The Resolution of Active and Inactive Xanthine Oxidase by Affinity Chromatography*

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SUMMARY

The resolution of fully functional xanthine oxidase from nonfunctional enzyme by the technique of affinity chromatography is described. Both forms of the enzyme possess the full complement of the oxidation-reduction components molybdenum, FAD, iron, and acid-labile sulfur. Nonfunctional xanthine oxidase does not possess the cyanolyzable persulfide group and can be partially activated by incubation with sodium sulfide. The presence of the active center persulfide is essential for catalytic activity with xanthine as substrate but is not required for DPNH-ferricyanide reductase activity. It is also required for the following properties of xanthine oxidase: (a) rapid bleaching of the oxidation-reduction chromophores of the enzyme by xanthine or by sodium borohydride, (b) development of the "rapid" molybdenum electron paramagnetic resonance signals by xanthine or NaBH₄, (c) rapid development of the electron paramagnetic resonance signals of the reduced iron sulfur chromophores by xanthine or NaBH₄, (d) interaction with the inhibitors arsenite and cyanide, (e) development of the molybdenum electron paramagnetic resonance signals associated with inactivation of the enzyme by formaldehyde.

A reaction mechanism for xanthine oxidase involving the persulfide linkage is proposed.

EXPERIMENTAL PROCEDURE

General Procedures

Milk xanthine oxidase was isolated as described previously (7). Xanthine-oxygen reductase activity was measured spectrophotometrically at 295 nm at 25° and expressed as AFR₂₅° values (10). Enzyme concentrations were estimated spectrophotometrically with an E₄₂₀ = 37,800 cm⁻¹ M⁻¹ per molecule of enzyme-bound FAD (7). Molybdenum was determined qualitatively with the dithiol method of Ringley (11) and quantitated as the o-phenanthroline chelate after trichloroacetic acid denaturation of the enzyme (12). Labile sulfide was determined by a modification of the method of Fogo and Popowsky (13). Active xanthine oxidase is rapidly reduced by substrate while inactive enzyme is only slowly reduced.

It would be of obvious advantage to have a method for separation of functional from nonfunctional enzyme so as to delineate their respective chemical, physical, and catalytic properties. A comparative study of the two forms would be expected to give information concerning the factor responsible for loss of functionality. Previous data show a stoichiometric complement of oxidation-reduction groups for enzyme preparations containing 30% of the inactive form (7), indicating the presence of another component essential for activity. Recent work in this laboratory (8) has shown that inactivation of xanthine oxidase by cyanide is due to cyanolysis of a persulfide group required for catalysis. The possibility was considered that lability of this persulfide might be responsible for nonfunctional enzyme.

The present work provides further evidence for this hypothesis. In addition, the isolation of >95% functional enzyme is described. The resolution of functional from nonfunctional enzyme was achieved by taking advantage of the strong complex-forming of active enzyme with the pyrazolo(3,4-d)pyrimidines (4, 5, 9). This property was utilized with a derivatized Sepharose gel, resolution thus being achieved by affinity column chromatography.

Preparation of Allopurinol Analog Used in Sepharose Derivatization

3-(1-H-Pyrazolo(3,4-d)pyrimidin-4-ylamino)-1-propanol (I)—The compound was prepared by an adaptation of the procedure of Robins (14). N,N-Dimethylaniline (60 ml, 0.48 mole) was

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* This work was supported by Grants GM11106 and GM12176 from the United States Public Health Service.
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added to a suspension of allopurinol (20 g, 0.147 mole) in 600 ml of phosphoric oxychloride. The reaction mixture was refluxed on a heating mantle for 14 hours, producing a clear, orange-colored solution. Most of the solvent was removed in vacuo, and the resulting black syrup was poured onto crushed ice, stirred well, and extracted with diethyl ether (4 × 400 ml). The ethereal phase was washed once with water, dried over sodium sulfate, and filtered. The slightly yellow solution was then refluxed in volume to about 200 ml. The resulting precipitate was dissolved in 150 ml of absolute ethanol, and 3-aminopropanol (12 ml, 0.16 mole) was added. The reaction mixture was warmed on the steam bath until the remaining ether had evaporated, and the ethanolic solution was then refluxed for 1½ hours. The ethanol was removed in vacuo, producing a thick yellow slurry, which was diluted with 25 ml of water and stored at 0° for 2 days. The resulting solid was collected, dissolved in 100 ml of hot water, treated with charcoal, filtered, cooled, and the resulting off-white precipitate (11.48 g, 40%) collected, m.p. 213-215°. \( \Delta_{\text{max}}^{50} = 277 (9400) \)

\[ \text{C}_{12}\text{H}_{14}\text{N}_{2} \text{O} \]
Calculated: C 59.99, H 6.41, N 19.08
Found: C 60.12, H 6.46, N 19.01

S-(1-N-Pyrazolo(3,4-d)pyrimidin-4-ylamino)-1-propyl-N-benzyloxycarbonyl-L-arginine (II)—A solution of Compound I (1.2 g, 6.2 mmoles) in 4 ml of dimethylformamide was treated with N,N'-carbonyldiimidazole (1.01 g, 6.2 mmoles), producing vigorous gas evolution. When this ceased, N-benzyloxycarbonyl-L-arginine (15) (1.66 g, 6.26 mmoles) and a catalytic amount (0.05 g) of sodium imidazolate were added, and dissolved by gently warming. After stirring for 20 hours at room temperature, the solution was diluted with 100 ml of ethyl acetate, then washed with 5% sodium bicarbonate (2 × 100 ml) and water (100 ml). The organic phase was dried over magnesium sulfate and the solvent removed in vacuo, producing an oil. This was chromatographed on silica gel and eluted with chloroform-methanol, 20:1. The resulting product was crystallized from ethyl acetate-hexane, (1.13 g, 42%), m.p. 87-89°.

C\(_{12}\)H\(_{14}\)NO\(_{4}\).O.0.5H\(_2\)O
Calculated: C 53.32, H 7.35, N 26.65
Found: C 53.01, H 7.43, N 26.53

Preparation of Activated Sepharose

A solution of cyanogen bromide (5.0 g, 0.047 mole) in 50 ml of water was added to a rapidly stirred suspension of 50 ml of Sepharose 6B in 50 ml of water. The pH of the solution was maintained at 11 by addition of 4 N sodium hydroxide. After 3 min no further change was observed in the pH, and the reaction mixture was diluted with ice, filtered on a coarse sintered glass filter, and washed with cold sodium bicarbonate buffer, pH 9.0 (3 × 200 ml). A solution of compound III (60 mg, 0.20 mmole) in 50 ml of cold buffer was added at once, with stirring, and the suspension was transferred to a beaker and stirred at 4° overnight. The activated Sepharose was collected and washed with water. About 30 mg of Compound III were attached to the Sepharose in this manner as estimated from the ultraviolet

The sequence of reactions involved in the synthesis of Compound III is summarized below.

![Diagram](https://example.com/diagram.png)

**FIG. 1.** Elution profile of xanthine oxidase on affinity column. \( \Delta, A_{450}; \bullet, \text{AFR}^{50} \) value. Peak I was eluted after a 16-hour incubation in the column in 0.01 M Na\(_2\)SO\(_4\), 0.1 M PP\(_i\), pH 8.5. Peak II was eluted 24 hours after washing the column with O\(_2\)-saturated 0.1 M PP\(_i\), pH 8.5, containing 1 mM salicylate and 2 × 10\(^{-4}\) M EDTA. Peak III was eluted 72 hours after the elution of Peak II. For fuller details see text.
RESULTS

Isolation of Low and High Activity Xanthine Oxidase by Affinity Chromatography—The 4-substituted pyrazolo(3,4-d)pyrimidine attached to the Sepharose was converted to its 6-hydroxy derivative by incubation at room temperature with xanthine oxidase in 0.1 M PPi, pH 8.5. The conversion was assumed complete when a small sample of gel would no longer reduce an anaerobic sample of enzyme.

Anaerobiosis was established in the derivatized Sepharose column (1 × 15 cm) by equilibration under nitrogen with 0.01 M Na2S2O4 in 0.1 M PPi, pH 8.5. Approximately 0.6 μmole (expressed as E·FAD) of salicylate-free xanthine oxidase in a volume of 2 ml was reduced with a few crystals of dithionite, applied to and slowly washed into the column. (Preliminary results indicated interference by salicylate with the complex-forming properties of the column.) The flow was stopped and the charged column was equilibrated overnight at room temperature to allow sufficient time for complex formation with active enzyme. Low activity xanthine oxidase was then eluted with the dithionite-containing buffer (Fig. 1).

The column was then washed aerobically with cold 0.1 M PPi, pH 8.5, containing 1 mM salicylate and 0.2 mM EDTA. A great enhancement of color intensity of the reddish brown band of xanthine oxidase became apparent as the iron and flavin chromophores reoxidized. Flow was stopped and the column allowed to stand in the cold room for 3 to 4 days to permit the slow reoxidation of reduced enzyme-bound molybdenum and the accompanying release of xanthine oxidase from the gel (4, 5). Elution of the column with the same buffer results in a reddish brown band with very high activity. This activity is stable for several weeks in the presence of salicylate but is significantly less stable in its absence. Fractions with similar specific activities were pooled and concentrated by ammonium sulfate precipitation (0.35 g per ml). This step had no effect on the specific activity.

Spectral Properties—As shown in Fig. 2, the absorption spectrum of low AFR250 xanthine oxidase is quite similar to that of the high activity enzyme in the visible spectral region. Differences are apparent in the near-ultraviolet region with a maximum at 320 nm. The visible absorption spectra indicate identical iron and flavin compositions for both forms. Analysis showed 1 mole of molybdenum and 4 moles of iron and acid-labile sulfur per mole of FAD for both low and high AFR enzyme fractions. These
results indicate that the difference in activity is not due to a deficiency in any of these oxidation-reduction components.

**Interaction of Inhibitors with Xanthine Oxidase Fractions of Differing Specific Activities**

Recent work by Coughlan et al. (16) investigated the mode of inhibition of xanthine oxidase (and other related enzymes) by cyanide, arsenite, and methanol. Their results were interpreted as indicating the molybdenum atom as the common site for inhibition of enzymic activity. Previous work in this laboratory (8) showed that the liberation of SCN⁻ as well as the extinction change at 320 nm produced by cyanide treatment was proportional to the AFR²⁵⁰ of different enzyme preparations. This proportionality has also been found upon cyanide treatment of low and high AFR²⁵⁰ enzymes isolated from the affinity column (Fig. 3). These data indicate that the catalytically essential persulfide group (8) is not present in inactive enzyme.

The extent of extinction change at 380 nm upon arsenite treatment is also proportional to xanthine oxidase activity (Fig. 4). In agreement with the results of Coughlan et al. (16), arsenite treatment of cyanide-treated enzyme produced no spectral change. Similarly, arsenite inactivation of nonfunctional enzyme resulted in insignificant spectral changes. These data, coupled with the fact that arsenite interferes with cyanide inactivation of the enzyme, indicate the persulfide group to be one of the ligands in the arsenite-xanthine oxidase complex.

Recent work by Pick et al. (17) has shown that formaldehyde treatment of xanthine oxidase gives rise to inhibition of enzymic activity and to the appearance of an air-insensitive molybdenum electron paramagnetic resonance signal similar to that found previously on inactivation with methanol (18). The data in Fig. 4 show the intensity of this inhibited signal to be proportional to AFR²⁵⁰, indicating that only active enzyme is capable of reacting with formaldehyde to give the inhibited molybdenum electron paramagnetic resonance signal.

**Borohydride as Specific Reductant of Functional Enzyme**—Addition of substrate to an anaerobic solution of xanthine oxidase, produces an initial rapid phase of bleaching followed by a slow, secondary phase (7). If the secondary bleaching is due to reduction of inactive enzyme by reduced active enzyme (1), the degree of initial bleaching by substrate should be greater with enzyme of increasing specific activity. In agreement with previous results (4-6), Fig. 5 shows a linear dependence of the extent of substrate bleaching with increasing specific activity. High AFR²⁵⁰ enzyme (204 to 205) gave less than 5% secondary bleaching when incubated with an excess of xanthine under anaerobic conditions, supporting the previous conclusion that fully functional enzyme should have an AFR²⁵⁰ value of about 210.

**Fig. 5.** The correlation with AFR²⁵⁰ values of extinction change at 450 nm of the rapid reduction of xanthine oxidase with xanthine, sodium borohydride, and dithionite. Experiments were carried out anaerobically in 0.1 M PPi, pH 8.5, at 25° with xanthine (●) and at 7° with NaBH₄ (○) to minimize frothing. The rapid changes were measured within 1 min of addition of xanthine or NaBH₄. The extinction change with dithionite (Δ) was measured by adding a few crystals of dithionite to an aerobic solution of enzyme. The total extent of bleaching with xanthine (fast plus slow phases) was found to equivalent to a -Δε of 24,000 to 25,000 m⁻¹ cm⁻¹ for all values of AFR²⁵⁰.

**Fig. 6.** The intensities of the g = 2.11 and g = 2.02 iron electron paramagnetic resonance signals upon reduction of differing AFR²⁵⁰ xanthine oxidase preparations with either xanthine or NaBH₄. Xanthine; g = 2.11 (●), g = 2.02 (△). NaBH₄; g = 2.11 (●), g = 2.02 (○). Enzyme samples (~1 × 10⁻⁴ M E'FAD) were incubated anaerobically with substrate for 2 min before freezing. Electron paramagnetic resonance settings: field modulation, 6 gauss at 100 kHz; microwave frequency, 9.21 GHz; microwave power, 10 mwatts; temperature, 21°K. The intensities are expressed as the percentage of that obtained after adding a few crystals of dithionite to the same sample. A trace amount of octyl alcohol was added to the borohydride samples to reduce frothing.
The extent of bleaching of xanthine oxidase absorbance by dithionite was identical for enzyme solutions of different specific activities. However, when sodium borohydride was used as reductant, the extent of reduction was dependent on the specific activity. As shown in Fig. 5, the extent of borohydride bleaching was identical with that produced by xanthine, thereby indicating that borohydride can reduce only functional enzyme. Borohydride did not reduce cyanide-treated xanthine oxidase and did not affect the AFR250 of active enzyme.

In a companion series of experiments, the extent of non-heme iron reduction by either xanthine or borohydride was determined by electron paramagnetic resonance spectroscopy. The intensities of the g = 2.11 and 2.02 signals (produced by either reductant) relative to the signal obtained with dithionite increased linearly with increasing AFR250 values (Fig. 6).

Recently, McGartoll et al. (6) have reported the linear increase in the intensities of the rapid molybdenum electron paramagnetic resonance signals and the decrease in intensities of the slow molybdenum signals with increasing enzymic activity. The data in Fig. 7 substantiate their observations and emphasize again the similar behavior of xanthine and borohydride as reductants.

Lack of Requirement of Persulfide Group in Reactions with NADH—Deflavo xanthine oxidase is devoid of NADH-Fe(CN)6 reductase activity (19), thereby indicating the FAD as the acceptor of reducing equivalents from NADH. Therefore if the lesion causing inactivation of native enzyme was associated with the molybdenum site, one would expect the same diaphorase activity for both high and low AFR250 forms of xanthine oxidase. This was found to be the case; no difference in NADH-ferri-cyanide reductase activity was found, no matter the AFR250 value. In agreement with earlier reports (16, 19), cyanide treatment had no effect on diaphorase activity.

 Interaction of Pyrazolo(3,4-d)pyrimidines with Differing AFR Enzymes—Previous work has shown that alloxanthine forms a strong complex with reduced xanthine oxidase (4, 5). The fractional molar ratio of alloxanthine to active site needed for complete inhibition indicated that only functional enzyme could bind alloxanthine. The mode of inhibition was shown to be a tight complex between alloxanthine and MoIV since two oxidizing equivalents were required to reconstitute catalytic activity. The linear dependence of alloxanthine to FAD stoichiometry for complete inhibition of enzyme preparations of differing AFR250

![Fig. 8. Stoichiometry of alloxanthine inactivation of xanthine oxidase preparations of differing AFR250 values. The values on the abscissa are the amounts of alloxanthine needed to completely inhibit xanthine-oxygen reductase activity. End points were determined by incubation of various alloxanthine to enzyme ratios anaerobically in the presence of 3.33 × 10^-4 M xanthine for 60 min at 25° in 0.1 M PPi, pH 8.5.](image)

![Fig. 9. Difference spectral properties of the reduced xanthine oxidase-3,4-dimercaptoypyrazolo(3,4-d)pyrimidine complex minus reduced enzyme. Xanthine oxidase preparation of differing specific activities were reduced anaerobically with dithionite, then mixed with 1 mM 4,6-dimercaptoypyrazolo(3,4-d)pyrimidine at room temperature in 0.1 M PPi, pH 8.5. Left figure, Curve 1, xanthine oxidase, AFR250 = 52, immediately after addition of 4,6-dimercaptoypyrazolo(3,4-d)pyrimidine; Curve 2, after incubation for 24 hours; Curve 3, Curve 2 minus Curve 1; Curve 4, cyanide-inactivated xanthine oxidase after incubation with 4,6-dimercaptoypyrazolo(3,4-d)pyrimidine for 24 hours. Right figure, extinction change at 500 nm obtained immediately (x) and the extinction change of slow minus immediate difference spectra (●) at 540 nm as a function of AFR250.](image)
values (Fig. 8) indicates a maximal theoretical AFR<sup>25°</sup> value of 210 when extrapolated to a molar ratio of 1.0.

The binding of various pyrazolo(3,4-d)pyrimidines to reduced xanthine oxidase can be detected as a rapid increase in absorption in the visible region (4, 5). A particularly distinct difference spectrum with a maximum at 500 nm occurs rapidly when reduced xanthine oxidase is incubated with 4,6-dimercapto-pyrazolo(3,4-d)pyrimidine. As shown in Fig. 9, the extinction of this difference maximum is linearly related to xanthine oxidase activity. Evidence for a slow rate of binding of the 4,6-dimercapto compound to inactive enzyme is also presented in Fig. 9. The difference extinction maximum for the inactive enzyme-pyrazolo(3,4-d)pyrimidine complex is located at 540 nm. The identical difference spectrum of the cyanide-inactivated enzyme (as well as the slow rate of its formation) with that of inactive xanthine oxidase indicates a very similar type of binding. The persulfide group thus appears to influence the rate of binding of pyrazolo(3,4-d)pyrimidines to the molybdenum site as well as influencing the spectral properties of their complexes.

Reactivation of Low AFR<sup>25°</sup> Xanthine Oxidase—In view of the many similar properties of low AFR<sup>25°</sup> xanthine oxidase with those of the cyanide-treated enzyme, one would expect reactivation with Na<sub>2</sub>S as previously shown for cyanide-inactivated xanthine oxidase (8). As shown in Fig. 10 incubation of AFR<sup>25°</sup> = 3 enzyme with 0.01 M Na<sub>2</sub>S resulted in an increase in specific activity to AFR<sup>25°</sup> = 55. It should be noted that the conditions for activation are rather critical; the rate of activation is markedly dependent on temperature, and becomes significant only at temperatures where denaturation also begins to occur.

Dithionite Titration of Native and Cyanide-treated Xanthine Oxidase—In view of the indicated catalytic requirement for the persulfide group and to probe its possible role in catalysis, dithionite titrations were performed on the cyanide-treated enzyme (with no persulfide group) and on native enzyme of approximately 80% functionality with the method of Foust et al. (20). The results are shown in Fig. 11. Because of the lack of a sharp spectral end point for full reduction at 450 nm, end points were estimated from the appearance of dithionite absorption at 315 nm, a wavelength which is very close to the isosbestic point of oxidized and reduced xanthine oxidase. The results of duplicate titrations for both forms of enzyme were identical within experimental error. The extrapolated dithionite absorption indicates an end point at 4 moles of dithionite per mole of enzyme flavin, in agreement with previous data (7). These data indicate that no electron equivalents are taken up by the persulfide group on complete reduction.

DISCUSSION

The resolution of a xanthine oxidase preparation into low and high activity fractions by affinity chromatography provides
conclusive evidence for the existence of nonfunctional enzyme in preparations containing stoichiometric amounts of molybdenum, FAD, iron, and acid-labile sulfur. As indicated in Fig. 1, the activity increased across each eluted band. The small amount of activity in the first band is due presumably to the presence of a small amount of uncomplexed functional enzyme. The finding of less than theoretical specific activities in the subsequent bands is due probably to some spontaneous inactivation during chromatography and also to some complex forming of inactive enzyme to the gel. Spectral changes (Fig. 9) indicate that reduced inactive enzyme slowly forms a complex with 4,6-dimercaptopyrazolo(3,4-d)pyrimidine. Based on the assumption that there are two active sites per enzyme molecule (for which no contrary evidence exists), one can visualize three forms of xanthine oxidase molecules: a species with both sites nonfunctional, a species with one functional and one nonfunctional site, and a species with both sites functional. The enzyme molecules with 50% functionality could presumably be in both inactive and active fractions and thus may contribute to the difficulty of getting a complete separation of functional from nonfunctional enzyme.

The only difference in chemical composition which has been found between the active and inactive enzyme is the presence of the persulfide in functional enzyme. This is indicated by the linear correspondence of SCN⁻ production and ∆ε₂₃₀ with the AFR₃⁵⁵ value of the enzyme upon cyanide treatment. The near-ultraviolet spectral properties of the inactive enzyme and the identical spectral properties of the complexes formed with 4,6-dimercaptopyrazolo(3,4-d)pyrimidine and reduced nonfunctional enzyme or cyanide-inactivated enzyme also provide further evidence for their similarity. The finding that both inactive and cyanide-treated enzymes can be partially activated by incubation with Na₂S provides conclusive evidence that the loss of a persulfide group is responsible for nonfunctionality in enzyme preparations containing the full complement of oxidation-reduction components.

There are several pieces of evidence which indicate that the persulfide is close to or associated with the enzyme-bound molybdenum. The insensitivity of diaphorase activity to the AFR₃⁵⁵ value indicates that FAD reduction by NADH is unimpaired by destruction of the persulfide. The red shift of the 4,6-dimercaptopyrazolo(3,4-d)pyrimidine enzyme-bound molybdenum complex (Fig. 9) and its slow rate of formation in the absence of the persulfide is stronger evidence for the proximity of the

![Diagram](attachment:fig12.png)

**Fig. 12.** Proposed mechanism for the catalytic role of the persulfide in xanthine oxidase. The bar is taken to represent the protein molecule, Fe/S, the iron-sulfide chromophore, and FAD, flavin adenine dinucleotide.
molybdenum. Additional support from electron paramagnetic resonance studies is the correlation of intensity of the slow molybdenum signal with enzymes of decreasing AFR* (Fig. 7). The cyanide-treated enzyme gives the strongest "slow" molybdenum signal. The extrapolated intensities indicate that in fully functional enzyme preparations, no "slow" molybdenum signal should be observed, in full agreement with the results of McGartoll et al. (6). Electron paramagnetic resonance studies of the anaerobic reduction of the enzyme upon cyanide treatment (8) indicate molybdenum to be the initial site of reduction.

The linear correlation of xanthine-oxygen reductase activity with degree of substrate bleaching (Fig. 5), intensity of iron electron paramagnetic resonance signals (Fig. 6) and the intensity of rapid molybdenum electron paramagnetic resonance signals (Fig. 7) are in agreement with the results of McGartoll et al. (6). The results indicate that only functional enzyme can be rapidly reduced by substrate while dithionite reduces both functional and nonfunctional enzyme. Of particular significance is the observation that sodium borohydride reduces only functional enzyme; the rapid reduction of 450-nm absorbance, intensity of iron electron paramagnetic resonance signals and intensity of "rapid" molybdenum signals parallel the results with xanthine as a reductant. This finding has mechanistic significance in that the borohydride ion reduces by hydride ion transfer. The observation that borohydride, aldehydes, and purines reduce only functional enzyme indicates that the persulfide group is required to facilitate enzyme reduction (with the molybdenum being the initial acceptor of reducing equivalents). These data imply that reduction of xanthine oxidase by aldehydes or by purines might also occur by a hydride ion transfer. Dithionite titrations (Fig. 11) indicate that the persulfide group does not take up any reducing equivalents; hence its function is probably to facilitate the hydride ion transfer reaction.

In an attempt to explain these results, as well as to provide a model for future experimentation, we would like to propose the mechanism for substrate reduction of enzyme and hydroxylation as outlined in Fig. 12. The tautomeric form of xanthine shown is that proposed by Bergmann et al. (21) as the structure acted on by xanthine oxidase. With its δ-+ charge on the C-8 position, this tautomer would be in a suitable structure for attack by the persulfide.

The reaction intermediate of reduced enzyme and the persulfide-bound substrate would then be subject to displacement by hydride ion, resulting in the regeneration of the persulfide and liberation of the hydroxylated product. This mechanism has the attractive feature of separating the hydroxylation reaction from the reoxidation of the reduced enzyme, and would, in fact, permit several molecules of substrate to be hydroxylated under anaerobic conditions at the expense of still further reduction of the enzyme. Indeed, in a previous publication, the anaerobic formation of 23 molecules of uric acid per functional active site, has been reported (5). The proposed mechanism is also consistent with the findings of Pick and Bray (22) and Bray et al. (23), who have demonstrated that the "rapid" molybdenum electron paramagnetic resonance signal shows interaction with a proton derived from the C-8 position of xanthine. A mechanism involving hydride ion transfer from substrate to enzyme has also been suggested previously by Rajagopalan and Handler (24).

The proposed mechanism is attractive in that it can also account for the oxidation of aldehydes by addition of the persulfide to the electron-deficient carbonyl carbon. The observations that xanthine oxidase oxidizes preferentially unhydrated aldehydes (25) and that thiols add to unhydrated aldehydes rather than to the hydrated species (26), are also consistent with the proposed mechanism. Unfortunately, the available knowledge of persulfide chemistry is limited and, to our knowledge, the reactions of persulfides with aldehydes and nitrogen heterocyclic compounds have not been studied. Thiols are known to add to the carbonyl carbon of aldehydes and have recently been shown to catalyze the deuterium hydrogen exchange at the C-5 position of uracil (27). More studies are required, however, before the chemical properties of persulfides can be compared to those of the sulfides.

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The Resolution of Active and Inactive Xanthine Oxidase by Affinity Chromatography
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J. Biol. Chem. 1972; 247:1597-1604.

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