Inactivation of *Salmonella* Phosphoribosylpyrophosphate Synthetase by Oxidation of a Specific Sulphydryl Group with Potassium Permanganate

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**SUMMARY**

Phosphoribosylpyrophosphate synthetase from *Salmonella typhimurium* contains four cysteine residues per subunit. Three of these react readily with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), forming an active derivative with kinetic and physical properties similar to the native enzyme, but one reacts only under denaturing conditions. Stoichiometric amounts of KMnO₄ inactivate the DTNB-treated enzyme. The loss of activity is correlated with the oxidation of the remaining cysteiny1 group to cysteic acid by KMnO₄. Amino acid analysis indicates that no other residues are altered. The rate of inactivation of the enzyme is decreased 30-fold by saturating concentrations of the substrate ATP.

Inorganic phosphate also protects substantially against inactivation (3-5). The physical properties of the enzyme from *Salmonella typhimurium* have been studied in detail, but little is known about the functional groups involved in catalysis or in maintaining tertiary structure.

An unusual property of PRPP synthetase from all sources is an absolute and specific requirement for inorganic phosphate. Pᵢ does not participate chemically in the reaction (1). High concentrations of Pᵢ also inhibit the enzyme (4, 6). The permanganate ion resembles the phosphate ion in size and shape (7, 8), but unlike Pᵢ, it oxidizes a wide variety of organic compounds (9). These considerations prompted Benisek (10) to use KMnO₄ to modify the catalytic subunit of aspartate transcarbamylase from *Escherichia coli*. He demonstrated that the specific oxidation of a cysteine residue near the active site inactivated the enzyme. More recently, Datta et al. (11) have used KMnO₄ to modify spinach chloroplast coupling factor 1 and to alter its regulatory functions. In this work, KMnO₄ was used as an active site-directed reagent to modify a single cysteine residue of PRPP synthetase. The oxidation of this cysteine to cysteic acid resulted in inactivation of the enzyme.

**MATERIALS AND METHODS**

**Enzyme**

PRPP synthetase was purified from *Salmonella typhimurium* cells as described previously (12). The specific activity of the enzyme under standard assay conditions (0.05 M potassium phosphate, 0.05 M triethanolamine, 0.75 mM EDTA, pH 8.0; 10 mM MgCl₂, 5 mM ribose-5-P, 2 mM ATP; 37°C) varied between 80 and 125 pmol/min/mg of protein in the ³²P transfer assay. The enzyme was diluted prior to assays into a solution of 0.05 M phosphate buffer containing 1 mg/ml of bovine serum albumin. Protein concentration was determined by the method of Lowry (13) using an absorbance at 660 nm of 7.3 cm⁻¹/mg of enzyme based on the dry weight of PRPP synthetase.

**Reagents**

KMnO₄ (Fisher Scientific), DTNB, N-ethylmaleimide, and pHBMB (Sigma), and guanidine hydrochloride (ultrapure grade, Schwarz/Mann) were used without further purification. Methyl methanethiosulfonate was a gift from Dr. George Kenyon (University of California, San Francisco). ATP, ribose-5-P, and ADP were diluted prior to assays into a solution of 0.05 M phosphate buffer containing 1 mg/ml of bovine serum albumin. Protein concentration was determined by the method of Lowry (13) using an absorbance at 660 nm of 7.3 cm⁻¹/mg of enzyme based on the dry weight of PRPP synthetase.
were the best grades obtainable from Sigma. [γ-32P]ATP was prepared by the exchange procedure described previously (4).

**Determination of Thiol Groups**

Total thiol groups were determined with DTNB essentially as described by Sedlak and Lindsay (14) using a value of ε at 412 nm of 1.36 × 10^4 M^-1 cm^-1 for the TNB produced on reaction with excess DTNB (15). Titration of thiol groups with pHMB was done under both native and denaturing conditions (4 to 6 M guanidine hydrochloride, pH 8.0) (16).

**Preparation of Enzyme Derivatives**

**DTNB Treatment**—The (TNB)s-E derivative of PRPP synthetase was prepared by reaction of the enzyme (1 to 2.5 mg/ml) in 0.05 M potassium phosphate, pH 7.5, with 2 to 6 M DTNB for 1 to 2 hours at room temperature. The enzyme was dialyzed against 0.05 M phosphate to remove excess DTNB.

**KMnO₄ Treatment**—To 0.2 to 2.5 mg of enzyme/ml of 0.05 M potassium phosphate, pH 7.5, at 0°C was added 8 to 10 mol of aqueous KMnO₄/mol of subunit for the native enzyme and 2 to 2.5 mol/mol of DTNB-treated enzyme. After reaction for a given time, samples were removed and quenched with 10 or more volumes of phosphate buffer containing 0.01 M 2-mercaptoethanol (or 0.05 M hydrazine acetate if sulfhydryl content was to be determined directly with DTNB). Specific activity measurements were made on the quenched samples. The oxidized enzyme was dialyzed against phosphate buffer for physical characterization studies.

**Amino Acid Analysis**

Acid hydrolyses were performed as recommended by Moore and Stein (17). Since the enzyme contains less than 0.4 mol of tryptophan/mol as judged spectrophotometrically, by N-bromosuccinimide titration, or by alkali hydrolysis, acid hydrolyses were not performed. Half-cystine residues were determined by the procedure of Hirs et al. (18). Analyses of the hydrolysates were performed using a Beckman model 120 amino acid analyzer.

**Binding Studies**

A column (1 X 10 cm) of Sephadex G-25 fine was used for detecting binding of ATP to PRPP synthetase by the method of Hummel and Dreyer (19, 20). The column was equilibrated with 200 µM ATP radioactive solution in 0.05 M phosphate and 5 mM MgCl₂, pH 7.5. The elution profile was followed by counting the [γ-32P]-ATP fractions (0.2 ml each).

**Ultracentrifuge Studies**

Sedimentation velocity experiments were carried out at 20°C in a Beckman model E analytical ultracentrifuge equipped with a photoelectric scanner. Native enzyme and derivatives were centrifuged at a concentration of 1 to 3 mg/ml at 40,000 rpm, into D₂O, as described by Vinograd et al. (21). Dissociation of the initial species occurred as the enzyme sedimented through 1% D₂O. Therefore, the s₂₀,w values reported are based on data accumulated in the first 30 min when log r versus time plots appeared to be linear.

**RESULTS**

**Sulfhydryl Content of PRPP Synthetase**—Performic acid oxidation and subsequent amino acid analysis of PRPP synthetase indicated that each subunit of molecular weight 31,000 contains four half-cystine residues. The data presented in Table I show that without prior denaturation of the enzyme, three of the residues react readily with DTNB or pHMB. The time course of the reaction of native enzyme with excess DTNB indicated that these three groups differ in their reactivity and were completely reacted in 50 min. When enzyme was dissolved in 2% sodium dodecyl sulfate at 25°C, the three sulfhydryl groups reacted with DTNB within 2 min. In order to react the fourth sulfhydryl group in 2% sodium dodecyl sulfate, it was necessary to heat the solution at 100°C for 5 min. DTNB or pHMB titration of enzyme denatured in 6 M guanidine hydrochloride at room temperature also yielded four free sulfhydryl groups per subunit.

**Comparison of kinetic properties of (TNB)s-E and unmodified enzyme**

- **Kinetic constant**
  - Native enzyme
  - (TNB)s-E
  - V_max (µmol/min/mg ± S.E.)
    - Native enzyme: 122 ± 5
    - (TNB)s-E: 102 ± 5
  - K_m,ATP (mM ± S.E.)
    - Native enzyme: 0.037 ± 0.006
    - (TNB)s-E: 0.049 ± 0.008
  - K_m,ribose-5-P (mM ± S.E.)
    - Native enzyme: 0.18 ± 0.03
    - (TNB)s-E: 0.16 ± 0.004
  - K_i,ATP (mM ± S.E.)
    - Native enzyme: 0.05 ± 0.03
    - (TNB)s-E: 0.13 ± 0.06

- **Table I**
  - Sulphydryl content of PRPP synthetase
  - Method of analysis
  - Free sulfhydryl groups per subunit
  - Native enzyme
  - Denatured enzyme
  - DTNB
    - 2.8
    - 3.7
  - pHMB titration
    - 2.7
    - 4.1

* a The enzyme was dissolved in 6 M guanidine hydrochloride, pH 8.0.

**Properties of Tris-5-Thio-2-Nitrobenzoate Derivative of PRPP Synthetase [(TNB)s-E]**—Treatment of native PRPP synthetase with DTNB, followed by removal of excess reagent by dialysis yielded enzyme with 1.2 ± 0.1 free sulfhydryl groups per subunit as measured by DTNB titration in guanidine hydrochloride. When excess 2-mercaptoethanol was added to solutions of the derivative, 2.8 mol of TNB anion were released, as determined by the increase in absorbance at 412 nm. These properties indicate that the tris-5-thio-2-nitrobenzoate derivative was the product.

[(TNB)s-E] had 80 to 85% of the activity of control native enzyme. Kinetic properties of this derivative and the native enzyme are shown in Table II. An unusual property of PRPP synthetase is the apparent induction of ribose-5-P substrate inhibition by ADP. It has been suggested that this effect results from binding of ADP to an allosteric site on the enzyme (3). As shown in Fig. 1, (TNB)s-E retains this property, although the derivative is appreciably less sensitive to ADP-induced ribose-5-P inhibition. Treatment of the native enzyme with low concentrations of Ca²⁺ ions has been shown to reverse this substrate inhibition substantially (3); (TNB)s-E was affected similarly.

**Reaction of PRPP Synthetase and (TNB)s-E with KMnO₄**—In preliminary investigations, native PRPP synthetase was treated for 5 to 30 min with molar concentrations of KMnO₄ four times the total sulfhydryl concentration. Inactivation occurred rapidly and was accompanied by loss of all sulfhydryl groups. Since each subunit contains three cysteine residues that react readily with sulfhydryl reagents without inactivating the enzyme, it seemed likely that the oxidation of the single relatively unreactive sulfhydryl group by KMnO₄ caused inactivation. This 4 The activity of native enzyme treated with pHMB or methyl methanethiosulfonate was also 80 to 85% of the control. In both cases approximately three of the four sulfhydryl groups per subunit were modified.

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3 M. F. Roberts, unpublished experiments.

4 The activity of native enzyme treated with pHMB or methyl methanethiosulfonate was also 80 to 85% of the control. In both cases approximately three of the four sulfhydryl groups per subunit were modified.
suggestion was tested by reacting (TNB)$_2$-E with KMnO$_4$. The extent of oxidation of the remaining sulfhydryl group was followed by DNTB titration under denaturing conditions (6 M guanidine hydrochloride, pH 8.0). Fig. 2 shows that loss of enzyme activity was correlated with disappearance of the remaining sulfhydryl group. This indicates that oxidation of this relatively unreactive group inactivates the enzyme. Approximately 2.3 mol of KMnO$_4$ were required for oxidation of 1 mol of sulfhydryl group (Fig. 2). The expected stoichiometry for oxidation of cysteine to cysteic acid is 2.0 mol of KMnO$_4$. Subsequent experiments showed that the time of reaction with KMnO$_4$ in these experiments (1 hour) was not quite sufficient for complete reaction, so that failure to observe complete oxidation and inactivation at a molar ratio of KMnO$_4$ per sulfhydryl of 2.0 is probably a consequence of incomplete reaction. The permanganate ion is implicated as the inactivating species, because incubation of the enzyme with MnO$_2$ or Mn$^{2+}$ did not inactivate the enzyme. The KMnO$_4$-oxidized enzyme was not reactivated by incubation with 2-mercaptoethanol or NaBH$_4$. All of these observations suggest that KMnO$_4$ oxidizes a single cysteinyI residue to a cysteic acid residue. This conclusion was confirmed by amino acid analysis of KMnO$_4$-oxidized native enzyme and KMnO$_4$-oxidized (TNB)$_2$-E (Table III). Analytical data are shown for amino acid residues identified by Benisek (10) as sensitive to KMnO$_4$ oxidation, but no significant alterations in the content of any of the other amino acids were observed. All of the reaction of KMnO$_4$ is accounted for by oxidation of cysteine to cysteic acid. In particular, (TNB)$_2$-E oxidized with KMnO$_4$ contains only one cysteic acid per subunit and a normal content of other amino acids.$^5$

$^5$ The possibility that methionine residues were oxidized to methionine sulfoxide and converted back to methionine during acid hydrolysis is not excluded by the amino acid analyses. How-

\[ \text{Moles KMnO}_4/\text{Mole (TNB)}_2-\text{E Subunit} \]

Table III

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Native enzyme</th>
<th>KMnO$_4$-treated native enzyme</th>
<th>(TNB)$_2$-E</th>
<th>KMnO$_4$-treated (TNB)$_2$-E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteic acid</td>
<td>0.1</td>
<td>3.5</td>
<td>0.0</td>
<td>0.8</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>3.3</td>
<td>0.0</td>
<td>0.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>7.0</td>
<td>7.5</td>
<td>7.1</td>
<td>7.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.6</td>
<td>3.4</td>
<td>3.5</td>
<td>3.4</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.1</td>
<td>3.2</td>
<td>3.2</td>
<td>3.0</td>
</tr>
</tbody>
</table>

$^a$ Sensitive amino acids are those which react rapidly with KMnO$_4$ when present as N-acetyl amide compounds (10).

Comparison of Reactivity of (TNB)$_2$-E with KMnO$_4$ and with Other Sulfhydryl-derivating Reagents—Although the rate of reaction of the remaining sulfhydryl group of (TNB)$_2$-E in 0.01 M phosphate, pH 7.5, with KMnO$_4$ at 0$^\circ$ is slower than the comparable oxidation of 2-mercaptoethanol (10), it is much faster than the rate of reaction of the same sulfhydryl group with pHB or $N$-ethylmaleimide at room temperature. The latter reagents will react quickly with three cysteinyI residues per subunit of native enzyme, but only slowly with the remaining cysteine. At 25$^\circ$, using a 10-fold excess of reagent, 20 to 25% of the ever, since the quantity of KMnO$_4$ added would have been almost entirely consumed by oxidation of cysteinyI residues to cysteic acid residues, it is unlikely that stoichiometric oxidation of any other residue could have occurred.
strate or analogue in 0.05 M phosphate, pH 7.5, were treated with 0.19 mM KMnO₄ at 0°C. Prior to starting the reaction a sample was taken, and were rapidly mixed with 5 μl of 0.1 M 2-mercaptoethanol and 485 μl of enzyme diluent. The quenched reaction samples were assayed for PRPP synthetase activity.

In subsequent experiments in which second order rate constants are reported, only data describing the fast (0 to 60% reaction) phase of the oxidation were analyzed. Such constants determined in the presence of substrates or substrate analogues strongly protected against inactivation by KMnO₄ (Fig. 3). This suggests that the essential sulphydryl group is protected from reaction when the active site is occupied.

An attempt to analyze the kinetics of the reaction revealed that a second order plot is linear for only about 60% reaction (Fig. 3, inset). This biphasic behavior has been observed at 13°C and 23°C as well as at 0°C, and at different enzyme concentrations. The break in the plot occurred in all cases after 00 ± 6% reaction.

The cause of this anomaly is unknown. It could be due to an inherent asymmetry in the arrangement of subunits of PRPP synthetase, to the presence of more than one state of aggregation already, or to utilization of KMnO₄ in unknown side reactions.

In some experiments in which second order rate constants are reported, only data describing the fast (0 to 60% reaction) phase of the oxidation were analyzed. Such constants determined from Fig. 3 indicate a 30-fold decrease in the rate of reaction of (TNB)₃-E with KMnO₄ when substrates are present (Table IV).

Increasing the relative Pi/KMnO₄ ratio brought about a very marked inhibition of the oxidation of (TNB)₃-E, indicating a competition between these species for the reactive site (Table V).

Concentrations of Pi below 5 mM could not be investigated because the enzyme is much less stable under such conditions (12).

The competition between KMnO₄ and Pi suggests that the selectivity of KMnO₄ oxidation may be due to its acting in an active site-directed manner. Within the somewhat limited range examined, the rate of inactivation was inversely proportional to the Pi concentration to the 2.7th power. This unusual result suggests that more than one Pi ion per subunit may be involved in protection of the essential sulphydryl group.

Reaction of Native PRPP Synthetase with KMnO₄—The relative reactivity of the four sulphydryl groups of native PRPP synthetase toward KMnO₄ was probed in experiments in which limiting quantities of KMnO₄ were allowed to react with enzyme. After incubation for 1½ hours at 0°C, the number of sulphydryl groups oxidized was determined by DTNB titration, and the remaining enzyme activity was assayed. As shown in Fig. 4, when the enzyme is protected with ATP and 50 mM Pi, nearly 3 eq of KMnO₄ are consumed (corresponding to oxidation of slightly more than two sulphydryl groups under these conditions) before substantial activity is lost. The very small loss of activity at lower concentrations of KMnO₄ presumably corresponds to the 15% activity lost on treatment with many other reagents. However, when the active site is not protected with ATP and Pi, activity is lost at much lower ratios of KMnO₄ to enzyme. In

![Fig. 3. Effects of substrates and substrate analogues on the rate of inactivation of (TNB)₃-E by KMnO₄. Enzyme solutions 0.076 mM in subunits, containing the indicated concentration of substrate or analogue in 0.05 M phosphate, pH 7.5, were treated with 0.19 mM KMnO₄ at 0°C. Prior to starting the reaction a sample was removed for determination of initial specific activity. After addition of KMnO₄, 10-μl samples were taken at the time intervals indicated and were rapidly mixed with 5 μl of 0.1 M 2-mercaptoethanol and 485 μl of enzyme diluent. The quenched reaction samples were then assayed for enzymatic activity. ○, No additions; ●, 5 mM MgCl₂; □, 3 mM ATP plus 5 mM MgCl₂; ■, 3 mM α,β-methylene-ATP, 5 mM ribose-5-P, and 5 mM MgCl₂. Inset, a plot of the inactivation reaction (in phosphate buffer only) treated as a second order reaction where the integrated rate equation is

\[ \frac{1}{2A - B} \ln \frac{B(A - X)}{A(B - 2X)} = kd. \]

A is the initial concentration of enzyme sulphydryl groups, B is the initial concentration of KMnO₄, and X is the amount of enzyme oxidized.

remaining sulphydryl groups per subunit have reacted after 1½ hours. A corresponding loss of activity was also observed. With 2.5 mol of KMnO₄/mol of (TNB)₃-E subunit (0.024 mM), 60% of the sulphydryl groups were oxidized at 0°C in the same amount of time; at 0°C (TNB)₃-E did not react with pHMB or N-ethyl maleimide to a detectable extent after several hours. DTNB did not react at all with the remaining sulphydryl group of (TNB)₃-E at room temperature over a period of 6 hours. The much greater reactivity of the remaining sulphydryl of (TNB)₃-E with KMnO₄ as compared to N-ethylmaleimide, pHMB, or DTNB could reflect either the smaller size of KMnO₄ or special affinity of this site on the enzyme for the permanganate ion.

Effects of Substrates and Pi on Rate of Inactivation of (TNB)₃-E by KMnO₄—The rate of inactivation of 0.08 mM (TNB)₃-E with 0.20 mM KMnO₄ was sufficiently slow at 0°C to be followed by quenching samples and assaying for PRPP synthetase activity. The reaction rate was not altered when 5 mM Mg²⁺ was included, but 3 mM MgATP³⁻ or a mixture of 5 mM Mg²⁺, 3 mM α,β-

**Table IV**

<table>
<thead>
<tr>
<th>Substrate or analogue</th>
<th>Apparent k²*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.25 ± 0.15</td>
</tr>
<tr>
<td>3 mM ATP, 5 mM MgCl₂</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>3 mM α,β-methylene-ATP, 5 mM MgCl₂, 5 mM ribose-5-phosphate</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>5 mM MgCl₂</td>
<td>3.14 ± 0.13</td>
</tr>
</tbody>
</table>

*Conditions are described in Fig. 4. k² is the initial slope of the biphasic second order plot of the data shown in Fig. 3 for 0 to 60% reaction. Uncertainty is expressed as mean deviation.
from the fact that enzyme activity is destroyed at lower KMnO₄ reactivity of all the sulfhydryl groups. This conclusion follows under these conditions the reactivity of the sulfhydryl group near the active site appears to be greater than the average reactivity of all the sulfhydryl groups. One class consists of three cysteinyl residues per subunit which are relatively reactive. Their modification by various sulfhydryl reagents does not bring about major catalytic or structural changes in the enzyme, so that it is unlikely that these residues have important roles at the active site or governing tertiary or quaternary structure. The second class is made up of a single remaining cysteine per subunit, which is quite unreactive in the native enzyme. Modification of this residue results in loss of activity and major structural changes in the enzyme. Clearly, the results do not establish that this sulfhydryl group participates in the PRPP synthetase reaction, because a less drastic modification might permit catalytic activity to survive, as has been shown for other enzymes (24). The ability of ATP to protect the enzyme and the failure of KMnO₄-oxidized enzyme to bind ATP tightly suggest, however, that this sulfhydryl group may be near the active site.

**DISCUSSION**

Evidence presented in this paper demonstrates that PRPP synthetase from *Salmonella typhimurium* contains two classes of sulfhydryl groups. One class consists of three cysteinyl residues per subunit which are relatively reactive. Their modification by various sulfhydryl reagents does not bring about major catalytic or structural changes in the enzyme, so that it is unlikely that these residues have important roles at the active site or governing tertiary or quaternary structure. The second class is made up of a single remaining cysteine per subunit, which is quite unreactive in the native enzyme. Modification of this residue results in loss of activity and major structural changes in the enzyme. Clearly, the results do not establish that this sulfhydryl group participates in the PRPP synthetase reaction, because a less drastic modification might permit catalytic activity to survive, as has been shown for other enzymes (24). The ability of ATP to protect the enzyme and the failure of KMnO₄-oxidized enzyme to bind ATP tightly suggest, however, that this sulfhydryl may be near the active site.

Several observations indicate that the reaction of KMnO₄ with the essential sulfhydryl of PRPP synthetase proceeds at least in part by virtue of an interaction of this phosphate analogue with a specific site on the enzyme. The reaction was quite specific for a sulfhydryl group when low, stoichiometric amounts of KMnO₄ were used, even though other reactive amino acid residues are present. The affected sulfhydryl is much less reactive.

**TABLE V**

*The effect of Pi on rate of inactivation of (TNB)₃-E with KMnO₄.*

Enzyme solutions (0.008 mM in subunit) were treated with stoichiometric amounts of KMnO₄ (0.020 mM) at 0°. The concentration of potassium phosphate buffer varied from 5 to 25 mM.

<table>
<thead>
<tr>
<th>Moles of Pi/mole of KMnO₄</th>
<th>Apparent $k_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{mole}^{-1}\text{min}^{-1}$</td>
</tr>
<tr>
<td>260</td>
<td>3.0 ± 0.4</td>
</tr>
<tr>
<td>640</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>890</td>
<td>0.11 ± 0.02</td>
</tr>
<tr>
<td>1250</td>
<td>0.03 ± 0.01</td>
</tr>
</tbody>
</table>

**Fig. 4.** Titration of native PRPP synthetase with KMnO₄. To 100-μl portions of a 1.4 mg/ml solution of enzyme was added 10 μl of aqueous KMnO₄ solutions containing 9 to 9.0 mol of KMnO₄/mol of enzyme subunit. Reaction time was 1½ hours at 0°. Specific activity and thiol content were determined as described under "Materials and Methods." Enzyme reacted in 0.05 M phosphate, 2 mM ATP, and 5 mM MgCl₂, pH 7.5. □, fraction initial specific activity; △, sulfhydryl content. Enzyme reacted in 0.01 M phosphate, pH 7.5, is denoted by ■ (fraction initial specific activity) and ○ (sulfhydryl content).

**TABLE VI**

*Specific activity values for derivatives of PRPP synthetase.*

Enzyme (0.5 to 1 mg/ml in 0.05 M phosphate, pH 7.5) was sedimented into D₂O.

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>Range of $s_{20,	ext{w}}$ observed (Svedberg units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native enzyme</td>
<td>9-13*</td>
</tr>
<tr>
<td>(TNB)₃-E</td>
<td>10-11</td>
</tr>
<tr>
<td>KMnO₄-treated native enzyme</td>
<td>16-18b</td>
</tr>
<tr>
<td>KMnO₄-treated (TNB)₃-E</td>
<td>15-17b</td>
</tr>
<tr>
<td>KMnO₄-treated (TNB)₃-E, dialyzed to remove P_i</td>
<td>3*</td>
</tr>
</tbody>
</table>

$^a$ K. R. Schubert, unpublished data.

$^b$ Refers to average $s_{20,	ext{w}}$ values of major component; patterns clearly show multiple sedimenting species.

The effect of Pi on rate of inactivation of (TNB)₃-E with KMnO₄...
with a variety of other sulfhydryl-derivatizing reagents than the other sulfhydryl groups of the enzyme. The reaction of (TNB)\textsubscript{3}-E with KMnO\textsubscript{4} was strongly inhibited by MgATP\textsuperscript{--} or Pi. Finally, the unprotected sulfhydryl group was shown to compete effectively with the other sulfhydryl groups when the native enzyme reacted with limiting amounts of KMnO\textsubscript{4}.

Kinetic studies of the effects of Pi on PRPP synthetase have suggested the presence of two sites of Pi action (1). Low concentrations of Pi (0 to 5 mM) are essential for activity and structural integrity of the enzyme. Higher concentrations (5 to 100 mM) stimulate PRPP synthesis but inhibit the reverse reaction and increase the apparent Michaelis constants of substrates, especially ATP. Some of these effects may well be due to secondary effects of Pi at the active site of the enzyme. The evidence available in this work suggests that KMnO\textsubscript{4} is acting at these low affinity sites, rather than the high affinity (0 to 5 mM) sites. All of our experiments were performed at Pi concentrations above 5 mM (because of the extreme instability of the enzyme below 5 mM Pi); in fact, most experiments were conducted in 50 mM Pi. Thus, the high affinity sites may well have been protected. That this is the case is indicated by the observation that removal of Pi by dialysis results in further changes in aggregation in KMnO\textsubscript{4}-oxidized (TNB)\textsubscript{3}-E. The dependence of the Pi protection on the Pi concentration raised to the 2.7th power suggests that as many as three Pi anions may associate with the active site to protect it against KMnO\textsubscript{4}. Finally, it should be noted that even in 0.05 M Pi, ATP provided marked protection against KMnO\textsubscript{4} oxidation of the enzyme. All of these observations suggest that the site of oxidation is one to which Pi binds with low affinity, and that it is near the active site. If this interpretation is correct, our findings would indicate that KMnO\textsubscript{4} may be used as an active site-directed reagent not only for proteins that bind Pi, but also for enzymes that bind nucleotides. We suggest that this reagent has a much broader range of uses than has been generally recognized.

It is interesting to note that PRPP synthetases from higher animals are extremely sensitive to sulfhydryl reagents (25-27). Bacterial PRPP synthetase has appeared to be different from the animal enzymes, because it is not stimulated by thiols, nor readily inhibited with sulfhydryl-derivatizing reagents. Now it is clear that the various PRPP synthetases all possess essential sulfhydryl groups, but differ markedly in the reactivity of such groups.

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