Non-Classical Inhibition of Uricase by Cyanide

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The interactions of *Aspergillus flavus* uricase with the substrates O_2 and urate and the inhibitors xanthine, cyanide, periodate and hydroxylamine were investigated. Under equilibrium conditions O_2 does not bind directly to the enzyme, and the absence of O_2 had no effect on either the binding stoichiometry or binding constant for xanthine, as measured by equilibrium dialysis and microcalorimetry. Cyanide, periodate and hydroxylamine inhibit uricase in a non-classical manner. A decrease in initial velocity to a steady-state inhibited velocity can be observed on a time scale of minutes. The time-dependence, which is unaltered by prior incubation with the inhibitors, is consistent with a first-order transition. Rate constants for induction of inhibition are linearly dependent on inhibitor concentration, but independent of urate and O_2 concentrations. Radioactively labelled urate forms a stable but reversible complex with uricase in the presence of cyanide and O_2 . These results were used to deduce the nature of enzyme-bound intermediates and thus for the proposal of a novel mechanism for cyanide inhibition.

Uricase (urate–oxygen oxidoreductase, EC 1.7.3.3) catalyses the oxidation of uric acid, with molecular O_2 serving as the electron acceptor. The products of the enzymic reaction are H_2O_2 (Keilin & Hartee, 1936) and compound (I) (Praetorius, 1948), which hydrolyses to other products after release from the enzyme surface (Pitts & Priest, 1973).

reported to be a copper protein (Mahler *et al.*, 1955), and this metal was assumed to be the site of cyanide attack (Baum *et al.*, 1956). *Aspergillus flavus* uricase contains neither copper nor iron (Conley & Priest, 1980). On the basis of kinetic and equilibrium-binding analysis, an alternative mechanism for *A. flavus* uricase is now proposed.

Isotope studies (Brown *et al.*, 1947; Cavalieri & Brown, 1948; Bentley & Neuberger, 1952; Canelakis & Cohen, 1955; Hübscher *et al.*, 1957) have shown that C-6 of urate becomes the carboxy group of compound (I) and that molecular O_2 atoms are incorporated into H_2O_2 (Bentley & Neuberger, 1952).

Uricase is strongly inhibited by cyanide (Truszkowski, 1930). The pig liver enzyme is

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Materials and Methods

Uric acid, xanthine and potassium oxonate were purchased from Calbiochem (Los Angeles, CA, U.S.A.). KCN was obtained from Fisher Chemical Co. (Silver Spring, MD, U.S.A.), and $[2-^{14}C]$ uric acid (specific radioactivity 25 mCi/mmol) and $K^{14}CN$ (specific radioactivity 61 mCi/mmol) were from ICN (Irvine, CA, USA). *A. flavus* uricase was purchased from Boehringer Mannheim Corp., (New York, NY, U.S.A.) as a freeze-dried powder. Before

use, the enzyme was typically dissolved in 0.1M-glycine/NaOH buffer, pH 8.8, at a concentration of approx. 1 mg/ml, and dialysed extensively against the same buffer. The electrophoretically homogeneous enzyme preparation had a specific activity of 16.5 units ($\mu\text{mol}/\text{min}$)/mg of protein. Protein concentration was determined by using an absorption coefficient ($A_{281}^{1\%}$) of 16.9 (Conley & Priest, 1980).

Enzymic activity was determined by monitoring the disappearance of urate at 390 nm in the concentration range 5–100 μM . A molar absorption coefficient of $12300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Kalckar, 1947) was used. Unless otherwise noted 0.1M-potassium borate buffer, pH 8.8, was used. Over the urate concentration range 50–2000 μM activity was determined by using the increase in absorbance at 312 nm by the method of Priest & Pitts (1972). A molar absorption difference of $4850 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at this wavelength was used. The buffer system was 0.1M-glycine/NaOH, pH 8.8. Reaction rates estimated by the two methods in the overlapping range were identical. Measurements at other than atmospheric O_2 partial pressure were made in Thunberg cuvettes (Priest & Fisher, 1969). Concentrated uric acid and concentrated KCN solutions were prepared fresh daily and diluted to the desired concentrations as needed.

Equilibrium-dialysis and batch-microcalorimetry experiments were performed as described elsewhere (Conley & Priest, 1980). Changes in O_2 concentration were measured with a Yellow Springs Industries Clark-type oxygen electrode. Typically, 0.5 ml samples containing 77 μM -uricase were flushed with water-saturated N_2 for 1 h, and added to 2.5 ml of air-saturated buffer (0.1M-glycine/NaOH buffer, pH 8.8) in the sample chamber. Uricase activity remaining after N_2 bubbling for 1 h was greater than 95% of the original enzymic activity.

Binding of radioisotopically labelled urate and cyanide to the enzyme was measured by adsorbing the enzyme complex on nitrocellulose filters (Schleicher and Schuell). Filters were soaked in borate buffer for 5 min before use. Bray's (1960) solution was used to dissolve the filters and for counting of their radioactivity.

Amino acid-modifying reagents were purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.) and modification procedures were performed as previously described (Swenson & Boyer, 1957; Colman & Chu, 1970).

Results

Non-linear kinetics have been reported for pig uricase as a function of both urate concentration and O_2 concentration (Pitts & Priest, 1974). Under the assumption that the reported single copper atom per enzyme molecule (Mahler *et al.*, 1955) indicated a

single catalytic site for the pig enzyme, the non-linearity was interpreted in terms of a random-substrate-binding model (Pitts & Priest, 1974). Since *A. flavus* uricase contains no copper (Conley & Priest, 1980), a re-appraisal of the random-substrate-binding mechanism for uricase was undertaken by using equilibrium-binding methods.

One test for a potential random-substrate-binding mechanism is the ability of each substrate to bind to the enzyme in the absence of the other. With uricase it is very difficult to remove completely the ubiquitous substrate, O_2 , and thus prevent turnover when urate-binding experiments are attempted under equilibrium conditions. Urate on the other hand can be easily excluded, and O_2 binding tested.

No O_2 binding to *A. flavus* uricase was observed by oxygen-electrode methods. Further, when xanthine, a competitive inhibitor of uricase (Conley & Priest, 1980), was included in the enzyme solution at a sufficient concentration so that the purine-binding sites were saturated, binding of O_2 was still undetectable.

Since the electrode method may not be sufficiently sensitive to measure low extents of O_2 binding, other methods involving perturbation of easily detectable xanthine binding were used. If O_2 binding occurred to a significant extent to either the free enzyme or an enzyme-xanthine complex, then some perturbation of xanthine binding should occur as O_2 concentration is altered.

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Fig. 1. Measurement of xanthine binding to *A. flavus* uricase by equilibrium dialysis in the presence (Δ) and absence ($\bullet$) of O_2 .

A sample (1 ml) of uricase (2 mg/ml) in 0.1M-glycine/NaOH buffer, pH 8.8, was placed on one side of the cell, and 1 ml of the buffer containing [^{14}C]xanthine was placed on the other. After 18 h in a shaking water bath at 24°C, samples were removed and their radioactivities counted. O_2 was removed by flushing the system with water-saturated N_2 .

radioactively labelled xanthine could be detected by equilibrium dialysis. The results, which are plotted according to the method of Scatchard (1949), yield identical binding stoicheiometry, 3.6 mol of xanthine/mol of enzyme, and binding constant, $2.33 \times 10^5 \text{ M}^{-1}$, when the O_2 concentration is lowered extensively by thorough N_2 equilibration. The lack of O_2 perturbation of xanthine binding has also been demonstrated microcalorimetrically (Conley & Priest, 1980). Neither the binding constant nor the enthalpy change associated with xanthine binding was significantly changed.

Fig. 2 shows that inhibition of urate oxidation by low concentrations of cyanide increases with time. The initial velocity, V_0 , is independent of cyanide concentration and is identical with the velocity obtained in the absence of cyanide. Under the conditions of the experiments shown in Fig. 2, initial velocity in the absence of cyanide is constant throughout at least 25% of the reaction. The reaction velocity decreases with time to a final inhibited state, V_f , the magnitude of which is dependent on cyanide concentration. As shown in the inset of Fig. 2, the transition from the initial velocity, V_0 , to the cyanide-inhibited state, V_f , follows first-order kinetics. When the apparent first-order rate constants derived from such plots were replotted as a function of cyanide concentration, a linear relationship was obtained (see Fig. 3). The rate equation that describes such behaviour can be expressed as:

$$-\frac{d[E_a]}{dt} = k'[E_a] = (k[\text{CN}^-])[E_a]$$

where $[E_a]$ is estimated from tangents to the reaction rate curve and k' is the apparent first-order rate constant. The value of the second-order constant, k , obtained from the slope of the line in Fig. 3 is $5.8 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$.

Table 1 shows that the apparent first-order rate constant for the transition to the inhibited state is essentially independent of urate concentration. Inhibited velocities, however, depend on urate concentration. Reciprocal plots constructed with these velocities exhibited both slope and intercept effects, consistent with classical non-competitive inhibition.

Fig. 4 shows that extensive preincubation of the enzyme in the presence of cyanide did not change either the initial velocity, V_0 (i.e. the uninhibited velocity), or the final, cyanide-inhibited, velocity, V_f . Occupancy of the purine-binding site in itself was not sufficient to cause cyanide binding. When

Table 1. Apparent first-order rate constants for acquisition of cyanide inhibition of *A. flavus* uricase as a function of urate concentration

Values were obtained at a cyanide concentration of $3 \mu\text{M}$. Apparent first-order rate constants (k') and standard deviations were estimated from five separate determinations (see the text for method of determination). Values were not significantly different at $\alpha = 0.05$.

[Uric acid]	$10^2 \times k' \text{ (s}^{-1}\text{)}$
10	1.30 ± 0.15
25	1.25 ± 0.18
50	1.64 ± 0.19
100	1.75 ± 0.28

Fig. 2. Time-dependence of the inhibition of *A. flavus* uricase by cyanide

The initial urate concentration was $100 \mu\text{M}$ in 3 ml of 0.1 M-potassium borate buffer, pH 8.8, containing $3 \mu\text{M}$ -KCN, at 24°C . The reaction was initiated by the addition of 2.5 ng of uricase. V_0 represents the velocity of the reaction at zero time, and V_f represents the final, cyanide-inhibited, reaction velocity. The inset is a first-order replot of velocities (V) estimated along the reaction curve.

Fig. 3. Apparent first-order rate constants for the cyanide-induced transition as a function of cyanide concentration

Each point represents the average of a minimum of five replicates and error bars show the standard deviation. Rate constants (k') were estimated as described in Fig. 2.

Fig. 4. Effect of cyanide pretreatment on inhibition of *A. flavus* uricase

Samples (3 ml) containing 2.5 ng of uricase in 0.1 M-potassium borate buffer, pH 8.8, and cyanide were incubated at 24°C. Urate was added to a final concentration of 100 μM to initiate the reaction. ▲ and ○, Initial velocities, V_0 , at 3 μM- and 5 μM-cyanide respectively; △ and ●, final cyanide-inhibited, velocities, V_f , at 3 μM- and 5 μM-cyanide respectively.

Fig. 5. Pretreatment of *A. flavus* uricase with cyanide in the presence of 40 μM-xanthine

Reaction conditions were similar to those given in Fig. 4, with the exception of added xanthine. ○ and ▲, Initial velocities with and without 3 μM-cyanide respectively; ● and △, final, cyanide (3 μM)-inhibited, velocities, V_f , with and without 40 μM-xanthine.

xanthine was included in preincubation mixtures, no change in either V_0 or V_f was observed (see Fig. 5). Likewise, no change could be observed in the apparent first-order rate constant for the transition when O_2 partial pressure was increased to 95%.

One of the products of the uricase reaction is an unstable intermediate. Previously, cyanide has been used to quench the uricase reaction and thus allow

study of the kinetic properties of this intermediate (Pitts & Priest, 1973). To test the possibility that cyanide interacts directly with the intermediate, the enzymic reaction was quenched with the competitive inhibitor oxonate, and the decay rate of the intermediate was monitored at 312 nm (Priest & Pitts, 1972) in the presence and absence of cyanide. The rate constant for intermediate decay was unchanged. The other reaction product, H_2O_2 , when included at a concentration of 10 mM, also had no effect on the transition.

Periodate and hydroxylamine also inhibit uricase in a time-dependent pseudo-first-order manner (see Table 2). Neither, however, was as effective as cyanide when compared at the same concentration.

Although inhibition is reversible and loss of radioactively labelled compounds is observed on dialysis, a relatively stable uricase-cyanide complex can be isolated on nitrocellulose filters (see Table 3).

Table 2. Comparison of apparent first-order rate constants for acquisition of inhibition of *A. flavus* uricase

A sample (5 ng) of uricase and the inhibitor were placed in 3.0 ml of 0.1 M-potassium borate buffer, pH 8.8, at 24°C. Urate was added to a final concentration of 100 μM to initiate the reaction. For details see the text.

Inhibitor	k' (s^{-1})
KCN (3.0 μM)	1.78×10^{-2}
KIO ₄ (30 μM)	2.80×10^{-1}
NH ₂ OH (0.83 mM)	2.53×10^{-1}

Table 3. Binding of radioisotopically labelled cyanide and urate to *A. flavus* uricase

Samples (1 ml) containing 2 mg of uricase, 1 mM-cyanide (when present) and 1 mM-urate (when present) in 0.1 M-sodium borate buffer, pH 8.8, were bubbled with water-saturated air for 30 min to maintain adequate O_2 concentrations. In other experiments O_2 was excluded by flushing all components with water-saturated N_2 before mixing. Samples (0.2 ml) of reaction mixtures were adsorbed on nitrocellulose filters and washed four times with 0.5 ml each of 0.1 M-sodium borate buffer, pH 8.8. Filters were dissolved in 15 ml of Bray's (1960) scintillation 'cocktail' before counting of radioactivity.

Reaction components	Labelled compound/uricase (molar ratio)
[¹⁴ C]Cyanide, urate, O_2	0.378 ± 0.042
Cyanide, [¹⁴ C]urate, O_2	0.425 ± 0.029
[¹⁴ C]Cyanide, O_2	0.012 ± 0.006
[¹⁴ C]Urate, O_2	0.010 ± 0.005
[¹⁴ C]Cyanide, urate, N_2	0.008 ± 0.003
Cyanide, [¹⁴ C]urate, N_2	0.010 ± 0.004

When urate, cyanide and O_2 were all present in the incubation mixture, both [^{14}C]urate and [^{14}C]-cyanide were bound to the enzyme. Because of the reversibility of the formation of the complex, the values shown in Table 3 do not represent the number of inhibitor-binding sites on the enzyme. However, the ratio of bound urate to bound cyanide, both initially and after extensive dissociation of the complex, remains approximately 1:1. Cyanide does not bind in the absence of urate. Likewise, a stable urate-enzyme complex cannot be isolated in the absence of cyanide.

The possibility that *A. flavus* uricase contains an essential thiol group with which cyanide could react was investigated by using thiol-modifying reagents. Neither *p*-chloromercuribenzoate (1 mM) nor *N*-ethylmaleimide (100 mM) caused a decrease in uricase activity after incubation with the enzyme for 1 h in either the presence or the absence of substrate.

Discussion

A random-binding mechanism has been proposed as an explanation for the extensive non-linear kinetics observed in reciprocal plots of initial velocity versus substrate concentration with uricase from pig liver (Pitts & Priest, 1974). This proposed mechanism has now been examined by using equilibrium techniques with the *A. flavus* enzyme. The microbial enzyme is much more soluble than the pig enzyme, but other properties appear to be identical (Conley & Priest, 1980). Since O_2 binding to the *A. flavus* enzyme in the absence of urate was not observed by equilibrium techniques, it is assumed that O_2 is also not able to act as the initial substrate under turnover conditions. Further, O_2 binding could not be demonstrated in the presence of the unreactive urate analogue xanthine. An ordered mechanism in which O_2 attacks enzyme-bound urate directly would be consistent with these results. Such a mechanism is particularly appealing, since no copper, iron or other common prosthetic group was found associated with this enzyme (Conley & Priest, 1980) to mediate electron transfer between urate and O_2 . Kosman (1978) has suggested the attack of O_2 on a forming carbanion at the bridge-head carbon atom of urate. Direct attack of triplet oxygen on singlet urate, with the subsequent formation of singlet products, is a spin-forbidden process. However, stabilization of an O_2 -urate complex by the enzyme could allow time for spin inversion to occur, thus making the mechanism more feasible.

The observed non-classical time-dependent inhibition by cyanide is consistent with the generation of an inhibitor site in the presence of substrates. The apparent first-order rate constant, k' , for the transition from fully active to cyanide-

inhibited enzyme was independent of initial urate concentration. Since varying the initial urate concentration would quantitatively change the steady-state distribution of enzyme species, cyanide probably interacts with only one enzyme form. If more than one intermediate were reactive, dependence of the apparent first-order rate constant on initial urate concentration would be expected.

Since a stable urate-cyanide complex could be isolated only in the presence of O_2 , it is assumed that this substrate is already present on the enzyme form that is attacked by cyanide.

Periodate and hydroxylamine also cause time-dependent inhibition of uricase. These inhibitors, as well as cyanide, are useful in elucidation of functional groups of reaction intermediates. Cyanide and hydroxylamine both are capable of attacking and forming stable complexes with aldehyde groups. A classic reaction of periodate is attack on vicinal keto carbon atoms. These functional groups are therefore implicated as uricase reaction intermediates. The adducts formed by the reaction of these non-classical inhibitors with the appropriate enzyme-bound urate intermediates are postulated to interrupt turnover by increasing the stability of resultant complexes.

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