Microbial Reduction of Ketopantoyl Lactone to Pantoyl Lactone

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Received for publication 31 August 1973

The results of a microbial survey study have shown that the ability to reduce added ketopantoic acid (or ketopantoyl lactone) and accumulate pantoic acid (or pantoyl lactone) in the growth medium is widespread among diverse fungi. The reductions generally proceeded with less than full stereoselectivity. However, specific strains of the ascomycete Byssoclamys fulva were found to form D-[-]-pantoic acid in unusually high yields and optical purity.

Pantoic acid (α-hydroxy-β, β-dimethyl-γ-hydroxybutyric acid) (Fig. 1) is an important intermediate in the synthesis of pantothenic acid. In the conventional synthetic process, pantoic acid is reacted with β-alanine to form the desired vitamin. When the process is conducted with a racemic DL(±) mixture of pantoic acid, the pantothenic acid thus formed is also racemic. However, since only the D-isomer is of value in nutrition, economic and process advantages could result if the synthesis were conducted with D-[-]-pantoic acid as starting material.

Since the conventional procedure for the production of D-[-]-pantoic acid or the corresponding γ-lactone (Fig. 1) involves a costly chemical resolution, we developed an interest in the report of Kuhn and Wieland (8) in 1942 demonstrating the reduction of ketopantoic acid (α-keto-β, β-dimethyl-γ-hydroxybutyric acid) (Fig. 1) to pantoyl by yeast cell suspensions. This suggested the possibility that D-[-]-pantoic acid could be produced efficiently by microbial stereospecific reduction of ketopantoic acid.

MATERIALS AND METHODS

Chemicals and substrates. Analytical samples of DL-pantoyl lactone, D-[-]-pantoyl lactone, and the substrate ketopantoyl lactone were generously supplied by Emil Lorz, Hoffman-Taff, Inc., Springfield, Mo. In all experiments, the ketone substrate was supplied to the various fermentation media as an aqueous solution prepared by dissolving crystalline ketopantoyl lactone in distilled water. Since the ketolactone in aqueous solution spontaneously hydrolyzes to form the free acid, the substrate solutions and also the fermentation reaction media contained equilibrium mixtures of the γ-lactones, γ-hydroxycarboxylic acids, and the corresponding bases, the relative proportions of which were, of course, pH dependent. The complete relactonization required for quantitative analysis, solvent extraction, and optical analysis was, therefore, readily accomplished by treatment with acid.

Cultures. Most microorganisms used in the survey study were obtained from the Syntex culture collection (accession number followed by "sy"). Strains having NRRL accession numbers were obtained from the Agricultural Research Station collection, Department of Agriculture, Northern Laboratory, Peoria, III. Strains having QM accession numbers were obtained from the U.S. Army Material Command, U.S. Army, Natick Laboratories, Natick, Mass. Those strains with CBS, IMI, and ATCC accession numbers were purchased from the Centraalbureau voor Schimmelcultures, Baarn, Netherlands, the Commonwealth Mycological Institute, Kew, Surrey, England, and the American Type Culture Collection, Rockville, Md., respectively. Red Star and Fleischmann's baker's yeast were obtained locally at retail outlets. Anheuser-Busch baker's yeast was kindly supplied by Walter Hunt, Anheuser-Busch, Inc., St. Louis, Mo.

Preparation of resting yeast cell suspensions. Yeast cells (150 g; compressed cake) were added to 500 ml of distilled water. In a second 500-ml portion of distilled water were dissolved 50 g of glucose, 37.5 g of NaH₂PO₄, and 25 g of ketopantoyl lactone. The yeast cell suspension was then mixed with the substrate solution. The final volume was approximately 1,200 ml. The final pH was 3.2 to 3.5. The reaction mixture was transferred to a glass fermentor jar (New Brunswick Scientific Co., New Brunswick, N.J.), agitated at 500 rpm, and aerated at 1 volume of air per volume of medium per min. Reaction temperatures were held at 30 to 32 C. Incubation was terminated after 24 h.

Media. Filamentous fungi used in the survey study were cultured in a medium that contained glucose (30 g), yeast extract (5 g), soybean meal (5 g), NaCl (5 g),...
The medium Byssochlamys with 2% lactone, pantoic acid, and pantoyl lactone was used to inoculate 50 ml of medium GP contained in a 250-ml Erlenmeyer flask. Incubation was continued as before, and, after 48 h, the ketopantoyl lactone was added as an aqueous solution (0.5 g/ml), and incubation continued. The concentration of ketolactone used varied depending on the strain studied (see text for details). Sampling for quantitative GLC analyses and recovery of pantoyl lactone for determination of optical rotation were as described below.

**Extraction, recovery, and purification.** Samples (1 ml) were aseptically withdrawn from test cultures and acidified to pH 0.8 to 1.0 with concentrated HCl and then held at 50 C for 15 min. This was to insure that all of the pantoatoes present in the sample were converted to the corresponding lactones before solvent extraction. The samples were then saturated with NaCl and extracted once with 4 volumes of ethyl acetate. Samples of the extraction solvent were then used for quantitative GLC (see below).

Recovery of pantoyl lactone from spent cultures was accomplished by differential extraction essentially as described by Kuhn and Wieland (8). Cells were removed from the culture broths by centrifugation. The supernatant fluid was then adjusted to pH 1.0 and held at 50 C for 15 min. The fluid was then titrated to pH 7.3 with NaOH and immediately extracted three times with 0.5 volume of ethyl acetate. The extracts were combined and dried over anhydrous Na2SO4 and solvent was stripped in vacuo. Portions of the crude residues that resulted were shown to be virtually free of unreacted ketopantoyl lactone by quantitative GLC. (The success of the differential extraction procedure lies in the fact that the α-ketolactone hydrolyzes to form the free acid very rapidly as the pH is raised from 1.0 to 7.3. However, the α-hydroxylactone hydrolyzes much more slowly and therefore may be partitioned with water-immiscible solvents leaving the polar keto acid in the aqueous phase.) Each 100 mg of crude residue was then taken up in 10 ml of a 60:40 mixture of benzene-ethyl acetate to which was added 300 mg of silica gel. The mixture was slurried and then filtered through a Teflon membrane (Millipore Corp., Bedford, Mass.). When necessary, samples were then decolorized with activated carbon. The solvent was stripped, and portions of the residues were used for determination of optical rotation.

**GLC.** Samples of solvent extracts containing from 0.1 to 5 mg of pantoyl lactones were combined with 1 ml of internal standard (0.1%, vol/vol) acetophenone in ethyl acetate). The mixture was brought to 10 ml final volume with ethyl acetate. This solution was analyzed on a Hewlett-Packard model 402B gas chromatograph equipped with a flame ionization detector. The glass column (1.8 m long, 3.0 mm inside diameter) was packed with 6% FFAP (Supelco, Inc.) on a 100- to 120-mesh Chromosorb W, acid washed (Johns-Manville). Operational parameters were: injection port, 210 C; detector, 255 C; column, 185 C;
helium carrier, 30 cm³/min; hydrogen, 25 cm³/min; and air, 500 cm³/min. Quantitation was made by reference to standard curves relating peak height ratios to concentration.

Optical rotation. The optical activity of pantoyl lactone recovered from yeast cell suspensions was estimated as the [α]₀ water (optically pure D-pantoyl lactone = -50.5°). Samples recovered during the survey studies were generally estimated as the [α]₀, methanol (-27.8° ± 0.4 as determined on a D-[-]-pantoyl lactone analytical standard). The instrument used was a Perkin-Elmer model 141 automatic polarimeter.

RESULTS

Conversion of ketopantoyl lactone to pantoyl lactone by yeast cell suspensions. The data presented in Table 1 show that, although initial rates of reduction of ketopantoyl lactone to pantoyl lactone varied somewhat among baker’s yeast cells from various commercial sources, the overall extent of reduction was quite uniform. In repeated trials, the 24-h percent conversions routinely fell between 90 and 93, irrespective of the source or batch of yeast cells used. However, although the yields of pantoyl lactone obtained in the yeast cell system were quite good, measurements of the optical activity of the recovered products showed that the reductions were not 100% asymmetric. In one series of experiments, the [α]₀ water obtained for crude samples of pantoyl lactone recovered from reaction mixtures by differential extraction were -23.3, -23.2, and -22.9° with Red Star, Fleischmann, and Anheuser-Busch baker’s yeast, respectively. When the crude samples were purified by slurring with silica gel and retested, the specific rotations were generally lower but within 0.5 to 0.9° of the crude sample determinations. Assuming an average [α]₀ water of -22.5°, it appeared that, of the pantoyl lactone produced by reduction of the ketopantoyl lactone, only about 72% was the D-[-]-isomer. (Note: When a crude sample of pantoyl lactone [α]₀ water = -26.6

± 2.7° recovered from a Red Star yeast cell reaction mixture was recrystallized from diethyl ether-petroleum ether and retested, the specific rotation increased to -41.7 ± 1.1°. When the first crystal crop was again recrystallized, the specific rotation increased to -49.6 ± 0.8, very near theoretical optical purity. It was subsequently possible to show that the process of recrystallization resulted in a selective concentration of the D-[-]-isomer in each crystal corp. For this reason, purification by recrystallization of crude pantoyl lactone samples was not suitable for estimating the degree of stereoselectivity with which various microbial systems reduced ketopantoyl lactone, and therefore resort was made to the nonselctive procedure of purification by silica gel and carbon absorption.)

Survey studies. At this point, it was of interest to determine whether microorganisms could be found that would reduce ketopantoyl lactone to pantoyl lactone with a greater degree of stereoselectivity than was obtainable in the baker’s yeast cell system. In all, 190 strains of filamentous fungi, yeasts, bacteria, and actinomycetes were included in the survey. In general, the ability to reduce added ketopantoyl lactone in the growth medium was nonexistent or negligible in 21 bacterial species, including members of the Bacillaceae, Brevibacteriaceae, Corynebacteriaceae, Enterobacteriaceae, Micrococaceae, Pseudomonadaceae, Actinomycetaceae, Mycobacteriaceae, and Strepnotomycetaceae. Included among the bacteria which showed no evidence of pantoyl lactone accumulation in the growth medium was a pantothenate auxotroph, Escherichia coli ATCC 14561 (M-99-4), which is able to grow well when supplied ketopantoyl lactone in the growth medium (9).

On the other hand, among fungi the ability to reduce added ketopantoyl lactone and accumulate pantoyl lactone in the growth medium was widespread. Of the 169 fungal strains examined, which included 55 different genera with representatives from the four major classes, 64 strains including 39 different genera were found to reduce ketopantoyl lactone and accumulate pantoyl lactone in the growth medium. In fact, two-thirds of the active fungi exhibited conversions of greater than 60%. However, as was the case with baker’s yeast, nearly all of the reductions proceeded with only partial stereoselectivity. Moreover, approximately half of those microorganisms reducing ketopantoyl lactone accumulated predominately the “unnatural” L-[+]-isomer. This was especially true among members of the order Mucorales, including several strains of Absidia, Mucor,

| Reaction time (h) | Conversion of ketopantoyl lactone to pantoyl lactone by baker’s yeast (%) | Fleischmann | Red Star | Anheuser-Bush |
|-------------------|--------------------------------------------------------------------------------|-------------|----------|---------------|
| 2                 |                                                                              | 35          | 47       | 26            |
| 4                 |                                                                              | 55          | 69       | 55            |
| 8                 |                                                                              | 76          | 81       | 71            |
| 12                |                                                                              | 85          | 85       | 82            |
| 24                |                                                                              | 93          | 91       | 92            |

a Average of four determinations.
*Mycotypha,* *Rhizopus,* and *Mortierella.* All strains of *Cunninghamella* examined produced predominantly the D[+]-enantiomorph, however.

At this point, attention was drawn to the ascomycete, *Byssochlamys fulva* (1119 sy), which accumulated nearly optically pure ([α]D water = --50.2°) D[-]-pantoyl lactone in the survey study.

To examine this phenomenon in more detail, several strains of *B. fulva* and *B. nivea* were obtained from other culture collections. Also included in these studies were several strains of the genus *Paecilomyces,* reportedly an imperfect form of the *Byssochlamys.* The data obtained with these cultures are listed in Table 2. All cultures were initially tested for the ability to reduce ketopantoyl lactone at a concentration of 30 mg/ml. If the percentage of conversions was 10 or less after 48 h of contact time, the experiment was rerun with lower concentrations (20 or 10 mg/ml) of ketopantoyl lactone. The extent of reduction after 48 h varied considerably among the various strains tested (Table 2).

| Microorganism          | Source      | Conversion to pantoyl lactone after 48 h (%) | [α]D Methanol | Reduction in optically pure D[-] pantoyl lactone |
|------------------------|-------------|---------------------------------------------|---------------|-----------------------------------------------|
| *Byssochlamys nivea*   | CBS100.11   | 21°                                         | --15.8        | Equally variable were the ratios of D- and L-pantoyl lactone produced. However, all strains of *Byssochlamys* accumulated predominately the D[-]-isomer. On the other hand, three strains of *Paecilomyces* produced predominantly the L[+] isomer as did a Syntex strain of *P. varioti* (1227 sy) used initially in the survey study ([α]D methanol = +22.2°). Several strains of *B. fulva* (CBS 135,62, QM 6766, NRRL 1125, IMI 163641, IMI 40,021, ATCC 10099) were, however, distinguished by their unique ability to reduce ketopantoyl lactone at high concentrations (30 mg/ml of medium) with the formation of nearly optically pure D[-]-pantoyl lactone. |
| *B. nivea*             | CBS134.37   | 23°                                         | --7.7         |                                               |
| *B. nivea*             | CBS140.65   | 51°                                         | --13.1        |                                               |
| *B. nivea*             | CBS136.67   | 43°                                         | --10.7        |                                               |
| *B. nivea*             | CBS133.37   | 76°                                         | --10.6        |                                               |
| *B. fulva*             | CBS135.62   | 92°                                         | --27.6        |                                               |
| *B. fulva*             | CBS132.33   | 79°                                         | --24.8        |                                               |
| *B. fulva*             | CBS146.48   | 89°                                         | --20.3        |                                               |
| *B. fulva*             | QM6766      | 86°                                         | --27.6        |                                               |
| *B. nivea*             | QM6816      | 16°                                         | --5.9         |                                               |
| *B. fulva*             | QM6994      | 34°                                         | --14.5        |                                               |
| *Byssochlamys*         | QM438       | 82°                                         | --12.8        |                                               |
| *B. fulva*             | NRR1125     | 87°                                         | --27.3        |                                               |
| *B. fulva*             | IMI163641   | 90°                                         | --27.5        |                                               |
| *B. fulva*             | IMI140.21   | 69°                                         | --28.9        |                                               |
| *B. fulva*             | ATCC10099   | 79°                                         | --28.5        |                                               |
| *Paecilomyces*         | QM6764      | 58°                                         | --10.2        |                                               |
| *P. varioti*           | QM9367      | 87°                                         | 0             |                                               |
| *P. terricola*         | QM9546      | 85°                                         | +11.7         |                                               |
| *P. elegans*           | QM7335      | 74°                                         | +19.4         |                                               |
| *P. elegans*           | QM9533      | 85°                                         | +2.4          |                                               |

*a* Ketopantoyl lactone supplied at 30 mg of medium per ml.

*b* Ketopantoyl lactone supplied at 20 mg of medium per ml.

*c* Ketopantoyl lactone supplied at 10 mg of medium per ml.

**DISCUSSION**

It is not as yet clear why the reduction of ketopantoyl lactone by yeast cells resulted in the formation of racemic (DL) mixtures of the pantoyl lactone. The apparently high yield of the D[-]-isomer reported by Kuhn and Wieland (8) with a similar system was most probably due to the selective resolution that occurs during recrystallization, since these authors purified their material by recrystallization from diethyl ether-petroleum ether. It is known that certain monofunctional, "unnatural" ketones are reduced by fermenting yeast with only partial stereoselectivity (10, 11). However, it was somewhat unexpected to find a bifunctional, α-keto acid such as ketopantoic acid, which is presumably a normal intermediate in the biosynthesis of D[+]-pantothenic acid (2), reduced with less than full stereospecificity. One explanation for this phenomenon is the existence in yeast cells of more than one carbonyl reductase able to reduce ketopantoic acid or ketopantoyl lactone, at least one of which produces predominantly the unnatural L[+] isomer. These may include alcohol dehydrogenase or α-glycerophosphate dehydrogenase or more probably an α-hydroxy acid dehydrogenase such as lactic dehydrogenase (10). Of particular significance in support of this supposition is a recent communication by King and Wilken (6) in which the authors describe the isolation and partial purification of two yeast cell reductases, one of which reduced ketopantoyl lactone, whereas the other reduced ketopantoic acid to the corresponding hydroxy derivatives. Unfortunately, the investigators did not report the specific rotations of the enzyme reaction products.

The inability of the bacterial cultures examined in the survey to reduce added ketopantoyl lactone and accumulate pantoyl lactone in the growth medium may have been due, in general,
to a more strict control of the related enzyme system(s), perhaps by a form of feedback inhibition or repression, than exists in fungi where this ability was found to be rather widespread.

The ability to reduce ketopantoyl lactone with formation of both D- and L-isomers, albeit of widely varying ratios, was so widespread that one may speculate on the existence of a natural physiological role for the L[+]-enantiomorph. It has been suggested by a number of investigators (1, 4, 5, 12) that pantoyl lactone plays a role in cell division, based on its ability to reverse cell division inhibition. This effect may be attributable to the L[+]-isomer present in the pantoyl lactones used in these investigations. Contrary evidence has come from a report by Kirby et al. (7) in which the authors report that D-pantoyl lactone is slightly more active that the DL mixture in protecting lysogenic E. coli strain T-44(λ) from induction, a phenomenon presumably closely related to cell division.

Nevertheless, it may be of interest to re-examine earlier work with the supposition that isoketovaleric acid (2), ketopantoyl lactone, and/or ketopantoic acid are intermediates in a branched biosynthetic pathway, one arm leading to the formation of D[-]-pantoic acid, pantothenate, and coenzyme A, and the other leading to L[+]-pantoic acid or pantoyl lactone ultimately to play a role in cell division. Examination of the data presented in Table 2 indicates that the ability to reduce ketopantoyl lactone in relatively high concentrations to form nearly optically pure D[-]-pantoyl lactone by cultures of Bysschoilamys fulva is quite strain specific. This strain specificity may be even more definitive than is immediately apparent, since B. fulva strains ATCC 10099, IMI 40,021, NRRL 1125, and QM 6766 are reportedly equivalent cultures (E. G. Simmons, personal communication).

It should be noted that B. fulva CBS 146.48 is also reported as equivalent to NRRL 1125 and ATCC 10099 (3). However, two subcultures of this strain purchased from the CBS on separate occasions failed to reduce ketopantoyl lactone at the higher test concentrations or exhibit the high degree of stereoselectivity observed in the other strains.

Acknowledgments

We thank Vernon Hayashida for optical rotation analyses and Roger Leibrand for help in developing the GLC analysis.

References

1. Adler, H. L., and A. A. Hardie. 1964. Analysis of a gene controlling cell division and sensitivity to radiation in Escherichia coli. J. Bacteriol. 87:720-726.
2. Brown, G. M., and J. J. Reynolds. 1963. Biogenesis of the water-soluble vitamins. Annu. Rev. Biochem. 32:419-462.
3. Centraalbureau voor Schimmelcultures. 1968. CBS list of cultures, 27th ed. Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.
4. Grula, E. A., and M. M. Grula. 1962. Cell division in a species of Erwinia. III. Reversal of inhibition of cell division caused by D-amino acids, penicillin, and ultraviolet light. J. Bacteriol. 83:981-988.
5. Grula, M. M., and E. A. Grula. 1962. Cell division in a species of Erwinia. IV. Metabolic blocks in pantotenate biosynthesis and their relationship to inhibition of cell division. J. Bacteriol. 83:989-997.
6. King, H. L., and D. R. Wilken. 1972. Separation and preliminary studies on 2-ketopantoyl lactone and 2-ketopantoic acid reductases of yeast. J. Biol. Chem. 247:4096-4098.
7. Kirby, E. P., W. L. Ruff, and D. H. Goldthwait. 1972. Cell division and prophase induction in Escherichia coli: effects of pantoyl lactone and various furan derivatives. J. Bacteriol. 111:447-453.
8. Kuhn, R., and T. Wieland. 1942. Zur Biogenese der Pantothensaure. Ber. Deut. Chem. Ges. 75B:121-123.
9. Lansford, E. M., Jr., and W. Shive. 1952. Microbiological activity of α-keto-β, β-dimethyl-γ-butyrolactone. Arch. Biochem. Biophys. 38:353-355.
10. MacLeod, R. H., Prosser, L., Fikentscher, J., Lanyi, and H. Mosher. 1964. Asymmetric reductions. XII. Stereo-selective ketone reductions by fermenting yeasts. Biochemistry 3:838-846.
11. Neuberg, C., and F. F. Nord. 1919. Die phytochemische Reduktion der Ketone. Biochemische Darstellung optisch-aktiver sekundärer Alkohole. Chem. Ber. 52:2237-2248.
12. van de Putte, P., C. Westenbroek, and A. Röhrs. 1965. The relationship between gene-controlled radiation resistance and filament formation in Escherichia coli. Biochim. Biophys. Acta 76:247-256.