Biochimica et Biophysica Acta, 480 (1977) 315—325

BBA 68015

A CIS-TRANS ISOMERISING ACTIVITY OF *ESCHERICHIA COLI*

ISOMERIZATION FROM 2-(2-FURYL)-3-*CIS*-(5-NITRO-2-FURYL) ACRYLAMIDE (FURYLFURAMIDE) TO ITS TRANS ISOMER

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(Received March 25th, 1976)
(Revised manuscript received August 13th, 1976)

Summary

The soluble enzyme fraction derived from *Escherichia coli* K-12 JE2100 cells was found to exhibit, in addition to NADH- and NADPH-dependent reductase activities, NADH-dependent cis-trans isomerising activity toward 2-(2-furyl-3-(5-nitro-2-furyl)acrylamide leading to a specific change in geometrical configuration of the vinyl group at the 2-position from cis to trans but not in the reverse direction. This furylfuramide-isomerising action of bacteria was dicoumarol insensitive, and did not require glutathione for full activity. The particulate enzyme fraction derived from JE2100 cells, although it showed little reductase activity toward furylfuramide in the presence of either NADH or NADPH, revealed an isomerising activity in the presence of NADH.

Introduction

Enzymatic cis-trans isomerizations about carbon-carbon double bonds may fall into two classes, i.e. those that isomerise without double bond migration in the final product and those that are accompanied with positional migration [1]. Several cis-trans isomerases which belong to the first class, are for example, maleate cis-trans isomerase from *Pseudomonas* [2—4] and *Alcaligenes* [5,6], maleylacetoacetate cis-trans isomerase from rat liver [7] and from *Vibro* [8], maleylpyruvate cis-trans isomerase from *Pseudomonas* [9,10] and *Bacillus*

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[11], maleylacetone *cis-trans* isomerase from *Vibrio* (Seltzer, S., cited in ref. 1), and all-*trans*-retinine 11-cis-trans isomerase from cattle retinas and frog pigment layers [12].

2-(2-Furyl)-3-*cis*-(5-nitro-2-furyl)acrylamide (furylfuramide) [13], which is a potent antibacterial nitrofuran and shows powerful mutagenic and DNA-modifying effects [14—17], has been reported to isomerise to the trans geometrical isomer when heated in alkaline methanol or when irradiated [18]. The present paper describes a specific isomerization from the cis to the trans isomer of furylfuramide by both soluble and particulate enzyme fractions of an *Escherichia coli* K-12 derivative, strain JE2100 [19].

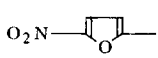
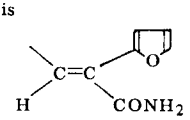
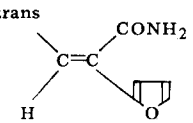
Materials and Methods

Materials. *Cis*-furylfuramide as orange needles was a gift from Professor T. Murata of the Pharmaceutical College of Shizuoka. *Trans*-Furylfuramide was prepared by heating *cis*-furylfuramide in 33 mM phosphate buffer (pH 7.0) at 90°C for 2.5 h followed by usual work up of the reaction mixture. The structure of the compound obtained as dark red prisms was identified by a mass spectroscopic analysis affording *m/e* 248 ($C_{11}H_8O_5N_2$) as M^+. Melting points, absorption spectral properties and R_F values on thin-layer chromatography of *cis*- and *trans*-furylfuramide are shown in Table I and Fig. 1.

Reduced *cis*- and *trans*-furylfuramide were prepared by catalytic hydrogenation of authentic specimens of furylfuramide with 10% palladium-charcoal as catalyst. Absorbance spectra of reduced materials thus obtained are shown in Fig. 1.

TABLE I

PHYSICAL PROPERTIES OF 2-(2-FURYL)-3-*CIS*- AND *TRANS*-(5-NITRO-2-FURYL)ACRYLAMIDES

Compound  R	m.p.	λ_{max} nm (ϵ)	R_f on silica gel thin-layer chromatography [e]
cis 	152—153°C [a]	306 (10 210) [c] 394 (12 790) [c]	0.26
trans 	178—180°C [b]	303 (13 040) [d] 402 (21 610) [d]	0.36

[a] 151°C [13].
[b] 177°C [13].
[c] 306 394 [14].
[d] 306 416 [13].
[e] Silica gel used: silica gel GF254 according to Stahl.
Eluent used: ethyl acetate/benzene (1 : 2, v/v).

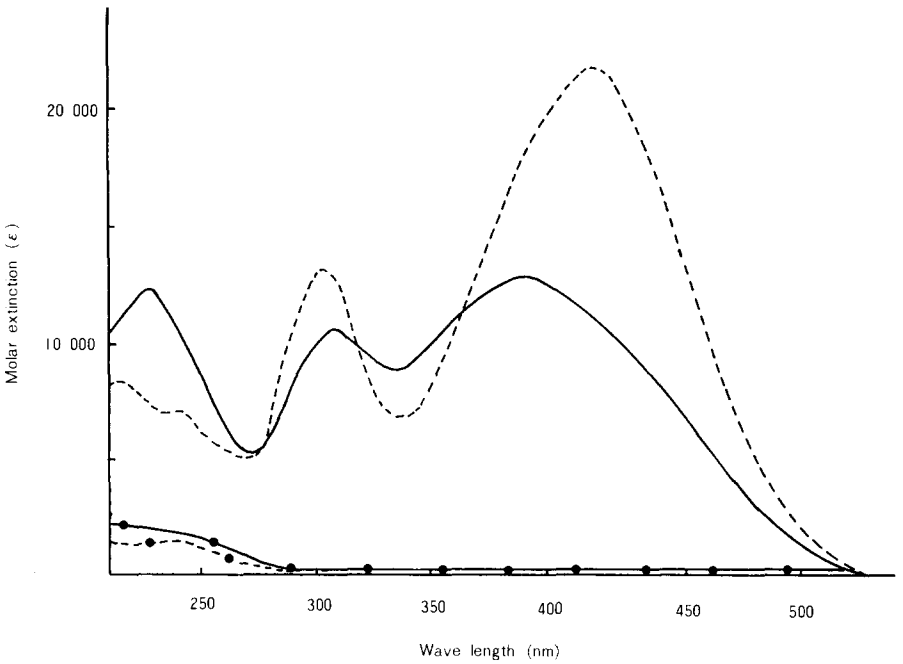


Fig. 1. Absorbance spectra of *cis*- and *trans*-furylfuramide and their reduced specimens in 0.1 M phosphate buffer at pH 7.0. The solid and dotted lines represent the spectra of *cis*- and *trans*-furylfuramide, respectively, and those with closed circles, the spectra of reduced *cis*- and *trans*-furylfuramide, respectively.

Bacterial strain and media. The bacterial strain used is *E. coli* K-12 JE2100 harboring an R plasmid R100-1, showing resistance to dihydrostreptomycin sulfate, 10 μg/ml; chloramphenicol, 25 μg/ml; tetracycline hydrochloride, 50 μg/ml; and sulfanilamide, 400 μg/ml, respectively.

Penassay broth (Difco), a complete medium, and Davis glucose medium, a synthetic medium composed of K_2HPO_4 (0.7%), KH_2PO_4 (0.2%), $(NH_4)_2SO_4$ (0.1%), sodium citrate (0.05%), M_gSO_4 (0.01%) and glucose (0.2%), were used for cultivation of bacterial cells. A complete Eosin/Methylene Blue/sugar/agar or synthetic Eosin/Methylene Blue/sugar/agar with one of four drugs was used for the identifying the drug-resistant marker of the R plasmid. The pH of media was 7.6.

Measurement of reduction and isomerization of furylfuramide by intact E. coli JE2100R$^+$ cells. 0.1 ml of a culture ($1 \cdot 10^8$—$5 \cdot 10^8$ cells per ml) of JE2100 cells in Penassay broth was diluted with 100 ml of Davis glucose medium and incubated overnight with shaking at 37°C until it reached about 10^8 cells per ml. The cells were collected by centrifugation at 10 000 × *g* for 15 min and suspended in 100 ml of the same medium. *Cis*- or *trans*-furylfuramide was then added to reach the final concentration of 10 μg per ml or $4.0 \cdot 10^{-4}$ M, and the incubation with shaking at 37°C was continued. As a control experiment, the incubation without furylfuramide was also carried out. At appropriate intervals, 0.1-ml and 4.5-ml samples were removed from the incubation mixture. To each 4.5 ml sample, 0.5 ml of 50% aqueous $HClO_4$ was added, and the suspension was centrifuged at 1500 × *g* for 30 min to remove

cells and cellular debris. The difference spectrum of the supernatants with and without furylfuramide was taken in the range 200 to 500 nm, using a 323 Hitachi Recording Spectrophotometer with quartz cubic cells (two compartments) and with a perchloric acid supernatant of bacterial cells with furylfuramide in the reference compartment. 0.1-ml samples were plated after appropriate dilutions on Eosin Methylene Blue/glucose/agar to count viable cells of the incubation mixture.

Preparation of enzyme fractions. An overnight culture of JE2100 cells ($1 \cdot 10^8$—$5 \cdot 10^8$ cells per ml in 200 ml Penassay broth supplemented with 25 μg/ml of chloramphenicol) was diluted to 2 l of the same broth without chloramphenicol and incubated with shaking at 37° C for 3 h to reach about the same titer of the cell. Cells harvested from the culture by centrifugation at 10 000 × *g* for 15 min were suspended in 300 ml of saline and kept at 0° C overnight. Cells were then centrifuged at the same spin speed for 15 min, and suspended in 25 ml of 0.1 M phosphate buffer (pH 7.0). The suspension was sonicated for two periods of 15 min each with full power from a UL Transonic Disruptor Model UR-200P (Tomy Seiko Co.), and centrifuged at 2000 × *g* for 15 min to remove cells and cellular debris. The supernatant was then centrifuged at 100 000 × *g* for 60 min. The supernatant thus obtained was dialysed against 6 l of 5 mM phosphate buffer/50 mM KCl/10 Mm $MgCl_2$ (pH 7.0) for 20 h, and is referred to as the bacterial soluble enzyme fraction. The precipitate obtained by the centrifugation at 100 000 × *g* was suspended in 5 ml of 0.1 M phosphate buffer (pH 7.0) and centrifuged at 2000 × *g* for 15 min to remove any remaining cells and cellular debris. The supernatant thus obtained is referred to as the bacterial particulate enzyme fraction. Specific reduced nicotinamide adenine nucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidation activities (mol per min per protein mg) of those fractions by unknown endogeneous substrate(s) were determined by decrease of absorbance at 340 nm, using a 356 Hitachi Dual Beam Spectrophotometer with a single quartz cubic cell (one compartment) and with a slit width of 1.25 nm and scanning speed of 10 nm per min, by scanning samples at a pair of wavelengths, 340 nm and 500 or 600 nm of reference light.

Enzyme assay conditions. Furylfuramide cis-trans isomerising activity of enzyme fractions toward *cis*-furylfuramide was routinely assayed at 21 ± 1° C in 3 ml (final volume) of 0.1 M phosphate buffer (pH 7.0) (protein concentration, about 1 mg per ml) containing $2.5 \cdot 10^{-8}$—$8 \cdot 10^{-5}$ M *cis*-furylfuramide, with or without being supplemented by 30 μl of NADH or NADPH ($6.7 \cdot 10^{-6}$—$1.6 \cdot 10^{-4}$ M final concentration), by measuring the increase in the first 30 s in absorbance at 420 nm (the absorbance maximum of *trans*-furylfuramide). The observed increase in absorbance per min was converted to the amount in mol of *cis*-furylfuramide isomerised per min. Furylfuramide reductase activity was assayed at the same temperature and in the same buffer by measuring in the first 30 s the absorbance change at the absorption maximum of *cis*-furylfuramide (394 nm) or *trans*-furylfuramide (420 nm) in the absence or presence of NADH or NADPH. The observed decrease in absorbance per min at 394 nm for *cis*-furylfuramide and at 420 nm for *trans*-furylfuramide was converted to the amount in mol of each furylfuramide reduced per min. Determination of

absorbances was carried out, using the 356 Hitachi Dual Beam Spectrophotometer (one compartment) under conditions mentioned before, by scanning samples at a pair of wavelengths, 394 or 420 nm and 500 or 600 nm of reference light.

Analytical methods. Protein was determined by the Lowry et al. modification [20] of the Folin procedure. The absorbances were converted to milligrams of protein by reference to a standard curve prepared by using bovine serum albumin. Mass spectroscopic analysis was performed on a JEOL JMS-OISG double-focusing spectrometer. Methods of spectrophotometric experiments have been described previously.

Thin-layer chromatography. Using Silica gel GF_{254} according to Stahl (type 60) (E. Merck, Darmstadt), samples were developed in the solvent containing ethyl acetate/benzene (1 : 2, v/v). *Cis-* and *trans*-furylfuramide were detected by their own orange to dark red colors.

Results

Change of absorbances and absorption maxima of furylfuramide induced by incubation with E. coli K-12 JE2100 cells

Incubation of *cis*-furylfuramide with JE2100 cells (incubation time 60 min) led to a rapid decrease of the absorbance of the compound at an absorbance maximum or 394 nm, accompanied by a shift of the absorbance maximum toward longer wavelengths, i.e. 416, 418, 418, and 418 (shoulder) nm after 3, 5, 10, and 30 min, respectively. In the case of *trans*-furylfuramide (incubation time, 180 min), a rapid decrease in intensity at an absorbance maximum or 420 nm only was observed; any shift of the absorbance maximum to shorter wavelengths was not observed.

Cis → trans isomerization and reduction of furylfuramide induced by incubation with the soluble enzyme fraction derived from JE2100 cells and their dicoumarol sensitivity

(a) *With four molar equivalents of NAD(P)H against furylfuramide.* A rapid decrease of absorbances at absorption maxima was observed with both *cis-* and *trans*-furylfuramide (Fig. 2). The reduction rates of *cis*-furylfuramide with NADH or NADPH were 31.1 or 24.2 μmol per min, respectively, and those of *trans*-furylfuramide with NADH or NADPH were 2.64 or 4.67 μmol per min, respectively. A slight increase of absorbance at 394 nm observed with *cis*-furylfuramide before the addition of NADH or NADPH to the incubation mixture, might reflect an enzymatic isomerization of *cis*-furylfuramide enhanced possibly by unknown endogeneous cofactor(s) derived from bacterial cells.

(b) *With one molar equivalent of NAD(P)H against furylfuramide.* Results are shown in Fig. 3. In the case of *trans*-furylfuramide, the absorbance at 420 nm decreased, as expected, at a slower rate than the case with four molar equivalents of NADH or NADPH. The reduction rates of *trans*-furylfuramide with NADH or NADPH were 1.76 or 2.56, μmol per min, respectively. When *cis*-furylfuramide was assayed with NADH, however, the decrease of absorbance at 420 nm (and 394 nm also) stopped shortly after the addition of NADH; then the absorbance began to increase rapidly. The observed reduction

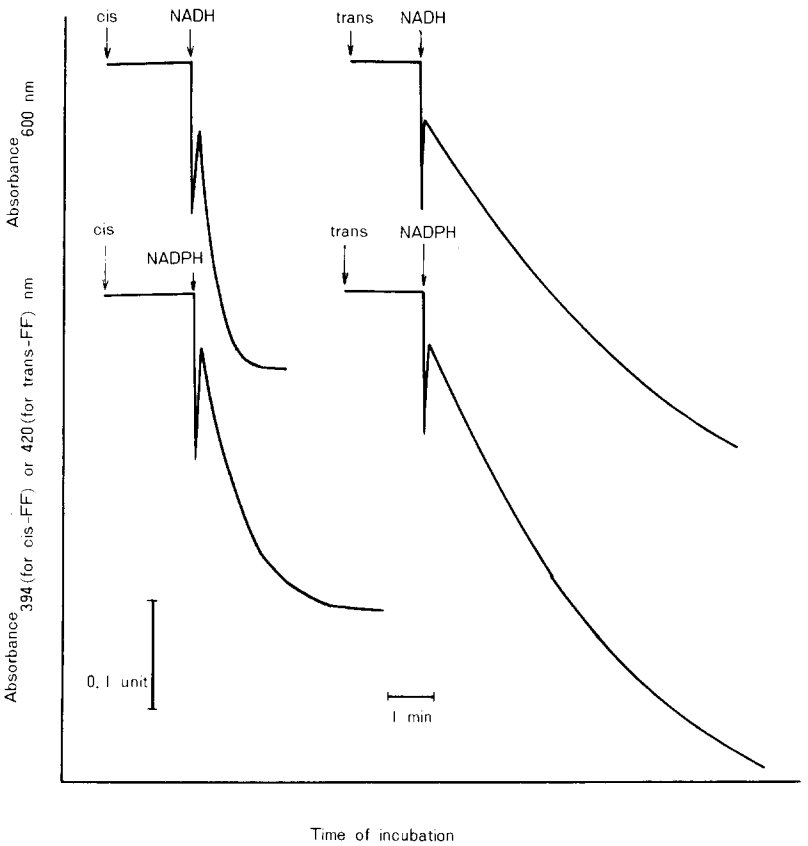


Fig. 2. Change of absorbances at the absorbance maxima of *cis*- and *trans*-furylfuramide (FF) by incubation with the soluble enzyme fraction, derived from JE2100 cells, supplemented with four molar equivalents of NADH or NADPH. Protein concentration, 1.0 mg per ml; final concentration of *cis*- and *trans*-furylfuramide added, $4.0 \cdot 10^{-5}$ M; final concentration of NADH and NADPH added, $1.6 \cdot 10^{-4}$ M. Specific NADH and NADPH oxidation activities (nmol/min per protein mg) of the fraction by endogeneous substrate(s), $3.8 \cdot 10^{3}$ and $7.0 \cdot 10^{2}$, respectively.

and isomerization rates of *cis*-furylfuramide were 11.9 and 15.6 μmol per min, respectively. In the assay of *cis*-furylfuramide with NADPH, the absorbance at 420 nm decreased for a longer time than for the case with NADH (the reduction rate: 8.48 μmol per min); it then stopped and began to increase at a rate as low as that observed before the addition of NADPH.

(c) With one twelvth molar equivalent of NAD(P)H against furylfuramide. When NADH were added to the incubation mixture of *cis*-furylfuramide, a rapid increase of absorbance at 420 nm was solely observed (the isomerization rate: 3.77 μmol per min). NADPH was found not to induce any greater increase of absorbance at 420 nm than that which was occurring before the addition of the cofactor.

In order to confirm that the isomerization and reduction phenomenons of furylfuramide determined by increase and decrease of absorbances at 420 or 394 nm are enzyme catalyzed, incubation of *cis*- and *trans*-furylfuramide in 0.1 M phosphate buffer (pH 7.0) supplemented with NADH or NADPH was

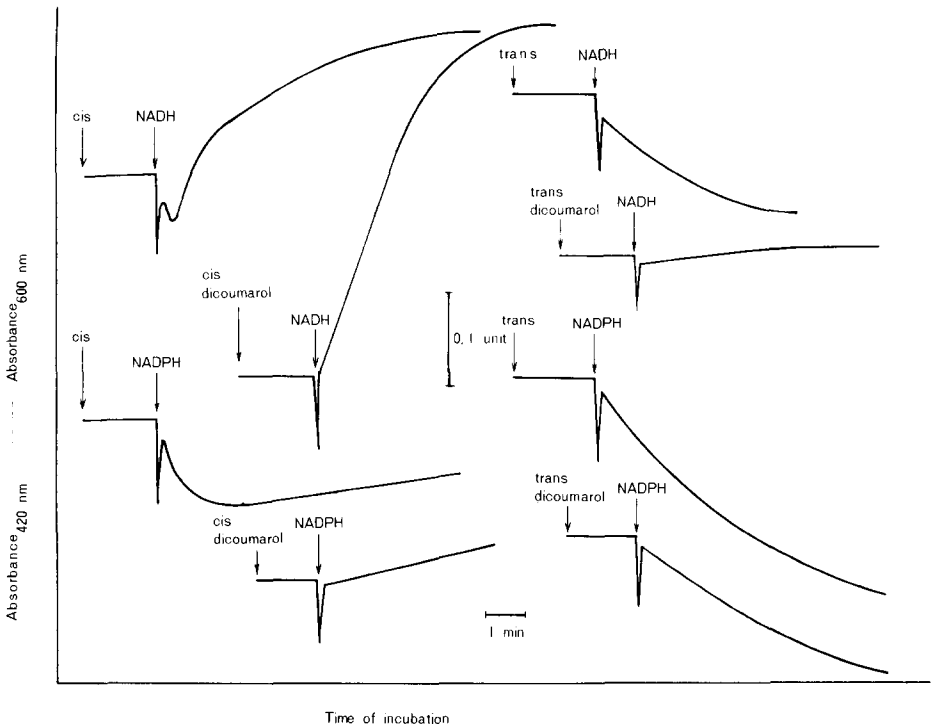


Fig. 3. Change of absorbances at 420 nm of *cis*- and *trans*-furylfuramide by incubation with the soluble enzyme fraction, derived from JE2100 cells, supplemented with one molar equivalent of NADH or NADPH and dicoumarol effect. Protein concentration, 1.0 mg per ml; final concentration of *cis*- and *trans*-furylfuramide, and NADH and NADPH added, $4.0 \cdot 10^{-5}$ M; final concentration of dicoumarol added, $1.3 \cdot 10^{-4}$ M. Specific NADH and NADPH oxidation activities (nmol/min per protein mg) of the fraction by endogeneous substrate(s), $3.8 \cdot 10^{3}$ and $7.0 \cdot 10^{2}$, respectively.

carried out. No increase or decrease of absorbances at 420 nm of *cis*- and *trans*-furylfuramide was observed.

Second, the supernatant enzyme fraction was incubated at 95°C for 20 s and isomerising activity of the fraction toward *cis*-furylfuramide with one equivalent of NADH toward furylfuramide was checked. Any increase of absorbance at 420 nm was not observed.

Isolation and identification of material absorbing at 420 nm derived from cis-furylfuramide as trans-furylfuramide

Cis-furylfuramide (500 μg) in 0.1 ml of propylene glycol was mixed with 0.2 ml of soluble enzyme fraction (protein concentration 4.5 mg per ml) and 0.1 ml of $4 \cdot 10^{-3}$ M NADH (final concentration $1.0 \cdot 10^{-3}$ M), and the mixture was incubated at 37°C for 5 min. The incubation mixture was thin-layer chromatographed as described previously, affording a spot of R_F 0.36, which was identical with that of authentic *trans*-furylfuramide.

Methanolic extract of material from this spot of R_F 0.36 gave absorbance maxima at 301 and 402 nm which were proved to be identical within experimental errors to those in methanol (302 and 401 nm) of an authentic specimen of *trans*-furylfuramide. The observed value, 1.64, of absorbance at

401 nm per absorbance at 302 nm of the material was in good agreement with that (1.62) of the authentic specimen of *trans*-furylfuramide. Furthermore, the spectrum of the material in 0.1 M phosphate buffer (pH 7.0) showed absorbance maxima at 305 and 402 nm which were identical to those (304 and 420 nm) of authentic trans-furylfuramide. The value, 1.60, of absorbances at 420 nm per 304 nm was also in good agreement with that (1.62) of *trans*-furylfuramide.

Dicoumarol sensitivity of enzymatic isomerization and reduction of furylfuramide

$NADH_2$: lipoamide oxidoreductase (diaphorase), known to be associated with a quinone or related compounds as electron acceptor, has been isolated from bacteria [21,22] and can be inhibited by dicoumarol [23].

With this observation in mind, possible dicoumarol sensitivity of an enzyme in the soluble fraction responsible for the observed reduction of furylfuramide was checked. In the assay with *trans*-furylfuramide as substrate and with one molar equivalent of NADH, addition of three molar equivalents of dicoumarol against furylfuramide and NADH was efficient for completely inhibiting the decrease in absorbance at 420 nm of the compound which had occurred in the absence of dicoumarol. In the reductase assay with NADPH, the decrease in absorbance was not greatly inhibited by dicoumarol (the reduction rate: 2.56 μmol per min) (Fig. 3).

In the case of *cis*-furylfuramide, addition of dicoumarol to the incubation mixture supplemented with NADH induced a marked increase of absorbance at 420 nm of the compound; any decrease of absorbance which had occurred immediately after incubation started in the assay without dicoumarol, was not observed (Fig. 3). The observed isomerization rate of *cis*-furylfuramide was 12.5 μmol per min. Assay with NADPH indicated that whereas dicoumarol inhibited an apparent decrease of absorbance of *cis*-furylfuramide at 420 nm, it enhanced a small increase in the absorbance (the isomerization rate: $8.77 \cdot 10^{-1}$ μmol per min) (Fig. 3).

Assay with the particulate enzyme fraction derived from JE2100 cells

Examination of reductase activity of the particulate fraction toward *trans*-furylfuramide was carried out. Little reductase activity was observed in the presence of one molar equivalent of NADH or NADPH (Fig. 4).

Cis-trans isomerising activity of the particulate enzyme fraction toward *cis*-furylfuramide was then checked. When one molar equivalent of NADH was added, a remarkable increase in absorbance at 420 nm was observed. Meanwhile, addition of NADPH induced a much smaller increase in absorbance at 420 nm. The isomerization rates of *cis*-furylfuramide with NADH and NADPH were $13.0 \cdot 10^{-1}$ and $3.50 \cdot 10^{-1}$ μmol per min, respectively.

Possible effect of dicoumarol on the isomerization rate of *cis*-furylfuramide with this enzyme fraction was checked. In the assay with NADH as cofactor, addition of dicoumarol effected the isomerization rate of the compound little (the isomerization rate: 10.8 μmol per min). It appeared, however, that the total amount of isomerization of *cis*-furylfuramide increased in the presence of dicoumarol. In the assay with NADPH as cofactor, a slight enhancing effect of

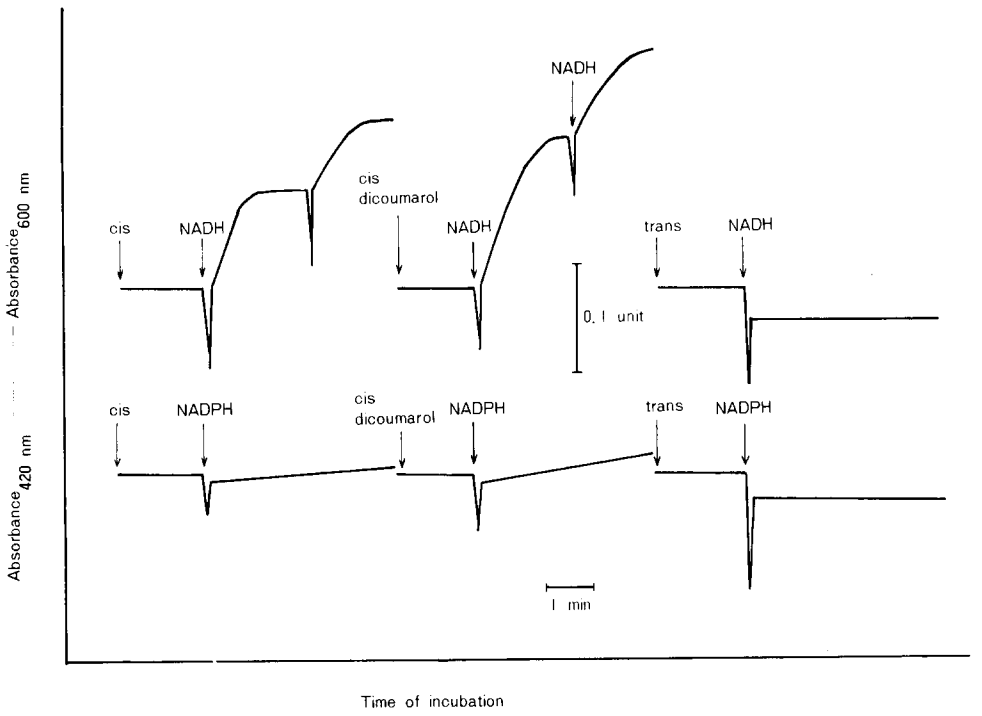


Fig. 4. Change of absorbances at 420 nm of *cis*- and *trans*-furylfuramide by incubation with the particulate enzyme fraction, derived from JE2100 cells, supplemented with one molar equivalent of NADH or NADPH and dicoumarol effect. Protein concentration, 1.0 mg per ml; final concentration of *cis*- and *trans*-furylfuramide and of NADH and NADPH added, $4.0 \cdot 10^{-5}$ M; final concentration of dicoumarol added, $1.3 \cdot 10^{-4}$ M. Specific NADH and NADPH oxidation activities (nmol/min per protein mg) by endogeneous substrate(s), $1.0 \cdot 10^{5}$ and $5.4 \cdot 10^{3}$, respectively.

dicoumarol on the isomerization rate of *cis*-furylfuramide was observed; the rate was $8.80 \cdot 10^{-1}$ μmol per min which was twice as much as the rate observed in the absence of dicoumarol (Fig. 4).

Effect of glutathione on the cis-trans isomerising activity of bacteria on furylfuramide

Cis-Furylfuramide in 0.1 M phosphate buffer (final concentration, $4.0 \cdot 10^{-5}$ M) was incubated with the soluble enzyme fraction (final protein concentration 0.3 mg per ml) and the effect of glutathione on the increase of absorbance at 420 nm of the compound was checked. The increase in absorbance (and the isomerization rate) per min was found to be 0.01 ($8.77 \cdot 10^{-1}$ μmol) in the assay without glutathine (control experiment), and 0.01, 0.01 (8.77 μmol each), and 0.02 (17.5 μmol) in the assays with one, five, and ten molar equivalents of glutathione against *cis*-furylfuramide, respectively.

Second, the effect of glutathione on the isomerising activity of the soluble enzyme fraction in the presence of one molar equivalent of NADH was examined: little change of increase of absorbance at 420 nm was observed.

Third, the effect of glutathione on the isomerising activity of the particulate enzyme fraction on *cis*-furylfuramide in the presence of NADH was checked;

no substancial change in absorbance at 420 nm was induced by addition of up to ten molar equivalents of glutathione against furylfuramide.

Discussion

Preliminary studies with intact JE2100 cells followed by studies with soluble and particulate enzyme fractions derived from bacterial cells have revealed a specific cis-trans isomerising activity of bacterial cells toward furylfuramide (Figs. 2—4). Thin-layer chromatographic separation and spectroscopic characterization of homogeneous material isolated from the incubation mixture of *cis*-furylfuramide with the soluble enzyme fraction and with NADH as co-factor, proved the structure of the product to be *trans*-furylfuramide.

There were marked differences in the property of enzyme fractions responsible for the isomerization and reduction of furylfuramide with respect to the requirement of NADH or NADPH for enzyme activity. First, whereas NADH and NADPH showed rate-increasing effects of similar degree toward the reductase activity of furylfuramide, it was NADH and not NADPH that enhanced the isomerising activity toward furylfuramide substantially at about 10^{-1} lower concentration than that required for reductase activity (Figs. 2 and 3). In the assay of *cis*-furylfuramide with NADH at a concentration as low as 10^{-6} M, only the isomerising activity was observed.

Second, whereas the NADH-dependent soluble enzyme responsible for the reduction of furylfuramide was dicoumarol sensitive as diaphorase, the NADH-dependent furylfuramide isomerization appeared to be dicoumarol insensitive. Only the isomerization was observed with *cis*-furylfuramide in the assay with NADH at concentrations the same as those required for the reductase activity but supplemented with dicoumarol.

We have carried out further experiments using the soluble enzyme fraction derived from nitrofuran-sensitive *E. coli* K-12 RC85 strain and cells of their nitrofuran-resistant mutant RC85 Nf^r-1 (Tomoeda, M., Kitamura, R. and Minamibayashi, T., unpublished; bacterials strains were a gift of Professor T. Arai, Keio University). We have found that whereas mutation of bacteria from nitrofuran sensitivity to resistance led to the substantial loss of reductase activity toward *cis*- and *trans*-furylfuramide, the mutation did not induce the loss of isomerising activity of bacteria toward *cis*-furylfuramide. This observation also suggests that reducing and isomerising activities of bacteria toward furylfuramide may be due to enzymes which are different in nature.

The particulate enzyme fraction of bacteria may contain certain NADH- or NADPH-dependent oxidoreductases associated with the respiratory chain of bacteria. Results with this enzyme fraction indicated that whereas this enzyme fraction showed little reductase activity toward furylfuramide in the presence of either NADH or NADPH, it revealed some isomerising activity in the presence of NADH (Fig. 4).

Isomerases which catalyse the isomerization of maleic acid residue to fumaric acid residue have been reported to require reduced glutathione or cysteine for full activity (ref. 1; and refs. cited therein). This was proved not to be the case with the isomerase activity of both soluble and particulate enzyme fractions of *E. coli* toward *cis*-furylfuramide. This indicates that the

cis-trans isomerising activity of *E. coli* responsible for the cis → trans isomerization of furylfuramide, may have different cofactor requirements than maleic acid : fumaric acid cis-trans isomerases.

We very recently noticed that Sugimura and his colleagues [24] have found a similar isomerization of *cis*-furylfuramide to *trans*-furylfuramide by a rat liver microsomal enzyme fraction with NADPH or by rat liver cytosol enzyme and xanthine oxidase fractions with NADH or hypoxanthine. Cis-trans isomerising activity specific for furylfuramide appears to be general both in microorganisms and mammals.

Purification and characterization of furylfuramide cis-trans isomerase from *E. coli* cells is underway.

Acknowledgements

We are deeply indebted to Dr. E. Itagaki of the Faculty of Science, Kanazawa University, for valuable advices and discussions. We are also indebted to Drs. M. Inuzuka and T. Date of our laboratory for continued interest and helpful discussions. This work was supported by grants-in-aid from the Ministry of Education, Science and Culture, Japanese Government, to which our thanks are due.

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