OXIDATION OF 4-HYDROXYPYRAZOLO(3,4-d)PYRIMIDINE BY XANTHINE OXIDASE: THE ROUTE OF ELECTRON TRANSFER FROM SUBSTRATE TO ACCEPTOR DYES

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With molecular oxygen as electron acceptor, the sequence of internal electron transfer in the molybdoflavoprotein xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2) is: substrate-molybdenum-FAD-iron-oxygen (Bray et al., 1964). The participation of enzyme-bound FAD in reactions catalyzed by xanthine oxidase has been demonstrated by electron spin resonance (Bray et al., 1959, 1964) and by direct spectrophotometry (Ball, 1939; Horecker and Heppel, 1949; Morell, 1952; Gutfreund and Sturtevant, 1959; Ackerman and Brill, 1962).

Less is known concerning the pathway of electron transfer to acceptor dyes, although much of the available evidence suggests that the terminal site for electron transfer from enzyme to molecular oxygen and to dyes is identical (Fridovich and Handler, 1958). In a recent communication, however, we reported that, with optimum concentrations of the one-electron acceptor phenazine methosulfate (PMS), the hypoxanthine isomer 4-hydroxypyrazolo(3,4-d)-pyrimidine (4-HPP) was oxidized by xanthine oxidase at a rate approaching 2000-fold that seen with atmospheric oxygen (Johns, 1968). This and related observations indicated that the mode or site of electron transfer from the enzyme to appropriate dyes might differ from the mode or site of electron transfer to oxygen.

The purpose of the present communication is to report that xanthine oxidase-catalyzed electron transfer from 4-HPP to PMS occurs without the spectrophotometrically detectable reduction of the enzyme-bound flavin which

is observed with conventional substrates. It is proposed that the unusually low rate of electron transfer from 4-HPP to oxygen arises as a consequence of this inability of 4-HPP to reduce enzyme-bound FAD; and that the rapid enzyme-mediated electron transfer from 4-HPP to acceptor dyes therefore takes place from a site preceding FAD in the electron transport chain.

Materials and Methods: 4-HPP was provided by Dr. G. B. Elion of Wellcome Laboratories, and 4-aminopyrazolo(3,4-d)pyrimidine (4-APP) by Prof. R. K. Robins, University of Utah. Sources of other reagents were indicated previously (Johns, 1968). Assays were performed at 25° in potassium phosphate buffer, pH 7.8, 0.05 M, containing EDTA, 10^{-5} M. Anaerobic studies were carried out under nitrogen in Thunberg-type cuvettes (Hellma, Codes 190 and 197). A Gilford Multiple Sample Absorbance Recorder was used for assays at fixed wavelengths, and absorption spectra were obtained with a Cary 15 recording spectrophotometer. Commercial bovine milk xanthine oxidase was chromatographed on hydroxylapatite (Fridovich, 1962); fractions with an $A_{280}/A_{450\ m\mu}$ ratio of 5.9 or less were used for direct spectrophotometric observations of enzyme-bound flavin. Details of individual assay methods are given in the legends for figures and tables.

Results: Flavin reduction in resting enzyme. All conventional substrates for xanthine oxidase, when incubated with the enzyme under anaerobic conditions, bring about spectrophotometrically detectable reduction of enzyme-bound FAD (Gilbert, 1964). However, the enzyme flavin of the xanthine oxidase preparations used in the present studies, although showing the typical "immediate" or "rapid phase" reduction on addition of xanthine (Morell, 1952), did not show spectrophotometrically detectable reduction even on prolonged incubation with the substrate 4-HPP. In a typical experiment (Fig. 1A), no reduction of enzyme-bound FAD was noted when xanthine oxidase was incubated with 4-HPP for 17 hrs at 25°. In a control experiment, incubation with xanthine resulted in "immediate" reduction of 21% of the enzyme-bound flavin, and 92% reduction within 90 min. 4-APP, a pyrazolo(3,4-d)pyrimidine substrate for xanthine oxidase which, unlike 4-HPP, can utilize molecular oxygen as electron acceptor

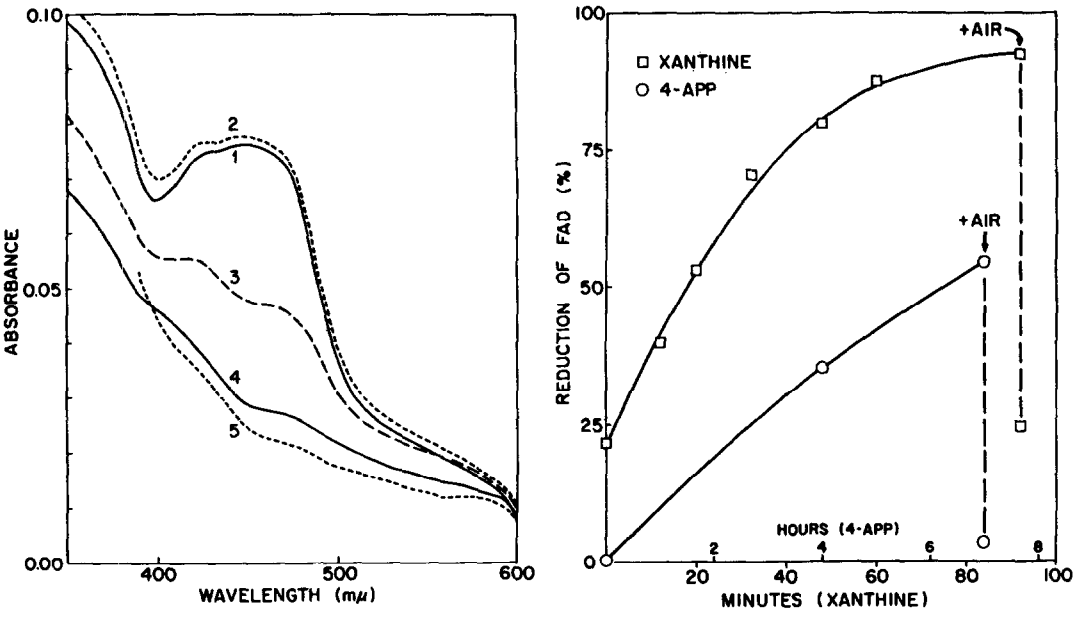
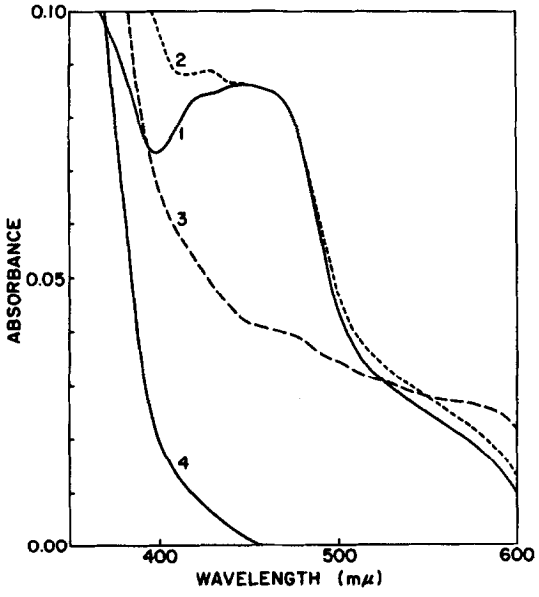


FIG. 1A (left). Anaerobic reduction of FAD of xanthine oxidase. Cuvettes contained potassium phosphate buffer, pH 7.8, 150 μmoles, EDTA, 0.03 μmoles and xanthine oxidase, 1.4 mg, in a total volume of 3 ml. Absorption spectra were recorded after anaerobic incubation at 25° as indicated. Curve 1: no substrate. Curve 2: 4-HPP, 0.50 μmoles, 17 hr. Curve 3: 4-APP, 0.50 μmoles, 7 hr. Curve 4: xanthine, 0.50 μmoles, 90 min. Curve 5: enzyme flavin reduced with sodium dithionite.
FIG. 1B (right). Time-course of anaerobic reduction of xanthine oxidase by substrate. Experimental conditions were as in Fig. 1A. Flavin reduction was followed at 450 mμ. Ordinate: flavin reduction as percentage of the total reduction observed with sodium dithionite. Note difference in time-scales for xanthine and 4-APP. No reduction was noted with 4-HPP.

(Feigelson et al., 1957), brought about 43% reduction of enzyme flavin over a period of 7 hr. Enzyme flavin bleached by anaerobic incubation with xanthine or with 4-APP was immediately reoxidized on exposure of the solutions to atmospheric oxygen (Fig. 1B).

Flavin reduction in actively cycling enzyme. In view of these results with resting enzyme, it was of interest to determine whether $FADH_2$ formation could be detected after several cycles of enzyme-mediated electron transfer from 4-HPP to an acceptor. The flavin spectrum of xanthine oxidase was recorded, and PMS and substrate (4-HPP or xanthine) were then added. Immediately after



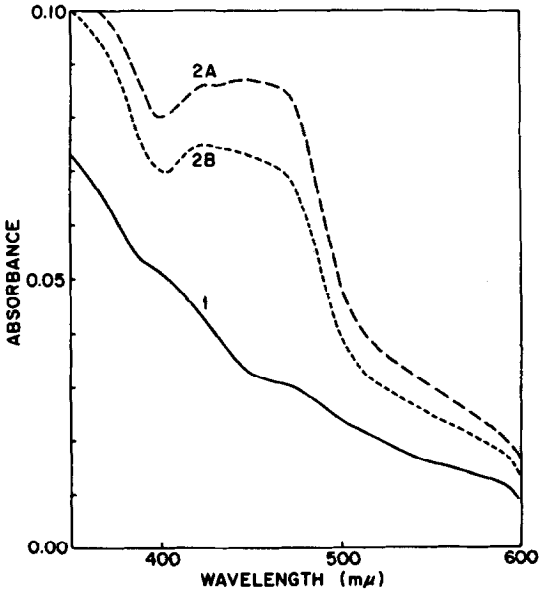


FIG. 2A (left). Flavin spectrum of xanthine oxidase after enzyme-catalyzed reduction of PMS. Cuvettes contained potassium phosphate buffer, pH 7.8, 150 μmoles, EDTA, 0.03 μmoles and xanthine oxidase, 1.4 mg, in a total volume of 3 ml. The spectrum of enzyme flavin was recorded (Curve 1); substrate, 0.5 μmoles, and PMS, 0.1 μmoles, were then added under anaerobic conditions. The enzyme spectrum was recorded again after decolorization of the PMS. Curve 2: enzyme spectrum 1 hr after addition of 4-HPP and PMS. Curve 3: enzyme spectrum 30 min after addition of xanthine and PMS. Curve 4: $PMSH_2$, 0.1 μmoles/3 ml, prepared by chemical reduction of PMS with sodium dithionite.
FIG. 2B (right). Effect of 4-HPP on reoxidation of FAD of xanthine oxidase. Enzyme flavin was reduced by anaerobic incubation with the substrate xanthine, 0.5 μmoles (Curve 1). The reduced enzyme was then reoxidized by exposure to atmospheric oxygen. Curve 2A: spectrum of enzyme to which 0.5 μmoles 4-HPP was added anaerobically before reoxidation. Curve 2B: reoxidized enzyme without added 4-HPP.

reduction of the PMS and at 5 min intervals thereafter, the absorption of the enzyme flavin was determined. With xanthine as substrate, the initial value after reduction of the PMS indicated 42% reduction of the enzyme flavin, proceeding to almost complete (> 90%) reduction in 30 min (Fig. 2A). 4-HPP completely reduced the PMS in less than 60 sec, but no reduction of the enzyme-bound flavin was detectable over an observation period of 1 hr.

Relative rates of oxidation of xanthine and pyrazolo(3,4-d)pyrimidines with PMS as electron acceptor. As the foregoing experiments indicate, 4-HPP, although unable to bring about detectable reduction of xanthine oxidase-bound

flavin, is a readily oxidized substrate for the enzyme when PMS is used as electron acceptor. With PMS at a concentration of 1.3×10^{-4} M, the ratio of the rates of anaerobic oxidation of the three substrates studied, xanthine, 4-HPP and 4-APP, 1.6×10^{-4} M, was 100 : 22 : 0.6. Using an alternate method, with PMS as "carrier" to cytochrome c, 8.2×10^{-5} M (Muraoka et al., 1965), the ratio of the three rates was xanthine : 4-HPP : 4-APP :: 100 : 111 : 13. It is evident therefore that the anaerobic oxidation of 4-HPP is sufficiently rapid that reduction of the flavin of the resting enzyme would not be overlooked if there were a positive correlation between the latter and reaction rate.

Effect of 4-HPP on reoxidation of $FADH_2$. It was previously suggested (Johns, 1968) that the anomalous dye/oxygen ratio of 4-HPP could arise from affinity of 4-HPP for a secondary (inhibitory) substrate-binding site at or near the site of electron transfer from enzyme to molecular oxygen. Reduction by substrate of enzyme-bound flavin could then take place, with only the final transfer to oxygen being inhibited. It is now possible to exclude this explanation, since 4-HPP does not prevent reoxidation by air of reduced enzyme-bound flavin (Fig. 2B).

Discussion: The foregoing experiments indicate that xanthine oxidase-catalyzed reduction of PMS by the substrate 4-HPP takes place without the intermediation of enzyme-bound dihydroFAD. They also render unlikely the possibility that a partially reduced semiquinone or free radical form of the flavin participates in the transfer. In the case of xanthine oxidase, the formation of such free radical flavin intermediates, unlike the formation of dihydroFAD, is not accompanied by consistent and well-defined spectral changes (Ackerman and Brill, 1962). However, since the semiquinone and dihydro forms of xanthine oxidase are interconvertible and the latter accumulates on incubation of the enzyme with excess substrate (Bray et al., 1964), 4-HPP, under the conditions of the present studies, would give rise to detectable dihydroFAD if the FAD semiquinone were formed in amounts sufficient to account for the rapid rates observed.

Although the evidence argues against participation of FAD in the xanthine oxidase-catalyzed reduction of PMS by 4-HPP, it does not necessarily follow that 4-HPP bypasses the electron transport chain entirely. A likely alternative is that PMS bound to enzyme, perhaps in the form of a molybdenum-PMS complex, acts as the acceptor species in place of the molybdenum-FAD complex of the conventional chain.

These studies demonstrate that the anomalous dye/oxygen ratio previously reported for the xanthine oxidase-catalyzed oxidation of 4-HPP is associated with inability of the latter substrate to reduce the enzyme-bound flavin. It is suggested therefore that electron transfer from xanthine oxidase to dyes, unlike electron transfer to molecular oxygen, can occur from a site preceding FAD in the internal electron transport chain of the enzyme.

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