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INFLUENCE OF 8-SUBSTITUENTS ON THE OXIDATION OF HYPOXANTHINE AND 6-THIOXOPURINE BY BOVINE MILK XANTHINE OXIDASE

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Summary

1. The influence of 8-substituents was studied on the rate of oxidation of hypoxanthine and 6-thioxopurine by bovine milk xanthine oxidase (EC 1.2.3.2).

2. An 8-methyl group does not alter the rate of oxidation of hypoxanthine materially, but an 8-phenyl substituent reduces it markedly. This is ascribed to inhibition of the tautomerisation process, responsible for substrate activation, prior to oxidation.

3. In contrast, the 8-phenyl group in 3-methyl-8-phenylhypoxanthine enhances the rate, presumably by binding to a hydrophobic site near the enzymic center.

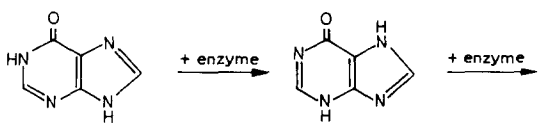
4. An 8-phenyl group in 6-thioxopurine markedly increases the rate of enzymic oxidation. Probably the aromatic substituent diverts anion formation to the imidazole ring. In contrast, ionisation of 8-methyl-6-thioxopurine involves the pyrimidine moiety, thus rendering enzymic attack at position 2 more difficult.

Introduction

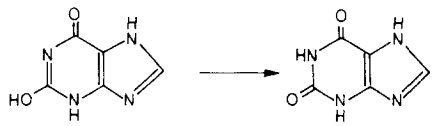
Previous experiments have shown that hypoxanthines and 6-thioxopurines are attacked by bovine milk xanthine oxidase (EC 1.2.3.2) along different pathways [1,2]. Hypoxanthine is first oxidised at C-2, but 6-thioxopurine at C-8. Since at pH 8, which is optimal for the enzyme, both substrates are present as mixtures of uncharged molecules and anions, we have proposed the following schemes for these reactions:

Scheme 1. Enzymic oxidation of hypoxanthine to xanthine

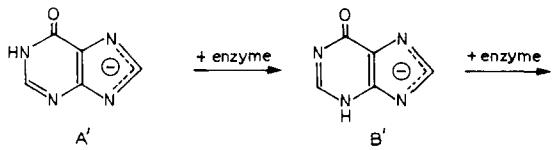
(a) as uncharged molecule.

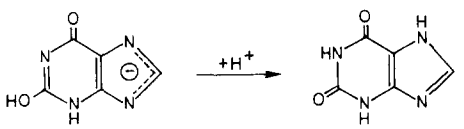


A B



(b) as anion



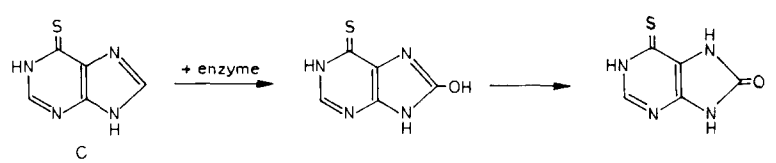


In aqueous solution, hypoxanthine is present exclusively in the lactam forms A and A' [3]. The enzyme induces tautomerisation to B or B', and the latter then serve as substrates. It has been shown previously that introduction of a 3-NH-group into the pyrimidine moiety of purines causes a shift of the tautomeric proton in the imidazole ring to N-7, as shown for B in scheme 1 [4]. Tautomer B can attach itself to the active center both via (6)CO, (7)NH and via 3(NH), (9)N [2].

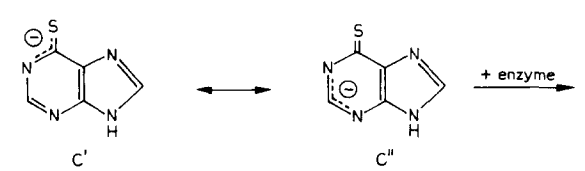
The monoanion of hypoxanthine (pK 8.4) is formed by dissociation of the imidazole NH-group [5]. This is of importance if the enzyme attacks the anion, since the reaction may involve abstraction of a hydride ion from and binding of OH^- to position 2 [6]. Presumably, such a process would be impeded by a negative charge in the pyrimidine moiety.

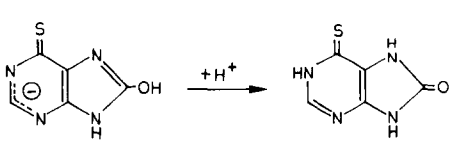
Scheme 2. Enzymic oxidation of 6-thioxopurine to 8-hydroxy-6-thioxopurine

(a) as uncharged molecule.



(b) as anion





In 6-thioxopurine, the monoanion is formed by dissociation of the 1-NH-group [5,7]. Since at pH 8, a solution of this compound contains about 76% of anions (Table II) and thus may react preferentially by enzymic attack on the ionised forms C′ and C″, the presence of a negative charge in the pyrimidine ring presumably is the main reason for diverting the first oxidation step to C-8. In addition, the driving force for tautomerisation, analogous to the process A → B, apparently is missing.

Materials and Methods

Purines

Unless stated otherwise, all substrates and their oxidation products (xanthines or 6-thioxanthines) were synthesised according to known methods.

(a) Hypoxanthines. Cmpd. 1 [8]; cmpds. 2 and 3 [9]; cmpd. 4 [10].

(b) 6-Thioxopurines. Cmpd. 5 [9]; cmpds. 6 and 7 [11].

(c) Xanthines. 8-Methylxanthine [8]; 8-phenylxanthine [9]; 3,8-dimethylxanthine [12] (λ_{max} at pH 8, 274 nm). 3-Methyl-8-phenylxanthine is unknown and was identified by comparison of its spectral properties with those of 8-phenylxanthine. The oxidation product of cmpd. 4 was isolated in pure form from paper chromatograms and its ultraviolet spectrum determined: λ_{max} (pH 8.0) 308 nm (8-phenylxanthine shows 309 nm).

(d) 6-Thioxanthines. 8-Methyl- and 8-phenyl-6-thioxanthine [9].

In addition, the following purines were used (see tables), but were all found refractory towards xanthine oxidase: 1,8-dimethylhypoxanthine [13]; 1-methyl-8-phenylhypoxanthine [14]; and 1-methyl-8-phenyl-6-thioxopurine [15].

Bovine milk xanthine oxidase

Two enzyme preparations were used:

(1) A highly purified xanthine oxidase (30 000 units/ml; 115 mg of protein/ml) was a gift of Prof. R.C. Bray, Dept. of Biochemistry, University of Sussex, England [16]; ratio of activity/E_{450} at 25° C ≈ 120.

(2) The commercial milk xanthine oxidase of Sigma Co., Saint Louis, Missouri, U.S.A., had 2000 units/ml (55.2 mg protein/ml) and a ratio of activity/E_{450} ≈ 60 at 25° C. This enzyme exhibited essentially the same specificity as the preparation of Dr. Bray.

Definition of enzyme unit: At pH 8 and 29° C, 1 unit of xanthine oxidsase produces 1.5 μg/ml of uric acid, using $5 \cdot 10^{-5}$ M xanthine as substrate.

Catalase (Worthington) had an activity of 50 000 units/ml; 3 μg/ml protein. At a concentration of 0.06 M H_2O_2, one unit of catalase decomposes 1 μmol of H_2O_2/min at 25° C and pH 7. In all enzymic assays, the final concentration of catalase was 10 units/ml.

Determination of enzymic rates

All components were dissolved in 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 the table refer to final concentrations of the components; total volume was 3 ml. The solutions were placed into the

thermospacer of a Cary 14 ultraviolet spectrophotometer at 29°C. Readings were taken at the wavelengths, specified in Table I. The enzymic rates were obtained from the initial linear portion of the curves, showing ΔA as function of time. Relative rates were calculated by dividing the rate of a given substrate by the maximal rate of xanthine at the same enzyme concentration. A linear relationship between enzyme concentration and rate of oxidation was assumed. V and K_m values were obtained from Lineweaver-Burke plots. Maximal rates were also determined from the bell-shaped curves, showing rates as a function of log (enzyme concentration) (see Table I).

Enzymic oxidation products were purified by paper chromatography. The reaction mixture was brought to dryness in vacuo and the residue extracted with warm Me_2SO. The extract was spotted on Whatman paper No. 1. The following solvents were used for descending chromatography: A, *n*-butanol/5 M acetic acid (67 : 33, v/v); B, 95% ethanol/12.5% ammonia (4 : 1, v/v); C, isopropanol/dimethylformamide/water (13 : 5 : 2, v/v).

Ultraviolet spectra were measured on a Cary recording spectrophotometer; pK values were determined from a plot of λ_{max} as function of pH (Fig. 1). The following buffers were used: pH −3, 6.75 M H_2SO_4; pH −2, 4.5 M H_2SO_4; pH −1, 2.5 M H_2SO_4; pH 0, 1 M HCl; pH 1, 0.1 M HCl; pH 2, 0.01 M HCl; pH 3, 0.1 M glycine buffer; pH 4—5, 0.1 M acetic acid/acetate; pH 6—8, 0.1 M phosphate buffer; pH 9—10; 0.1 M borate buffer; pH 11, 0.001 M NaOH; pH 12, 0.01 M NaOH; pH 13, 0.1 M NaOH; pH 14, 1 M NaOH.

Results

On the basis of the reaction schemes 1 and 2, substitution at C-8 should have different effects on the enzymic oxidation of hypoxanthine and 6-thioxopurine. This is borne out by the results in Table I. 8-Methylhypoxanthine (cmpd. 1) is attacked at a rate approaching that of hypoxanthine itself [2]. Oxidation of the 8-phenyl derivative (cmpd. 2) is ≈80 times slower than of hypoxanthine. This is surprising in view of the observations of Baker et al. that the enzymic center of milk xanthine oxidase has a hydrophobic region with high affinity for phenyl at position 8 or 9 [17,18]. At pH 8, cmpd. 2 is present to more than 90% in the uncharged form (Table I) and thus probably undergoes activation via the tautomerisation process A → B (scheme 1). The adverse effect of the 8-phenyl substituent in cmpd. 2 on the enzymic rate may be due to inhibition of this tautomerisation. In general the latter may be achieved by direct binding of N-7 to a specific H-donor in the active center or may require participation of a water molecule, approaching position 7. In either case, in the transition state the 8-phenyl group impedes attachment of a proton to N-7. Steric interference between the 8-substituent and the groups attached to the imidazole nitrogens in cmpd. 2, whether protons or alkyl groups, has been demonstrated previously [15,19].

For the 3-methylhypoxanthines (cmpds. 3 and 4), the reverse situation is encountered: The 8-phenyl derivative (cmpd. 4) is oxidised about 20 times faster than cmpd. 3. Both these substrates are present in solution in the "proper" tautomeric form corresponding to B (scheme 1), and thus can combine as such with the active center. It should be noted, that at pH 8, cmpd. 3

TABLE I

ENZYMATIC OXIDATION OF HYPOXANTHINES AND 6-THIOXOPURINES

Cmpd. No.	Substituents	λ_{max}(nm) [a]		pK for mono-anion formation	% anion at pH 8	Wavelength used for rate measurement (nm)	Xanthine oxidase, final concn. (units/ml)	V [b] (%) (± SD)	S_{opt} [b] ($\times 10^5$ M)	V [c] (%)	K_m ($\times 10^6$ M)	No. of experiments
		N	A_1									
		249	258	8.4 (im) [d]	28.6	280	0.33	117 ± 8	2–8	121	3.3	4
1	8-Methyl	252	259	10.2 (im)	0.6	290	0.67	94 ± 7	4	99	9.6	3
	1,8-Dimethyl	252	262	10.3 (im)	0.5	w.s. [e]	10	— [f]	—	—	—	2
2	8-Phenyl	288	298	9.0 (im)	9	310	4	1.4 ± 0.1		~1.4	—	3
	1-Methyl-8-phenyl	289	303	9.3 (im)	4.8						—	2
3	3,8-Dimethyl	265	268	9.2 (im)	6	245 270	10	0.56		4.3	—	3
4	3-Methyl-8-phenyl	298	311	8.5 (im)	24	240	0.67	118 ± 6	20	145	50	3
B. 6-Thioxopurines												
		322	312	7.5 (pyr)	76	345	4	1.3 ± 0.5		0.9	—	6
5	8-Methyl	325	312	7.9 (pyr)	56	340	1.33	2.4	11	3.9	64.5	3
6	8-Phenyl	346	343	7.5 (im?)	76	370	0.67	60 ± 2	9.5	64	4.4	3
7	1-Methyl-8-phenyl	347	344	9.5 (im)	3.1						—	2
8	3,8-Dimethyl	340	338	9.0 (im)	9	w.s. [e]	4	— [f]		—	—	2

[a] N = neutral form; A_1 = monoanion.

[b] Maximal rates at S_{opt}, relative to the rate of xanthine (=100) at its S_{opt}.

[c] Relative maximal rates from Lineweaver-Burke plots.

[d] im = dissociation in imidazole ring; pyr = dissociation in the pyrimidine moiety.

[e] w.s. = whole spectrum.

[e] Not attacked.

forms about 94% and cmpd. 4 about 76% of uncharged molecules (Table I).

Cmpd. 3 can attach itself only via the grouping (6)CO, (7)NH, while the 3-methyl substituent interferes sterically with the approach of reactive groups in the enzymic center to C-2. This may explain the low rate of oxidation of cmpd. 3. In cmpd. 4, the 8=phenyl group increases affinity and rate considerably. Indeed the K_m value of cmpd. 4 is strikingly lower than that of cmpd. 3 (see Table I).

In the 6-thioxo series, the 8-substituent diverts attack from C-8 to C-2. At pH 8, the 8-methyl derivative (cmpd. 5) is present to about 44% as neutral molecule, i.e. both uncharged and anionic particles may serve as substrates. The question arises whether oxidation of these derivatives requires tautomerisation to a form analogous to B or B′ (see scheme 1). Lack of such tautomerisation in the 6-thioxo series is evident from the fact that the 3-methyl derivative (cmpd. 7) is refractory. Evidently in 6-thioxopurines, the driving force for tautomerisation in the enzyme-substrate complex is missing. It appears that cmpd. 5 can attach itself to the enzyme only via the grouping (3)N, (9)NH. This may be the reason why the rate of cmpd. 5 is so small (about 1/40 of that of cmpd. 1).

In contrast, the 8-phenyl derivative (cmpd. 6) is oxidised about 25 times faster than cmpd. 5, the K_m indicating again the increased affinity of cmpd. 6 (Table I).

The pK of 6 is ≈7.5, i.e. at pH 8 this purine is ionised to about 76%. As was discussed before, the presence of a negative charge in the pyrimidine ring should reduce the enzymic rate. Therefore if the structure of the monoanion of cmpd. 6 resembles that of 6-thioxopurine (see C′ and C″ in scheme 2), then the high speed of oxidation of cmpd. 6 is surprising. This led us to investigate more

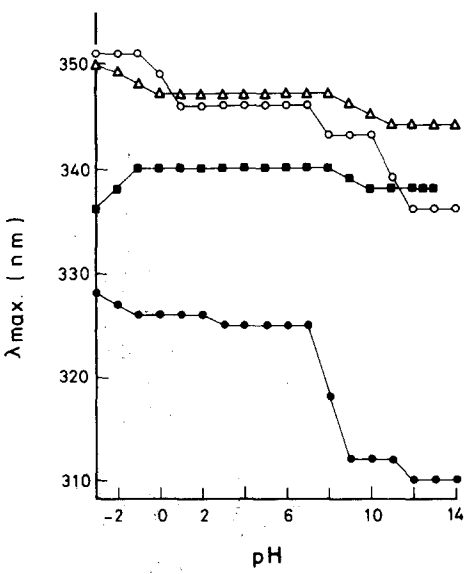


Fig. 1. Ultraviolet long-wave absorption maxima as function of pH. For buffers see Experimental. ●———●, 8-methyl-6-thioxopurine (cmpd. 5), $5.4 \cdot 10^{-5}$ M; ■———■, 3,8-dimethyl-6-thioxopurine (cmpd. 7), $1.7 \cdot 10^{-5}$ M; ○———○, 8-phenyl-6-thioxopurine, $0.2 \cdot 10^{-5}$ M (cmpd. 6); △———△, 1-methyl-8-phenyl-6-thioxopurine.

thoroughly the transformation of cmpd. 6 into its monoanion. Fig. 1 demonstrates that in the 8-methyl derivative 5, formation of the monoanion closely resembles the analogous process in 6-thioxopurine, involving a hypsochromic shift of 13 nm of the long-wave absorption maximum. This is characteristic for the ionisation sequence 1-NH → 9-NH [5]. In contrast we find for the 8-phenyl derivative 6 and its 1-methyl homolog a small value of $\Delta\lambda_{max}$ of only −3 nm, accompanying monoanion formation. For the dianion of 6, the shift is −7 nm. Thus the first ionisation step of cmpd. 6 closely follows the dissociation of the imidazole NH in the 1-methyl homolog (Fig. 1). These data suggest that in contrast to 6-thioxopurine and cmpd. 5, the ionisation sequence of cmpd. 6 is 9 → 1. Therefore the negative charge in the monoanion of cmpd. 6 is located predominantly in the imidazole ring and does not impede enzymic oxidation at C-2. On the other hand, binding of the 8-phenyl group to the hydrophobic site near the enzymic center increases the affinity of cmpd. 6 considerably.

If the ionisation sequence of cmpd. 6 can be substantiated by further experiments, it would appear that dissociation of the imidazole NH-group relieves the steric strain between this NH and the ortho-hydrogens of the aromatic ring. This probably is the reason why dissociation in the imidazole ring competes successfully with the ionisation of the 1-NH-group, characteristic in general for 6-thioxopurines [5].

Discussion

The present experiments demonstrate that an 8-methyl substituent does not alter much the rate of oxidation of hypoxanthine at position 2. An 8-phenyl group, however, can exert two opposite influences:

(a) By binding to a hydrophobic region near the active center of xanthine oxidase it can increase substrate affinity and rate of oxidation. Such an effect has been reported previously for a variety of phenylated purines. Thus 9-phenylguanine is a potent inhibitor of xanthine oxidase [20,21], and in this respect 8-phenylhypoxanthine is even seven times stronger [18], i.e. the aromatic substituent at C-8 appears to be most effective. 2-Phenylhypoxanthine is oxidised at position 8 about 50 times faster than the 2-methyl analog [22]. This relationship resembles the ratio found in the present study for the rates of cmpds. 4 and 3 (see Table I).

(b) On the other hand, the aromatic substituent may also reduce the enzymic rate. In the case of cmpd. 2, this effect may be ascribed to the difficulty to perform the enzyme-induced tautomerisation process A → B (see scheme 1).

It has been shown previously that xanthine oxidase can attack both anions and uncharged molecules with high speed [23]. The present observations indicate, however, that location of the charge is of great importance and that an aromatic substituent may have a decisive influence by changing the site of ionisation. Thus in the imidazole moiety of 8-phenylpurines, the *o*-hydrogens of the aromatic ring show strong interference with adjacent NH-groups [9]; this strain is relieved by anion formation in the imidazole ring. In cmpd. 6, the sequence of ionisation is the reverse of that in cmpd. 5. The acceleration of enzymic oxidation, when passing from cmpd. 5 to 6, supports the mechanism proposed by Edmondson et al. [6] for the action of xanthine oxidase.

Acknowledgment

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