Biochimica et Biophysica Acta, 481 (1977) 359—363

BBA 68098

ENZYMIC OXIDATION OF 3-HYDROXYXANTHINE TO 3-HYDROXYURIC ACID

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(Received September 27th, 1976)

Summary

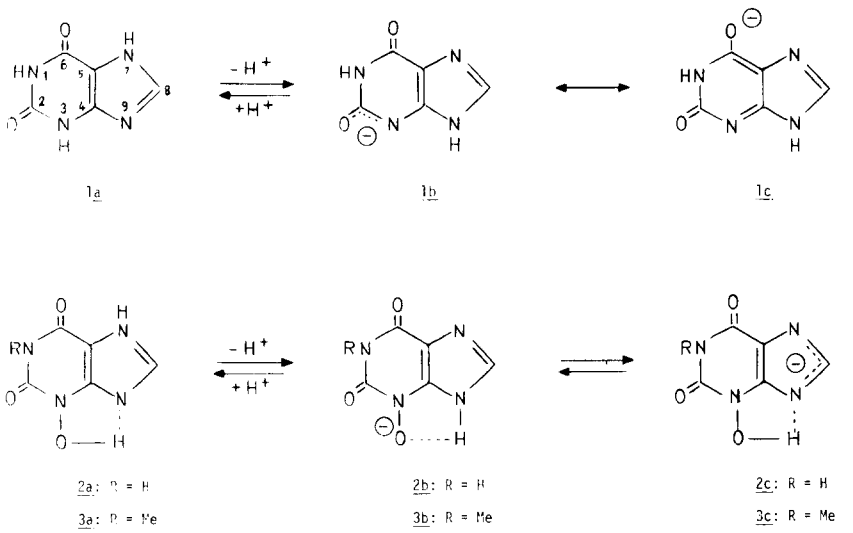
1. Bovine milk xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) oxidises 3-hydroxyxanthine slowly to 3-hydroxyuric acid; the 1-methyl derivative of 3-hydroxyxanthine is attacked about twice as fast.

2. The pH optimum for the reaction of 2-hydroxyxanthine is near 5, i.e. the neutral form of this substrate is attacked much faster than the anion. Probably in the 'active' form of the latter, the negative charge is located mainly in the imidazole ring, thus inhibiting nucleophilic attack at C-8.

Introduction

In a recent study we have discussed the 'active' form of xanthine, serving as substrate of bovine milk xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2) [1]. Since at the optimal pH of 8, xanthine (pK = 7.5) is present to 76% as uncharged molecule and to 24% as anion, both forms may serve as substrates for the enzyme. For the neutral molecule (compound 1a in Scheme 1), we have proposed complexation with the enzymic center via two groupings: (3)NH,(9)N and (6)C=0,(7)NH. For the anion (compound 1b and 1c), this role was assigned to the groupings (3)N,(9)NH and (6)C-0^-,(7)N in 1c. Our conclusions were based on the fact that 1-methylxanthine is oxidised faster than compound 1, because unfavorable binding via the partial structures (1)NH,(6)C=0 or (1)NH,(2)C=0 is now prevented.

3-Hydroxyxanthine (compound 2) may allow another test for the validity of these assumptions. The neutral form (compound 2a) bears a 7-NH group [2], similar to compound 1a, because of steric interference of 3- and 9-substituents in the purine system [3,4]. In addition, compound 2a is stabilised by intramolecular hydrogen bonding (see Scheme 1). Monoanion formation takes place by dissociation of the 3-OH group [5]. In compound 2b, tautomerisation in the imidazole ring places the proton at position 9, as can be seen by



SCHEME 1

comparing the ultraviolet spectrum of compound 2 with those of its 7- and 9-methyl derivatives (Table I). A similar process takes place upon monoanion formation in xanthine [6]. Form 2b is in tautomeric equilibrium with com-

TABLE I

PHYSICAL PROPERTIES OF DERIVATIVES OF XANTHINE AND URIC ACID

Compound	λ_{max} (nm)			pK		R_F in solvent [b]			Fluorescence
	N [a]	A_1 [a]	A_2 [a]	A_1	A_2	A	B	C	
Xanthine (1)	267	276	282	7.5	11.0	Str. [d]	0.17	0.25	purple
1-Methylxanthine	268	277	283	7.9	11.8	0.55	0.35	—	purple
3-Hydroxyxanthine (2)	272	299	297	6.7	9.65	0.25	0.05	0.19	blue
3-Hydroxy-1-methyl-xanthine (3)	272	298	297	6.8	9.7	0.49	0.15	—	blue
3-Hydroxy-7-methylxanthine	274	309	304	6.9	10.9				
3-Hydroxy-9-methylxanthine	271	297	290	6.3	11.2				
Uric acid	285	293	294	5.75	10.3	Str.	0.09	0.03	purple
1-Methyluric acid	283.5	292	294	5.75	10.6	Str.	0.15	—	purple
3-Hydroxyuric acid	289	309	300	5.5		0.17	—	0.12	blue
3-Hydroxy-1-methyluric acid		309 [e]				0.43	0.06	—	blue

[a] N, neutral form; A_1, monoanion; A_2, dianion.
[b] Descending paper chromatography; for solvents see Materials and Methods.
[c] Under a Mineralight ultraviolet lamp ($\lambda \sim 254$ nm).
[d] Str., streaking.
[e] For this compound, only the spectrum at pH 8 could be measured, because the amount available was very small.

pound 2c (Scheme 1), because of the more pronounced electronegative character of oxygen [7]. Thus both the neutral form and the monoanion of compound 2 resemble the corresponding states of xanthine, but should bind via (3)OH,(9)N and (6)C=0,(7)NH.

Materials and Methods

Purines. 3-Hydroxyxanthine (compound 2) and its 1-methyl derivative (compound 3) were a gift of Prof. G.B. Brown [2,8]. Because of the sensitivity of these purines to light, their solutions and chromatograms were always kept in the dark. The physical properties of the compounds, pertinent to the present study, are given in Table I. 3-Hydroxy-1-methyluric acid has not been described previously. Its properties are very similar to those of 3-hydroxyuric acid and differ characteristically from those of 1-methyluric acid (see Table I).

Enzymes. Highly purified bovine milk xanthine oxidase was supplied by Prof. R.C. Bray, Department of Biochemistry, University of Sussex, England. This preparation contained 30 000 units/ml and 115 mg protein/ml; ratio of activity/$A_{450\,nm}$ at 25°C was 120. One unit of xanthine oxidase produces at 29°C and pH 8 (0.01 M phosphate buffer) 1.5 μg/ml of uric acid, when $5 \cdot 10^{-5}$ M xanthine serves as substrate.

Catalase (Worthington) had an activity of 50 000 units/ml and contained 3 μg protein/ml. In all enzymic assays, the final concentration of catalase was 10 units/ml.

Determination of enzymic rates. All components were dissolved in 0.01 M phosphate buffer of pH 8. Substrate and catalase were incubated together for 3 min and the reaction was started by adding xanthine oxidase. All values in Table II refer to final concentrations; total volume was always 3 ml. The solutions were placed at once into the thermospacer of a Cary 14 ultraviolet spectrometer at 29°C. Readings were taken at the wavelengths specified in Table II. The enzymic rates were obtained from the initial linear portion of the curves,

TABLE II

ENZYMIC OXIDATION OF XANTHINES BY BOVINE MILK XANTHINE OXIDASE

All substrates were used at $5 \cdot 10^{-5}$ M concentration. For the first three compounds, this value is close to S_{opt}, but for compound 3 the optimal concentration was not determined because of the scarcity of material available.

Substrate	Xanthine oxidase, (units/ml, final concentration)	Wavelength used (nm)	Relative rate ± S.D.	No. of experiments
Xanthine (1)	0.67	300	100	12
1-Methylxanthine	0.67	300	132 ± 18 [a]	8
3-Hydroxyxanthine (2)	5.0	300	0.4 ± 0.08	4
3-Hydroxy-1-methylxanthine (3)	5.0	305	1.0 ± 0.08	3

[a] The following data show that the pH optimum for this substrate is near 8: relative rate at pH 7, 121.1; at pH 9, 24.7.

showing ΔA as function of time, and are expressed relative to the rate of xanthine oxidation at the same enzyme concentration.

For the pH dependence of enzymic activity, a series of buffers was used, as described in a previous paper [9].

Reaction products were characterised by their spectral properties, after chromatographic purification. For paper chromatography (descending method), the following solvents were used: A, *n*-butanol/5 M acetic acid (67 : 33, v/v); B, 95% ethanol/glacial acetic acid/water (17 : 1 : 2, v/v); C, isopropanol/dimethylformamide/water (13 : 5 : 2, v/v).

Results

Conversion of compound 2 into 3-hydroxyuric acid has been demonstrated by Myles and Brown [10], but the rate was not reported. Aerobic oxidation of compound 2 by xanthine oxidase is a slow process, leading to 3-hydroxyuric acid as sole product (Table II). Chromatographic analysis showed the absence of uric acid at the termination of the enzymic reaction. This is important since compound 2 can be reduced by xanthine oxidase to xanthine [10,11]; the latter then could serve as source of uric acid by enzymic oxidation.

The 1-methyl derivative (compound 3) reacts about three times faster than compound 2, in parallel with the relative rates of xanthine and its 1-methyl homolog [1] and of lumazine and its 3-methyl derivative [12]. Again no trace of 1-methyluric acid could be detected.

The most important feature of these reactions is their pH dependence, which differs from the data known so far for purines, which have a pH optimum at 8.0. This is well known for xanthine [1] and has now been confirmed also for 1-methylxanthine. In contract, the rate of compound 2 increases nearly 3-fold, when the pH is lowered from 8 to 5 (Fig. 1). The pH optimum is near 5, i.e. when compound 2 is present about 98% as neutral molecule, while at pH 8 it forms 95% anions. Clearly in this case the uncharged form is the better substrate. We have not determined the pH optimum of compound 3, because of

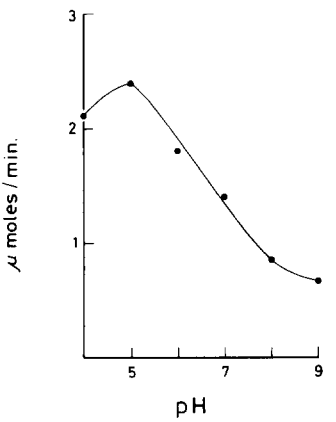


Fig. 1. pH dependence of the enzymic oxidation of 3-hydroxyxanthine (compound 2). Substrate: $5 \cdot 10^{-5}$ M; xanthine oxidase: 10 units/ml; 29°C. For each pH, the readings at 300 nm were corrected for the changing absorbance.

lack of material, but have observed that its rate increases when the pH is lowered from 8 to 6.

Discussion

Several explanations should be considered for the unusual pH dependence of the oxidation of compound 2: (a) 3-Hydroxyxanthine does not complex with the same groups in the enzymic center that combine with xanthine. Therefore the pH dependence of these two substrates is not comparable. (b) The "active" form of the anion of compound 2 (and likewise of compound 3) bears the negative charge mainly in the imidazole ring (compound 2c in Scheme 1). Therefore nucleophilic attack at position 8 (by RSS^- or OH^-), which is involved in the enzymic oxidations [13], is inhibited. If this were the sole reason for the low pH optimum of compound 2, the rate should increase from pH 8 to 5 in proportion to the percentage of anions present, i.e. about 20-fold. However as shown in Fig. 1, the maximal enhancement is about 3-fold. (c) 3-Hydroxyxanthine becomes attached to the same groups in the enzymic center that combine with xanthine, but the activity of the enzyme decreases at lower pH values, as is the case with xanthine or hypoxanthine as substrates.

It appears that a combination of b and c may satisfactorily explain the limited increase in the rate of compound 2, when the pH is lowered. If this assumption is correct, we should expect an "abnormal" pH dependence for other substrates which are completely ionised at pH 8 and bear the charge in the same ring that is attacked by xanthine oxidase. New examples of such behavior will be reported separately.

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