ANOMALOUS DIFFERENTIAL EFFECTS OF 4-HYDROXYPYRAZOLO(3,4-d)-PYRIMIDINE ON ELECTRON TRANSFER FROM XANTHINE OXIDASE TO MOLECULAR OXYGEN AND TO ACCEPTOR DYES

David G. Johns

Department of Pharmacology, Yale University, New Haven, Conn.

Received March 12, 1968

The internal electron transport chain of the flavoprotein xanthine oxidase (xanthine:O_2 oxidoreductase, EC 1.2.3.2) is complex, with at least three sites of electron egress being distinguishable (Fridovich and Handler, 1962). Available evidence suggests, however, that the terminal site for electron transfer from enzyme to molecular oxygen and to dyes is identical. Fridovich and Handler (1958) examined the possibility that phenazine methosulfate (PMS), a dye with a relatively stable semiquininoid state, and a consequent ability to act as a one-electron acceptor (Singer et al., 1957), might utilize a site of electron egress preceding that for conventional two-electron acceptors. An increase over the aerobic rate of oxidation of xanthine was, in fact, noted; the increase was slight, however (2.1-fold, at optimum PMS concentration) and, in other respects, PMS behaved as a conventional two-electron acceptor for xanthine oxidase. The result was attributed, therefore, not to an additional site of electron egress, but to more rapid reoxidation of the terminal step of the electron transport chain by PMS than by atmospheric oxygen. The purpose of the present communication is to report that, with PMS at optimum concentration as electron acceptor, the hypoxanthine isomer 4-hydroxypyrazolo-(3,4-d)pyrimidine (4-HPP) is oxidized by bovine xanthine oxidase at a rate approaching 2000-fold that seen with atmospheric oxygen as electron acceptor. This result, together with additional evidence presented below, supports the alternative hypothesis of the earlier authors, that electron egress to appro-

priate acceptor dyes precedes or otherwise differs significantly from that to molecular oxygen; and, furthermore, localizes the site of action of 4-HPP as a xanthine oxidase inhibitor, previously undefined, to the site of electron transfer from the enzyme to molecular oxygen.

Materials and Methods: Commercial bovine xanthine oxidase (Worthington, Code XO) was chromatographed on hydroxylapatite (Fridovich, 1962). The specific activity of the fractions used in the present experiments was 6.2 (as defined by the latter author) and the $A_{280}/A_{450\ m\mu}$ ratio was 6.0. Preliminary chromatographic purification was not essential, similar results being obtained with commercial xanthine oxidase preparations subjected only to dialysis before use (Worthington, Code XO; Boehringer, Code XOD 15347). Enzyme assays were carried out at 25°, using a Gilford Multiple Sample Absorbance Recorder; ultraviolet absorption spectra were obtained with a Cary 15 recording spectrophotometer. All assays were carried out in potassium phosphate buffer, pH 7.8, 0.05 M, containing EDTA, 10^{-5} M. Cytochrome c, Type III, was obtained from the Sigma Chemical Co. 4-HPP was provided by Dr. G. B. Elion of Wellcome Laboratories, and 4,6-dihydroxypyrazolo(3,4-d)pyrimidine (4,6-diHPP) by Prof. R. K. Robins, University of Utah.

Results and Discussion: In a typical experiment (Fig. 1), the addition of PMS at a "catalytic" or sub-stoichiometric level (0.04 µmoles/3 ml), resulted in an 80-fold increase in the rate of aerobic oxidation of 4-HPP (0.25 µmoles/3 ml) by xanthine oxidase. The identity of the reaction product as 4,6-diHPP was confirmed by cellulose thin-layer chromatography of the deproteinized reaction mixture, using the systems butanol:acetic acid:water::58:24:18, and butanol:water::86:14 with ammonia in the vapor phase. No oxidation of 4-HPP by PMS or by other dyes was observed in the absence of xanthine oxidase. The aerobic recording of serial spectra in the presence of PMS was made possible by a property of this dye previously utilized in a spectrophotometric assay method of Fridovich and Handler (1958): reduced PMS is reoxidized by atmospheric oxygen at a rate such that reduced dye does not accumulate in a concen-

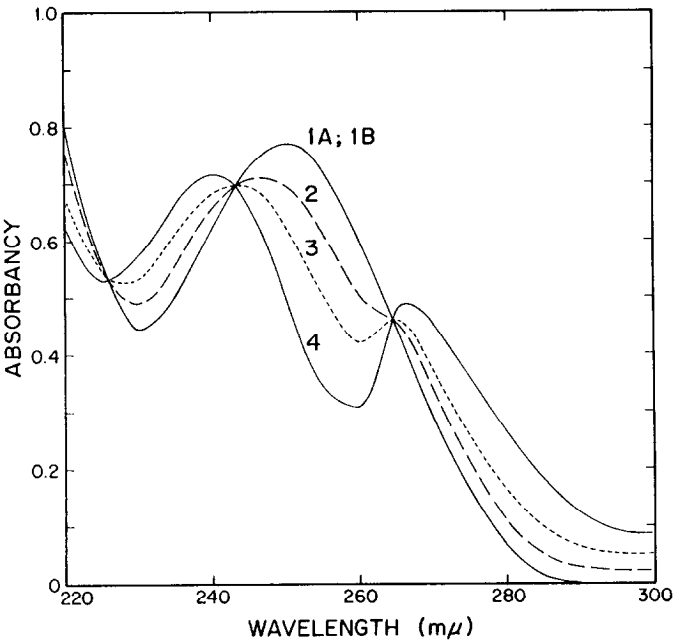

FIG. 1. Stimulation by PMS of the rate of enzymic oxidation of 4-HPP. The reference cuvette contained potassium phosphate buffer, pH 7.8, 150 µmoles, EDTA, 0.04 µmoles, and catalase, 25 units, in a total volume of 3 ml. The sample cuvette contained the same constituents plus 4-HPP, 0.25 µmoles. At 0 min, xanthine oxidase, 0.5 units, was added to both cuvettes and spectrum 1A recorded. After aerobic incubation for 50 min at 25°, spectrum 1B was recorded. A perceptible difference, too slight for illustration, was noted between spectra 1A and 1B; the maximum change was a decrease in absorbancy of 0.009 OD units at 255 mµ. PMS, 0.04 µmoles, was then added to both cuvettes, and spectra recorded at 10 min (2), 20 min (3), and 45 min (4). No further spectral change was detectable; spectrum 4 is identical with that of 4,6-di-HPP at pH 7.8. No reaction was observed when xanthine oxidase was omitted from the complete system. Catalase, although present in this experiment, was not an essential component of the reaction mixture.

---

tration sufficient to contribute to the absorption spectrum of the product. Qualitatively identical results were obtained with catalytic amounts of 2,6-dichlorophenolindophenol (DCIP); with higher concentrations of the latter, however, sufficient reduced dye accumulated in the reaction cuvette to contribute significantly to the total absorption spectrum.

Considerable difference was noted in the relative effectiveness of PMS and DCIP as electron acceptors for the enzymic oxidation of 4-HPP. With PMS (10^{-3} M), the maximal rate observed was 1720-fold that with atmospheric oxygen alone, and only slight inhibition was noted at even higher concentrations

of the dye. With DCIP, the maximal rate was only 120-fold that with atmospheric oxygen, and marked inhibition was noted at dye concentrations above 2×10^{-4} M. Cytochrome c possessed no detectable activity as an electron acceptor in this system, although, as noted below, it was fully active as an acceptor when xanthine was used as substrate.

With physiologic substrates for xanthine oxidase, only minor differences in rate have been reported with oxygen and with dyes, when conditions were otherwise comparable. As noted above, Fridovich and Handler noted a slight increase in the rate of aerobic oxidation of xanthine with PMS. Mackler et al. (1954) found the rates of oxidation of xanthine with oxygen and with DCIP to be approximately equal; while Avis et al. (1956) reported the aerobic DCIP rate to be about half that with oxygen. The enzyme preparations used in the present studies gave almost identical rates of xanthine oxidation with atmospheric oxygen and with DCIP. 4-HPP, however, in addition to being a substrate for xanthine oxidase, is a potent inhibitor of the enzyme in the usual aerobic assay systems (Elion, 1966; Hitchings, 1966); thus, a likely interpretation of the anomalous dye:oxygen ratios for 4-HPP described above is specific inhibition by this substrate of electron transfer from xanthine oxidase to molecular oxygen. Previous authors (Hofstee, 1955; Fridovich and Handler, 1958) in studying the unusual susceptibility of xanthine oxidase to substrate inhibition, have presented kinetic evidence that this enzyme possesses a secondary (inhibitory) substrate-binding site late in the electron transport chain. Since 4-HPP is itself a substrate, and is, furthermore, an isomer of one of the natural substrates for the enzyme, an affinity of 4-HPP for this secondary site would not be unexpected, and could account for the results presented above.

If this interpretation is correct, 4-HPP should lose much of its activity as a xanthine oxidase inhibitor when PMS or DCIP, rather than oxygen, are used as electron acceptors, since the data above indicate that electron transfer to these dyes precedes, or in some other fundamental way differs from,

electron transfer to oxygen; and since 4-HPP appears to inhibit principally the latter process. To test this assumption, the activity of 4-HPP as an inhibitor of the enzymic oxidation of xanthine was determined, with oxygen, PMS, DCIP, and cytochrome c as electron acceptors (Table I): it was indeed found

TABLE I. Effect of electron acceptor on activity of 4-HPP as an inhibitor of the enzymic conversion of xanthine to uric acid by xanthine oxidase

Electron acceptor	Concentration of 4-HPP required to reduce the rate of oxidation of xanthine, 3.3×10^{-4} M, to 50% of the control rate
Oxygen, 2.4×10^{-4} M	7.0×10^{-7} M
Cytochrome c, 8.0×10^{-5} M	5.6×10^{-7} M
DCIP, 8.0×10^{-5} M	1.1×10^{-4} M
PMS, 8.0×10^{-5} M	5.0×10^{-4} M

Control cuvettes contained potassium phosphate buffer, pH 7.8, 150 µmoles, EDTA, 0.04 µmoles, xanthine, 1 µmole, xanthine oxidase, 0.05 units, catalase, 25 units, and electron acceptor at the concentration noted; the concentration indicated for oxygen is that of dissolved oxygen in an aqueous solution in equilibrium with air. Total volume was 3 ml. Xanthine oxidation was monitored directly at 290 mµ, except in the assays with cytochrome c as electron acceptor, where the rate of formation of reduced cytochrome c was followed at 550 mµ; under the reaction conditions used here, the reduction of cytochrome c proceeds at about one-fifth the total rate of oxidation of xanthine (Fridovich, 1962). All assays were carried out under aerobic conditions. In the assays with DCIP, reduced dye accumulated in the reaction cuvettes; the absorption spectra of oxidized and reduced DCIP were determined separately, however, and the difference at 290 mµ was found to be sufficiently small to disregard ($\epsilon_{290\ m\mu}$, oxidized DCIP: 9560; $\epsilon_{290 m\mu}$, DCIP reduced with sodium dithionite: 8630). Experimental cuvettes contained the same components, plus 4-HPP. Buffer, EDTA, xanthine oxidase, catalase, and, in the experimental cuvettes, 4-HPP, were pre-incubated for 3 min at 25°; the reaction was then started by the addition of xanthine and electron acceptor. Initial rates were used for all calculations.

that far higher concentrations of 4-HPP (as great as 900-fold) were required to produce equivalent inhibition of electron transfer to the dyes. Cytochrome c behaved in this system as did oxygen, showing extreme sensitivity to inhibition by 4-HPP; this latter result is compatible with the observation (Horecker and Heppel, 1949; Fridovich and Handler, 1962) that electron trans-

fer from xanthine oxidase to cytochrome c requires the participation of molecular oxygen. Parallel experiments with other xanthine oxidase inhibitors (2,4-diamino-6-hydroxy-s-triazine (ammeline) (Fridovich, 1965), 2-amino-4-hydroxypteridine-6-carboxaldehyde, cyanide, and p-hydroxymercuribenzoate) failed to show comparable differential effects with oxygen and with acceptor dyes.

The experiments reported here indicate that 4-HPP, with its anomalous and possibly unique properties both as a substrate and inhibitor of xanthine oxidase, is a potentially useful compound for further elucidation of the internal electron transport sequence of this enzyme.

Acknowledgements: This work was supported by Grants CA-02817 and CA-08010 of the U. S. Public Health Service. The technical assistance of Mrs. Lucilla Yang is gratefully acknowledged.

REFERENCES

Avis, P. G., Bergel, F. and Bray, R. C., J. Chem. Soc., 1219 (1956).
Elion, G. B., Ann. Rheum. Dis., 25, 608 (1966).
Fridovich, I., J. Biol. Chem., 237, 584 (1962).
Fridovich, I., Biochemistry, 4, 1098 (1965).
Fridovich, I. and Handler, P., J. Biol. Chem., 233, 1581 (1958).
Fridovich, I. and Handler, P., J. Biol. Chem., 237, 916 (1962).
Hitchings, G. H., Ann. Rheum. Dis., 25, 601 (1966).
Hofstee, B. H. J., J. Biol. Chem., 216, 235 (1955).
Horecker, B. L. and Heppel, L. A., J. Biol. Chem., 178, 683 (1949).
Mackler, B., Mahler, H. R. and Green, D. E., J. Biol. Chem., 210, 149 (1954).
Singer, T. P., Kearney, E. B. and Massey, V., Adv. in Enzymol., 18, 65 (1957).