria. Recently, we found that AF-2, a potential mutagenic nitrofuran derivative, was converted from its cis form to its trans form* during the course of reduction by mammalian nitroreductases (6). Yamada et al.(7) also briefly reported such isomerization of this compound by bacterial enzymes.

In the present study, we provide further evidences of cis-trans isomerization of nitrofurans, AF-2 and NFBFA by xanthine oxidase (EC 1.2.3.2) and discuss a possible mechanism of this reaction.

MATERIALS AND METHODS

Chemicals. Cis- and trans-AF-2 were kindly donated by Ueno Pharmaceutical Co.,Ltd. (cis isomer, mp 151 - 152°; trans isomer, mp 176 - 177°). Cis- and trans-NFBFA were prepared according to the method of Hirao et al.(8) (cis

*Cis or trans form means the isomer in which the furan rings attached to the olefinic double bond lie on the same or opposite sides of the molecule,respectively.

The abbreviation used are: AF-2, 3-(5-nitro-2-furyl)-2-(2-furyl)acrylamide; NFBFA, 3-(5-nitro-2-furyl)-2-(5-bromo-2-furyl)acrylamide; FFA, 2,3-bis(2-furyl)-acrylamide.

isomer, mp 181°; trans isomer, mp 173 - 174°). Cis- and trans-FFA were prepared from furfural and potassium furylacetate by the method of AF-2 synthesis (9) (cis isomer, mp 118 - 119°; trans isomer, mp 117 - 119°). The chemical purity of these isomers was proved by thin layer chromatography prior to use.

Enzyme. Purified butter milk xanthine oxidase was purchased from Sigma Chemical Co.,Ltd.

Enzyme assays. A Thunberg tube was used for anaerobic incubations. An incubation mixture consisted of 0.075 μmol of substrate in 5 μl of methanol or acetone, 0.15 μmol of electron donor and 0.005 or 0.05 units of milk xanthine oxidase in a final volume of 2.5 ml of 1/30 M phosphate buffer (pH 7.4). Xanthine was added as a solution in 30 μl of 0.1 N NaOH. The tube was evacuated with an aspirator for 3 min. After the tube was preheated for 5 min at 37°, the reaction was started by addition of the enzyme solution from the side arm to the tube and continued for 10 min in the dark. In aerobic experiments, the above incubation was carried out in open vessels.

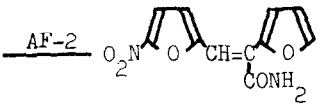
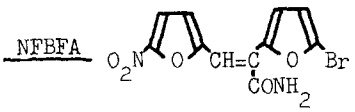
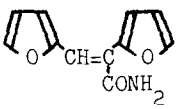
After incubation, the mixture was immediately extracted twice with an equal volume of ethyl acetate, and the combined extracts were evaporated to dryness under vacuum. The residue was then applied to a Silica gel G plate and developed with benzene-acetone (7 : 3,v/v) or ether-acetone (7 : 3, v/v). After removal of solvents, the plate was developed again with the same solvent system in order to separate better the isomers which were visualized under uv light (3650 Å). The areas corresponding to the cis and trans isomers of AF-2, NFBFA or FFA were scraped and eluted separately with 2.5 ml of ethanol. The Rf values of the isomers after the second development are as follows: Cis-AF-2 0.57, trans-AF-2 0.62, cis-NFBFA 0.50 and trans-NFBFA 0.85 in benzene-acetone (7 : 3,v/v); cis-FFA 0.80 and trans-FFA 0.85 in ether-acetone (7 : 3,v/v). The amount of each isomer in these ethanolic extracts was determined spectrophotometrically at 386 nm for cis-AF-2, 402 nm for trans-AF-2, 395 nm for cis-NFBFA, 400 nm for trans-NFBFA, 320 nm for cis-FFA or 324 nm for trans-FFA.

The reduction of trans-AF-2 by milk xanthine oxidase was also investigated using a Thunberg type cuvette and the same incubation mixture as that in the isomerization reaction. Xanthine (0.15 μmol) was used as an electron donor. Prior to incubation, the cuvette containing the substrate and xanthine in phosphate buffer was gassed for 3 min with nitrogen, fitted with the side arm containing the enzyme preparation and then evacuated with an aspirator for 3 min. The cuvette was placed in the cuvette chamber of a Hitachi 124 spectrophotometer and preheated for 5 min at 37°. The reaction was started by addition of the enzyme preparation and continued for 10 min. In aerobic experiments, the incubation was carried out in open vessels. The amount of reduced trans-AF-2 was calculated from the observed decrease in the absorbance at 402 nm.

RESULTS

Isomerase activity. As shown in Table I, AF-2 and NFBFA were easily isomerized from the cis to the trans form, but not in the reverse direction, by the xanthine oxidase-xanthine system. When the enzyme in this system was inactivated by boiling or when xanthine as an electron donor was omitted from the system, the isomerization ratios decreased markedly, indicating that this isomerization was catalyzed by xanthine oxidase supplemented with an electron donor, namely by the enzymatic oxidoreduction system involved in nitroreduction. On the contrary,

Table I. Cis-trans isomerization of nitrofurans and related compound by milk xanthine oxidase* under various conditions

	Cis → Trans (%)	Trans → Cis (%)
AF-2 		
Xanthine oxidase-xanthine	86.9 ± 1.5	11.3 ± 1.0
Boiled xanthine oxidase-xanthine	5.5 ± 1.1	5.4 ± 0.9
Xanthine oxidase only	7.2 ± 1.1	7.7 ± 0.6
Phosphate buffer (pH 7.4)	3.4 ± 0.5	4.8 ± 0.5
NFBFA 		
Xanthine oxidase-xanthine	86.2 ± 4.2	14.3 ± 2.6
Boiled xanthine oxidase-xanthine	9.8 ± 2.9	11.0 ± 2.0
Xanthine oxidase only	10.6 ± 2.0	13.0 ± 2.9
Phosphate buffer (pH 7.4)	7.4 ± 1.9	9.5 ± 1.9
FFA 		
Xanthine oxidase-xanthine	1.0 ± 0.6	7.0 ± 3.7

* 0.005 units

Each value represents mean ± S.D. of four experiments.

when cis- or trans-FFA was used as a substrate, no enzymatic isomerization could be observed in either case, indicating the importance of the nitro group in such isomerization.

Cofactor requirements. The effects of hypoxanthine, acetaldehyde, NADH, NADPH, NAD and NADP on the isomerization of AF-2 by xanthine oxidase were compared. Hypoxanthine, NADH, and to a lesser extent acetaldehyde and xanthine, were more effective than NADPH in promoting isomerization of cis-AF-2 to the trans form (Table II); NAD and NADP were virtually ineffective. On the other hand, the

Table II. Cofactor requirements for cis-trans isomerization of AF-2 by milk xanthine oxidase*

Cofactor	Cis → Trans (%)	Trans → Cis (%)
Hypoxanthine	91.2 ± 3.9	7.0 ± 1.8
Acetaldehyde	40.8 ± 6.9	6.7 ± 2.5
NADH	81.5 ± 5.4	5.4 ± 2.1
NADPH	11.8 ± 1.4	3.1 ± 0.3
NAD	3.7 ± 1.1	6.5 ± 2.0
NADP	3.3 ± 0.6	3.6 ± 1.3

* 0.005 units

Each value represents mean ± S.D. of four experiments.

enzymatic isomerization of trans-AF-2 to the cis form was not observed in any case (Table II). All effective cofactors described above are also the preferrred electron donors of xanthine oxidase. These results also support the participation of xanthine oxidase in cis-trans isomerization of AF-2 and NFBFA.

Relation between isomerase and reductase activities of xanthine oxidase. Xanthine oxidase, supplemented with an appropriate electron donor, is known to reduce nitrofuran derivatives (10-14). Therefore, the relative activities of the enzyme for isomerization and nitroreduction were compared.

In the system, xanthine oxidase (0.005 units) - xanthine, only about 3 % of the added trans-AF-2 was reduced (Table III), while in the same system the isomerization of cis-AF-2 to the trans isomer occurred to a much greater extent (about 87 %) as shown in Table I. When 0.05 units of the enzyme were used, the extent of reduction increased up to about 70 % (Table III). These results indicate that with AF-2 as substrate, the isomerase activity of xanthine oxidase is much higher than its reductase activity.

Effect of air on isomerization and reduction. Unlike the reduction of trans-AF-2, the isomerization of cis-AF-2 to the trans isomer occurs in air (Table III). However, the extent of isomerization with 0.005 units of the enzyme in air was lower than that observed anaerobically (Table I).

Table III. Reduction and cis-trans isomerization of AF-2 by milk xanthine oxidase under aerobic or anaerobic conditions

Unit of xanthine oxidase	Anaerobic	Aerobic		
	Reduction	Reduction	Cis → Trans	Trans → Cis
		(%)		
0.005	3.3 ± 0.5	1.1 ± 0.1	41.5 ± 4.5	7.6 ± 1.4
0.05	69.3 ± 0.1	7.9 ± 2.3	86.3 ± 3.2	7.2[a]

Each value represents mean ± S.D. of four experiments.

a) Value represents mean of two experiments.

DISCUSSION

This study provides the first evidences for enzymatic cis-trans isomerization of compounds that do not occur naturally. The isomerization of AF-2 and NFBFA can be catalyzed by milk xanthine oxidase supplemented with an electron donor, whereas such isomerization is not observed in the compound containing no nitro group (FFA). These results strongly suggest that the reduction of the nitro group of nitrofuran derivatives by xanthine oxidase system is an important first step in isomerization. A new isomerization mechanism, based on transfer of a single electron to nitrofurans to give the anion free radical, is tentatively proposed as shown in Fig.1.

Mason and Holtzman (15) demonstrated by electron spin resonance examination that nitrofurantoin is reduced anaerobically by microsomal reductase to form its one-electron reduction product, i.e. nitrofurantoin anion free radical, which may rapidly react with O_2 to give the original nitrofuran derivative and superoxide anion free radical under aerobic condition. Assuming the formation of such nitrofuran anion free radical, the quick isomerization observed under both aerobic and anaerobic conditions can be well explained as shown in Fig.1.

Our preliminary experiments show that microsomal nitroreductase and

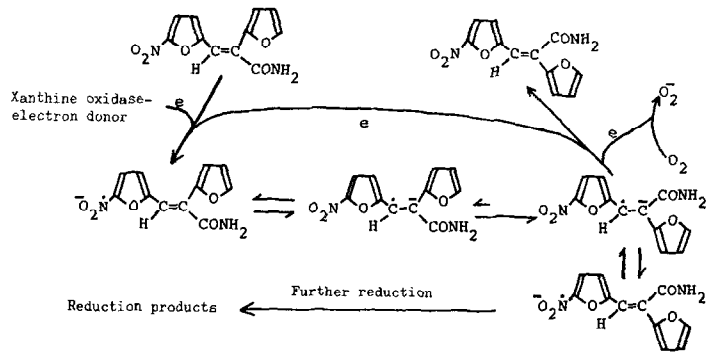


Fig.1. Postulated mechanism for the cis-trans isomerization of AF-2 by xanthine oxidase.

cytosol xanthine oxidase from rat liver, like milk xanthine oxidase, also isomerize AF-2 from its cis to its trans form. The study of these reactions is now under way.

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