4-HYDROXYPYRAZOLO(3,4-d)PYRIMIDINE AS A SUBSTRATE FOR XANTHINE OXIDASE: LOSS OF CONVENTIONAL SUBSTRATE ACTIVITY WITH CATALYTIC CYCLING OF THE ENZYME

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SUMMARY. The 6-hydroxylation of 4-hydroxypyrazolo(3,4-d)pyrimidine (4-HPP) by xanthine oxidase takes place by a mechanism which, initially, utilizes the complete internal electron transport chain of the enzyme; electron transfer to molecular oxygen and to cytochrome c can be demonstrated, together with spectrophotometrically detectable reduction of the enzyme flavin by substrate. Conventional substrate activity is rapidly lost, however, the rate of aerobic oxidation of 4-HPP showing an exponential decline with a half-time of 18 sec at 25°. The anomalous behavior of 4-HPP as a xanthine oxidase substrate becomes manifest only on catalytic cycling of the enzyme.

The hypoxanthine isomer 4-hydroxypyrazolo(3,4-d)pyrimidine (4-HPP; allopurinol) has been of value in studies of electron transfer from the internal electron transport chain of the molybdoflavoprotein xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2) to external electron acceptors (1-3). 4-HPP is a substrate for the enzyme, being converted to 4,6-dihydroxypyrazolo-(3,4-d)pyrimidine; its behavior as a xanthine oxidase substrate is anomalous, however, since with artificial electron acceptors (dyes, ferricyanide), the rate of enzymic oxidation of 4-HPP is rapid, exceeding the rate of oxidation of xanthine under the same conditions, while with physiologic acceptors such as molecular oxygen and cytochrome c, the rate of enzymic oxidation of 4-HPP is extremely slow, less than 0.5% that for xanthine (1,2).

In an attempt to elucidate this disparity, studies of the slow aerobic oxidation of 4-HPP were initiated, using assay methods suitable for the measurement of low rates of product formation. These studies have revealed that the aerobic oxidation of 4-HPP by xanthine oxidase is characterized by a fast initial rate previously undetected because of its rapid exponential decline ($t_{1/2}$ = 18 sec at 25°). Enzyme-catalyzed electron transfer from 4-HPP to cytochrome c can also be demonstrated during this brief initial phase, as can substrate-induced anaerobic reduction of the enzyme flavin. These results

suggest that the initial interaction between 4-HPP and the resting, fully oxidized enzyme is conventional in type, and that the almost complete inability to utilize molecular oxygen as electron acceptor subsequently observed, together with the other anomalous properties of the reaction, arise as a consequence of complex formation between 4-HPP and one of the reduced or otherwise altered forms of the enzyme generated during the catalytic cycle.

METHODS. Bovine xanthine oxidase was purified from cream by the method of Nelson and Handler (4) or was purchased from a commercial source (Seravac Laboratories, Code 5P); the latter preparation had been purified by the method of Gilbert and Bergel (5). The specific activities of the two preparations were 8.4 and 9.3 units/mg respectively (as defined by Fridovich (6)); and the 280/450 mμ ratios were 7.4 and 6.2. Enzymes were stored in the presence of salicylate, 2 mM, and were dialyzed before use. Similar results were obtained with enzyme prepared by either method. Assays were performed at 25° in potassium phosphate buffer, pH 7.8, containing EDTA, 10^{-5} M. The conversion of 4-HPP to 4,6-diHPP was measured from the rate of increase in absorption at 285 mμ as previously described (2); the reaction was followed by means of a Gilford Model 2000 spectrophotometer coupled to a Honeywell strip chart recorder with a full-scale response time of 0.75 - 1 sec. A full-scale setting of 0.25 absorbance units was used for most experiments. The reaction was started by adding the substrate directly to the sample cuvette by means of a rapid-injection syringe, without removing the cuvette from the light path. The anaerobic studies were carried out under nitrogen, in Thunberg-type cuvettes, using an Aminco-Chance Dual-Wavelength Split-Beam recording spectrophotometer. Nitrogen was deoxygenated by the vanadous sulfate method of Meites and Meites (7).

RESULTS. Formation of 4,6-diHPP. As shown in Fig. 1, addition of 4-HPP to xanthine oxidase under aerobic conditions results in the generation of 4,6-diHPP at a rate which, while initially comparable to that seen with rapidly oxidized substrates for this enzyme, shows an exponential decline with a half-

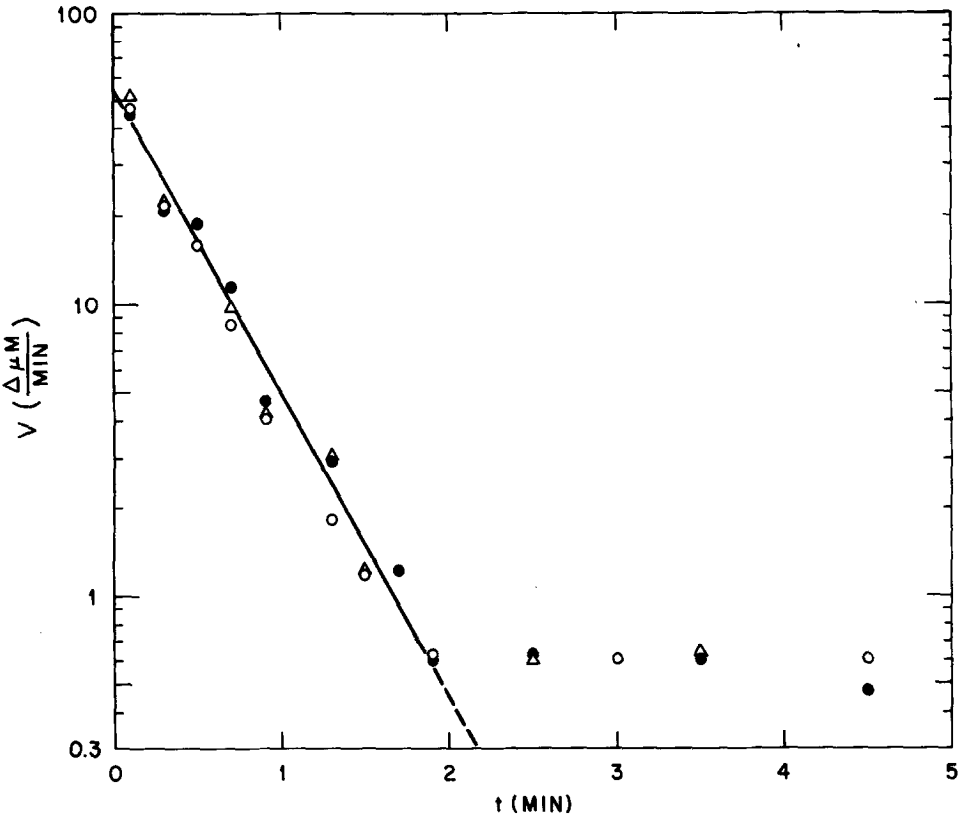

FIG. 1. Rate of aerobic oxidation of 4-HPP by xanthine oxidase. Cuvettes contained potassium phosphate buffer, pH 7.8, 0.5 mmole, EDTA, 0.1 µmole, and xanthine oxidase, 0.9 mµmole, in a total volume of 3 ml. The reaction was started by the rapid addition of 4-HPP (see Methods), and 4,6-diHPP formation was monitored at 285 mµ. Initial concentration of 4-HPP: △——△ : 0.75 µmole/3 ml; ●——● : 0.50 µmole/3 ml; ○——○ : 0.25 µmole/3 ml. Reaction temperature was 25°.

time of 18 sec at 25°, until terminating in a low constant value at approximately 100 sec. Experiments at three initial levels of substrate (Figs. 1 and 2A) resulted in identical rates of product formation, indicating that, over the concentration range studied, the decline in rate with time was not attributable to depletion of substrate. The addition of more 4-HPP or an increase in the oxygen tension of the reaction mixture after termination of the fast phase failed to bring about reversion to the rapid initial rate (Fig. 2C). The initial turnover number for 4-HPP with this enzyme preparation was 163 min^{-1} at 25°, pH 7.8; and the total or cumulative turnover of 4-HPP during

the fast phase was 73 - 77 molecules of substrate per molecule of enzyme.

Effect of product concentration on the rate of oxidation of 4-HPP. Since the rapid anomalous decline in the rate of 4-HPP oxidation was not related to the decrease in substrate concentration during the reaction, the possibility was considered that the slowing of the rate resulted from inhibition by one of the reaction products. It was found, however, that the addition of 4,6-diHPP to the reaction mixture at a concentration equimolar to the initial concentration of 4-HPP did not result in detectable alteration in either the rate or duration of the fast phase (Fig. 2B); nor did the addition of catalase to the reaction mixture affect the transition from the fast to the slow phase.

Reduction of cytochrome c. Under aerobic conditions, cytochrome c can serve as an electron acceptor for the xanthine oxidase-catalyzed oxidation of conventional substrates (8). With 4-HPP as substrate, the duration and rate of the aerobic fast phase paralleled that seen with oxygen alone; the efficiency of electron transfer to cytochrome c was lower, however, a total of 21 - 28

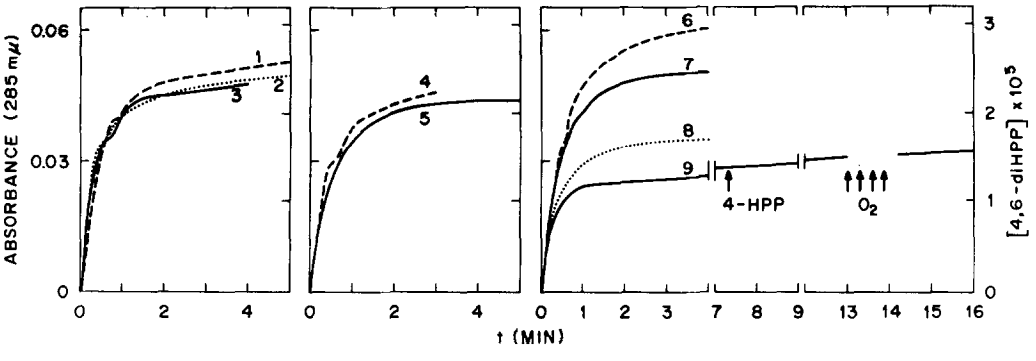

FIG. 2A (left). Effect of substrate concentration on the rate of aerobic oxidation of 4-HPP by xanthine oxidase. Experimental conditions were as in Fig. 1. Initial concentration of 4-HPP: curve 1: 0.083 mM; curve 2: 0.167 mM; curve 3: 0.250 mM. FIG. 2B (center). Effect of product concentration. Experimental conditions were as in Fig. 1. Curve 4: 4-HPP, 83 μM, plus 4,6-diHPP, 83 μM; curve 5: 4-HPP, 83 μM. FIG. 2C (right). Effect of enzyme concentration. Cuvettes contained potassium phosphate buffer, pH 7.8, 0.5 mmole, EDTA, 0.1 μmole, 4-HPP, 0.25 μmole, and enzyme as indicated, in a total volume of 3 ml. Xanthine oxidase concentration: curve 6: 1.2 mμmoles/3 ml; curve 7: 0.9 mμmole/3 ml; curve 8: 0.6 mμmole/3 ml; curve 9: 0.3 mμmole/3 ml. Subsequent additions to curve 9: at 7.3 min, 4-HPP, 0.25 μmole; at 13 min, oxygen bubbled through reaction mixture for 1 min.

molecules of cytochrome c being reduced per molecule of enzyme during the fast phase.

Anaerobic reduction of the flavin spectrum of xanthine oxidase by 4-HPP. In previous studies, it was not possible to demonstrate reduction of the flavin spectrum of xanthine oxidase by 4-HPP under conditions sufficiently anaerobic for reduction to be readily detectable with the physiologic substrate xanthine (3). The results described above suggested an explanation for this difference: since the fast phase of 4-HPP oxidation is transient and self-limited,

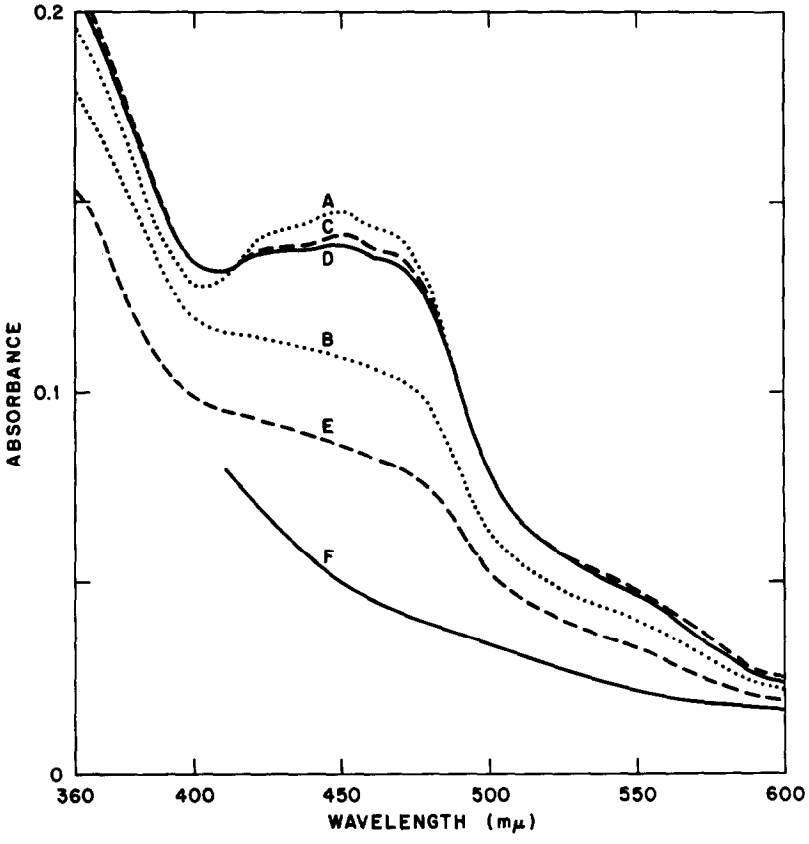

FIG. 3. Anaerobic reduction of FAD of xanthine oxidase by 4-HPP and by xanthine. Cuvettes contained potassium phosphate buffer, pH 7.8, 0.15 mmole, EDTA, 0.1 μmole, and xanthine oxidase, 4.8 mμmoles, in a total volume of 2.3 ml. Absorption spectra were recorded after anaerobic incubation at 25°, as indicated: curve A: no substrate; curve B: 4-HPP, 1 μmole, 15 sec after addition of substrate; curve C: same conditions as B, after reoxidation by atmospheric oxygen, followed by re-deoxygenation; curve D: same conditions as C, after addition of more substrate (4-HPP, 1 μmole); curve E: xanthine, 1 μmole, 15 sec after addition of substrate; curve F: enzyme flavin reduced with sodium dithionite.

the oxidation of this substrate, unlike that of a conventional substrate like xanthine, could terminate before residual traces of oxygen are utilized. These experiments were therefore repeated under conditions more nearly anaerobic than those previously employed, with particular care being taken to minimize oxygen contamination of the nitrogen used in gassing the reaction mixture (see Methods). It was found that, under these conditions, spectrophotometrically detectable reduction of the enzyme flavin could be demonstrated with 4-HPP as substrate (Fig. 3). In none of these experiments, however, was the "immediate" phase of reduction as great with 4-HPP as with xanthine; furthermore, with xanthine, the cycle of reoxidation by air and re-reduction by substrate could be repeated indefinitely, while with 4-HPP, aerobic reoxidation could be demonstrated, but a second reduction of the flavin on redeoxygenation did not occur (Fig. 3).

DISCUSSION. From the experiments described above, it appears on several grounds (the catalytic activity with molecular oxygen and with cytochrome c as electron acceptors, and the substrate-induced flavin reduction) that the initial interaction between xanthine oxidase and the substrate 4-HPP is conventional in type, i.e., that the initial reaction utilizes the complete internal electron transport chain of the enzyme. The subsequent anomalous slowing of the reaction rate is not due to product inhibition in the usual sense; the alternative explanation, therefore, is that the exponential decline in oxygen acceptor activity is dependent on the generation of reduced or otherwise altered forms of the enzyme during the catalytic cycle. Complex formation between substrate and reduced xanthine oxidase has been demonstrated (9), and is thought to account for the marked sensitivity to substrate inhibition which is characteristic of this enzyme (10,11).

It is of interest that, although the iron and flavin components of the 4-HPP-inhibited enzyme can be readily reoxidized by air (Fig. 3), reoxidation of these terminal components of the chain does not result in reversal of the inhibition, as evidenced by the almost complete loss of activity with molec-

ular oxygen and cytochrome c as acceptors, and the inability of substrate to bring about a second reduction of the enzyme flavin. The reducible component of the chain with which 4-HPP interacts must therefore lie proximal to the flavin. The retention of normal substrate activity with artificial acceptors (1-3), would indicate either that electron transfer to the latter can occur through a pathway which bypasses the electron transport chain, or that these acceptors, unlike oxygen, can reoxidize the component of the chain involved in complex formation with 4-HPP.

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