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Canaline

Characterization of Enzyme-Pyridoxal Phosphate Complex

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A method of preparation of canaline is described. Canavanine, isolated from Jack bean meal, is enzymatically hydrolyzed with commercial arginase, chromatographed on an cation exchange resin and prepared saltfree. The yield from canavanine obtained suggests losses during the preparation not in excess of approximately 10 % of that expected.

The mode of canaline inhibition of the ornithine ketoacid aminotransferase reaction was studied (a) by analyzing spectrophotometrically the complex formed between canaline and pyridoxal phosphate, the coenzyme of this transamination, and (b) by characterization of the inhibition under various conditions. The results suggest the formation of a covalent bond between canaline and pyridoxal phosphate, stable within a wide range of ionic strenght and temperature, but unstable at high pH. Competitive inhibition of the transamination was demonstrated when the ornithine concentration of the assay medium was the variable factor and uncompetitive when α-ketoglutarate concentration was varied. This finding is in accord with the concept that the formation of an oxime-type compund with pyridoxal phosphate is the mechanism of canaline-induced inhibition of transamination.

Canaline, α-amino-γ-aminooxybutyrate, an analogue of ornithine inhibits ornithine-α-ketoglutarate transamination. In addition, a chemical homologue of canaline canavanine (α-amino-γ-guanidinooxybutyrate) has been shown to competitively inhibit a number of reactions of arginine, a homologue of ornithine. Ornithine is involved in a number of enzymic reactions, some of which are inhibited by canaline;[1,2] and of these which are inhibited all require pyridoxal phosphate as coenzyme.[2] Other reactions catalyzed by pyridoxal enzymes are also inhibited by canaline.[2] In addition it has been demonstrated that canaline alters the specific adsorption maximum of pyridoxal phosphate in a manner suggestive of a chemical reaction between the two molecules.[2] Thus we suggested that the effect of canaline in the reactions involving ornithine may be due to a monovalent non-catalyzed reaction between canaline

and pyridoxal phosphate, possibly *via* the formation of an oxime, rather than to the structural similarity of ornithine and canaline.

This study describes the purification of canaline, characterizes the canaline-pyridoxal phosphate complex and the kinetics of the canaline-induced inhibition of transaimination. Due to the strong and irreversible inhibition of ornithine-α-ketoglutarate transamination by canaline,[2] and to the detailed knowledge of its kinetic proporties,[3–5] this enzyme, ornithine ketoacid aminotransferase (OKT) (EC 2.6.1.13), was chosen as a model for the inhibition studies.

MATERIALS AND METHODS

Ornithine ketoacid aminotransferase (OKT). OKT was partially purified from rat liver according to Peraino *et al.*[5] Comparison of the specific enzymic activity of the final preparation with that of the crude homogenate indicated a 600-fold purification.

Enzyme assay. OKT-activity was measured with *o*-aminobenzaldehyde as described by Matsuzawa *et al.*[6]

Preparation of canaline from Jack bean seeds. A 200 g portion of finely ground Jack bean seeds was defatted with petroleum ether and extracted with 50 % ethanol.[7] Canavanine was precipitated from the extract with absolute ethanol at 4°C and the precipitate was redissolved in water. The resulting solution was deproteinized by the addition of a saturated solution of lead acetate in water. Excess lead ions in the supernatant fluid were precipitated as the sulphate by the addition of 5 M H_2SO_4 to a final pH of 3.0.

The supernatant fluid, rich in canavanine but containing amino acid impurities, was purified from the neutral amino acids by a cation exchange resin (Dowex 50 × 8, NH_4^+-form) according to Thompson *et al.*[8] Preliminary experiments indicated that canavanine, like the other dibasic amino acids, was eluted quantitatively from the column with 4 M NH_4OH.[8] The eluate containing approximately 4 g of canavanine, was evaporated *in vacuo* at ambient temperature. The yellow residue was dissolved in a 0.1 M phosphate buffer pH 7.8, the solution heated to 100°C, and covered with a layer of liquid paraffin. Fifty mg of arginase (Sigma Chemical Corporation, St. Louis, Mo., USA) was added and hydrolysis of canavanine to canaline and urea was carried out at 42°C. The reaction was followed by serial determinations of urea concentration [9] in aliquots of the incubation system at 6 h intervals. The incubation was discontinued after the urea concentration had reached a maximum.[10] Dissolved canaline was dialyzed from the incubation mixture, concentrated *in vacuo*, and chromatographed on Dowex 50 × 8 (Na^+-form).[11] All of the canaline-containing fractions, identified with high voltage electrophoresis and ninhydrin staining, were then purified from acid and neutral impurities with cation exchange resin (Dowex 50 × 8, NH_4^+-form),[8] reprecipitated with ethanol, dried *in vacuo* and weighed. The purity of the preparate was tested using a small lot of canaline purchased from Sigma as a reference standard.

Characterization of the pyridoxal phosphate-canaline complex. The absorption spectrum of an equimolar solution of canaline and pyridoxal phosphate was analyzed between 700 nm and 300 nm with a Coleman 124 spectrophotometer. The effect of varying pH on the complex was tested in 0.1 M phosphate buffer in the pH range of 6.0 to 7.5 and in 0.1 M tris-HCl buffer in the pH range of 7.5 to 9.0. The stability of the complex was also examinated in tris-HCl buffer at pH 8.0 with a concentration from 1×10^{-3} M to 2 M. The effect of temperature on the complex was studied at pH 8.0 using 0.1 M tris-HCl buffer and temperatures from 20 to 60°C.

Analysis of the inhibition of OKT by canaline. The inhibition of canaline in transamination of ornithine was estimated according to the graphic method of Lineweaver and Burk by varying the concentration of canaline in the reaction mixture from 0 to 2.5×10^{-6} M. In these experiments, the concentrations of ornithine and α-ketoglutarate were 1×10^{-4} M. When examining the type of canaline inhibition, the concentration of ornithine and α-ketoglutarate was varied from 0 to 5×10^{-3} M.

Characterization of the OKT-pyridoxal phosphate-canaline complex. The stability of the enzyme-inhibitor complex was tested under similar conditions to those used for the

pyridoxal phosphate-canaline complex. In these studies the concentration of canaline selected was that which resulted in a 50 % inhibition of OKT in optimal enzyme assay conditions (at pH 8.0, in 0.1 M tris-HCl buffer concentration and at 37°C).

RESULTS

The yield and characteristics of the purified canaline. Based on the amount of urea produced by the arginase reaction, the content of canavanine in Jack bean meal was 1.8 % (w/w). This agrees well with earlier findings.[12,13] The final canaline yield was 2.5 g, which indicates that about 10 % of canaline produced by the arginase reaction was lost during the purification procedures.

The final purity of the product was studied with ascending paper chromatography using three different solvent systems and with high voltage electrophoresis at two different pH's. When butanol – glacial acetic acid – water (120:30:50) was used as the solvent, the R_F value of canaline was 0.11; using butanol – glacial acetic acid – pyridine – water (120:30:30:60) the R_F value was 0.20; and using propanol – NH_4OH – water (70:10:20) the R_F value was 0.53. With high voltage electrophoresis (50 V/cm) at pH 3.4 the R_F value was 11.8 (the R_F value of glutamine is chosen as 1.0) and at pH 2.1 the R_F value was 2.2. All of these systems contained only one ninhydrin positive spot which had a mobility identical to that of the Sigma canaline standard.

Stability of the pyridoxal phosphate-canaline complex. In agreement with earlier studies,[5] pyridoxal phosphate exhibited an absorption maximum at 420 nm at pH ≥ 7.0. At lower pH's the maximum shifted to 390 nm. Interaction with canaline obliterates both of the maxima. When the pH was raised up to 9.0 an absorption peak reappeared at 420 nm (Fig. 1). Neither the ionic strength nor the temperature of the buffer affected the absorption spectrum of the complex within the limits tested (Fig. 1). Canaline alone did not have any absorption maxima between 300 nm and 700 nm.

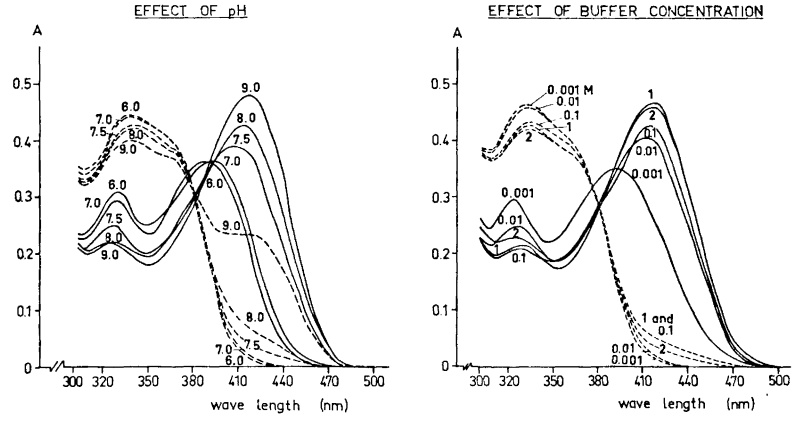

Fig. 1. The absorption spectra of pyridoxal phosphate and pyridoxal phosphate-canaline complex: left, at different pH's; right, at different buffer concentrations. ——— pyridoxal phosphate. – – – pyridoxal phosphate-canaline complex.

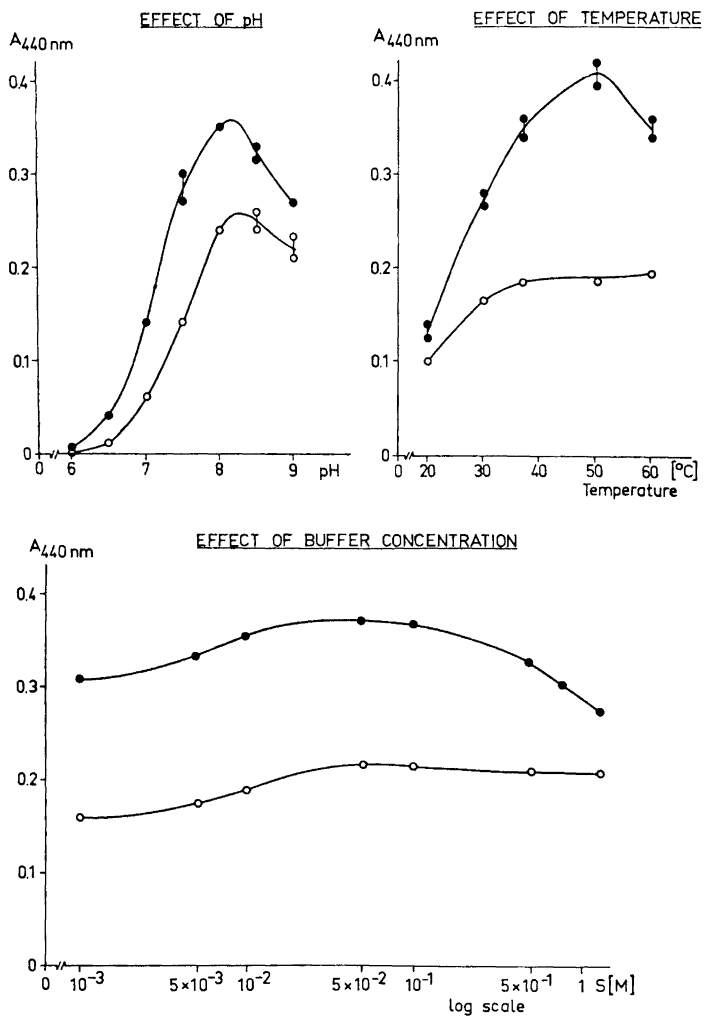

Fig. 2. The effect of pH, temperature, and buffer concentration on the stability of the OKT-canaline complex. The concentration of canaline used was 1.9×10^{-7} M.

Stability of the OKT-pyridoxal phosphate-canaline complex. The addition of canaline did not change the pH-, temperature- or buffer concentration optima of OKT (Fig. 2). The canaline inhibition was strongest at optimal OKT-assay conditions.

Inhibition of OKT by canaline. The inhibition constant (K_i) for canaline is between $1 - 2 \times 10^{-7}$ M (Fig. 3). The inhibition of ornithine α-ketoglutarate transamination was competitive when the concentration of ornithine was varied and uncompetitive when varying α-ketoglutarate (Fig. 4).

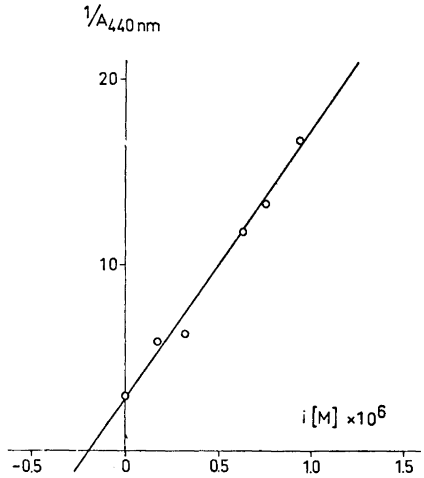

Fig. 3. The inhibition with canaline (i) under optimal ornithine ketoacid aminotransferase assay conditions (see text for details).

DISCUSSION

The results of this study suggest that the canaline induced inhibition of OKT may result from covalent binding of canaline to the pyridoxal phosphate cofactor. The addition of canaline obliterates the typical absorption maximum of pyridoxal phosphate at 390 nm (pH $\leq$ 7.0) or at 420 nm (pH $\geq$ 7.0) between pH 6.0 and 8.5. The complex is not stable at pH 9.0, which reflects dissociation of the pyridoxal phosphate-canaline complex in alkaline medium. Altering the concentration of the buffer between 1×10^{-3} M and 2 M or the incubation temperature between 20 and 60°C provided no evidence for any dissociation of the complex. The OKT-canaline complex was also stable at pH $\leq$ 8.5, and the per cent OKT inhibition due to canaline did not change between pH 6.0 and 8.5, but at pH > 8.5 the per cent inhibition was less, perhaps a result of the dissociation of the enzyme-inhibitor complex. In 1 M buffer the per cent inhibition was also less than at concentrations between 1×10^{-3} M and 1 M. The incubation temperature had no effect on the per cent inhibition. According to earlier studies [2] the activity of OKT could not be restored by additional pyridoxal phosphate. This was interpreted as evidence for both the stability of the pyridoxal phosphate-canaline complex, and the irreversibility of the binding between OKT and pyridoxal phosphate, in clear contrast to that of pyridoxal phosphate and other apoenzymes. These results are consistent with our earlier findings.

OKT was inhibited irreversibly by canaline with an inhibition constant (K_i) between $1 - 2 \times 10^{-7}$ M.* Complete inhibition of this transamination was found in the presence of 1×10^{-6} M canaline, using a highly purified enzyme preparation.[3] With crude liver homogenate 3×10^{-6} M canaline resulted in a 50 % inhibition,[2] indicating canaline binding by the homogenate in addition to

* Because of a very complicated inhibition kinetics in this enzyme system, only an approximate value of the inhibition constant can be given.

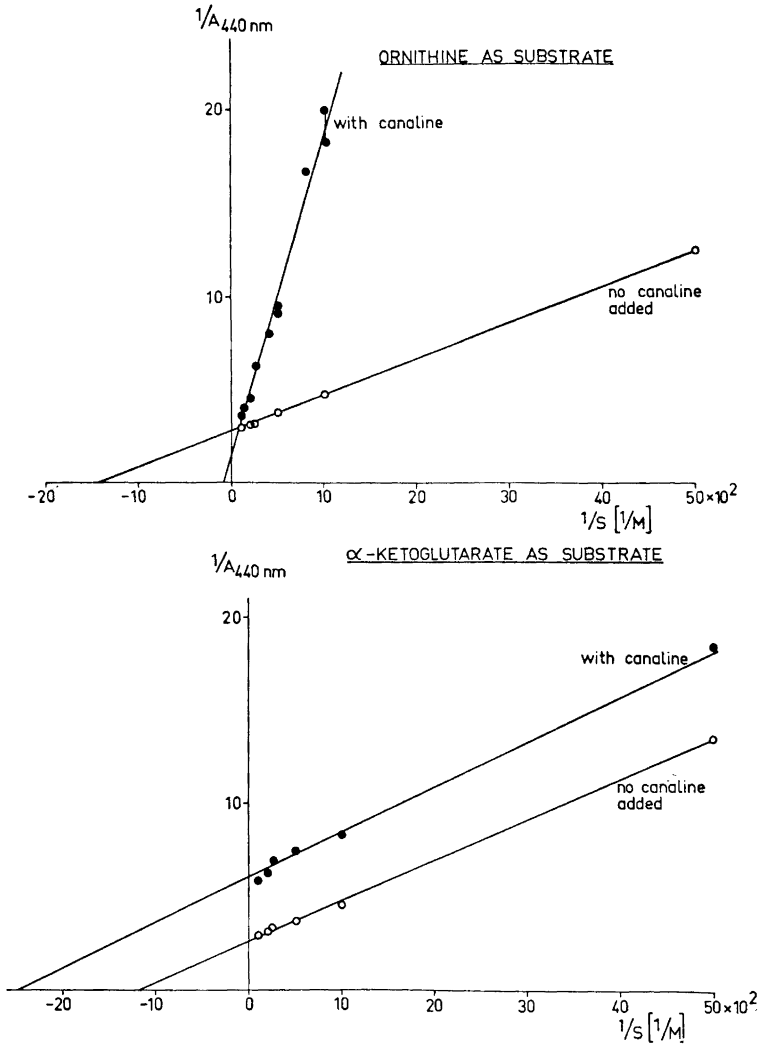

Fig. 4. Estimation of the type of canaline inhibition: upper, the concentration of ornithine was varied; and lower, the concentration of α-ketoglutarate was varied. The concentration of canaline in the assay system was 1.9×10^{-7} M.

OKT. The graphic analysis of the kinetic of canaline-induced inhibition of OKT, using varying concentrations of either ornithine or α-ketoglutarate, demonstrates different patterns of inhibition (Fig. 4). When the concentration of ornithine was varied the inhibition was competitive, whereas it was uncompetitive when varying the concentration of α-ketoglutarate. This result is consistent with the earlier hypothesis that the inhibitory action of canaline is based on the formation of an oxime with the aldehyde group of pyridoxal

phosphate.[2] Similar to the action of other aminooxy compounds [2,14,15] canaline combines with the same enzyme form as ornithine during transamination, thus acting as a dead-end inhibitor for OKT. In contrast, α-ketoglutarate reacts with the enzyme when the prosthetic group, pyridoxal phosphate, is in the aminoform ($-C-NH_2$). Therefore α-ketoglutarate does not compete with canaline, and the resulting inhibition is uncompetitive in type.[16] These results can be interpreted to indicate the formation of an oxime in the reaction between canaline and pyridoxal phosphate, the chemical basis of inhibition of the pyridoxal phosphate dependent enzymes by canaline.

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