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Note

Use of isotachophoresis to monitor the synthesis and purification of L-α-amino-ε-ketopimelate

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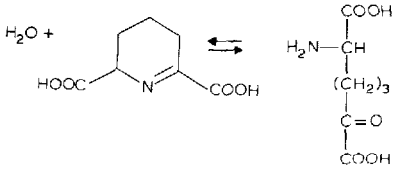
L-α-Amino -ε-ketopimelate (L-AKP) is regarded as an important intermediate in the bacterial pathway of L-lysine. In aqueous solution it exhibits an equilibrium between the linear and the cyclic form:



From AKP, many bacteria like *Escherichia coli* follow a rather complex pathway, the first step of which is the formation of a succinyl intermediate from succinyl-coenzyme A[1], whereas others, *e.g.*, some of the genus Bacillus incorporate an acetyl group from acetyl-coenzyme A[2,3]. Finally , some other strains exhibit another recently demonstrated enzymatic activity[4] consisting of a one-step reduction of AKP to *meso*-α,ε-diaminopimelate (DAP) using NADPH as a cofactor and incorporating free ammonia. Whatever the pathway may be, the study of the above enzymes in biosynthesis requires purified AKP.

At the present time, two methods of preparation are available:

(i) conversion of *meso*-DAP by *meso*-α-ε-diaminopimelate D-dehydrogenase[5]; which is very tedious and requires large amounts of NADP (the last point was resolved by White[6])

(ii) oxidation of L-DAP with L-amino acid oxidase according to Farkas and Gilvarg[7], Tosaka and Takinami[8] or Shapshak[9] involving the same kind of problems as above if purified AKP is needed.

The aim of this study was to find a non-enzymatic method of preparing AKP having specific advantages over the previously available ones. The method described is simple, rapid and inexpensive.

Isotachophoresis (ITP), a very easy analytical technique for anionic compounds [10–12], proved to be a powerful tool in the study of AKP, allowing us to follow the course of the synthesis and to estimate in a very simple manner the purity and the stability of the isolated compound. AKP can be stored indefinitely under the conditions described.

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MATERIALS AND METHODS

Chemicals

DL-α-ε-DAP was from Calbiochem. The *meso*-isomer was separated from the other isomers by recrystallization from ethanol–water[13]. L-DAP was prepared and purified according to the procedure of Saleh and White[13].

o-Aminobenzaldehyde, pyridoxal hydrochloride, $NADP^+$ and NADPH (highest quality available) were purchased from Sigma Chemical Company. Water and the reagents used in isotachophoretic experiments were of the highest available purity from Fisons or Sigma. All other chemicals were of analytical grade.

meso-α-ε-Diaminopimelate D-dehydrogenase was purified from *Bacillus sphaericus* NCTC 9602 as described by Misono *et al.*[4]. A crude extract of *Escherichia coli* W (ATCC 9637) was used to assay tetrahydrodipicolinate: succinyl-coenzyme A (CoA) succinyltransferase.

Analytical isotachophoresis

Isotachophoresis was carried out with a Model IP-2A (Shimadzu, Kyoto) instrument equipped with both potential gradient and UV (254 nm) detectors. Analytical separation was carried out in a two-stage capillary tube (first stage, 100 × 1 mm I.D.; second stage, 100 × 0.5 mm I.D.) at 15°C. The leading electrolyte consisted of 5 m*M* hydrochloric acid and 0.01 *M* β-alanine containing 0.05% hydroxypropylmethylcellulose in water. The terminal electrolyte was 0.013 *M* sodium acetate in water. The final current was 50 μA and the chart speed was 20 min/min.

Determination of AKP

Enzymatic assay. The reductive amination of L-AKP by *meso*-α-ε-diaminopimelate D-dehydrogenase was monitored spectrophotometrically at pH 7.5 using the system of Misono *et al.*[4]. The transfer of succinate from succinyl-CoA to L-AKP by tetrahydrodipicolinate: succinyl-CoA succinyltransferase was determined in crude extracts of *E. coli* W by measuring the rate of increase of the absorbance at 412 nm in the following system (total volume 1 ml): Tris–HCl buffer, pH = 8.0, 10 μmol; succinyl-CoA, 0.1 μmol; AKP, 1 μmol; 5,5′-dithiobis(2-nitrobenzoic acid), 0.1 μmol; and extract containing 0.5 mg protein[14].

Colorimetric assay. The *o*-aminobenzaldehyde adduct of AKP was prepared according to White[6] and the absorbance measured at 440 nm.

Chromatography. Samples containing about 20 μg of AKP were chromatographed on silica gel thin layers in the solvent system methanol–chloroform–water–acetic acid (70:20:10:2, v/v/v/v) and revealed with ninhydrin or dinitrophenylhydrazine in acidic solution as a faint colouration. The R_F value was 0.55.

Preparation of AKP

AKP was prepared from DAP according to the type of reaction described by Metzler and Snell[15], *i.e.*, the reversible transamination between pyridoxal and glutamate. DAP undergoes partial transamination when heated with pyridoxal and aluminium ammonium sulphate as a catalyst:

$$\text{DAP} + \text{pyridoxal} \rightleftarrows \text{AKP} + \text{pyridoxamine}$$

The reaction mixture (50 ml) containing 0.5 mmol of DL-DAP, 0.5 mmol of pyridoxal hydrochloride, 0.5 mmol of aluminium ammonium sulphate and 5 mmol of sodium acetate buffer (pH = 4.5) was incubated at 100°C for 45 min. The reaction was stopped by rapidly cooling the flask in an ice-bath.

Authentic L-AKP was made according to Misono *et al.*[4] by oxidative deamination of *meso*-DAP using *meso*-α,ε-diaminopimelate D-dehydrogenase with $NADP^+$ as a cofactor, and purified according to the procedure of the same author.

Purification of AKP

The reaction mixture was acidified to pH = 1.0 and applied to a Dowex 50-X8 (H^+) column (20 × 2.5 cm). The column was washed with 120 ml of 0.1 *M* hydrochloric acid, the product eluted with a linear gradient (300 ml) of 0.1–2.0 *M* hydrochloric acid and the effluent was monitored in the UV range (210 nm). The first peak, eluted with *ca.* 1.5 *M* hydrochloric acid, was collected and evaporated to dryness at 30°C and stored at −20°C under a nitrogen atmosphere. As the compound is unstable, it is necessary to employ stringent operating conditions during the purification (see also stability).

RESULTS AND DISCUSSION

An isotachophoretic analysis of purified AKP is shown in Fig. 1A. Under such analytical conditions, only AKP and some of its degradation products appear as anions in the preparations (Fig. 1B), plus sulphate when present, *e.g.*, in the incubating mixtures.

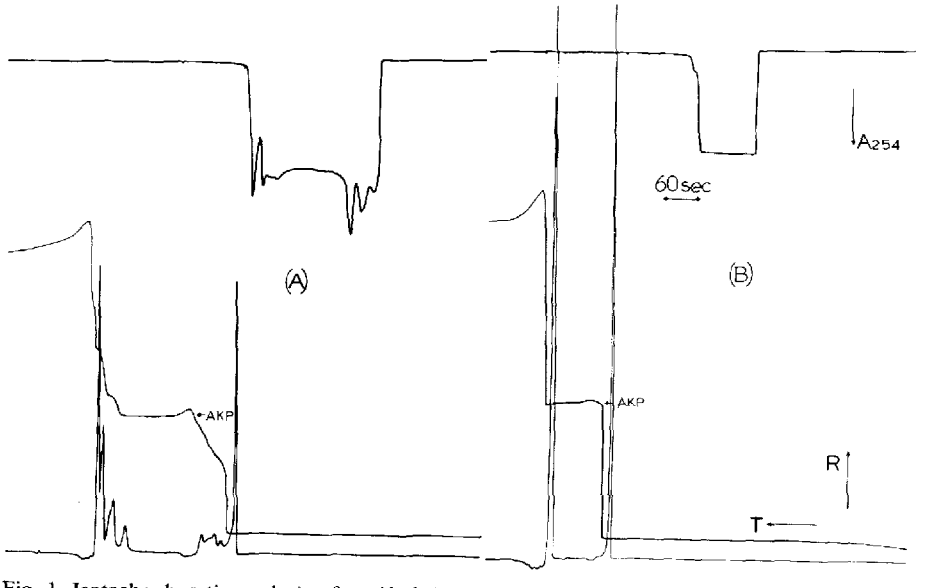

Fig. 1. Isotachophoretic analysis of purified AKP: A, stored for 2 months in solution (1 mg/ml), 5 μl injected; B, stored for 6 months in the dry state, 10 nmol injected. Analytical conditions as described under Materials and Methods. R = Resistance; T = time; A_{254} = absorbance at 254 nm.

Evidence of structure

The mixture during incubation was monitored by ITP: in the isotachopherograms, the potential gradient step which appears and increases has the same height as that of authentic AKP. Furthermore, a typical mixed zone is obtained when AKP is added to the mixture. AKP and the incubation product have the same UV absorbance at 254 nm.

The product eluted from the Dowex column reacts with *o*-aminobenzaldehyde to give a compound whose visible spectrum is similar to that of the AKP adduct.

The incubation product is a substrate of both *meso*-α,ε-diaminopimelate D-dehydrogenase assayed at pH = 7.5 and tetrahydrodipicolinate: succinyl-CoA succinyltransferase. It migrates with an R_F value of 0.55 on silica gel similar to that of authentic AKP in the solvent system described above.

Choice of the reaction conditions

Fig. 2 shows the yield of the AKP formed as a function of the operational conditions. It is seen that the maximum yield requires stoichiometric amounts of DAP, pyridoxal and aluminium ammonium sulphate. A temperature of 100°C is optimal.

Obviously, the amount of AKP present in the incubating mixture is a result of the counterbalance between its formation and degradation. The maximum yield is obtained between 45 and 60 min of incubation.

Purity and stability

We successfully assessed the breakdown of AKP during its purification by ITP. It could thus be shown that Dowex chromatography must be performed below 4°C. The subsequent drying under reduced pressure at 30°C causes no substantial loss of

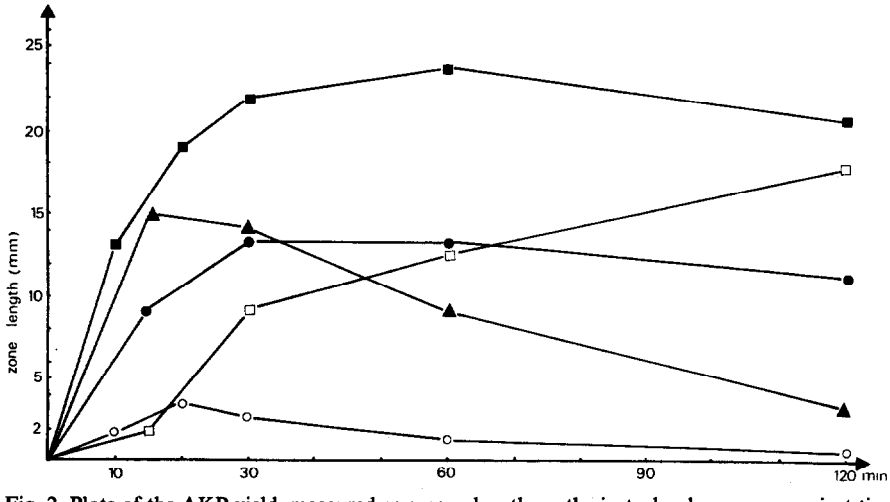

Fig. 2. Plots of the AKP yield, measured as a zone length on the isotachopherogram, against time under different incubation conditions. Analysis was carried out as described in Materials and Methods; 5 μl of the incubation mixture were injected. Millimolar concentrations of DL-DAP, pyridoxal and Al^{3+} and the temperature of the reaction (°C): ■, (10, 10, 10; 100); □, (10, 10, 10; 80); ●, (10, 10, 20; 100); ▲, (10, 20, 10; 100); ○, (10, 20, 1; 100).

compound provided it is carried out at that temperature for the shortest possible time. This stage of purification results in less than 2–3% of degradation.

Any attempt to purify AKP without the evaporation step, *e.g.*, chromatography with ammonium formate and freeze-drying or preparation of precipitable lithium or barium salts, resulted in a significant decrease in purity. In solution AKP breaks down and polymerizes to a complex set of compounds. The speed of degradation is temperature dependent and can be reduced only by storage at 4°C[5]. On the other hand, the purified compound, stored dry under a nitrogen atmosphere at −20°C, does not show any degradation after 6 months (see Fig. 1B). AKP prepared in this manner is free of the anions of the reaction mixture and cations ($<0.05\%$ Al^{3+}; 0.5% Na^{+}). The degradation products of AKP as estimated from the ITP zone lengths represent less than 5% of its total weight and are the main impurities in the isolated compound. The overall molar yield of DL-AKP is 50% of the initial DAP. The L-isomer comprises 50% of the racemic mixture. With L-DAP as starting material, the overall yield of L-AKP is 25%, showing that this procedure results in racemization.

DISCUSSION

The method of synthesis described has been shown to be an effective, simple and inexpensive way of preparing AKP for subsequent purification. Although very unstable, AKP was purified and stored for several months under the conditions described without significant degradation.

Isotachophoresis, a useful analytical technique when working with anionic species, proved to be a very powerful and irreplaceable tool in this study, allowing us to follow the course of the synthesis reaction. Its ease of application to the assessment of the purity and the stability of the isolated compound was demonstrated. This technique appears to be very useful in the study of such unstable species.

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REFERENCES

1 C. Gilvarg, *J. Biol. Chem.*, 236 (1961) 1429–1431.
2 G. Sundharadas and C. Gilvarg, *J. Biol. Chem.*, 242 (1967) 3983–3984.
3 S. Weinberger and C. Gilvarg, *J. Bacteriol.*, 101 (1970) 323–324.
4 H. Misono, H. Togawa, T. Yamamoto and K. Soda, *J. Bacteriol.*, 137 (1979) 22–27.
5 S. A. Simms, W. H. Voige and C. Gilvarg, *J. Biol. Chem.*, 259 (1984) 2734–2741.
6 P. J. White, *J. Gen. Microbiol.*, 129 (1983) 739–749.
7 W. Farkas and C. Gilvarg, *J. Biol. Chem.*, 240 (1965) 4717–4722.
8 O. Tosaka and K. Takinami, *Agr. Biol. Chem.*, 42 (1978) 95–100.
9 P. Shapshak, *Ph. D. Thesis*, Princeton University 1969.
10 F. M. Everaerts, J. L. Beckers and Th. P. E. M. Verheggen, *Isotachophoresis: Theory, Instrumentation and Applications (J. Chromatogr. Library*, Vol. 6), Elsevier, Amsterdam, New York, 1976.
11 P. Boček, K. Lekova, M. Deml and J. Janák, *J. Chromatogr.*, 117 (1976) 97–104.
12 V. Madajová, D. Kaniansky, Z. Radëj and A. Eszéniova, *J. Chromatogr.*, 216 (1981) 313–320.
13 F. Saleh and P. J. White, *J. Gen. Microbiol.*, 96 (1976) 253–261.
14 S. P. Chatterjee and P. J. White, *J. Gen. Microbiol.*, 128 (1982) 1073–1081.
15 D. E. Metzler and E. E. Snell, *J. Amer. Chem. Soc.*, 74 (1952) 979–983.