The Radical Chemistry of Milk Xanthine Oxidase as Studied by Radiation Chemistry Techniques

Robert F. Anderson‡, Russ Hille§, and Vincent Massey¶

From the ‡Gray Laboratory of the Cancer Research Campaign, Mount Vernon Hospital, Northwood, Middlesex HA6 2RN, Great Britain, the §Department of Physiological Chemistry, Ohio State University, Columbus, Ohio 43210, and the ¶Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109

The kinetics of electron transfer within the molybdoflavoenzyme xanthine oxidase has been investigated using the technique of pulse radiolysis. Subsequent to one-electron reduction of native enzyme at 20 °C in 20 mM pyrophosphate buffer, pH 8.5, using the CO₂ species as reductant, a spectral change is observed having a rate constant of ~290 s⁻¹. From its wavelength dependence, this spectral change is assigned to the transfer of an electron from flavin semiquinone (formed on reaction with the CO₂ species) to one of the iron-sulfur centers of the enzyme in an intramolecular equilibration process. The value for this rate constant agrees well with the 330 s⁻¹ observed in previous stopped-flow pH-jump experiments carried out at 25 °C (Hille, R., and Massey, V. (1986) J. Biol. Chem. 261, 1241-1247). Experimental results with fully reduced enzyme reacting with the radiolytically generated N₃ species also support the conclusion that the equilibration of reducing equivalents among the oxidation-reduction centers of xanthine oxidase is a rapid process. Evidence is also found that xanthine oxidase possesses an unusually reactive disulfide bond that is reduced rapidly by radiolytically generated radicals. The ramifications of the present results with regard to the interpretation of experiments involving chemically reactive radical species, generated either by photolysis or radiolysis, are discussed.

Milk xanthine oxidase containing four redox centers (one molybdenum, one FAD, and a pair of 2Fe/2S centers) serves as a useful system in which to study biological electron transfer processes. Whereas previous work (1) studying the thermodynamic properties of the enzyme has shown that equilibration of reducing equivalents is rapid and is governed by the relative oxidation-reduction potentials of the centers, a recent report (2) using flash photolysis has concluded that reducing equivalents equilibrate between the molybdenum center and Fe/S center I at a rate of 77 s⁻¹ and among the remainder of the centers at 12 s⁻¹ (22-25 °C). These slow rates cannot be reconciled with kinetic simulation of both the reductive (1) and oxidative (3, 4) half-reactions which suggest that the lower limit for the rate of internal equilibration is approximately 100 s⁻¹ nor with pH-jump experiments (5, 6), which put the rates of equilibration at 330 s⁻¹ at pH 9.1 and 155 s⁻¹ at pH 6.0.

The radiolysis of aqueous solutions containing solutes is routinely used to generate and study qualitatively the chemistry of inorganic and organic free radicals as well as metal ions. In the present study, we have applied the techniques of pulse radiolysis to observe directly the properties of the transient radical species formed in xanthine oxidase and steady-state radiolysis to help elucidate mechanisms. Our results (i) support the view that intramolecular electron transfer is fast in xanthine oxidase and (ii) provide evidence that the slow rates reported using flash photolysis most likely arise from an experimental artifact.

MATERIALS AND METHODS

Xanthine oxidase was isolated from fresh milk by the method of Massey et al. (7). The stabilizing salicylate was removed by elution of enzyme with the radiolysis buffer (20 mM pyrophosphate, pH 8.5) on a Sephadex G-25 column. The enzyme solutions were diluted by the addition of various reagents required for radiolysis experiments (see below) and the concentration of the enzyme was determined spectrophotometrically using a value of 37,800 M⁻¹ cm⁻¹ for the absorbance per enzyme-bound FAD at 450 nm (7).

An aerobic solution was prepared by holding high purity N₂O or N₂ gas (BOC Special Gases, Enfield, United Kingdom) for 1 h over the surface of the enzyme solution contained in a Pyrex syringe (for pulse radiolysis experiments) or in a silica spectrophotometric cell fitted with an airtight stop (for ²⁴Co γ-ray experiments). Reduced enzyme was prepared by titration with sodium dithionite and when required was alkylated at the flavin by incubation with iodoacetamide for 1 h (8) or on the disulfide bond using iodoacetate (9). In some experiments, alloxanthine (50 μM) was also added to block the molybdenum site (10).

Details of the Gray Laboratory’s 4-MeV Van de Graaff accelerator pulse radiolysis and radical detection system have been published (11). All irradiations (2-150 Gy) were delivered to fresh solutions contained in a small volume cell of 0.5-cm path length at 20 ± 0.5 °C. Dosimetry was carried out using aerated KSCN solution assuming the (SCN⁻); radical produced has a radiation chemical yield,2 G, of 0.29 μmol J⁻¹ with an extinction coefficient (e) of 7580 M⁻¹ cm⁻¹ at 472 nm (12). Water was purified (δ = 0.06 μS cm⁻¹) by a Milli-Q system (Millipore) and purified free of oxygen with N₂ or N₂O gas. Changes in optical absorption were recorded on a Tektronix 7612D digitizer interfaced to a PDP11/34 computer. In all figures displaying kinetic traces, the time of the electron pulse is

*This work was supported by the Cancer Research Campaign, a grant from the Wellcome Foundation, and Grant DBM-8521645 (to R. H.) and PCM-8208240 (to V. M.) from the National Science Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1The abbreviations used are: Gy, gray; V⁺⁺, oxidized viologen compound; V⁻⁻, one-electron-reduced viologen radical; V32, 7,8-dihydro-2,3,11,12-tetramethyl-6H-dipyrido[1,2-a,2',1'-c][1,4]diazepinediium dibromide; V40, tetraquat, 6,7,8,9-tetrahydrodipyrido[1,2-a,2',1'-c][1,4]diazocinediium dibromide; V41, 6,7,8,9-tetrahydro-2,13-dimethyl dipyrido[1,2-a,2',1'-c][1,4]diazocinediium dibromide; MV⁺⁺, methyl viologen (1,1'-dimethyl-4,4'-bipyridinium dichloride).

The 3f unit of radiation chemical yield is used throughout this work expressed in μmol J⁻¹; a G-value of 1 molecule (100 eV⁻¹) corresponds to a yield, G = 0.1036 μmol J⁻¹.
shown by an arrow. Radical absorption spectra are presented as the product of the radiation chemical yield and extinction coefficient assuming a density of 1.0.

A 60Co γ-ray source of ~37 TBq (1000 Ci) was used to irradiate enzyme solutions at a low dose rate (~12 Gy min⁻¹) in sealed spectrophotometric cells. Spectra were recorded using a Pye Unicam SP8-200 spectrophotometer. Dosimetry was carried out using the standard Fricke dosimeter (ferrous sulfate (1 mM) and sulfuric acid (0.4 M) in aerated aqueous solution), and the absorbed dose was calculated using G(Fe³⁺) produced = 1.61 μmol J⁻¹ (13).

The radiolysis of water results in a variety of reducing and oxidizing species:

\[ \text{H}_2\text{O} \rightarrow e_\text{aq}^-, \text{H}^+, \text{OH}, \text{H}_2\text{O}_2, \text{H}_2, \text{H}_2\text{O}^+, \text{OH}^- \]  

To study specifically the one-electron reduction of the enzyme, it is necessary to scavenge the oxidizing radicals by (i) adding 2-methylpropan-2-ol (0.2 M) to form a radical that does not undergo electron transfer at a significant rate with the oxidants of interest here,

\[ '\text{OH}(\text{H}^+) + (\text{CH}_2\text{OH}) \rightarrow '\text{CH}_2(\text{CH}_2)\text{O} + \text{H}_2\text{O}(H_2) \]  

or (ii) adding sodium formate (0.1 M) or propan-2-ol (0.2 M) to convert the reducing species CO₂⁻ or (CH₂OH)COH by

\[ '\text{OH}(\text{H}^+) + \text{HCOO}^- \rightarrow \text{CO}_2 + \text{H}_2\text{O}(H_2) \]  

Enhanced yields of CO₂⁻ and (CH₂OH)COH were obtained in solutions saturated with N₂O where

\[ \text{N}_2\text{O} + e_\text{aq}^- \rightarrow '\text{OH} + \text{N}_2 + \text{OH}^- \]  

is followed by Reaction 3 or 4.

Low potential viologen radicals, V⁺ (14), used in this work were generated through the fast electron transfer reactions

\[ \text{CO}_2^- + V^+ \rightarrow V^+ + \text{CO}_2 \]  

\[ (\text{CH}_2\text{OH}) + V^+ \rightarrow V^+ + (\text{CH}_3)\text{CO} + \text{H}^+ \]  

In the course of these studies, it was found that formate was a reducing substrate for xanthine oxidase, although not nearly as effective as formamide in this regard (15). In the course of the 2-h preparation time for the experiments described in the present studies, it was found that, in the presence of formate, enzyme was reduced by approximately 10% (on the basis of the fractional absorbance change at 450 nm). In order to avoid this potential source of artifacts, anaerobic solutions of sodium formate were added to the anaerobic enzyme immediately prior to radiolysis, rather than have the formate present in the course of making the enzyme solution anaerobic.

One-electron oxidation of fully reduced enzyme was carried out by the N₂⁻ radical species (16) produced in N₂O saturated solution containing sodium azide (0.2 M).

\[ '\text{OH} + \text{N}_2^- \rightarrow \text{N}_2 + \text{OH}^- \]  

The total radical yield per absorbed dose (Gy) is taken as 0.65 μmol J⁻¹ (17).

RESULTS

Reduction of Native Enzyme—In order to determine the reactivity of native xanthine oxidase toward the hydrated electron, anaerobic enzyme in a solution made 0.2 M in 2-methylpropan-2-ol (to scavenge HO⁻ and H') was examined by pulse radiolysis. Because the hydrated electron is known to react in a nonspecific manner with peptide bonds, disulfide bonds, and a variety of amino acid residues in proteins, large radiolytic doses of 150 Gy (sufficient to reduce enzyme by approximately 30% at the enzyme concentration used in the experiment) were used to ensure that the oxidation-reduction chromophores of the enzyme became reduced. Immediately following the initial reduction of enzyme (occurring in less than 10 μs), a spectrum was obtained that was characterized by a rising absorption below 400 nm and a smaller absorption in the 450-600 nm region (Fig. 1A). The lower wavelength absorption has been attributed to both the one-electron reduction of disulfide bonds, forming RS—SR, and also of peptide bonds (18). The latter absorbance is indicative of formation of the blue neutral semiquinone of xanthine oxidase. This initial spectrum decays to an end-point spectrum with a minimum at 470 nm and a shoulder at 430 nm that is characteristic of the reduction of the oxidation-reduction chromophores (flavin and iron-sulfur centers) present in the enzyme. Similar spectral features are observed at much lower radiation doses (20 Gy, or ~5% enzyme reduction) when the experiment was repeated with CO₂⁻ as reductant (i.e., in the presence of 0.1 M formate under an atmosphere of N₂O to scavenge the hydrated-electron formed in the pulse (Fig. 1B)). Because the radiation chemical yield for CO₂⁻ is approximately twice that for the hydrated electron and because CO₂⁻ reacts only slowly with peptide bonds, larger changes in absorption are expected and observed in this reaction compared with that using the hydrated electron as reducing agent. The large absorption below 400 nm in the spectrum of the intermediate species observed in the CO₂⁻ reaction indicates that this absorbance is not likely to be only due to reduction of peptide bonds.

The initial intermediate decayed in a wavelength-dependent manner to the final spectrum. First-order kinetic plots showed that whereas the faster decay at 550 nm (Fig. 2A) could be fitted to a single exponential yielding \( k_f = 287 \pm 3 \text{ s}^{-1} \), the apparent slower decay at 375 nm (Fig. 2B) could not. This is interpreted as two radical species being observed to decay simultaneously with rate constants \( k_1 \) and \( k_2 \). Fitting the loss in radical absorption to AEXP(−kₙₜ) + BEXP(−k₂t) (Fig. 2B, inset) using nonlinear regression analysis with \( k_f = 287 \text{ s}^{-1} \) gave \( k_2 = 62 \pm 7 \text{ s}^{-1} \).

**Reduction of Xanthine Oxidase with Pulse-generated Viologen Radicals**—In an attempt to introduce reducing equiva-

![Fig. 1. A, absorption spectra following the reaction of \( e_\text{aq}^- \) with xanthine oxidase. Spectra are shown following pulse radiolysis of the enzyme (48 μM) in deaerated solution containing 2-methylpropan-2-ol (0.2 M) buffered at pH 5.5 with 20 mM pyrophosphate. O, 30 μs; □, 35 ms after pulse (150 Gy). B, absorption spectra following the reaction of CO₂⁻ with xanthine oxidase. Spectra are shown following pulse radiolysis of the enzyme (38 μM) in solution saturated with N₂O and containing sodium formate (0.1 M) buffered at pH 8.5 with 20 mM pyrophosphate. O, 100 μs; □, 35 ms after pulse (40 Gy).
Radical Chemistry of Milk Xanthine Oxidase

Fig. 2. A, kinetic transient from Fig. 1B at 550 nm. Percentage absorption change versus time is shown. Dashed lines indicate the asymptote and time frame used to construct the first-order kinetic plot; inset yielding a value of $k = 287 \pm 3$ s$^{-1}$. B, kinetic transient from Fig. 1B at 375 nm. Percentage absorption against time is shown. Inset, decay of radical concentration with time fitted to the expression given in the text using nonlinear regression analysis.

Fig. 3. Absorption spectra following pulse radiolysis of solutions containing disulfide-alkylated xanthine oxidase and viologens. Spectra were produced in solutions saturated with N$_2$O containing sodium formate (50 mM) buffered at pH 8.5 with 20 mM pyrophosphate. Open symbols represent absorption changes for the enzyme (40 $\mu$M) and V40 (2 mM): O, 50 $\mu$s; □, 30 ms after the pulse (25 Gy). Closed symbols represent absorption changes for the enzyme (55 $\mu$M) and V32 (2 mM): ●, 50 $\mu$s; ■, 6 ms after the pulse (20 Gy).

Fig. 4. Kinetic transient from Fig. 5 at 470 nm. Percentage absorption change versus time following pulse radiolysis of the enzyme in the presence of V40. Top trace displays the stability of the analyzing light.

Electron transfer from CO$_2^-$ to the viologens V40 or V32 (present at a concentration of 2.0 mM) in the presence of alkylated enzyme produced the viologen radical in full radical yield ($G = 0.65 \mu$mol J$^{-1}$), as determined from the extent of positive absorbance change in the near-ultraviolet and the known extinction coefficients of the radicals (14), as is shown.

R. Hille, unpublished results.
in Fig. 3. In both cases, the viologen radical thus formed decayed exponentially at a rate that was independent of wavelength and dependent on enzyme concentration. The viologen radical of lower reduction potential, V32 (E = -777 mV (14)), underwent faster electron transfer, with a second-order rate constant (calculated from rates observed at two different enzyme concentrations) of 2 x 10^9 M^-1 s^-1 compared to V40 (E = -640 mV (19, 20)). The V40 radical reduced enzyme with a second-order rate constant (again calculated from data with two different enzyme concentrations) of 5.5 x 10^9 M^-1 s^-1. These relative reactivities are consistent with the relative reactivities of these radicals in cross-reactions with one another, indicating a free energy correlation between reduction potential and rate of reaction. The final spectrum observed with both V32 and V40 indicates bleaching of the oxidation-reduction chromophores to the extent anticipated for the amount of reducing equivalents generated in the form of the viologen radicals with no indication of slower reactions taking place (Fig. 4).

Reduction of Flavin-alkylated, Alloxanthine-blocked Enzyme—The radiation chemistry of xanthine oxidase containing the intact reducible disulfide bond was studied using flavin-alkylated, alloxanthine-blocked enzyme. The rate constants for electron transfer to enzyme from a range of V^+ exhibit a dependence on the one-electron reduction potential of the viologen, with the compounds of lowest potential reacting at a diffusion-controlled rate of ~10^9 M^-1 s^-1 (Fig. 5). Repetitive pulsing using V-22 as the electron transfer agent (Fig. 6) shows that the fast decay of V^+ ceased after the introduction of 1 eq of electrons into the enzyme. It is to be noted from a comparison of the initial and final absorbance spectra of the time course shown in Fig. 6 that there is no net bleaching of the enzyme iron-sulfur centers (the only intact chromophores in this chemically modified form of the enzyme) on the time scale of the initial reaction of the viologen radical with enzyme. Whereas subsequent radiolytic titrations of flavin-alkylated, alloxanthine-blocked enzyme (data not shown) indicate that on a time scale of many minutes such reduction does eventually take place, there is no rapid reduction of the iron-sulfur centers by the viologen radicals. These observations indicate that perhaps for accessibility or steric reasons the iron-sulfur centers are not particularly reactive toward the viologen radicals used in the present studies.

The reoxidation by enzyme of the radical of methyl viologen formed after the initial pulse in a sequence such as that shown in Fig. 6 was observed to be incomplete, and the extent of reoxidation was found to depend on the concentration of methyl viologen (Fig. 7). These results are consistent with the establishment of the equilibrium

\[ \text{MV}^{2+} + \text{XO}^- \rightarrow \text{MV}^+ + \text{XO} \]  

(9)

where XO^- is one-electron reduced enzyme. The difference
in one-electron reduction potential is \( \Delta E = (RT/nF)\ln(K^+)/(f_i) = 59(\log K + \log f_i) \) at \( T = 295 \) K, where \( f_i \) is the activity coefficient. At constant radiation dose, \( (G_t)_{\text{abs}} \) is proportional to the optical density observed when both MV\(^+\) and XO\(^-\) are present at equilibrium and is proportional to the optical density of solutions containing only MV\(^+\) or XO, \( (G_t)_{\text{MV}^+} \) and \( (G_t)_{\text{XO}^-} \), by

\[
K' = \frac{[\text{MV}^+][\text{XO}^-]}{[\text{MV}^+]^2} = \frac{(G_t)_{\text{abs}} - (G_t)_{\text{XO}^-}}{(G_t)_{\text{MV}^+} - (G_t)_{\text{abs}}} \cdot \frac{[\text{XO}^-]}{[\text{MV}^+]^2}
\]

From two mixtures of enzyme (30-27 \( \mu \)M and MV\(^+\) (2-4 mM) (Fig. 7), we measure \( K' = 0.027 \pm 0.003 \) and calculate \( \log f_i = -0.254 \) (assuming the radical on the enzyme is unprotonated). Hence, \( \Delta E = -77 \) mV and \( E \) of the redox center under study is \( -370 \pm 10 \) mV. This is unusually high for a disulfide, and this point is considered further under "Discussion."

**Steady-state Irradiation Studies**—The importance of the reducible disulfide bond (9) in the overall radical chemistry of xanthine oxidase has also been studied by observing spectral changes following irradiation by \( ^{60}\text{Co} \gamma\)-rays. This technique permits the titration of the enzyme with specific concentrations of radicals. Such a titration is presented in Fig. 8, which shows a progressive reduction of native enzyme arising by electron transfer from the \( V^+ \) of V41. A plot of the percentage change in optical density against dose, normalized for enzyme concentration, clearly shows a lag phase of \( \sim 1.5 \times 10^6 \) J mol\(^{-1}\) and complete reduction at \( \sim 13.0 \times 10^6 \) J mol\(^{-1}\) (Fig. 9). Since the concentration of one-electron reducing equivalents produced is 0.65 \( \mu \)M J\(^{-1}\), the initial lag phase corresponds to 1 reducing eq, whereas, in agreement with the previous study (9), 8 reducing eq are required for complete reduction. Experiments with disulfide-alkylated enzyme and V41 did not exhibit the initial lag phase seen for native enzyme, and complete reduction was observed at \( \sim 10 \times 10^6 \) J mol\(^{-1}\), corresponding to 6 reducing eq (Fig. 10). Similar results were found when using V32. Experiments using triblocked enzyme (flavin-alkylated, alloxanthine-blocked, and disulfide-alkylated) showed that the \( V^+ \) species slowly reduced the enzyme, consistent with direct reduction of the Fe/S centers, albeit on a time scale of many minutes.

**Oxidation of Reduced Disulfide-alkylated Enzyme by Pulse Radiolysis**—It has been known for some time that the inorganic radical anions \( \text{Br}_2^- \) and \( (\text{CNS})_2^- \) can oxidize NADH to a pyridine-centered radical, NAD\(^+\) (21); and subsequent work has shown that a similar reaction occurs with FADH\(_2\) to produce flavosemiquinone, FADH\(^+\) (22). Using the \( ^{60}\text{Co} \gamma\)-ray procedure, it was found that neither \( \text{Br}_2^- \) nor \( (\text{CNS})_2^- \) could oxidize fully reduced enzyme, whereas the neutral radical \( \text{N}_3 \) does.

Pulse radiolysis of the reduced disulfide-alkylated enzyme

![Image](https://via.placeholder.com/150)
(36 μM) in N₂O-saturated solution containing sodium azide (0.2 M) showed the fast formation (<100 μs) of a transient which decayed to a final absorption (Fig. 10). The intermediate exhibits the expected characteristics of absorption due to flavosemiquinones, whereas the final product indicates some oxidation of the Fe/S centers. Greater yields were found with higher enzyme concentrations, indicating that the low radiation chemical yield is due to some loss of N₃ radicals through fast bimolecular reaction (2k = 8 × 10⁶ M⁻¹ s⁻¹) at the radiation doses used (25 Gy). The rate of conversion of the intermediate to the product was independent of enzyme concentration (32-47 μM), giving a rate constant of 170 ± 15 s⁻¹.

**DISCUSSION**

It is clear from our results that the initial reduction of native oxidized xanthine oxidase and disulfide-alkylated enzyme by low potential electron donors (e.g. V?) occurs at different sites. Whereas reduction of the native enzyme requires the addition of 1 reducing eq before reduction of the oxidation-reduction chromophores associated with enzyme turnover occurs (Figs. 6 and 9), the disulfide-alkylated enzyme does not (Fig. 9). This difference appears to arise from the reduction of a center in the native enzyme associated with the intact disulfide bond at several orders of magnitude higher than reduction at the oxidation-reduction chromophores (Fig. 5). It should be emphasized that the formation of a singly reduced disulfide bond is a kinetic phenomenon due in large part to the extremely high chemical reactivity of the pulse-generated radicals produced in these experiments and is not a thermodynamically governed process. The disulfide radical is formed in the present experiments not because it is a stable chemical species but because it is reduced most rapidly of all the potential electron-accepting sites in the enzyme.

The one-electron reduction of disulfide bonds by radiolytically generated radicals is a well-documented phenomenon, and RS—SR⁻ is known to form in both small molecules (24) and proteins (18). The decay rates of one-electron reduced disulfide derivatives of amino acids (RS—SR⁻) at pH 7 are 1.5-6.0 × 10⁹ s⁻¹ (24).

\[ \text{RS—SR}^- \rightarrow \text{RS}^- + \cdot \text{SR} \quad (11) \]

A better model for disulfide bridges in enzymes is lipote whose disulfide bond is part of a cyclic structure which imparts stability to the —S—S— structure. The lipote radical anion decays with second-order kinetics, 2k = 1.4 × 10⁸ M⁻¹ s⁻¹ (24). As the one-electron reduction potential of lipote is less than ~1 V, it is hard to understand how protein structure alone could raise the potential of a disulfide bond to ~370 mV, measured here for blocked enzyme, without some involvement of other oxidation reduction centers, located in close proximity to a metal ion. However, it has been pointed out that the 5-deazaflavin photoreduction procedure, which generates 5-deazaflavin semiquinone, cannot in general reduce disulfide bonds but does so in the case of xanthine oxidase. This observation can be taken as support for the view that the disulfide bond of xanthine oxidase is of higher one-electron reduction potential than ~650 mV as determined for 5-deazaflavin (26). It cannot be overemphasized that one-electron transfer from low potential radicals such as formed by viologens and 5-deazaflavin will be generally governed thermodynamically by the difference in one-electron reduction potential of the reductants and oxidants, not their two-electron reduction potentials.

The rates of electron addition to xanthine oxidase by e⁻ (standard reduction potential ~−2.9 V) and from CO₂ (approximately ~−2.0 V) would be expected to be diffusion-controlled. The wavelength-dependent decay of the transient formed indicates that at least two species are initially formed which decay independently of each other. The single exponential at 550 nm (Fig. 2A) is consistent with the electron transfer from blue flavosemiquinone to another oxidation-reduction center occurring at ~287 ± 35 s⁻¹, which, when corrected to 25 °C, is similar to ~330 s⁻¹ as observed by the pH-jump technique under otherwise identical conditions (5, 6). At shorter wavelengths, this decay is superimposed on a much slower decay, 62 ± 7 s⁻¹ (Fig. 2B). The wavelength dependence of this process indicates that it most likely arises from the decay of the disulfide radical (which would be expected to undergo slow bond scission).

The rate constants for the reaction of V? with the disulfide center of xanthine oxidase are several orders of magnitude higher than with the oxidation-reduction centers of the enzyme (Fig. 5). Similar differences in rate constants would be expected for the reactions of the 5-deazaflavin semiquinone with the enzyme and may account for the slow decay rates observed (approximately 80 and 12 s⁻¹) in the flash photolysis results of Battacharyya et al. (2). These workers used native enzyme containing the intact disulfide, and it is possible that the slow reactions these workers observed involved the transfer of a reducing equivalent from the disulfide bond to the oxidation-reduction chromophores in either an intramolecular process.

The present results indicate the need to consider the possibility that reactions which might otherwise seem unlikely when studying the reactions of proteins with extremely reactive species such as those that are generated in radiolytic or photolytic techniques. The formation of species such as the disulfide radical in reactions that occur rapidly despite the thermodynamic instability of the products cannot be excluded a priori, and control experiments such as those with the disulfide-alkylated enzyme reported here are necessary to ensure that the experimental results are correctly interpreted. Particularly in the case of photolytic experiments, it is necessary to characterize carefully both the reactivity of the

---

*It should be noted that the rates observed in the flash photolysis experiments are not fast enough to account for the observed turnover or oxidation of the iron-sulfur centers of xanthine oxidase under the conditions in which the flash experiments were performed at pH 7.0, enzyme turnover is approximately 20 s⁻¹; and the rate of iron-sulfur reoxidation, a reaction that must occur by electron transfer from the iron-sulfur centers to the flavin for reaction with O₂, is 90 s⁻¹ (1).*

---

*F. Anderson and K. B. Patel, personal communication.*
deazaflavin radical with amino acid residues and disulfide bonds as well as the reactivity of secondary radicals and products generated (e.g. EDTA radicals) in most photolytic experiments with the protein.

Acknowledgments—We wish to thank Boris Vojnovic for continual development of the pulse radiolysis facility, Brian Hall and Keith Allen for operating the Van de Graaff accelerator, and Darshan Sehmi for help with data processing.

REFERENCES