ISOLATION AND CHARACTERIZATION OF AN HYDROXYKYNURENINASE FROM HOMOGENATES OF ADULT MOUSE LIVER[1]

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SUMMARY

A kynureninase-type enzyme was isolated from adult mouse liver. With kynurenine as the substrate, this enzyme has a K_m of 300 μM; when the substrate is hydroxykynurenine, the K_m is 6 μM. We conclude that this enzyme is an hydroxykynureninase. No enzyme which we could characterize as a kynureninase was found in this preparation. This suggests that tryptophan metabolism in the mouse occurs primarily through pathways that use hydroxykynurenine rather than kynurenine. Preliminary studies indicate that the enzyme is inhibited by its reaction product, hydroxyanthranilate, which is an intermediate in the synthesis of NAD. Such control of the hydroxykynureninase reaction may be of physiological importance in regulating the synthesis of NAD and/or in preventing the accumulation of hydroxyanthranilate, a putative carcinogen.

It has long been recognized that a "kynureninase" catalyzes the conversion of kynurenine to anthranilate and of hydroxykynurenine to hydroxyanthranilate in Neurospora crassa and other organisms. Gaertner et al. (1) have recently shown that in N. crassa this "enzyme" is actually two enzymes, which are readily separated by chromatography on DEAE-cellulose. One can be kinetically characterized as a distinct kynureninase, catalyzing the formation of anthranilate, which is subsequently excreted or reutilized for the synthesis of L-tryptophan. It is induced by tryptophan, consistent with its primarily degradative role. The second enzyme is an hydroxykynureninase which catalyzes the formation of hydroxyanthranilate, an intermediate in the biosyn-

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thesis of nicotinamide adenine dinucleotide (NAD). It is present in only small amounts and is constitutive, consistent with its biosynthetic role.

Shetti and Gaertner (2) have found a different enzyme system in the yeast Saccharomyces cerevisiae. Only a single kynureninase-type enzyme is demonstrable by chromatographic analysis. The enzyme is kinetically characterized as an hydroxykynureninase and is not inducible by tryptophan. Thus it is similar to the constitutive enzyme of N. crassa.

We have recently isolated and characterized a single kynureninase-type enzyme from mouse liver. Its kinetic properties suggest that it is an hydroxykynureninase similar to that of N. crassa and S. cerevisiae.

MATERIALS AND METHODS

Animals—The livers of male (C3H X C57BL)F_1 mice, 21-28 days old, were used as the source of the enzyme. Specific activity of the enzyme appears to be maximum by this age.

Preparation of extracts—Fifty-one grams of diced mouse liver were homogenized with about 50 ml of 0.05 M potassium phosphate, pH 7.0, at 4° C in a ground-glass homogenizer. The homogenate was centrifuged at 10,000 X g for 30 min to remove particulate material. The resulting crude extract was purified by treatment with protamine sulfate and ammonium sulfate as previously described (3). The pellet obtained after precipitation with ammonium sulfate and centrifugation was dissolved in 14 ml of 0.05 M potassium phosphate, pH 7.0, and frozen in two 7-ml aliquots.

Column chromatography—The extract was thawed and desalted on a Sephadex G-25 column (2 X 30 cm) with 0.05 M potassium phosphate, pH 7.0, containing 0.2 mM dithiothreitol and 0.01 mM ethylenediaminetetraacetic acid. All buffers subsequently used contained these protective agents. The pigmented fraction was collected, heated to approximately 26°C in a water bath, and centrifuged at 10,000 X g for 15 min to remove precipitated impurities.

The desalted extract was applied to a DEAE-cellulose column (2 X 70 cm).

A 1500-ml gradient from 0.01 to 0.15 M potassium phosphate, pH 7.0, was used for chromatography, followed by elution with 500 ml of 0.2 M potassium phosphate, pH 7.0. Fractions of approximately 9 ml were collected every 10 min.

Molecular weight determination—Enzyme (0.15 ml) and catalase (0.05 ml) solutions were layered on 4-ml gradients of 5 to 20% sucrose. The gradients were centrifuged at 39,000 X g for 11 hr. Fractions were collected and assayed for kynureninase and hydroxykynureninase activity as described below. Catalase was located by measuring the absorbance of the fractions at 405 nm on a Gilford recording spectrophotometer. The sedimentation coefficient and molecular weight of the enzyme were estimated by use of the method of Martin and Ames (4).

Assays—Kynureninase and hydroxykynureninase activities were assayed as described previously (1), except that the standard reaction mixture contained no $MgSO_4$. The standard amounts of reagents were varied as indicated in specific experiments. Reaction rates were corrected for the quenching of fluorescence by the substrate as previously described (1).

Pyridoxal-5'-phosphate dependency—The dependence of the reaction on pyridoxal-5'-phosphate was determined by measuring the reaction rate in the presence or absence of this cofactor.

Kinetic studies—The initial reaction rate was measured as a function of substrate concentration over a 1 μM to 1 mM range. K_m and V_{max} were determined for both kynurenine and hydroxykynurenine by plotting the velocity versus substrate concentration data according to the method of Lineweaver and Burke (5).

Anthranilate or hydroxyanthranilate, at concentrations from 0.05 to 1.0 μM, was added to the reaction mixtures to investigate the possibility of product inhibition. In these experiments the enzyme was incubated with the inhibitor for 15 min at room temperature before the addition of the substrate.

RESULTS

A single peak[4] of enzymatic activity is observed when extracts of mouse liver are chromatographed on DEAE-cellulose (Fig. 1). This peak contains both

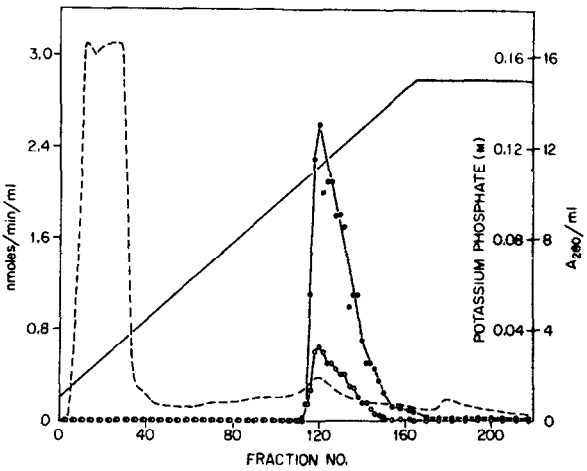

Figure 1. DEAE-cellulose chromatography of extracts from adult mouse liver. Symbols: kynureninase assay (O—O), hydroxykynureninase assay (●—●), absorbance 280 nm (----), and potassium phosphate gradient (——).

kynureninase and hydroxykynureninase activities, but under our assay conditions the latter activity is much higher. Recovery of hydroxykynureninase from the crude extract after DEAE-cellulose chromatography was ~80%. The results of the kinetic studies of the enzyme recovered from the peak sample are shown in Figure 2. The K_m for kynurenine is 300 μM, whereas that for hydroxykynurenine is 6 μM. The maximal velocity is approximately the same for both substrates. The enzyme depends on the presence of pyridoxal-5'-phosphate for its activity. The initial velocity of the reaction in the presence of 1 μM pyridoxal-5'-phosphate is more than twofold higher than that in the absence of the cofactor. From the position of the recovered enzyme in a sucrose gradient relative to that of catalase [an enzyme of known sedimentation con-

[4] Although we presently interpret this to be a single peak of activity, its somewhat heterogeneous appearance may reflect the presence of different molecular forms of the enzyme. However, no evidence was obtained indicating differences in catalytic properties.

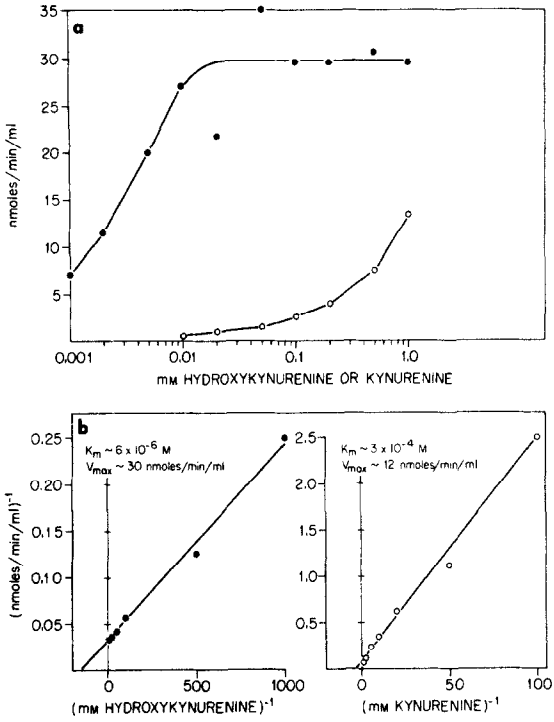

Figure 2. Dependence of the rate of the reactions catalyzed by the enzyme isolated from adult mouse liver on the concentration of the substrates L-kynurenine and L-3-hydroxykynurenine. The reaction rates have been corrected for the quenching of fluorescence by these substrates. (a) Semilogarithmic plots of these reactions, from which a point-for-point comparison of rates at various substrate concentrations can be made. (b) Lineweaver-Burke plots of these reactions, from which K_m and V_{max} for the two substrates can be estimated. Symbols: kynurenine (o—o), and hydroxykynurenine (●—●).

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stant (11.2 S) and molecular weight (250,000)], a sedimentation constant of 5.5 S was estimated for this enzyme. From these values, the molecular weight of the enzyme was estimated to be 85,000 daltons.

Preliminary inhibition studies indicate that the hydroxykynurenine-to-hydroxyanthranilate reaction is significantly inhibited by low concentrations of the reaction product hydroxyanthranilate. When the substrate concentration was 4 μM, the reaction rate in the presence of 1 μM hydroxyanthranilate was approximately half the uninhibited reaction rate.

DISCUSSION

The kinetic constants we have measured for the kynureninase-type enzyme of mouse liver indicate that it acts primarily as an hydroxykynreninase. It appears to be quite similar to the hydroxykynureninases isolated from S. cerevisiae and N. crassa. All three have (i) a K_m for L-3-hydroxykynurenine of ~1 μM, (ii) a K_m for L-kynurenine of ~100 μM, and (iii) approximately equal maximal velocities for both substrates. In addition, the molecular weight (L. L. Pritchard, unpublished results) and dependence upon pyridoxal-5'-phosphate (1) are similar for both mouse and N. crassa enzymes.

Mice appear to lack an active kynureninase. This is consistent with the observation often made that mammals excrete very little anthranilate. Experiments to determine whether mice excrete anthranilate are now in progress.

The most important role of the hydroxykynureninase in all three organisms may be biosynthesis of NAD, a cofactor for many enzymes. Hence, the inhibition of mouse hydroxykynureninase may be of significance in the regulation of NAD synthesis. It may also be of interest that this product inhibition would hinder the buildup of pools of hydroxyanthranilate, a substance which is thought to have carcinogenic properties (6).

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REFERENCES

1. Gaertner, F. H., Cole, K. W., and Welch, G. R., J. Bacteriol. 108: 902-909 (1971).
2. Shetty, A. S., and Gaertner, F. H., J. Bacteriol., in press.
3. Gaertner, F. H., and DeMoss, J. A., Meth. Enzymol. 17a: 387-401 (1970).
4. Martin, R. G., and Ames, B. N., J. Biol. Chem. 236: 1370-1379 (1961).
5. Lineweaver, H., and Burke, D., J. Amer. Chem. Soc. 56: 658-666 (1934).
6. Boyland, E., and Williams, D. C., Biochem. J. 64: 578-582 (1956).