BBA 65777

PURIFICATION AND PROPERTIES OF RAT LIVER HISTIDASE

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(Received April 11th, 1968)

SUMMARY

Rat liver histidase (L-histidine ammonia-lyase, EC 4.3.1.3) has been purified 200-fold. The final preparation was estimated to be about 80% pure and was used to evaluate cofactor requirements and other reaction parameters. Histidase has a molecular weight of 226 000, a K_m for histidine of $2.0 \cdot 10^{-3}$ M, and a pH optimum of 8.8–9.2. The enzyme is stimulated by glutathione although the latter appears to function as a bivalent anion, not as a sulfhydryl-protecting reagent. Histidase is inhibited by versene, and the inhibition is effectively reversed by both manganese and zinc ions; magnesium is slightly less effective in this regard.

Some physiological implications of the histidase reaction are discussed.

INTRODUCTION

The enzyme histidase, (L-histidine ammonia-lyase EC 4.3.1.3) is, in two ways, an indicator of development in the rat. First, histidase is absent from the liver during fetal life and becomes detectable either just before or at the time of birth. After its initial appearance the levels of liver histidase increase at similar rates in immature male and female animals, but these rates diverge as sexual maturation proceeds. Ultimately, mature female rats have from 2 to 5 times the male level of enzyme activity[1,2].

As part of a study of its developmental characteristics, we have purified histidase and attempted to characterize the enzyme protein and the reaction it catalyzes. The studies reported here were felt desirable in view of the ambiguities in reported properties of histidase.

MATERIALS AND METHODS

Female albino rats obtained from Charles River Animal Laboratories and weighing 150–175 g were killed by decapitation. The livers were removed, washed in ice-cold buffer containing 0.01 M Tris (pH 7.8), 0.014 M $MgCl_2$ and 0.06 M KCl (TMK

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buffer), blotted on filter paper, sliced into thin sections and added to 4 vol. TMK buffer. Tissue slices were homogenized in a Lourdes Multi-Mix homogenizer operated at half maximum voltage for 2 min. Unless designated otherwise, all operations were performed at 0–4°. Centrifugations were conducted either in a Servall refrigerated centrifuge (SS-1 rotor) or a Spinco Model L (40 rotor).

During the purification histidase was assayed by a spectrophotometric procedure similar to that described for the bacterial enzyme[3]. The assay mixture contained 30 μmoles sodium pyrophosphate (pH 9.2), 5.0 μmoles glutathione, 10 μmoles L-histidine (pH 9.0), and a volume of enzyme solution containing approx. 30 histidase units in a final assay volume of 3.0 ml. Incubations were conducted at 37° for 10 min and the absorbance read at 277 mμ using a blank treated in the same manner but lacking histidine. In all other cases, unless noted, histidase was assayed in a Beckman DK-2 ratio recording spectrophotometer with the sample compartment maintained at 37°. A unit of histidase, in this report, is the amount of enzyme which catalyzes the formation of 1.0 mμmole of urocanic acid per min at 37°. The molecular weight of histidase was determined by the method of MARTIN AND AMES[4] using linear gradients (20–5%) of sucrose dissolved in TMK buffer. Protein was determined by the procedure of LOWRY *et al*[5]. Sephadex G-200 was obtained from Pharmacia and reduced glutathione from Nutritional Biochemicals. The oxidized, disulfide form of glutathione was prepared from an aqueous solution of the reduced compound by adjusting the pH to 7.5 with NaOH and incubating for 1 h at 37° with 95% oxygen. Other chemicals were reagent grade commercial products.

RESULTS

Purification

Histidase was purified from a crude homogenate prepared as described in MATERIALS AND METHODS. The procedure used and results obtained are outlined in Table I. Histidase remained soluble during operations 1–4. After step 4, the pH was returned to 7.8 with NaOH and the enzyme precipitated by making the solution 40% (v/v) with regard to ethanol. In step 6, the material precipitating between 0.24 and 0.50 saturation with ammonium sulfate was collected by centrifugation and dissolved in TMK buffer for further purification.

TABLE I

PURIFICATION OF HISTIDASE FROM RAT LIVER

Fraction	*Volume (ml)*	*Total activity histidase units*	*Total protein (mg)*	*Specific activity units/mg protein*
1. 20 000 × g	110	41 750	2460	17
2. 105 000 × g	86	31 000	508	61
3. 55°, 10 min	78	26 200	179	146
4. pH 5.0	82	19 700	98	201
5. 40% ethanol	50	14 000	40	350
6. $(NH_4)_2SO_4$	10	12 800	5	2560
7. Freezing and thawing	10	12 100	4	3025
8. Sephadex	9	6 300	1.8	3500

At all stages of the procedure through step 5, the enzyme preparation could be stored at - -10° with little or no change in histidase activity. Following step 6, however, freezing and thawing resulted in the aggregation of superfluous material and, consequently, an additional purification of histidase.

The solution from step 7 was placed in dialysis tubing and packed in powdered confectioner's sugar to reduce the volume to 1.2 ml. The concentrated enzyme solution was put on a column of Sephadex G-200 equilibrated with TMK buffer which was also used as the eluting solvent. Fractions containing 1.5 ml each were collected and assayed for histidase activity. Approx. 50% of the activity placed on the column was found in the first three fractions collected after the void volume. Another 30% of the activity was contained in fractions 4–15. Fractions 1–6 were pooled and used in subsequent assays. As described below, the material eluted from Sephadex was estimated to be about 70% pure.

Molecular weight

The molecular weight of histidase was estimated by the procedure of MARTIN AND AMES[4]. Linear gradients, 20–5%, of sucrose were prepared in TMK buffer. Histidase and human hemoglobin were layered on the gradient and centrifuged in the SW 39 rotor for 8 h at 150 000 × *g*. Fractions of 0.12 ml were collected and assayed for histidase activity and hemoglobin (by absorbance at 540 mμ). With a value of 4.31 as the sedimentation coefficient of hemoglobin[6], the results of 4 separate runs gave a sedimentation coefficient for histidase of 9.70 ± 0.24.

The assumption involved in this calculation is that histidase has a partial specific volume of 0.725 cm^3/g. Since the partial specific volume of enzyme proteins is generally between 0.700 and 0.750 cm^3/g (refs. 7, 4), the error arising from that assumption should be no greater than 5%.

In each trial 0.5 ml of histidase solution, to which had been added 10 mg of hemoglobin, was layered on 4.4 ml of the sucrose gradients. On fractionating the centrifuged gradient, histidase was located in a single symmetrical band containing all of the added enzyme activity and 71 μg of protein. Since the aliquot of enzyme solution placed on the gradient contained 100 μg of protein, these data suggest that the histidase was, at most, about 70% pure.

pH optimum

After first ascertaining in the DK-2 that the reaction proceeded at a linear rate for at least 30 min, the pH optimum was determined in the following way: complete reaction mixtures, including histidine, were prepared, adjusted to the desired pH with either HCl or NaOH, and then two aliquots were removed. To one aliquot was added 0.5 ml of 30% $HClO_4$. Then approx. 50 units of enzyme activity were added to each aliquot followed by incubation at 37° for 20 min. The reaction was stopped in the second aliquot with 0.5 ml of 30% $HClO_4$. Both samples were filtered and the absorbance read at 268 mμ, the acid absorption maximum for urocanic acid[3]. These results are given in Table II.

K_m

To determine the Michaelis constant for histidine, the reaction velocity was measured at a variety of substrate concentrations. Double reciprocal plots of $1/v$ *vs.*

TABLE II

ACTIVITY OF PURIFIED HISTIDASE FROM RAT LIVER AS A FUNCTION OF pH

pH	*Activity units*
7.2	2.7
7.6	4.8
8.0	6.9
8.4	33.0
8.8	48.4
9.2	48.0
9.4	37.1
9.8	36.8

$1/[S]$ (ref. 8) were linear. The K_m obtained in this fashion is $2.0 \cdot 10^{-3}$ M. It has been pointed out by DOWD AND RIGGS[9] that, instead of the usual Lineweaver–Burk transformation, more reliable analysis of kinetic data may result from the use of the following transformation of the Michaelis–Menten equation:

$$v = V - K_m(v/[S])$$

Graphs of the same histidase data reported above were linear when v was plotted against $(v/[S])$. The K_m obtained in this manner was $1.3 \cdot 10^{-3}$ M for histidine.

Co-factor requirements

I. Glutathione. The assay mixture for histidase commonly includes GSH which has been reported to stimulate the bacterial enzyme[10]. On the other hand, SPALTER AND BALDRIDGE[11] concluded that GSH was without effect on rat liver histidase. Since previous studies were conducted with crude or slightly purified enzyme preparations, we have re-examined the role of GSH using our most highly purified preparation. It was found that enzyme activity in the presence of 6.0 μmoles of GSH was 2.2 times greater than that observed in the absence of GSH. Table III shows histidase activity as a function of GSH added to the assay mixture. The product in these reaction mixtures had an ultraviolet spectrum identical to that of authentic urocanic acid[10].

TABLE III

ACTIVITY OF PURIFIED HISTIDASE FROM RAT LIVER AS A FUNCTION OF THE CONCENTRATION OF GLUTATHIONE (GSH)

GSH added to assay mixture (μmoles)	*Activity units*
None	17.6
2.0	22.7
4.0	32.4
5.0	38.2
6.0	38.5
8.0	36.0
10.0	30.5
15.0	30.8

TABLE IV

EFFECTS OF SULFUR-CONTAINING COMPOUNDS ON THE ACTIVITY OF HISTIDASE

Assay mixture	*μmoles*	*Activity units*
Complete, *minus* GSH	—	17.6
Complete, with GSH	5.0	38.2
Complete, *minus* GSH with mercapthoethanol	5.0 10.0	17.5 17.0
Complete, *minus* GSH, with cysteine	5.0	6.1
Complete, *minus* GSH, with GSSG	5.0	35.9
Complete, *minus* GSH, with cystine	5.0	5.7

Table IV contains data obtained from assays conducted with GSH or with one of several sulfur-containing compounds substituted for GSH. Unlike GSH, β-mercaptoethanol is virtually without effect on histidase while cysteine is a very effective inhibitor. Moreover, it was found that GSSG was nearly as effective GSH in stimulating histidase, *i.e.*, either the sulfhydryl or the disulfide compound will stimulate the enzyme. In contrast, cystine inhibits histidase as effectively as does cysteine.

II. Metal ion activation. To our knowledge no definitive information is available in previously published reports concerning the activation of histidase by metal ions. Cobalt, among others, has been suggested as a stimulator[10]. In the present study, it was found that addition of cobalt to histidase assay mixtures immediately causes the formation of a strongly absorbing product which has none of the spectral properties of urocanic acid. The cobalt-induced product is formed even when histidase is omitted from the assay mixture.

Further tests for metal ion activation of histidase were conducted as follows. Histidase was dialyzed against 250 vol. of 0.01 M Tris (pH 7.8), containing 0.0001 M versene. After 6 h the dialysis sack was transferred to a fresh 250 vol. of Tris–versene and dialysis continued for 12 h. Samples of dialyzed histidase were assayed in the absence or in the presence of various metal ions added to the assay mixture. For comparison a sample of histidase which was not dialyzed was assayed at the same time. Dialysis resulted in a 80% reduction of histidase activity which was fully restored on addition of manganese. Zinc was nearly as effective as manganese in reactivating

TABLE V

EFFECTS OF METAL IONS ON THE ACTIVITY OF HISTIDASE

Metal ions were added as the chloride salts. In all cases the final metal concentration was $2 \cdot 10^{-3}$ M. See text for EXPERIMENTAL PROCEDURE.

Enzyme preparation	*Metal ion added*	*Activity units*
Non-dialyzed	None	42.0
Dialyzed	None	8.1
	Mg^{2+}	34.2
	Mn^{2+}	47.6
	Fe^{2+}	15.2
	Fe^{3+}	8.3
	Zn^{2+}	42.7

histidase, and magnesium restores the activity to 80% of the non-dialyzed level. These data are given in Table V.

III. Folic acid. With liver preparations from folic acid-deficient animals, ICHIHARA *et al.*[12] observed a decreased rate of histidine degradation and concluded that histidase requires folic acid for activity. We have, however, been unable to detect any direct effect of folic acid on the purified enzyme. The latter has none of the spectral properties of a folate-containing enzyme. In addition, neither dihydro- nor tetrahydrofolic acid stimulates the activity of purified histidase, even after the enzyme has been dialyzed 24 h against 500 vol. of TMK buffer. Finally, dihydroaminopterin, a folate antagonist, is without effect on histidase when added at a final concentration of 0.001 M to the assay mixture.

DISCUSSION

Histidase catalyzes the first reaction of a pathway in which histidine, an essential amino acid, is degraded to glutamic acid. With restricted diets or other physiological conditions which limit the availability of essential amino acids, the degradative enzymes could become effective in controlling biosyntheses requiring such amino acids. Thus it seems likely that histidase might be subject to regulatory influences. In this regard, SAHIB AND MURTI[13] have recently shown that either histidine or casein hydrolyzate increased the levels of histidase in protein-starved rats. These authors proposed that histidase levels may be controlled by a type of catabolite repression in the well-fed animal. Moreover, the reaction catalyzed by histidase is, under physiological conditions, irreversible. It has been suggested[14] that regulation of the rate of an irreversible step in a metabolic sequence could determine the physiological activity of that sequence. The significance of the histidase reaction, both in metabolic controls and in the course of development (see INTRODUCTION) was the impetus for the purification and characterization studies reported here.

The Sephadex elution pattern indicates that histidase has a molecular weight greater than 200 000. That is consistent with the sedimentation data which lead to a calculated molecular weight of 226 000. The enzyme, as others have observed[10], is strongly inhibited by versene. Manganese most effectively reverses the inhibition, although zinc also restores activity to the untreated level. Magnesium gives an 80% restoration of activity lost by versene treatment (Table V).

The role of glutathione still cannot be precisely defined. The data with cysteine, which inhibits, and with mercaptoethanol, which is without effect (Table IV), suggests that GSH may not function simply as a sulfhydryl reagent. That suggestion is supported by the data obtained with cystine and especially with GSSG. The latter may be acting as a bridge between parts of the histidase molecule. This hypothesis can be tested with ophthalmic acid, an analogue of GSH which lacks a sulfhydryl group. Others have observed that pyrophosphate activates histidase in an unexplained manner[10]. Thus, the activity of histidase may involve a structural feature which is induced or stabilized by multi-valent anions. This could explain why SPALTER AND BALDRIDGE[11] were unable to find an effect of GSH on histidase in a crude preparation.

Finally, a sex-related influence on histidase activity is indicated by the different levels observed in mature male and female rats[1]. A variety of explanations can be offered *a priori* and, in a preliminary report[15], it has been suggested that synthesis of

histidase is stimulated by estradiol and suppressed by cortisol, testosterone and possibly hypophysial hormones. In addition it can be suggested that the sex-related difference reflects an inhibition of histidase activity in male rats. This suggestion stems from our observation that extracts of male rat liver show an increase in total histidase activity during the course of purification. That is, an inhibitor of histidase is apparently removed when the enzyme is purified from male animals. In future experiments this possibility will be examined.

REFERENCES

1 V. H. AUERBACH AND H. A. WAISMAN, *J. Biol. Chem.*, 234 (1959) 304.
2 R. MAKOFF AND R. C. BALDRIDGE, *Biochim. Biophys. Acta*, 90 (1964) 282.
3 H. TABOR AND A. H. MEHLER in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 2, Academic, New York, 1955, p. 228.
4 R. G. MARTIN AND B. N. AMES, *J. Biol. Chem.*, 236 (1961) 1372.
5 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
6 G. KEGELES AND F. J. GUTTER, *J. Am. Chem. Soc.*, 73 (1951) 3770.
7 H. K. SCHACHMAN in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 4 Academic, New York, 1957, p. 32.
8 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
9 J. E. DOWD AND D. S. RIGGS, *J. Biol. Chem.*, 240 (1965) 863.
10 H. TABOR, in W. D. MCELROY AND H. B. GLASS, *Amino Acid Metabolism*, Johns Hopkins Press, Baltimore, 1955, p. 373.
11 P. D. SPALTER AND R. C. BALDRIGE, *J. Biol. Chem.*, 238 (1963) 2071.
12 K. ICHIHARA, M. UCHIDA, K. MATSUDA, N. KUMAGAI, AND H. KIKUOKA, *Z. Physiol. Chem.*, 295 (1953) 220.
13 M. K. SAHIB AND C. R. KRISHNA MURTI, *Biochim. Biophys. Acta*, 149 (1967) 615.
14 H. A. KREBS, *Enzymologia*, 12 (1946) 88.
15 MURIEL FEIGELSON, *Federation Proc.*, 26 (1967) 806.