Vit C·Fe(III) Induced Loss of the Covalently Bound Phosphate and Enzyme Activity of Phosphoglucomutase*

Vasanti V. Deshpande and Jayant G. Joshi†
From the Department of Biochemistry, University of Tennessee, Knoxville, Tennessee 37916

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Rabbit muscle phosphoglucomutase was irreversibly inactivated upon preincubation with vitamin C (Vit C)·Fe(III), NADH·NADH oxidase·Fe(III), or ferritin·Vit C. Substrate, glucose 1-phosphate and Mg2⁺ afforded partial protection. No altered amino acid could be detected in the inactive enzyme. Enzyme so inactivated was more susceptible to trypsin. More importantly, during inactivation, the enzyme lost up to 70% of its enzyme-bound phosphate; the completely inactivated enzyme retained the remainder of the bound phosphate which was isolatable as phosphoserine residing in the 22-amino acid long tryptic peptide.

Free phosphoserine as well as those in phosphorylase a and phosphocasein were resistant to the oxidizing system, suggesting that the phosphoserine of phosphoglucomutase is uniquely vulnerable to these treatments. Alternatively, a fraction of the total 1 mol of phosphate in the phosphoform of phosphoglucomutase may not be associated with phosphoserine.

Phosphoglyceromutase, which has phosphohistidine at its active site, was also inactivated by the oxidizing system. However, it did not release any of the bound phosphate.

The function of a cellular protein is controlled by the rates of its synthesis and degradation as well as modification of the synthesized protein. Covalent modifications of specific amino acid residues of proteins are known to alter their functions. These modifications could be reversible or irreversible. They include phosphorylation (1), methylation (2), adenylylation (3), uridylylation (4), ADP-ribosylation (5), etc. or oxidative alteration of an amino acid residue. Stadtman et al. (6) suggested that oxidative modifications also determine the half-life of a protein. Using glutamine synthetase from Escherichia coli as a model, the following interesting observations were made. (i) A unique histidine residue in each of the 12 subunits of this enzyme was susceptible to oxidation by the NADH·NADH oxidase system, the cytochrome P-450 system, or O₂ vitamin C, and Fe(III). (ii) Histidine in the adenylylated protein was more susceptible to such modifications. (iii) The modification irreversibly inactivated the enzyme. (iv) In addition to glutamine synthetase, all other enzymes which were susceptible to such a modification required a divalent metal ion and had a histidine residue at the active site (7–10). This led to the suggestion that in vivo such a covalent modification of an enzyme "marks" the molecule for subsequent proteolysis and contributes to maintaining a dynamic equilibrium of protein levels. In the aging cells, the regulation between the marking process and proteolysis is disturbed, giving rise to elevated levels of inactive proteins as determined by their antibody reactivity (7). Recently, Shinar et al. (11) reported the inactivation of the membrane-bound acetylcholine esterase caused by superoxide in the presence of copper as an explanation for the biochemical damage of proteins.

Our attempts to seek regulatory properties of phosphoglucomutase have been unsuccessful. Indeed, of all the compounds tested, only citrate inhibited the enzyme from E. coli; Mg(II) reversed the inhibition (12). The observed inhibition of rabbit muscle phosphoglucomutase by lithium salts of several nucleotides (13) was found to be due to the lithium rather than the nucleotide moiety (14). Insulin seemed to increase the activity of the enzyme in vivo (15–17). This increase was suggested to be due to the conversion of a less-active form of the enzyme, the E·Zn complex (0.3% active), to the fully active form of the enzyme, E·Mg complex (100% active), without any net increase in the number of enzyme molecules (17). Phosphoglucomutase from diverse origin such as E. coli, yeast, human muscle, flounder muscle, and rabbit muscle is a single polypeptide chain, requires a divalent metal ion for activity, and contains a unique serine residue (15) which apparently undergoes alternate phosphorylation and dephosphorylation during catalysis (18). The amino acid sequence of the pentapeptide around the active serine is Thr-Ala-Ser-P-His-Asp– (19). Therefore this enzyme appeared well-suited for the extension of the studies made with the other enzyme (7–10).

The present study shows that phosphoglucomutase from rabbit muscle is completely and irreversibly inactivated by either Fe(III) or ferritin in the presence of vitamin C or by the NADH·NADH oxidase system. No evidence for the modified histidine could be obtained. Substrate, Mg2⁺, or catalase afforded partial protection. Inactive phosphoglucomutase is more susceptible to trypsin than its active counterpart. Most significantly, the completely inactivated enzyme had lost only about half of its covalently bound phosphate, suggestive of the possibility of two phosphate-binding sites even on phosphoform of phosphoglucomutase.

EXPERIMENTAL PROCEDURES

Materials

Glucose 1-phosphate, glucose 1,6-diphosphate, glucose-6-phosphate dehydrogenase, NADP, NADH, 3-phospho-L-glyceric acid, 2,3-diphosphoglyceric acid, enolase, catalase, O-phospho-L-serine, horse spleen ferritin, phosphoglyceromutase, phosphohistidine a, a-casein, 5,5'-dithiobis(2-nitrobenzoic acid), and 2,4-dinitrophenylhydrazine were purchased from Sigma. Trypsin t-1-tosylamido-2-phenylthethyl chloromethyl ketone was obtained from Worthington. H₃²³PO₄ (car-

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† To whom all inquiries may be addressed.
Phosphoglucomutase from rabbit muscle was isolated and assayed by a coupled assay according to Joshi et al. (20). The enzyme had a specific activity of at least 500. Phosphoglucomutase was assayed by coupling to enolase and determining the absorption at 240 nm due to phosphohexosepyruvate (21). The protein concentration of phosphoglucomutase was measured spectrophotometrically, \( \varepsilon_{280} = 0.70 \) (22). Inorganic phosphate in the protein samples digested with \( \text{H}_2\text{SO}_4 \) was determined by the method of Barlett (23). Horse spleen ferritin was purified as described previously (24). Radioactivity of liquid samples was measured by using Bray's solution (Research Products International Corp.) with Beckman liquid scintillation counter.

**Methods**

Phosphoglucomutase requires a divalent metal ion for activity. It binds a maximum of 1 g atom of a metal ion. The \( E\cdot\text{Mg} \) form is most active, \( E\cdot\text{Zn} \) form is 0.3% as active, and complexes with other metal ions show varying activities. The enzyme is invariably contaminated with metal ions. Thus, the enzyme is routinely preactivated by preincubation at pH 7.4 with 0.1 mM EDTA and assayed in the presence of excess Mg(II). A nonprofessional enzyme shows progressively increasing reaction rates which parallel the replacement of inhibitory metal ion by Mg(II) present in the assay mixture.

Preliminary experiments showed that the rate of inactivation by the oxidizing systems employed here of the metal-free phosphoglucomutase was more reproducible in the presence of EDTA and were similar to that of the metal-contaminated enzyme in the presence of EDTA. Thus, for subsequent studies, the enzyme as isolated was used in the presence of 0.1 mM EDTA. Accordingly, for inactivation by \( \text{Vit C} \cdot \text{Fe(III)} \), 0.4 units of purified phosphoglucomutase was incubated at 25°C in 40 mM Tris-\( \text{HCl} \) buffer, pH 7.4, with 15 mM vitamin C, 0.1 mM EDTA, and 0.2 mM FeCl₃ in a total volume of 100 \( \mu \)l. At timed intervals, 10-\( \mu \)l samples were taken directly into the assay mixture and assayed immediately. Inactivation by ferritin and vitamin C was also carried out as above except that the concentration of vitamin C in the incubation mixture was 105 mM and FeCl₃ was substituted by ferritin (0.5 mg/ml corresponding to 0.2 mM Fe(III)). While studying the inactivation of phosphoglucomutase by NADH-NAD oxidase, the incubation mixture of 100 \( \mu \)l contained 0.4 units of phosphoglucomutase, 0.1 mM EDTA, 0.2 mM FeCl₃, 2 mM NADH, and 3.2 units of NADH oxidase.

\(^32\text{P}\)-labeled phosphoglucomutase was prepared using potato phosphophorylase and carrier-free \( \text{H}_3\text{PO}_4 \) as described by Miletin and Sandstrom (26). The labeled enzyme was used in a phosphate-coupled assay. The specific activity of at least 500. Phosphoglucomutase was assayed in the presence of 0.1 mM EDTA, 1 mM MgCl₂, and 40 mM Tris-\( \text{HCl} \) buffer, pH 7.4. The enzyme was precipitated with ammonium sulfate as before, dissolved in buffer and dialyzed as above and the amount of \( ^{14}\text{C} \) was determined.

**Isolation of the Active-site Peptide from Vit C \( \cdot \) Fe(III)-treated \(^{32}\text{P}\) Phosphoglucomutase**—4 mg of phosphoglucomutase was incubated at room temperature for 30 min in a final volume of 1 ml with 0.1 \( \mu \)mol each of unlabelled and \([\text{U}^\text{32}\text{P}]\) glucose 1-phosphate, 2 \( \mu \)mol of glucose 1,6-diphosphate, 0.1 mM EDTA, 1 mM MgCl₂, and 40 mM Tris-\( \text{HCl} \) buffer, pH 7.4. The enzyme was precipitated with ammonium sulfate as before, dissolved in buffer and dialyzed as above and the amount of \( ^{32}\text{P} \) was determined.

**Detection of Serine, Phosphoserine, and Other Phosphocompounds**—After the chromatography, the dried paper was sprayed with 0.2% ninhydrin in 95% ethanol and dried again in a current of hot air. Serine and phosphoserine appeared as purple spots. Inorganic phosphate, pyrophosphate, glucose 6-phosphate, and glucose 1,6-diphosphate were located by spraying the dried paper with FeCl₃ (5 mg/ml in 80% ethanol) followed by drying and spraying with sulfosalicylic acid (1% in 80% ethanol). The background is stained pink while the phosphate spots remain white. Subsequently the paper was cut into 1 x 7.5-cm strips and counted for radioactivity.

**Gel Electrophoresis**—The gel electrophoresis was carried out in 7% acrylamide gels at pH 8.3 according to Davis (26).

**Binding of Glucose Phosphates to Phosphoglucomutase**—Fe(III) inducement was carried out at room temperature for 30 min in a final volume of 1 ml with 1 \( \mu \)mol each of unlabelled and \([\text{U}^\text{32}\text{P}]\) glucose 1-phosphate and \([\text{U}^\text{32}\text{P}]\) glucose 6-phosphate. The enzyme was inactivated by treatment with vitamin C and FeCl₃ and the excess reagents were dialyzed. The incubation was initiated by adding 1 \( \mu \)mol of this inactive enzyme (0.1 \( \mu \)mol) to 5 \( \mu \)l of the reagents and the mixture was incubated for 30 min at room temperature.

**Isolation of Dehydrogenases**—The enzyme was precipitated with inactivating reagents at 24°C, and the amino acid composition of the hydrolysate was determined.

**Estimation of 2,4-Dinitrophenyl Derivative of Phosphoglucomutase**—The reaction was prepared by mixing 9 volumes of 8 M guanidine in 26.7 mM potassium phosphate, pH 6.83, with 1 volume of 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl (28). Phosphoglucomutase was inactivated by treatment with vitamin C and FeCl₃ and the excess reagents were dialyzed. The reaction was initiated by adding 1 \( \mu \)mol of the inactive enzyme (0.1 \( \mu \)mol) to 5 \( \mu \)l of the reagents and the mixture was incubated for 30 min at room temperature.

**RESULTS**

Preliminary experiments showed that glucose-6-phosphate dehydrogenase is not inactivated by 2 \( \mu \)M Fe(III) and 15 \( \mu \)M vitamin C. These were the concentrations during phosphoglucomutase assay. This permitted the use of coupled assay for phosphoglucomutase.

**Inactivation of Phosphoglucomutase**

Incubation of enzyme with vitamin C and Fe(III) or with NADH-NAD oxidase inactivated the enzyme within 10 min (Fig. 1). Under similar conditions, vitamin C, FeCl₃, NADH, or NAD oxidase when present alone did not cause any significant decrease in activity even after 30 min. FeSO₄ (0.2 mM) alone produced less inhibition than did equivalent amount of FeCl₃ along with vitamin C (Fig. 1). Ferritin stores iron as Fe(III). It is released as Fe(II) whenever required. The mechanism of this reduction and release in vivo is yet unclear. However, in vitro vitamin C at high concentrations is able to release Fe(II) from ferritin (32) and, as expected, ferritin together with vitamin C inactivated phosphoglucomutase. The concentration of vitamin C required was much higher.
(105 mM) and the rate of inactivation was much slower than the Vit C·Fe(III) and NADH·NADH oxidase systems (Fig. 2). As expected, apoferritin together with vitamin C did not inhibit the enzyme. Phosphoglucomutase purified from yeast (33) and E. coli (34) was also inactivated by Vit C·Fe(III) (data not shown). Dialysis of Vit C·Fe(III)-inactivated enzyme against 1000 volumes of buffer did not result in any recovery of the enzyme activity, but about 10% of the activity could be recovered by dialysis of the enzyme which was inactivated by the NADH·NADPH oxidase system.

Fig. 3 shows the effect of the presence of substrate and Mg$^{2+}$ in the incubation mixture containing the inactivation agents and the enzyme. Both glucose 1-phosphate and Mg$^{2+}$ (2 mM each) afforded partial protection for the enzyme inactivation. The result was more pronounced when they were added together. Cysteine, histidine, and catalase partially protect the enzyme against the oxidative inactivation by Vit C·Fe(III), whereas histidine and mannitol protected, to some extent, the inactivation by NADH·NADH oxidase (Table I).

Since it was easier to dialyze out the excess of inactivating agents in the case of the Vit C·Fe(III) system, this system was used as a model inactivating system in most experiments. The term "modified enzyme" refers to the phosphoglucomutase from rabbit muscle treated with Vit C·Fe(III), the "oxidizing system."

**Attempts to Detect Altered Histidine in the Modified Phosphoglucomutase**

Total amino acid composition of the native and the modified enzyme was, within the experimental error, identical (data not shown). Rabbit muscle phosphoglucomutase contains ten histidine residues (20), and a reduction of a single residue is likely to escape detection by this technique. Therefore, two additional approaches were used to detect modification of histidine.

**Reaction with 2,4-Dinitrophenylhydrazine**—The oxidative modification of glutamine synthetase leads to an introduction of a carbonyl group in histidine which reacts with 2,4-dinitrophenylhydrazine to yield a dinitrophenylhydrazone derivative of enzyme (9). When modified phosphoglucomutase was treated with 2,4-dinitrophenylhydrazine, no phenylhydrazone could be detected.

**Amino Acid Analysis of the Tryptic Peptide**—In order to determine whether treatment of phosphoglucomutase with Vit C·Fe(III) could lead to the modification of histidine or of any other amino acid in the vicinity of the phosphoserine at the active site, modified 32P-labeled enzyme was digested with trypsin and the 22-amino acid long tryptic peptide containing serine phosphate was isolated (27). The amino acid composition of this peptide showed no deviation from the similar peptide isolated from the native enzyme (27).

**Table I**

<table>
<thead>
<tr>
<th>Compound added to the system</th>
<th>Concentration</th>
<th>% inactivation of phosphoglucomutase by mixed function oxidation systems</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>/</td>
</tr>
<tr>
<td>Cysteine</td>
<td>$1 \times 10^{-3}$ M</td>
<td>52</td>
</tr>
<tr>
<td>Methionine</td>
<td>$1 \times 10^{-3}$ M</td>
<td>72</td>
</tr>
<tr>
<td>Histidine</td>
<td>$1 \times 10^{-3}$ M</td>
<td>60</td>
</tr>
<tr>
<td>Catalase</td>
<td>60 units/ml</td>
<td>54</td>
</tr>
<tr>
<td>Superoxide</td>
<td>60 units/ml</td>
<td>72</td>
</tr>
<tr>
<td>Mannitol</td>
<td>$2.5 \times 10^{-3}$ M</td>
<td>70</td>
</tr>
</tbody>
</table>

**Determination of Sulphydryl Groups**

1.14 mg each of the native and modified enzyme in 0.5 ml of 0.05 M K-phosphate buffer, pH 7.5, were treated with 10 μl of 0.01 M 5,5′-dithiobis-(2-nitrobenzoic acid). The change in
optical density after 2 h of incubation at room temperature corresponded with 4.4 mol of —SH groups/mol of native enzyme and 3.3 mol of —SH groups/mol of modified enzyme.

Thus, inactivation of phosphoglucomutase by the oxidizing system leads to the loss of 1 eq sulfhydryl group/mol of the enzyme.

Inactivation Accompanied by the Partial Loss of Protein-bound $^{32}$P

During the inactivation experiments it was observed that the modified $[^{32}P]$phosphoglucomutase upon dialysis lost a significant amount of its covalently bound phosphate. The complete loss of catalytic activity, but only a partial loss of radioactivity, implied two types of phosphate binding site in phosphoglucomutase. This was unexpected and needed to be confirmed.

Accordingly, a $[^{32}P]$phosphoglucomutase was prepared as described above and the excess radioactivity was removed by repeated precipitations with ammonium sulfate followed by exhaustive dialysis (see “Experimental Procedures”) and the phosphate content of the enzyme was determined. A $[^{32}P]$phosphoglucomutase containing 0.98 mol of phosphate/mol of enzyme ($M_r = 62,000$) was treated in triplicate with Vit C-$\text{Fe(III)}$, dialyzed, and assayed for enzyme activity, radioactivity, and the protein content. In each instance the modified enzyme was devoid of catalytic activity and contained 0.4 to 0.6 mol of phosphate/mol of enzyme. It was essential to rule out the possibility of the bound carbohydrate because it has been shown that a significant amount of glucose 1,6-diphosphate bound to the enzyme survives precipitation with acetone, acid, or treatments with urea followed by exhaustive dialysis (35).

The radioactivity lost during modification of phosphoglucomutase is dialyzable and could be filtered through PM-10 filter (Table IIb). For its identification, an aliquot of $[^{32}P]$phosphoglucomutase was exposed to Vit C-$\text{Fe(III)}$ for 4 h rather than 30 min. Aliquots of this completely inactive enzyme as well as the untreated control were electrophoresed in duplicates in polyacrylamide gels and stained for protein and radioautographed to detect the distribution of phosphate. The results showed that the native enzyme had a single band for protein which also contained 95% of the radioactivity. By contrast, the oxidized enzyme which also had a single band for protein had only 37% of the total radioactivity associated with it; the remainder migrated as orthophosphate. The above results were confirmed by paper chromatography (Fig. 4). The solvent system employed for paper chromatography retained the native enzyme at the origin and also distinguished between free orthophosphate, phosphoserine, glucose phosphates, and glucose 1,6-diphosphate. None of these except orthophosphate were detected in the oxidized enzyme.

In a separate series, aliquots of $[^{32}P]$phosphoglucomutase were treated with NADH-NADH oxidase which is probably more akin to physiological environment than the Vit C-$\text{Fe(III)}$ system. The aliquots of the incubation mixture were assayed for enzyme activity, and after paper chromatography the distribution of radioactivity was determined (Table III). The results show that although exposure of enzyme to different constituents of the oxidizing system does release some protein-bound phosphate as orthophosphate, the complete system was most effective, liberating up to 63% of the total radioactivity.

Fig. 5 shows that release of orthophosphate did not parallel the loss of catalytic activity. In these studies the concentration of the enzyme used was more than that used for the data in Fig. 1. This reduced the rate of inactivation and permitted withdrawal of aliquots for the quantitation of orthophosphate and enzyme activity. As seen, within 20 min of preincubation, although 50% of the $^{32}P$ was released, only 30% of the catalytic activity was lost. Thereafter the inactivation continued in a steady manner and at the end of 12 h the enzyme had no measurable activity and lost a total of about 70% of the bound phosphate. As expected, the dephosphoenzyme was more resistant to inactivation. The probable significance of these results is discussed later.

![Fig. 4. Identification by paper chromatography of $^{32}P$ released during inactivation. 50 µg of $[^{32}P]$phosphoglucomutase was incubated with 0.2 mM FeCl$_3$, 15 mM vitamin C, 0.1 mM EDTA, and 40 mM Tris-HCl, pH 7.4, in a total volume of 50 µl at room temperature for 4 h. 25 µl each of the untreated and treated enzyme were subjected to paper chromatography and the distribution of radioactivity was determined.](image-url)
TABLE III
Effect of different compounds on the release of orthophosphate from phosphoglucomutase

50 µg of [32P]phosphoglucomutase (1 × 10^4 cpm) was incubated for 8 h in 50 mM buffer, pH 7.4, in a total volume of 50 µl with the indicated components and 25-µl aliquot subjected to paper chromatography (see text).

<table>
<thead>
<tr>
<th>Addition</th>
<th>Radioactivity cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>At the origin</td>
</tr>
<tr>
<td>None</td>
<td>95</td>
</tr>
<tr>
<td>FeSO₄ (0.2 mM)</td>
<td>83</td>
</tr>
<tr>
<td>FeCl₃ (0.2 mM)</td>
<td>84</td>
</tr>
<tr>
<td>Vitamin C (15 mM)</td>
<td>83</td>
</tr>
<tr>
<td>FeCl₃ (0.2 mM) + vitamin C (15 mM)</td>
<td>37</td>
</tr>
<tr>
<td>FeCl₃ (0.2 mM) + NADH (2 mM)</td>
<td>78</td>
</tr>
<tr>
<td>FeCl₃ (0.2 mM) + NADH (2 mM) + NADH oxidase</td>
<td>42</td>
</tr>
<tr>
<td>Zn²⁺ (5 × 10⁻⁴ M)</td>
<td>95</td>
</tr>
<tr>
<td>Zn²⁺ + FeCl₃ + vitamin C</td>
<td>95</td>
</tr>
<tr>
<td>Be²⁺ (5 × 10⁻⁵)</td>
<td>95</td>
</tr>
<tr>
<td>Be²⁺ + FeCl₃ + vitamin C</td>
<td>95</td>
</tr>
<tr>
<td>Urea (8 M)</td>
<td>95</td>
</tr>
</tbody>
</table>

Incubation of [32P]phosphoglucomutase with Zn²⁺ or Be²⁺ alone or followed by incubation with Vit C•Fe(II1) did not release any detectable amounts of orthophosphate as determined by the distribution of the radioactivity on chromatography of the treated samples (Table III). These results indicate that the preincubation of enzyme with these metals prevents the access of Vit C•Fe(II1) to the enzyme phosphate. However, only 54% of the enzyme activity was recovered by the dialysis of the enzyme treated with Zn²⁺ followed by Vit C•Fe(II1).

Proteolytic Degradation of Phosphoglucomutase

To determine the susceptibility of the modified enzyme to trypsin, 0.25 mg each of native and modified [32P]phosphoglucomutase were treated with trypsin at room temperature in the protein-to-trypsin ratio of 50:1 for 4 h at pH 7.4. At timed intervals, 50-µl aliquots of trypsin-treated control and modified enzyme were subjected to paper chromatography. The rate of release of radioactivity from the modified enzyme and its subsequent appearance in the peptide, formed due to proteolysis, is faster than the corresponding rate for the trypsin-treated control enzyme. Thus phosphoglucomutase, like glutamine synthetase of *E. coli* (8), upon oxidation becomes more susceptible to proteolysis. These results were also confirmed by electrophoresis of the identical aliquots of the trypsin-treated control and modified enzyme and by scanning the radioautograms with Beckman DU8 to determine the 32P distribution in the bands generated therein. The appearance of more radioactivity in the faster moving band in the modified enzyme indicates that the modified enzyme is more susceptible to trypsin than the native enzyme.

Treatment of Dephosphoenzyme with Vit C•Fe(II1)

Since the inactivation of [32P]phosphoglucomutase by the oxidizing systems was accompanied by the partial loss of the protein-bound phosphate it was desirable to determine the effect of this system on the dephospho form of the enzyme. The dephosphoenzyme, like the [32P]phosphoglucomutase, is inactivated by Vit C•Fe(II1) but the rate of inactivation of the dephosphoenzyme is much slower than that of the phosphoenzyme (Fig. 1). Thus this reagent not only releases the
phosphate from the phosphoenzyme but also slowly and steadily destroys either the site at which the phosphorylation can occur or modifies the enzyme in such a way the recycling of the enzymes becomes impossible.

Effect of the "Oxidizing System" on O-Phosphoserine, Phosphorylase a and a-Casein

The fact that even the control enzyme did release very small but detectable radioactivity suggested that perhaps all the orthophosphate released in the modified enzyme arose from its O-phosphoserine. To verify this possibility O-phosphoserine (10 pmol), phosphorylase a (0.15 mg, corresponding to 50 ng of phosphate), and a-casein (5 mg, corresponding to 13 μg of phosphate) were incubated with Vit C-Fe(III) for 4 h at room temperature followed by 20 h at 4°C. The aliquots of the incubation mixture were assayed for orthophosphate. In each instance no detectable orthophosphate was released. Furthermore, there was no inhibition of enzyme activity when 50 μg of phosphorylase a was incubated with 0.2 mM FeCl₃, 15 mM vitamin C, and 0.1 mM EDTA in 50 mM phosphate buffers at room temperature up to 40 min.

Inactivation of Phosphoglyceromutase by the Oxidizing System

Phosphoglyceromutase is a single polypeptide chain and has -phosphoserine-His- at the active site. By contrast, phosphoglyceromutase is a dimer and contains -Ser-phosphohistidine-on each of its subunits (36). To determine whether the latter enzyme loses its phosphate, 5 mg of [³²P]phosphoglyceromutase (490 cpm/mg of protein) was exposed to the oxidizing system and aliquots were assayed at timed intervals. The results showed that such a treatment caused over 80% loss of catalytic activity (Table IV) without any detectable loss of system and aliquots were assayed at timed intervals. The fact that even the control enzyme did release very small but detectable radioactivity suggested that perhaps all the orthophosphate released in the modified enzyme arose from its O-phosphoserine. To verify this possibility O-phosphoserine (10 pmol), phosphorylase a (0.15 mg, corresponding to 50 ng of phosphate), and a-casein (5 mg, corresponding to 13 μg of phosphate) were incubated with Vit C-Fe(III) for 4 h at room temperature followed by 20 h at 4°C. The aliquots of the incubation mixture were assayed for orthophosphate. In each instance no detectable orthophosphate was released. Furthermore, there was no inhibition of enzyme activity when 50 μg of phosphorylase a was incubated with 0.2 mM FeCl₃, 15 mM vitamin C, and 0.1 mM EDTA in 50 mM phosphate buffers at room temperature up to 40 min.

Discussion

The studies reported here began with a limited objective of extending the observations of Stadtman et al. (6) to the enzyme phosphoglyceromutase for which no regulatory factors have been defined unequivocally. The data resulting from such studies presented here appear significant for four reasons. 1) It extends the oxidatively induced inactivation of enzymes observed for other enzymes to phosphoglyceromutase and phosphoglyceromutase. 2) It suggests that this oxidative modification could be of some amino acid other than histidine. 3) Complete and irreversible loss of catalytic activity but only a partial loss of the bound phosphate of phosphoglyceromutase reveals a new mode of oxidative modification. 4) Inactivation of its 0-phosphoserine. To verify this possibility O-phosphoserine (10 pmol), phosphorylase a (0.15 mg, corresponding to 50 ng of phosphate), and a-casein (5 mg, corresponding to 13 μg of phosphate) were incubated with Vit C-Fe(III) for 4 h at room temperature followed by 20 h at 4°C. The aliquots of the incubation mixture were assayed for orthophosphate. In each instance no detectable orthophosphate was released. Furthermore, there was no inhibition of enzyme activity when 50 μg of phosphorylase a was incubated with 0.2 mM FeCl₃, 15 mM vitamin C, and 0.1 mM EDTA in 50 mM phosphate buffers at room temperature up to 40 min.

**Table IV**

**Inactivation of phosphoglyceromutase by Vit C-Fe(III)**

<table>
<thead>
<tr>
<th>Preincubation with</th>
<th>Activity after 20 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>FeCl₃ (0.2 mM)</td>
<td>110</td>
</tr>
<tr>
<td>Vitamin C (15 mM)</td>
<td>70</td>
</tr>
<tr>
<td>Vitamin C (15 mM) + FeCl₃</td>
<td>15</td>
</tr>
</tbody>
</table>

More recent studies from Dr. Stadtman’s laboratory (personal communication) show that the histidine which was lost during oxidative inactivation of glutamine synthetase is not converted to a carbonyl derivative. The carbonyl group generated in the oxidation of glutamine synthetase is associated with the oxidation of an as yet unidentified amino acid residue.

1. Additional information from our laboratory (personal communication) shows that the histidine residue is involved in the active site of the enzyme. The histidine residue is the only histidine residue in the protein that is susceptible to oxidation by the oxidizing system.

2. Apparently, histidine in the peptide chain is susceptible to the oxidizing system (9, 10). In our hands, free histidine was quite resistant to this reagent.
because sulfhydryls are not essential for the catalytic activity (41, 42).

Perhaps the most unexpected finding is only the partial loss of the enzyme-bound phosphate phosphate which occurred simultaneous to the complete loss of the catalytic activity. The relative rates of the two were significantly unequal, however. All of this phosphate is presumably attached to serine 116 (22). Simple hydrolytic removal of this phosphate should yield an equivalent amount of dephosphoenzyme which is inactive in the absence of glucose 1,6-diphosphate. Since the assay mixture contained the latter, full activity should be restored in milliseconds (43). The facts that the inactivation is irreversible and the dephosphoenzyme is also inactivated, albeit more slowly than the phosphoenzyme, suggest that the inactivation may involve more than a single amino acid residue. The data in Fig. 5 suggest that during the initial period of inactivation, the modification of the protein includes loss of phosphate and alteration of some other residue(s). After about 1 h, the modification at the site(s) other than serine 116 continues, making this phosphorylation site inaccessible to glucose 1,6-diphosphate for repHosphorylation, the event which is obligatory for enzyme activity. Here again, as expected, the dephosphoenzyme was inactivated more slowly than its phospho form. The rate of inactivation of the phosphoenzyme after it had ceased losing its phosphate and of dephosphoenzyme during similar time period were similar but not identical, the phosphoenzyme had retained about 15% of its activity after 18 h (data not shown). Several attempts were made to detect differences in the modified enzyme. None were successful. These included differences in the total amino acid composition, spectrophotometric detection of the modified histidine if any, finger print maps of the tryptic digest of the 32P-labeled enzyme, and comparative UV and visible difference spectroscopy of the native and modified enzyme alone and in the presence of added glucose 1-phosphate and Mg2+. Admittedly with the exception of the direct assay for sulfhydryl using 5,5'-dithiobis-(2-nitrobenzoic acid), none of the methods employed were sufficiently sensitive to establish unequivocal modification of a single residue.

Another explanation for the partial loss of enzyme-bound phosphate is to resurrect the concept of the second phosphate binding site probably implied by Jagannathan and Luck (44). Britton and Clark (45) proposed isoforms of the enzyme, E,P and E, P which are rapidly interconverted during each catalytic cycle of the enzyme. Ray and Roscelli (46) deduced that the existence of isoforms is probable but mechanistically unnecessary. Their subsequent elegant studies (47) indicated that indeed the dephosphoenzyme has two different phosphate binding sites, both of which are involved in forming a glucose diphosphate-enzyme complex,

\[
\begin{align*}
\text{E} & \quad \text{G} & \quad \text{P} \\
\space & \quad \text{6 or 1} & \quad \text{1 or 6}
\end{align*}
\]

where G represents glucose.

It is tempting to speculate that even the phosphoenzyme has two such sites, both of which are catalytically essential and at equilibrium with each other. Under such circumstances, loss of one site would produce an inactive species. In view of this we attempted anew the search for the second phosphate binding site, but found only the single site already discovered earlier by Milstein and Milstein (35). The amino acid composition of the tryptic peptide containing active serine in the modified enzyme was identical to that of the native enzyme. By hindsight this should be the case because the procedure for the isolation of this peptide depended upon monitoring labeled phosphoserine.

In this connection it is noteworthy that the recovery of the phosphoserine from the hydrolysates of [32P]phosphoglucomutase obtained by digestion with trypsin or acid has always been far less than theoretical despite the stability of free phosphoserine to such treatment. Thus Milstein and Milstein (35), who first carefully deduced the amino acid sequence of the phosphoserine containing tryptic peptide of phosphoglucomutase from rabbit muscle, observed that as much as 36% of the total radioactivity was separable as orthophosphate. They also clearly visualized the possibility of the migration of a phosphoryl group to other sites. Their cautious interpretation of the meticulous study and the complete loss of catalytic activity associated with only a partial loss of enzyme-bound phosphate reported here may further lend support to the second phosphate binding site even on the phosphoenzyme.

The release of phosphate appears to be related to the metal ion present. Thus Be2+ and Zn2+, which bind very tightly to this enzyme, also prevented the release of phosphate by Vit C-Fe(III), but their subsequent removal by chelating agent generated enzyme which had retained 54% of the original activity. This underscores the possibility that the observed inactivation of the enzyme is due to the modification at more than one site. Since the tryptic peptide maps of the native and modified enzymes were similar, identification of the modified residues entails either comparative study by diagonal electrophoresis or detailed sequence analysis and it is likely that, despite this, an altered residue may escape detection.

In summary, the data presented here clearly demonstrates that the oxidizing system is not specific to histidine residues as suspected earlier (9). The inactivation of phosphoglucomutase was also accomplished by using ferritin as a source of Fe(III) and vitamin C as the reductant. Although only one of the total of five sulfhydryls is susceptible to the oxidizing system, the loss of enzyme activity is not due to the loss of this amino acid because this enzyme does not require sulfhydryls for activity, and presence of cysteine during inactivation afforded only partial protection. If the orthophosphate released by the oxidizing system originated at serine 116, this phosphoserine at the active site of phosphoglucomutase appears uniquely vulnerable to the oxidizing system because, although it lost its phosphate, neither the free phosphoserine nor those contained in phosphorylase a nor phosphocasein were dephosphorylated. Despite these studies, the inactivation of phosphoglucomutase by this system remains obscure. Nevertheless, this report adds phosphoglyceromutase and phosphoglucomutase to the list of enzymes which are irreversibly inactivated in vitro by the oxidizing system described by Levine et al. (7). It is well-established that the biological activity of several phosphoproteins are different from their dephospho counterparts. The differences in the susceptibility of phospho and dephospho forms of phosphoglucomutase to the inactivating system reported here adds another refinement to the metabolic regulation.

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REFERENCES
Vit C-Fe(III) Induces Loss of Phosphate and Enzyme Activity