DENATURATION, RENATURATION AND OTHER STRUCTURAL
STUDIES ON PHOSPHOGLUCOSE ISOMERASES

THESIS

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By

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Structural properties of phosphogluco\textsuperscript{se} isomerases isolated from a variety of species have been compared by peptide fingerprinting, predicted amino acid sequence homologies and by denaturation and renaturation studies. The enzymes are more readily denatured in guanidinium chloride than in urea, and the isomerase isolated from yeast is more stable toward acid pH than the rabbit muscle enzyme. The rates of guanidinium chloride-induced denaturation are markedly increased by ionic strength and decreased by substrates, competitive inhibitors or glycerol. The enzyme can be renatured, but only in the presence of glycerol. The renaturation process is dependent on protein concentration and temperature and provides a method for the formation of mixed species hetero\textsuperscript{dimers}. 
INTRODUCTION

Discovered by Lohmann (1933), phosphoglucose isomerase, D-glucose-6-phosphate ketol isomerase (EC 5.3.1.9), catalyzes the reversible isomerization of glucose 6-phosphate and fructose 6-phosphate.

\[
\begin{align*}
\text{D-glucose 6-phosphate} & \quad \text{D-fructose 6-phosphate} \\
\end{align*}
\]

In 1957 Yale Topper postulated that the mechanism of the above isomerization reaction involved the labilization of a proton alpha to the carbonyl and proceeded through an enediol intermediate. Topper also suggested that the proton on C-1 of fructose 6-phosphate is lost to the media, and then a proton from the media returns to the adjacent carbon C-2 to form glucose 6-phosphate.

In 1971 Rose and O'Connell showed that at lower temperatures the proton migration occurs by an intramolecular
transfer in which the proton is directly abstracted from
the alpha carbon (e.g., C-1) and transferred to a nucleo-
phile in the active center of the enzyme. Subsequently,
the same proton is transferred back to the accepting car-
banion (e.g., C-2).

Data from several other laboratories (Dorrer et al.,
1966; Fedtke, 1968; Gonzales de Galdeano and Simon, 1970;
Simon et al., 1964; Simon et al., 1968) clearly show that
the glucose 6-phosphate isomerase reaction proceeds both by
intramolecular and intermolecular proton migration and that
at 25°C, the proton is transferred directly in more than half
of the catalytic turnovers.

Rose (1961) suggested that this was the result of a
higher activation energy for the exchange reaction compared
with the intramolecular transfer reaction, thus favoring
direct transfer at lower temperatures.

\[
\text{Fructose 6-P} \quad \begin{array}{c}
\text{k}_0 \\
\text{B:T-C-OH} \\
\text{C=O} \\
\text{k}_0
\end{array} \quad \begin{array}{c}
\text{H} \\
\text{B-T} \\
\text{C-OH} \\
\text{C=O}
\end{array} \quad \begin{array}{c}
k_1 \\
\text{B-T} \\
\text{C-OH} \\
\text{C=O}
\end{array} \quad \begin{array}{c}
k_2 \\
\text{B:} \\
\text{T-C-OH}
\end{array} \quad \begin{array}{c}
k_3 \\
\text{Glucose 6-P}
\end{array}
\]

\[
\text{Glucose 6-P} \quad \begin{array}{c}
k_{+3} \\
\text{H} \\
\text{B:H} \\
\text{C-OH} \\
\text{C-0}^-
\end{array} \quad \begin{array}{c}
k_{-3} \\
\text{H} \\
\text{B:H} \\
\text{C-OH} \\
\text{C-0}^-
\end{array}
\]

\[
\text{H}^+ \quad \begin{array}{c}
k_x \\
\text{T}^+ \\
\text{B-H} \\
\text{C-OH} \\
\text{C-0}^-
\end{array} \quad \begin{array}{c}
k_{-4} \\
\text{k}_4
\end{array} \quad \text{Glucose 6-P}
\]
At higher temperatures, $k_x$ in the above diagram competes with $k_2$. Hence, tritium (T) is replaced with a proton ($H^+$) from the media leading to the non-labelled product in this case.

Phosphoglucose isomerase occupies a strategic position at the branching point of several metabolic pathways (Figure 1), including glycolysis, gluconeogenesis, and the hexose monophosphate shunt. With this strategic role in the intermediary metabolism of living organisms, it is not surprising that phosphoglucose isomerase is found ubiquitously distributed in nature.

Deficiencies of this enzyme in man result in a hereditary nonspherocytic hemolytic anemia (Baughan et al., 1967). This association was first recognized by Baughan et al. (1967), who observed an adolescent boy with a life-long nonspherocytic hemolytic anemia associated with lowered levels of phosphoglucose isomerase activity. From 1967 to 1972 twelve other patients from nine different families were reported suffering from a hemolytic anemia caused by phosphoglucose isomerase deficiency (Baughan et al., 1968; Paglia et al., 1969; Cartier et al., 1969; Schroter et al., 1970; Leger et al., 1970; Arnold et al., 1970; Oski and Fuller, 1971). In 1972 Blume and Beutler (1972) reported a method for the rapid detection of phosphoglucose isomerase deficiencies, and the use of these rapid screening techniques has proven to be a useful tool in the differential
Figure 1. Glycolysis, gluconeogenesis, and general reductive pentose phosphate shunt. Double-headed arrows \( \leftrightarrow \) represent reversible reactions, while single-headed arrows \( \rightarrow \) represent essentially irreversible reactions. The branched pentose phosphate shunt is shown as a cycle.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Compound Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHAP</td>
<td>dihydroxyacetone phosphate</td>
</tr>
<tr>
<td>1,3-DPG</td>
<td>1,3-diphosphoglycerate</td>
</tr>
<tr>
<td>2,3-DPG</td>
<td>2,3-diphosphoglycerate</td>
</tr>
<tr>
<td>E-4-P</td>
<td>erythrose 4-phosphate</td>
</tr>
<tr>
<td>F-D-P</td>
<td>fructose 1,6-diphosphate</td>
</tr>
<tr>
<td>F-6-P</td>
<td>fructose 6-phosphate</td>
</tr>
<tr>
<td>G-6-P</td>
<td>glucose 6-phosphate</td>
</tr>
<tr>
<td>G-3-P</td>
<td>glyceraldehyde 3-phosphate</td>
</tr>
<tr>
<td>Pi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>LAC</td>
<td>lactate</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenol pyruvate</td>
</tr>
<tr>
<td>6-PG</td>
<td>6-phosphogluconate</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenol pyruvate</td>
</tr>
<tr>
<td>3-PG</td>
<td>3-phosphoglycerate</td>
</tr>
<tr>
<td>2-PG</td>
<td>2-phosphoglycerate</td>
</tr>
<tr>
<td>PYR</td>
<td>pyruvate</td>
</tr>
<tr>
<td>Ru-5-P</td>
<td>ribulose 5-phosphate</td>
</tr>
<tr>
<td>R-5-P</td>
<td>ribose 5-phosphate</td>
</tr>
<tr>
<td>S-7-P</td>
<td>sedaheptulose 7-phosphate</td>
</tr>
<tr>
<td>Xu-5-P</td>
<td>xylulose 5-phosphate</td>
</tr>
</tbody>
</table>
diagnosis of hemolytic diseases. At the present time phospho-
glucose isomerase deficiency disease is the third most com-
mon enzyme deficiency responsible for hemolytic anemia
(glucose 6-phosphate dehydrogenase and pyruvate kinase defi-
ciencies being more common, Blume and Beutler, 1972).
Through a complete understanding of phosphogluco-
se isomerase's structural-functional relationships, insights toward possible
treatment of phosphoglucone isomerase deficiency disease
may be possible. In order to better understand these rela-
tionships, a comparison of the structural-functional proper-
ties from a variety of phosphoglucone isomerases from
different species has been undertaken.

It is now clear that phosphoglucone isomerase is composed
of two identical subunits. In the presence of dissociating
conditions, the non-covalently bound monomers of the active
dimeric enzyme dissociate to form a solution of inactive
monomers. These monomers have been found to have one-half the
molecular weight found for the native enzyme. The rabbit
muscle enzyme with a molecular weight of 132,000 daltons
dissociates to form two subunits each with a molecular weight
of 64,400 (Pon et al., 1970). Similarly, the enzymes iso-
lated from human muscle (Carter and Yoshida, 1969), human
erthrocytes (Tsuboi; 1971; Tilley and Gracy, 1975; and
Tilley et al., 1974); and catfish liver and muscle and
conger muscle (Mo et al., 1975) were all found to be of
molecular weight of 131,000 - 134,000 and composed of
subunits of approximately 63,000 - 65,000 daltons. The enzyme isolated from Brewers' yeast seems to be significantly smaller (60,000), but is still dimeric (Kempe and Noltmann, 1971). The above examples firmly establish the quaternary structure of phosphoglucoisomerase and are summarized in Table I.

In addition to subunits of equal size, several other lines of evidence have pointed out that the amino acid sequences of the subunits are probably identical. James (1971) found that the terminal alpha-amino groups of rabbit muscle phosphoglucoisomerase are blocked by an acetyl group. Further studies using standard amino acid analysis and gas chromatography showed that 1.6 - 1.9 acetylalanine residues per enzyme molecule of molecular weight 132,000 were present (James, 1971). Carboxyl terminal analysis of the native enzyme by three different methods, carboxypeptidase digestion, tritium exchange, and hydrazinolysis also agreed with the presence of two subunits and yielded 1.7 - 2.1 glutamine residues per 132,000 daltons (Noltmann, 1972A).

Peptide mapping studies of phosphoglucoisomerase from various sources also are consistent with identical subunits. Peptide maps prepared from tryptic digests of fully carbamylated and carboxymethylated rabbit muscle enzyme showed 20 - 22 ninhydrin-positive spots (James and Noltmann, 1970). This finding is in good agreement with the native enzyme composed of two identical subunits
### TABLE I

**COMPARISON OF PHYSICAL PROPERTIES OF PHOSPHOGLUCOSE ISOMERASES**

<table>
<thead>
<tr>
<th>Source</th>
<th>$S_{20,w}$</th>
<th>M.W.</th>
<th>Subunit M.W.</th>
<th># Subunits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catfish muscle</td>
<td>7.04S</td>
<td>132,000</td>
<td>65,000</td>
<td>2</td>
</tr>
<tr>
<td>Catfish liver</td>
<td>7.05S</td>
<td>131,000</td>
<td>65,000</td>
<td>2</td>
</tr>
<tr>
<td>Conger muscle</td>
<td>7.00S</td>
<td>132,000</td>
<td>65,000</td>
<td>2</td>
</tr>
<tr>
<td>Human blood (normal)</td>
<td>7.2 S</td>
<td>132,000</td>
<td>63,000</td>
<td>2</td>
</tr>
<tr>
<td>Human blood (Singh variant)</td>
<td>7.2 S</td>
<td>131,000</td>
<td>64,000</td>
<td>2</td>
</tr>
<tr>
<td>Human muscle</td>
<td>6.15S</td>
<td>134,000</td>
<td>61,000</td>
<td>2</td>
</tr>
<tr>
<td>Rabbit muscle</td>
<td>7.19S</td>
<td>132,000</td>
<td>64,000</td>
<td>2</td>
</tr>
<tr>
<td>Yeast</td>
<td>6.7 S</td>
<td>120,000</td>
<td>60,000</td>
<td>2</td>
</tr>
</tbody>
</table>

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*a* Mo et al., 1975.

*b* Tilley and Gracy, 1974.

*c* Carter and Yoshida, 1968.

*d* Pon et al., 1970.

*e* Kempe and Noltmann, 1971.
each containing 21 arginine residues (Pon et al., 1970). Peptide maps of the fully carboxymethylated and carbamylated human enzyme similarly supported the concept of two identical subunits (Tilley et al., 1974).

Dyson and Noltmann (1969A) demonstrated that the rabbit muscle phosphoglucone isomerase has a "rigid core" structure which requires severe denaturing conditions to produce a completely unfolded molecule. Dyson and Noltmann (1969B) have also proposed that this enzyme has several levels of structural integrity which have different degrees of resistance to denaturing reagents. In the presence of denaturants the first transition the enzyme undergoes is dissociation to folded monomers. Later transitions are thought to lead to the unfolding of the individual subunits.

Several enzymes of dimeric structure have been shown to regain catalytic activity in vitro after reassociation of the monomeric units (Waley, 1973; Kohn, 1970). However, the conditions necessary for renaturation and subsequent reassociation vary widely. This regain of activity in the case of triosephosphate isomerase (EC 5.3.1.1), a dimeric enzyme of 53,000 daltons, requires only a 10-fold dilution of the denaturant (3 M guanidinium hydrochloride). Regain of activity is easily monitored after only 8 minutes (Waley, 1973). On the other hand, the reassociation of spinach leaf glyoxylic acid reductase, a dimer of 90,000 daltons, requires the presence of glycerol (Kohn, 1970),
and under optimal conditions the half-time for maximal renaturation was seven to ten hours.

One of the objectives of the present study was to evaluate the denaturation-dissociation process of phosphoglucone isomerase from various species and to establish various parameters which effect the dissociation and unfolding of this protein. Although no previous attempts have been made to study the reassociation process of this enzyme, the observation of mixed heterodimers in individuals heterozygous at the phosphoglucone isomerase locus (Tilley et al., 1974) strongly suggests that dissociation and reassociation do occur in vivo. Therefore, this study was also designed to elucidate the mechanism of renaturation and the factors influencing the reassociation-refolding process.
MATERIALS

Enzymes

Glucose 6-phosphate dehydrogenase and yeast phosphoglucone isomerase were obtained from Sigma. The catfish muscle, human blood, and rabbit muscle phosphoglucone isomerases were isolated in our laboratory as described by Mo et al. (1975), Tilley and Gracy (1974), and Phillips and Gracy (1975), respectively. All the isomerases were dialyzed exhaustively against 50 mM TEA, pH 8.4, before use. Trypsin (L-1-tosylamido-2-phenyl-ethyl chloromethyl ketone-treated) was obtained from Worthington Biochemicals.

Chromatographic and Electrophoretic Supplies

Precoated, plastic-backed thin layer cellulose sheets were from Eastman (Eastman Chromatogram Sheets #60640, 160 μ thickness). Pyridine, acetic acid, and butanol were obtained from Mallinckrodt. The pyridine and acetic acid used in the electrophoresis and chromatography were freshly redistilled from ninhydrin. Fluorescamine (4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione) from Hoffman-LaRoche was used as a 0.02% (w/v) solution in peroxide-free dioxane.

Buffer and Assay Reagents

Tris(hydroxymethyl)-aminomethane (Tris), and triethanolamine were from Aldrich. Sodium acetate was from Fisher,
while imidazole and glycine were obtained from Sigma. The sodium salts of NADP and fructose-6-phosphate were obtained from Sigma.

Miscellaneous Reagents

Iodoacetic acid (Eastman Chemicals) was recrystallized prior to use. Sodium chloride, urea, and 88% formic acid were obtained from Baker. Ethylenediamine tetraacetic acid (EDTA) and magnesium chloride were obtained from Mallinckrodt. Guanidine hydrochloride, β-mercaptoethanol, and 6-phosphogluconate were obtained from Sigma. Reagent grade glycerol was obtained from Matheson, Coleman and Bell.

METHODS

Enzyme Activity Assay

Phosphoglucone isomerase was assayed in the reverse direction (fructose 6-P → glucose 6-P) by coupling the product, glucose 6-P, to glucose-6-phosphate dehydrogenase and measuring the rate of reudction of NADP at 340 nm (Noltmann, 1972). All measurements were carried out in a recording spectrophotometer with temperature controlled at 30.0°C. The assay mixture was composed of 50 mM triethanolamine buffer, pH 8.3, 4.0 mM fructose 6-phosphate, 0.5 mM NADP, and 1.0 unit of glucose-6-phosphate dehydrogenase.
injected into the solution through the septum. The reaction with iodoacetic acid was allowed to progress in the dark for 15 - 20 minutes, then terminated by the addition of 5 μl of 2-mercaptoethanol. Stirring was continued for 10 minutes; then the solution was diluted two-fold with 1.0 M formic acid and transferred to a small dialysis bag. The mixture was dialyzed in the dark at 40 against 1% formic acid for at least 12 hours. The sample was subsequently dialyzed against water and lyophilized to dryness. At this point, the enzyme was ready for either trypsin digestion or carbamylaition.

\[ \text{N}^{\alpha,\varepsilon} \text{-Carbamylation:}\] After S-carboxymethylation, 1.0 ml of buffered guanidine hydrochloride solution (8.6 g of guanidine hydrochloride added to 5.0 ml of 0.1 M N-ethylmorpholine, pH 8.5) was added to the dried S-carboxymethylated enzyme. Water and 12 N HCl were then added to a final volume of 15.0 ml and pH 8.0. The ε- and α-amino groups were then carbamylated by reaction with 8.1 mg of potassium cyanate (Stark, 1967). The mixture was incubated at 50° for 12 hours, dialyzed exhaustively against water and then lyophilized. At this point, the enzyme was ready for trypsin digestion.

**Tryptic Hydrolysis**

After \( \text{N}^{\alpha,\varepsilon} \) -carbamylaition, enough 2% trimethylamine was added to produce a 5 mg/ml protein solution. Then, the sample was digested with TPCK-treated Sepharose-bound
trypsin at 1:50 trypsin:phosphoglucose isomerase weight ratio. The digestion was allowed to proceed for 8 - 10 hours with continuous stirring at room temperature, then the matrix-bound trypsin removed by centrifugation. Finally, the peptides were lyophilized to dryness.

Peptide Mapping

Following digestion, the peptides were redissolved in electrophoresis buffer (pyridine: acetic acid: water, 1:10:89, at pH 3.5) and 25 - 75 µg spotted on 20 x 20 cm cellulose coated thin layer sheets. Thin layer peptide fingerprinting was carried out in a varsol-cooled microscale mapping chamber at 0 - 4°C using the above described electrophoretic buffer. Electrophoresis was followed by chromatography in butanol: pyridine: acetic acid: water (50:33:1:40), and the peptides were located by spraying with fluorescamine.

Composition Coefficients

Composition coefficients were calculated by the method of Dedman et al. (1974) using a Fortran IV computer program adapted to the IBM 360-50 computer.

Inactivation

Urea and Guanidine Hydrochloride Titration: "Standard" concentrations (0.0795 mg/ml) of catfish muscle, rabbit muscle, human, and yeast phosphoglucose isomerases were
prepared by dilution. At "zero time" a 10 μl aliquot of the 0.0795 mg/ml enzyme solution was added to 500 μl of urea or guanidinium hydrochloride solution and incubated for 30 minutes at 30°C. Aliquots (5 - 50 μl) of the incubation mixture were withdrawn and assayed.

**pH Stability:** pH stability studies were carried out in 20 mM solutions of the following buffers: sodium acetate (pH 4 - 6), imidazole (pH 6 - 7), Tris (pH 7 - 8), TEA (pH 8 - 9), and glycine (pH 9 - 11). Five microliters of 0.227 mg/ml yeast phosphoglucoisomerase (specific activity = 582.2) and 0.214 mg/ml reabbott muscle phosphoglucoisomerase (specific activity 691.4) were added to 1.00 ml of the various buffers and incubated at 30°C. After 30 minutes 5 μl samples of the above mixtures were withdrawn and added to 960 μl of assay buffer. The pH of the solutions were determined immediately after each incubation.

**Salt Effects on Inactivation:** Ten microliters of a 0.0795 mg/ml solution of phosphoglucoisomeras (yeast, human, catfish, rabbit) were added to 500 μl of 0.0 to 2.0 M NaCl and 0.0 to 0.66 M MgCl₂ in 1.02 M guanidinium hydrochloride, 50 mM TEA, pH 8.4. At 1 - 5 minute intervals 10 μl aliquots were removed from the 30°C incubation solution and directly assayed for phosphoglucoisomerase activity.
Effects of Substrate and Inhibitors on Inactivation:

Phosphoglucose isomerases were added to varying concentrations (0 - 10 mM) of the disodium salt of fructose 6-phosphate in the presence of 0.88 M guanidine hydrochloride. Proteins were all at a final concentration of $1.56 \times 10^{-3}$ mg/ml. At 1 - 3 minute intervals 10 µl aliquots were removed from the 30° incubation solution and directly assayed for phosphoglucose isomerase activity.

The enzymes were also incubated with gluconate 6-phosphate in the presence of 1.00 M guanidine hydrochloride, 50 mM TEA buffer, pH 8.4. All proteins were present at a final concentration of $1.56 \times 10^{-3}$ mg/ml. At 1 minute intervals 10 µl aliquots were removed from the 30° incubation mixture and directly assayed for phosphoglucose isomerase activity.

Effects of Glycerol on Inactivation: Ten microliters of a 0.0795 mg/ml solution of phosphoglucose isomerase with an initial specific activity of 650 units/mg were added to 500 µl of 1.27 M guanidine hydrochloride (in 5 mM TEA buffer, pH 8.85) with varying glycerol concentrations (0 - 40%). Ten microliter aliquots were removed at the various times and added to 530 µl of assay mixture and directly assayed.

Reactivation

Rabbit muscle phosphoglucose isomerase was inactivated by adding 100 µl of the enzyme (5.3 mg/ml - 523 units/mg)
to 900 μl of 6 M guanidine hydrochloride in 0.2 M Tris, pH 7.7, containing 5 mM 2-mercaptoethanol. This mixture was dialyzed against the 6 M guanidine hydrochloride for 17 hours at 3°C. Subsequently, aliquots of the inactive enzyme mixture were diluted to 1.0 ml in the cold (3°C) with renaturation buffer, which consisted of 0.2 M Tris, 5 mM TEA, pH 7.7, 25% glycerol (v/v). The mixture was allowed to reach room temperature (23.5°C), and aliquots were removed at various times and assayed. Ten microliters of the inactivated (as described above) rabbit muscle phosphoglucose isomerase was diluted (at 3°C) to 1.0 ml with the standard renaturation buffer containing various concentrations of glycerol (0 - 40%). The resulting sample was then incubated at 23.5°C and aliquots removed at various times and assayed for phosphoglucose isomerase activity.
RESULTS

Structural Studies and Comparison of Several Species of Phosphoglucone Isomerase

Tryptic digests of the S-carboxymethylated catfish muscle, catfish liver, and conger muscle phosphoglucone isomerasers yielded peptide fingerprints with many unresolved spots. In order to better compare the structures of these enzymes, tryptic digests on the S-carboxymethylated and N-carbamylated enzymes were performed. The peptide maps of the catfish liver enzyme and muscle isozymes are shown in Figure 2. The peptide map of catfish muscle enzyme resulted in 18 fluorescent spots representing 17 arginine residues per subunit. This finding compares reasonably well with the reported 15.3 arginine residues per subunit (Mo et al., 1975). The peptide map of catfish liver phosphoglucone isomerase resulted in 19 fluorescamine-positive spots and compares with the 16.5 arginines per subunit from amino acid analysis (Mo et al., 1975).

A comparison of the peptide maps of catfish muscle and liver phosphoglucone isomerasers clearly indicated that a number of structural differences in the two types of isozymes exist. The catfish liver and muscle isozymes were more similar to each other than either isozyme was to the enzyme isolated from conger muscle (Figure 3). The amino
TABLE II

COMPARISON OF PREDICTED AMINO ACID SEQUENCE HOMOLOGIES OF PHOSPHOGLUCOSE ISOMERASES

<table>
<thead>
<tr>
<th>Species</th>
<th>Human</th>
<th>Rabbit</th>
<th>Catfish Muscle</th>
<th>Catfish Liver</th>
<th>Conger Muscle</th>
<th>Yeast A</th>
<th>Yeast B</th>
<th>Yeast C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit</td>
<td>0.983</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Catfish Muscle</td>
<td>0.929</td>
<td>0.925</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Catfish Liver</td>
<td>0.936</td>
<td>0.945</td>
<td>0.951</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Conger Muscle</td>
<td>0.907</td>
<td>0.922</td>
<td>0.896</td>
<td>0.917</td>
<td></td>
<td></td>
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<tr>
<td>Yeast A</td>
<td>0.925</td>
<td>0.960</td>
<td>0.909</td>
<td>0.923</td>
<td>0.934</td>
<td></td>
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<tr>
<td>Yeast B</td>
<td>0.918</td>
<td>0.957</td>
<td>0.900</td>
<td>0.916</td>
<td>0.925</td>
<td>0.999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yeast C</td>
<td>0.907</td>
<td>0.948</td>
<td>0.888</td>
<td>0.909</td>
<td>0.916</td>
<td>0.996</td>
<td>0.999</td>
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</tr>
<tr>
<td>B. Stearothermophilis</td>
<td>0.892</td>
<td>0.881</td>
<td>0.879</td>
<td>0.934</td>
<td>0.926</td>
<td>0.870</td>
<td>0.861</td>
<td>0.855</td>
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</table>
acid composition coefficient (c.c.) between the catfish liver and muscle isozymes was calculated to be 0.951 (Table II). On the other hand, the composition coefficient between catfish liver and conger muscle was 0.917 and between catfish muscle and conger muscle was 0.896. The conger muscle enzyme was essentially as different in its amino acid composition from the two catfish isozymes as it was from rabbit muscle (c.c. = .922) or human (c.c. = .907). The three isozymes of yeast phosphoglucose isomerase (A, B, and C) show almost a 100% agreement of predicted sequence homology.

Composite peptide maps of normal conger muscle phosphoglucose isomerase and a genetic variant of the conger muscle enzyme showed a single peptide difference at peptides 7 and 7A (Figure 3). The observation of a single peptide difference in the variant parallels the recent findings with a human phosphoglucose isomerase variant (Tilley et al., 1974), and is consistent with a point mutation affecting a single amino acid residue.

Denaturation Studies

Background

Denaturation has been defined as a major change from the original native structure of a protein without alteration of the amino acid sequence. This definition is, of course, not an absolute one. What one chooses to call a "major" change in conformation remains a matter of degree. For the
Figure 3. Composite peptide fingerprints from wild type conger muscle phosphoglucose isomerase and the genetic variant. The enzymes were S-carboxymethylated and α- and ε-amino carbamylated as described in Methods prior to tryptic digestion. Simultaneous thin layer peptide mapping was carried out, and the plates were sprayed with fluorescamine. The figure represents composite tracings of eight and seven maps of the normal and variant proteins, respectively. The variant protein was identical with the normal protein with the exception of peptide 7, which was present in the wild type and was replaced by peptide 7A in the genetic variant. The most highly fluorescent peptides are shown by stippling.
cases described in the following section, a major change from the original native structure will be defined as one which causes a loss of catalytic activity.

Care must be exercised in equating denaturation with loss of catalytic activity. The definition of denaturation is more accurately denoted in terms of physical changes relating to molecular conformation. Enzymes may be inactivated as a result of minor conformational changes, as well as major ones, or as a result of interaction between the inactivating agent and the catalytic site of the enzyme. In the case of phosphoglucone isomerase, the loss of activity does not appear to result from a major conformational change in the native enzyme structure leading to immediate inactivation (Dyson and Noltmann, 1969). This change is that of dissociation in which the active dimeric enzyme separates into inactive monomers. Hence, in the following discussed cases, the specific denaturation causing immediate loss of catalytic activity is that of dissociation.

**Guanidine Hydrochloride and Urea Titrations of Phosphoglucone Isomerase**

The molar concentrations of urea, [Urea], and guanidine hydrochloride, [GmHCl], which are required to give 50% inactivation of phosphoglucone isomerase after 30 min at 30°C varied from species to species as shown in Table III.
TABLE III

UREA AND GUANIDINE DENATURATION OF PGI

<table>
<thead>
<tr>
<th>Species</th>
<th>[Urea]₃₅</th>
<th>[GmHCl]₃₅</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast</td>
<td>3.82M</td>
<td>0.85M</td>
</tr>
<tr>
<td>Catfish muscle</td>
<td>4.10M</td>
<td>0.79M</td>
</tr>
<tr>
<td>Rabbit muscle</td>
<td>4.36M</td>
<td>0.85M</td>
</tr>
<tr>
<td>Human blood</td>
<td>4.44M</td>
<td>1.06M</td>
</tr>
</tbody>
</table>

The human enzyme showed the highest resistance to inactivation by urea or guanidine hydrochloride. The rabbit muscle enzyme showed a similar [Urea]₃₅ value, but the [GmHCl]₃₅ value of 0.85M, for rabbit muscle, differed significantly from the human (1.06M). The isomerase found to be most susceptible to inactivation by urea was the yeast enzyme, while the catfish muscle enzyme was slightly more easily denatured by GmHCl. Figures 4 and 5 also point out the sigmoidal nature typical of denaturation processes in which the transition from native to denatured states consists of a steep cooperative transition from the native to denatured states.

pH Stability of Phosphogluco Isomerases

With the ultimate goal of reassociation of phosphogluco isomerase subunits, a variety of denaturation conditions were studied in hopes that conditions could be found that would
lead to the reversible denaturation of the protein. One such parameter tested was that of pH. When the rabbit muscle and yeast phosphoglucone isomerase were incubated at 30° for 30 minutes, the data shown by Figure 6 were obtained. While both enzymes showed essentially the same susceptibility in the basic region, the yeast enzyme was found to be much more stable toward acid pH than was the rabbit muscle enzyme. It would seem that this functional difference may be of physiological importance, since it is well known that the physiological pH within a yeast cell varies a great deal, and is, in fact, quite acidic under a variety of growth conditions. Therefore, it would be essential for the glycolytic enzymes, including phosphoglucone isomerase, to be stable toward pH's in the region of pH 5 and even lower. However, in rabbit muscle the pH is never that low, and therefore, the rabbit muscle enzyme does not require this stability toward acidic conditions.

**Effect of Ionic Strength on the Denaturation of Phosphoglucone Isomerase**

When four different species of phosphoglucone isomerase were incubated at 30° C in the presence of varying ionic strengths, plots similar to that shown for the rabbit muscle enzyme in Figures 7 and 8 were obtained. It can be seen by the linear semilog plots that this inactivation process followed pseudo first order kinetics. More importantly is the observation that increasing concentrations
Figure 4. Urea denaturation titration of phosphogluco- 

ose isomerase from various species. Yeast (●), 

rabbit (▲), human (○), catfish (△) phosphogluco- 

ose isomerases were titrated with various concentrations 

of urea. Ten microliters of a 0.0795 mg/ml phosphoglu- 

 cose isomerase solution were added to 500 μl of 

varying urea concentrations and incubated for 30 min- utes at 30°. Aliquots (5 - 50 μl) of the incubation 

mixture were withdrawn and assayed as described in 

"Methods".
Figure 5. Guanidinium hydrochloride-induced titration of phosphoglucone isomerase from various species. Yeast (○), rabbit (●), human (▲), catfish (△) phosphoglucone isomerases were titrated with various concentrations of GmHCl. Ten microliters of a 0.0795 mg/ml enzyme solution was added to 500 μl of GmHCl. After incubating at 30°C for 30 minutes, 5 to 50 μl of the enzyme-guanidine hydrochloride mixture was added to 960 μl of assay mix and assayed as described in "Methods".
Figure 6. pH stability of yeast (●) and rabbit muscle (○) phosphoglucone isomerase. Five microliters of a 0.227 mg/ml sample of yeast phosphoglucone isomerase (specific activity = 582.2) was added to 1.00 ml of various pH solutions and incubated at 30° C. After 30 minutes a 5 µl sample of the above mixture was withdrawn and added to 960 µl of assay buffer and assayed as described under "Methods".

Five microliters of a 0.214 mg/ml sample of rabbit muscle phosphoglucone isomerase (specific activity = 691.4) was added to 1.00 ml of various pH solutions and treated as above. The pH of the solutions was determined immediately after each incubation.
Figure 7. Effects of NaCl on the rate of guanidine-induced inactivation of rabbit muscle phosphogluco

zyme. Ten microliters of a 0.0795 mg/ml solution of rabbit muscle phosphogluco isomerase with an ini-

ial specific activity of 650 units/mg was added to 500 µl of 1.02 M guanidine hydrochloride solutions with vary-

ing concentrations of NaCl: 0.00M NaCl (○); 0.050M NaCl (△); 1.00M NaCl (○); 2.00M NaCl (▲). At

the indicated times 10 µl aliquots were removed from the incubation solution (30° C) and assayed for phosphoglu-

co isomerase activity as discussed under "Methods".
Figure 8. Effects of MgCl₂ on the rates of guanidine-induced inactivation of rabbit muscle phosphoglucoisomerase. Ten microliters of a 0.0795 mg/ml solution of rabbit muscle phosphoglucoisomerase with an initial specific activity of 650 units/mg was added to 500 μl of 1.02 M guanidine hydrochloride solutions of varying concentrations of MgCl₂: 0.00M MgCl₂ (O); 0.33M MgCl₂ (●), 0.50M MgCl₂ (△); and 0.66M MgCl₂ (▲). At the indicated time 10 μl aliquots were removed from the incubation (30° C) solution and assayed for phosphoglucoisomerase activity as discussed under "Methods".
of either MgCl₂ or NaCl bring about an increase in the inactivation rates.

For each enzyme, increasing salt concentrations lead to higher rates of inactivation. This inactivation process was more pronounced in MgCl₂ than by NaCl even at identical ionic strengths. This can be seen from the ratio of the rate constant in the absence of salt (k₀') to the rate constant in the presence of an ionic strength of 3.0 (k₂'). The k₂'/k₀' value for human phosphoglucone isomerase in NaCl was found to be 22.2, as compared to the k₂'/k₀' value for MgCl₂, 99.5. In the case of the other three phosphoglucone isomerase enzymes studied, significantly higher values of k₂'/k₀' were also found for the MgCl₂ cases, as compared with the NaCl case (Table IV).

The MgCl₂ seemed to effect the inactivation of the different species of phosphoglucone isomerase to varying degrees. For example, the k₂'/k₀' value for yeast phosphoglucone isomerase was only 14.0, whereas the value for the human, catfish, and rabbit muscle enzymes were much higher. The NaCl concentration also tended to effect the different species to varying degrees. The k₂'/k₀' value for human blood phosphoglucone isomerase in the presence of NaCl was 22.2. However, the values for the rabbit muscle, catfish muscle, and yeast enzymes were found to be 7.1, 3.4, and 3.6, respectively.
### TABLE IV

EFFECT OF IONIC STRENGTH ON DENATURATION RATES OF PHOSPHOGLUCOSE ISOMERASE

<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>Rate of Inactivation $k_{\text{inact}}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k'_2/k'_0$ [NaCl]</td>
</tr>
<tr>
<td>Catfish muscle</td>
<td>3.4</td>
</tr>
<tr>
<td>Human blood</td>
<td>22.2</td>
</tr>
<tr>
<td>Rabbit muscle</td>
<td>7.05</td>
</tr>
<tr>
<td>Yeast</td>
<td>3.6</td>
</tr>
</tbody>
</table>

|                  | [MgCl$_2$] | 0.0 | 0.33M | 0.50M | 0.66M |
| Catfish muscle   | 106.2      | 0.098 | 1.48 | 3.45 | 10.4 |
| Human blood      | 99.5       | 0.038 | 0.22 | 0.59 | 3.78 |
| Rabbit muscle    | 128.1      | 0.027 | 0.53 | 0.92 | 3.46 |
| Yeast            | 14.0       | 0.292 | 1.08 | 2.13 | 4.08 |

*aDenaturation carried out in 1.0M guanidinium chloride at 30.0°, pH 8.0. Ionic strength was adjusted with NaCl.

*bPsuedo first order rate constants; values are min$^{-1}$. 
Effect of Fructose 6-Phosphate and Gluconate 6-Phosphate on Rates of Inactivation of Phosphoglucone Isomerase

Inactivation of four different species of phosphoglucone isomerases in the presence of varying fructose 6-phosphate concentrations (Table V) shows that in the presence of fructose 6-phosphate, only a slight protection of the enzyme from inactivation is offered when exposed to 0.88 M guanidine hydrochloride. With increasing fructose 6-phosphate concentrations, the rate of inactivation was generally found to decrease for all four species except with the human enzyme. When the ratio of the rate constants determined in the absence (k₀') and in the presence (k_f') of 10 mM fructose 6-phosphate were calculated (Table V), the greatest extent of protection was observed with the yeast enzyme (k₀'/k_f' = 2.02). The k₀'/k_f' ratio for human was found to be 1.34, whereas smaller extents of protection for the catfish muscle (k₀'/k_f' = 1.14) and rabbit (k₀'/k_f' = 1.26) enzymes were found.

Table VI shows the inactivation rate constants for the various species of phosphoglucone isomerase in the absence (k₀') and in the presence (k_g') of 10 mM 6-phosphogluconate. The k₀'/k_g' values were found to be close to 1, showing that the extent of protection was essentially negligible. These data suggest that the substrate, fructose 6-phosphate, and the competitive inhibitor, gluconate-6-phosphate, provide only a minimal protection of the enzyme from guanidine-induced inactivation.
<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>Rate of Inactivation $k_{inact} \times 10^3$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_0/k_f$</td>
</tr>
<tr>
<td>Human</td>
<td>1.34</td>
</tr>
<tr>
<td>Rabbit</td>
<td>1.26</td>
</tr>
<tr>
<td>Catfish</td>
<td>1.14</td>
</tr>
<tr>
<td>Yeast</td>
<td>2.02</td>
</tr>
</tbody>
</table>

Inactivation carried out in 0.88 M guanidine hydrochloride 30°, pH 8.0; proteins were all at a final concentration of 1.56 x $10^{-3}$ mg/ml.
TABLE VI

EFFECT OF GLUCONATE 6-PHOSPHATE ON RATES OF GUANIDINE-INuced INACTIVATION OF PHOSPHOGLUCOSE ISOMERASE

<table>
<thead>
<tr>
<th>Source of Enzyme</th>
<th>Rate of Inactivation $k_{inact} \times 10^3$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k'_0/k'_g$</td>
</tr>
<tr>
<td>Catfish muscle</td>
<td>1.18</td>
</tr>
<tr>
<td>Human blood</td>
<td>1.34</td>
</tr>
<tr>
<td>Rabbit muscle</td>
<td>1.48</td>
</tr>
<tr>
<td>Yeast</td>
<td>1.34</td>
</tr>
</tbody>
</table>

Inactivation carried out in 1.00 M guanidine hydrochloride, 30°, pH 8.0. Proteins were at a final concentration of 1.56 x 10$^{-3}$ mg/ml.
Inactivation of Phosphoglucone Isomerase with Guanidine Hydrochloride at Varying Glycerol Concentrations

When rabbit muscle phosphoglucone isomerase was denatured in 1.25 M guanidine hydrochloride, 50% loss of activity was observed after 1.48 minutes, giving a pseudo first order rate constant of $0.468 \text{ min}^{-1}$ (Figure 9). The addition of 10% glycerol lowered the rate constant by a factor of 7.8 to $0.060 \text{ min}^{-1}$. Further addition of glycerol lowered the rate constant of inactivation even more (e.g., 40% glycerol gave an inactivation rate constant of $0.0014 \text{ min}^{-1}$).

Reactivation of Phosphoglucone Isomerase

Effect of Enzyme Concentration

At $3.5^\circ C$ rabbit muscle phosphoglucone isomerase showed a definite concentration dependence upon reassociation of the inactive monomeric units to the active dimer. Over a ten-fold range of concentration (0.00270 mg/ml to 0.027 mg/ml native enzyme concentration) a greater amount of reactivation over a period of 72 hours was obtained at a concentration of 0.0107 mg/ml. At $23.5^\circ C$ the rabbit muscle enzyme also showed a concentration dependence of activation (Figure 10).

Effect of Temperature on Reactivation

The reassociation of phosphoglucone isomerase also showed a temperature dependence as demonstrated in Figure 11. When the dissociated rabbit muscle phosphoglucone
Figure 9. Effect of glycerol on rate of guanidine-induced inactivation on rabbit muscle phosphoglucone isomerase. Ten microliters of a 0.0795 mg/ml solution of rabbit muscle phosphoglucone isomerase with an initial specific activity of 650 units/mg was added to 500 µl of 1.27 M guanidine hydrochloride solutions with varying glycerol: 0.0% (○), 10.0% (▲), 20.0% (△), 30.0% (●), 40.0% (□). At the indicated times, 10 µl aliquots of the incubation solution (30°C) were removed and assayed for phosphoglucone isomerase activity as discussed under "Methods".
Figure 10. Reactivation of dissociated rabbit muscle phosphoglucone isomerase at 23.5°C as a function of enzyme concentration. Inactive rabbit muscle phosphoglucone isomerase was incubated at various [0.00270 mg/ml (O), 0.00533 mg/ml (A), 0.0107 mg/ml (O), and 0.0279 mg/ml (A)] concentrations of protein in 0.2 M Tris, pH 7.7, 5 mM 2-mercaptoethanol, and 25% glycerol (v/v). At the indicated times aliquots were withdrawn and assayed for phosphoglucone isomerase.
Figure 11. Renaturation of inactive phosphogluco- 
cose isomerase as a function of temperature. Ten 
microliters of 0.534 mg/ml phosphogluco isomerase 
(5.3 µg) was dialyzed against 6.0 M GmHCl and diluted 
to 1.0 ml in renaturation buffer consisting of 0.2 M Tris, 
PH 7.7, 5 mM 2-mercaptoethanol, 25% glycerol. The 
resulting sample of enzyme was then incubated at various 
temperatures: 3.5°C (●), 37.5°C (●●), and 23.5°C 
(▲), and a 10 µl aliquot of the incubating mixture 
removed at various times and assayed for phosphogluco 
isomerase activity.
isomerase was incubated at three different temperatures (3.5°, 23.5°, and 37.5°) in standard renaturation buffer, maximal regain of activity was observed at 23.5°.

**Effect of Glycerol on Reactivation**

The reactivation of dissociation rabbit muscle phosphoglucone isomerase also showed a glycerol concentration dependence (Figure 12). When the enzyme was incubated at 23.5° C at varying glycerol concentrations, a maximal regain of activity was achieved when the renaturation buffer contained 20% glycerol. Higher concentrations of glycerol tended to result in lower regain of activity after 102 hours.
Figure 12. Effect of glycerol on the reactivation of rabbit muscle phosphoglucose isomerase. Ten microliters of 0.534 mg/ml denatured enzyme (5.3 μg) was added to 790 μl of standard renaturation buffer containing various concentrations of glycerol [0% (▲), 5% (△), 10% (●), 20% (□), 40% (〇)]. At the indicated times 10 μl aliquots were removed from the incubating (23.5°C) solution and assayed for phosphoglucose isomerase activity.
DISCUSSION

The peptide maps of catfish muscle and catfish liver isozymes (Figure 2A and 2B) were very similar, suggesting a significant homology in their primary structures. Several peptides (e.g., numbers 1, 3, 4, 5, 6, 8, 10, 13, 14 in Figure 2A and 2B) exhibited essentially identical electrophoretic and chromatographic properties. However, distinctly different migrations of the other peptides and the relative fluorescence after reaction with fluorescamine clearly indicated a number of structural differences between the liver and muscle isozymes. A greater degree of structural homology was evident between the catfish liver and muscle isozymes (c.c. = 0.951) than between the conger and catfish muscle isozymes (c.c. = 0.896). When peptide fingerprints of the wild type conger muscle and variant conger muscle were compared, a single peptide (7 and 7A in Figure 3) was observed with altered electrophoretic mobilities. The altered peptide from the variant appears to be more basic and slightly more hydrophobic than the corresponding peptide from the wild type isozyme. These data indicate that the phenotype may result from a point mutation.

Peptide maps of the fully carboxymethylated and carbamylated catfish muscle, catfish liver, and conger muscle enzymes revealed the presence of two identical subunits.
Even though phosphoglucose isomerase may not require large changes in conformation to catalyze the reversible isomerization of the hexose phosphoate, the rapid process of dissociation and reassociation could play a part in the regulation of the activity of phosphoglucose isomerase levels in vivo. Allosteric effects (Stadtman, 1966) and feedback inhibition from intermediates of the pentose phosphate cycle (Racker, 1965) have been proposed as evidence of regulation of phosphoglucose isomerase in vivo. On the other hand, Rose and Rose (1969) have been skeptical about the possibility of the enzyme ever becoming rate-limiting, since there are no concrete data available to support the concept. Within the living cells the enzyme exists in a complex environment which may markedly affect the dissociation-reassociation equilibrium. What role, if any, the dissociation-reassociation event has upon levels of the active enzyme in vivo has not yet been determined.

At least four major types of weak interactions are likely to be involved in maintaining the non-covalently bonded subunits of phosphoglucose isomerase together: (1) hydrogen bonds between peptide groups; (2) hydrogen bonds between R groups; (3) hydrophobic interactions between nonpolar R groups; and (4) ionic bonds between positively charged and negatively charged groups, such as the $\text{--COOH}^-$ of aspartate or glutamate R groups and the $\text{--NH}_3^+$ of lysine R groups.
Hydrophobic interactions between the nonpolar R groups have, in general, been shown to contribute more to the total conformational stability of oligomeric systems. In the case of rabbit muscle phosphoglucone isomerase five tryptophan residues have been suggested to be situated between the subunits of the native enzymes (Dyson and Noltmann, 1969B). Other nonpolar amino acids also are undoubtedly buried in this interface region. In the dissociated state these nonpolar R groups (which would be protected from water in the dimeric state) are inserted into a water environment, creating a new interface which requires the adjacent water molecules to assume a more ordered arrangement than they would have in pure water. Thus, an input of energy is required to force a nonpolar R group into water. Subunits with their nonpolar R groups exposed will thus tend to associate to minimize the unfavorable exposure to water. The tendency of the surrounding water molecules to relax into their maximum entropy state thus provides the driving force needed for the association process. In this transition the enzyme system goes to a state of lower entropy. Yet, the increase in the entropy of the surrounding free water molecules leads to an overall increase in entropy.

In addition to these hydrophobic interactions conferring stability upon the dimeric enzyme, the specificity of fit between subunits is, at least in part, provided by hydrogen bonding and other ionic interactions. In the
case of rabbit muscle phosphoglucone isomerase, eight cysteiny1 residues have been postulated to be situated between the subunits of the native enzyme (Dyson and Noltmann, 1969B). These residues could allow for hydrogen bonding between subunits to confer a proper fit. Yet when compared with the stability offered by the hydrophobic effect, hydrogen bonding contribution only marginally increases the stability of the oligomer (Schellman, 1955). Regardless of the factors which energetically and sterically drive the dimerization process, it is clear that these interactions are highly specific, since the subunits seem to aggregate in single state, and higher degrees of aggregations have never been observed for the phosphoglucone isomerase case.

With some idea of the types of molecular interactions between the two subunits of phosphoglucone isomerase, our attention can be focused upon the denaturation, renaturation, dissociation, and reassociation processes of this enzyme. When different phosphoglucone isomerasers were incubated in urea, [Urea] values were found from 3.32 M to 4.44 M. On the other hand, under identical conditions [GmHCl] values were found from 0.79 M to 1.05 M. Tiffany and Krimm (1973) postulated that the denaturation effects upon proteins by urea and guanidine hydrochloride are primarily due to their hydrogen bonding to the polypeptide backbone. This hydrogen bonding was pointed out to occur preferentially to the carbonyl group rather than the NH group of the amide
bonds and is supported by studies which indicated that the C=O group is a better proton acceptor than the NH group is a proton donor (Cannon, 1955; Mizushima et al., 1955). The greater general denaturation ability of guanidine hydrochloride on proteins as compared with urea is believed to be due to the greater number of interactions of guanidine hydrochloride with the carbonyl group of the amide bond (Tiffany and Krim, 1973).

In the presence of 1.0 M guanidine hydrochloride, the rates of inactivation of phosphoglucone isomerase from several species increased with increasing concentrations of salt. The rate of the rabbit muscle enzyme inactivation increased 128-fold by increasing the MgCl₂ concentration from 0 to 0.66 M. The mechanism of action of these salts on the dissociation is not understood, but may be due to an alteration of the structure of water by the salt allowing the guanidine hydrochloride to more readily act upon the polypeptide backbone of the enzyme. When a salt such as NaCl is dissolved in water, the Na⁺ and Cl⁻ ions become surrounded by shells of water dipoles, resulting in an altered geometry of the water. As more salt is added, the water becomes more ordered. Thus, in the presence of salt, the hydrogen bonding which normally occurs between guanidine hydrochloride and water may be decreased, thus allowing for more hydrogen bonding of the denaturant with the polypeptide backbone.
In addition to the altered structure of water, these salts could be directly acting on the protein to decrease the ionic and hydrogen bonding interactions between the subunits. It is generally accepted that those polar amino acid residues which are responsible for ionic and hydrogen bonding interactions between the subunits are found on the outer perimeter of that area which is involved in the non-covalent binding of one subunit to another. On this outer perimeter the polar amino acid residues are closer to the polar aqueous environment which they favor. When the subunits are associated into the dimer form, these polar amino acid residues also tend to protect the hydrophobic areas. However, salt may compete for the ionic interactions between such groups as the $\text{--COO}^-$ of aspartate or glutamate R groups and the $\text{--NH}_3^+$ of lysine R groups and the hydrogen bonding interactions between two peptide chains or between R groups. In competing in these two types of interactions, the salt may tend to loosen the outer fringer ionic interactions of the dimer, thereby allowing the denaturant to more rapidly "leak into" the hydrophobic areas, leading to a more rapid dissociation process.

While salt caused a more rapid dissociation and, hence, a subsequent loss of activity, fructose-6-phosphate and gluconate-6-phosphate showed only a slight protection against the guanidine hydrochloride-induced inactivation process.
In both cases \((k_0'/k_f')\) and \((k_0'/k_g')\) were slightly above 1.00 for four different species of phosphoglucone isomerase (catfish, rabbit, yeast, and human), indicating only minimal protection. This slight protection may suggest an altered conformational state of the dimeric enzyme which would allow for a larger contact area between subunits when the competitive inhibitor, gluconate-6-phosphate, or substrate, fructose-6-phosphate is positioned in the active site of the molecule. Upon removal of the substrate or competitive inhibitor from the active site, the enzyme may return to its original conformation, leaving less contact area between the two subunits.

While fructose-6-phosphate and gluconate-6-phosphate only slightly protect the phosphoglucone isomerase enzyme from guanidine-hydrochloride-induced inactivation, glycerol markedly protects the enzyme from this inactivation. The inactivation rates in the presence of 1.25 M guanidine hydrochloride decreased as the enzyme was incubated in increasing amounts of glycerol. The inactivation rate was slowed over 300-fold in the presence of 40% (v/v) glycerol. In fact, 40% glycerol almost completely protected the enzyme from inactivation by the guanidine hydrochloride. Shifrin and Parrott (1975) suggested that the stabilization against urea-induced denaturation provided by polyhydric alcohols such as glycerol may come about as a result of direct interactions with the protein or by modifying the
structure of the solvent molecules. Levitt (1962) has proposed that these polyhydric agents interact directly with proteins and points out that glycerol may hydrogen-bond to a variety of hydrophilic sites on proteins, thereby serving to maintain protein conformation. By binding to the polypeptide backbone, as well as hydrophilic R groups, glycerol could substitute for the bound water around the protein and prevent denaturation by forming a protective coat around the protein molecule. If the above mechanism is correct, increasing amounts of glycerol could compete more successfully against the denaturant for the hydrogen bonding sites on the protein.

In addition to significantly protecting phosphoglucoisomerase from denaturation, glycerol aids in the reassociation process of this enzyme. This ability to enhance the rates and extent to which denatured subunits reassemble into oligomeric proteins has recently been demonstrated in several other systems (Bradbury and Jakoby, 1972; Shelanske, et al., 1973; Ruwart and Suelter, 1971; and Kohn, 1970). Kohn (1970) found that the dimeric spinach leaf glyoxylate reductase (molecular weight 95,500 ± 5,000) reassociated under the optimal conditions of 0.2 M Tris-chloride, pH 7.2 to 7.6, 25% (v/v) glycerol and 5 mM mercaptoethanol at 0°C. Under these optimal conditions the half-life for maximal renaturation was found to be 7 to 10 hours, and the recovery was 68% of the theoretical maximum.
When phosphoglucone was denatured in 6.0 M guanidine hydrochloride for 17 hours at 3.5°C and placed into the optimal renaturation conditions found for glyoxylic acid reductase, a small amount of phosphoglucone isomerase (1 unit/mg) was regained after 72 hours. This amount of renaturation only represented 0.2% regain of the theoretical possible activity of 523 units/mg. When the renaturation temperature was increased to 23.5°C, 15.6% recovery was observed. A further increase in the renaturation temperature to 37.5°C lead to only a 2.5% recovery. The finding of maximal recovery at 23.5°C was similar to the observations of an optimal renaturation temperature between 16°C and 23°C for yeast glyceraldehyde 3-phosphate dehydrogenase (Deal, 1965). This temperature dependency raises the question of whether the effect produced by increase in temperature is (1) to shift the equilibrium to favor dimers, (2) to provide the necessary activation energy for folding of association, or (3) both. If the temperature increase from 3.5°C to 23.5°C was shifting the equilibrium, then the overall free energy of the reaction would be strongly temperature-dependent, and in particular favor subunits at 3.5°C. If this were so, the native enzyme should dissociate into subunits upon standing in the renaturation mixture after dilution at 3.5°C. This loss of activity has never been observed. At 23.5°C the denatured subunit may have the necessary activation energy for folding, leading to more
subunits with the proper conformation to form an active dimer. Yet at higher temperature (37.5°C) the subunits may fold at a faster and less specific fashion to yield a higher percent of incorrectly folded subunits. In this incorrect conformation the subunit may fail to associate or may associate to form inactive dimers.

The reassociation process also was found to be dependent on the protein concentration (Figure 10). In general, the half-time for maximal renaturation increased as the concentration increases. A similar situation was found in the case of spinach leaf glyoxylate phosphoglucone isomerase (Kohn, 1970). At 2.67 μg/ml the half-time was 6 hours. At 5.33 μg/ml the half-time was 8 hours. At 10.7 μg/ml the half-time was 13 hours. Yet, at 26.7 μg/ml the half-time was only 10 hours. Most likely this latter concentration does not follow the pattern due to the greater concentration (0.3 M) of guanidine hydrochloride in the renaturation buffer. The data suggest the existence of a complex kinetic system. These data were not surprising considering the complicated side reactions leading to improperly folded subunits which are "frozen" into metastable conformations.

In conclusion, this study has pointed out several structural differences between catfish muscle, catfish liver, wild type conger muscle, and variant conger muscle phosphoglucone isomerases. These structural variances confirmed from peptide fingerprints were in agreement with
the predicted amino acid homologies as estimated by calculation of the composition coefficients. The number of fluorescent spots found in the peptide maps added additional support to the presence of two identical subunits forming the quaternary structure of phosphoglucone isomerase. Further structural studies of phosphoglucone isomerase led to the elucidation of several parameters which effect the enzyme's denaturation-dissociation and renaturation-association processes. The presence of different salts increased the guanidine hydrochloride-induced inactivation to varying degrees. On the other hand, fructose-6-phosphate and glucuronate-6-phosphate led to only a slight decrease in the inactivation rates. Glycerol markedly decreased the inactivation rates in phosphoglucone isomerase in all species thus far studied.

Prior to this study, renaturation of phosphoglucone isomerase had not been demonstrated. In fact, a large number of studies conducted in this investigation - varying substrates, competitive inhibitors, pH, ionic strength, reducing agents, EDTA, etc. - were unsuccessful in promoting the reactivation of dissociated phosphoglucone isomerases. Yet glycerol markedly stimulated the regain of enzymatic activity. This process was found to be dependent on the temperature and protein concentrations. Even though conditions were not found for the regain of 100% enzymatic activity, conditions were found in which the process of
phosphoglucose isomerase's denaturation-dissociation was shown to be reversible. It is clear that future studies on this system should not only provide a more quantitative recovery of activity, but should also provide clues as to the biochemical (and perhaps physiological) basis for dimerization of the isomerase polypeptides in vivo.
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