A study on the phosphoryl group in pepsinogen

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OLIVEIRA, Robert James, 1942-
A STUDY ON THE PHOSPHORYL GROUP IN PEPSINOGEN.

Iowa State University, Ph.D., 1970
Biochemistry

University Microfilms, A XEROX Company, Ann Arbor, Michigan
A STUDY ON THE PHOSPHORYL GROUP IN PEP SIN OGEN

by

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A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

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Dean of Graduate College

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Ames, Iowa
1970
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LIST OF ABBREVIATIONS

ANSA  Aminonapthol sulfonylic acid
ATP  Adenosine triphosphate
Cyclic AMP  3',5'-Cyclic adenosine monophosphate
DEAE cellulose  Diethylaminoethyl cellulose
DTNB  5,5'-dithio-bis(2-nitrobenzoic acid)
EDTA  Ethylenediaminetetraacetate
$^{32}$P  Radioactive phosphorous
$^{32}$P$_i$  Radioactive inorganic phosphate
$\gamma^{32}$P-ATP  Adenosine triphosphate labeled in $\gamma$ position with $^{32}$P
POPOP  1,4-bis-[2-(5-phenyloxazolyl)]-benzene
PPO  2,5-diphenyloxazole
TCA  Trichloroacetic acid
iv

DEDICATION

to
Deanna
and
Mom and Dad
INTRODUCTION

Pepsinogen, the zymogen of the proteolytic enzyme, pepsin, found in the stomach, is a single chain macromolecule with a molecular weight of about 42,000 (1). It has been extensively studied by protein chemists because of its historic availability, i.e. first crystallized in 1936 (2). This zymogen might be singled out among other globular proteins for its rather high content of hydrophobic residues (3), as well as its unusually low isoelectric point, approximately 3.7 (4).

Pepsinogen, compared to its active enzyme, pepsin, has approximately twenty percent more amino acids, and is stable to denaturation between pH 5.5 and pH 9.0 (4), whereas pepsin is denatured above pH 6.0 (5). Actually, pepsin is autocatalytically derived from pepsinogen by the release of peptide segments from the N-terminus (1) at pH's below 5.0 (6). Pepsin contains the three disulfide bridges (7) and phosphoserine residue present in pepsinogen (1).

The function of phosphoryl groups in proteins has been of interest to investigators since Lipmann isolated a phosphoserine residue from an acid hydrolyzate of casein in 1933 (8). Prior to Lipmann's discovery, phosphorous had been recognized as a constituent of several proteins, e.g. ovalbumin, 1900 (9); casein, 1924 (10); and pepsin, 1930 (11). In 1938 pepsinogen was described as containing one mole of phosphorous per mole of protein (4).
Perlmann studied the role of phosphoryl groups in proteins with dephosphorylation enzymes. She succeeded in dephosphorylating ovalbumin using different phosphatases (12) and was able to demonstrate, electrophoretically, that commonly isolated ovalbumin was heterogenous because it was made up of ovalbumin molecules that contain either 0, 1, or 2 phosphate groups (13). In 1952 she was successful in enzymatically removing phosphoryl groups from casein (14). Pepsin and pepsinogen were reported to be dephosphorylated by either potato or intestinal phosphatases with no loss of pepsin or potential pepsin activity as measured by the hydrolysis of hemoglobin (15).

In a review article in 1955, Perlmann suggested for some phosphoryl groups in phosphoproteins a role similar to that of disulfide bridges (16). Specifically, she proposed that 40% of the phosphoryl groups of casein and the phosphoryl group of pepsin are intramolecular phosphodiester bridges. That the phosphate group in phosphoproteins exists as a diester has been disputed by a number of investigators (1, 17), and most recently by Clement et al. (18).

Irrespective of the actual linkage of the phosphate, it has been shown by Perlmann that in the absence of the phosphoryl group in pepsin, the rate of autodigestion is increased considerably (19). It appears that the phosphate group contributes to the stability of pepsin. Recently, investigators have
shown that the phosphate on pepsin has little or nothing to do with the enzymatic catalysis of N-acetyl-L-phenylalanyl-L-tyrosine methyl ester (18) or ovalbumin (20).

Another possibility for the role of phosphoryl groups in phosphoproteins is a source of phosphate. This "source type" of phosphoprotein is clearly identifiable by its large amount of phosphate, i.e. about 10% of the weight of the protein, and by the high turnover of its phosphate (16). Fish roe phosphoproteins are typical of this class (21). Since pepsinogen contains only one phosphoryl group, the "source type" of phosphoprotein is clearly different and will not be discussed here.

With the role of phosphate in phosphoproteins considered, the question can be raised of how and when phosphate is introduced into a protein molecule. One might break this "how" and "when" of phosphorylation into three cases.

1) The phosphate is incorporated as a phosphoamino acid as the protein chain is being synthesized.

2) The phosphate is incorporated by a phosphorylating enzyme, i.e. kinase, into an amino acid in the protein chain, before the protein is folded into a native-like conformation.

3) The phosphate is incorporated by a kinase into the protein after it is folded into a native-like conformation.

An experiment in which dephospho-ovalbumin was isolated as an intermediate in the biosynthesis of ovalbumin (22) tends to rule out cases 1 and 2. Short-term oviduct incubations
produced an ovalbumin containing no phosphate. With long incubation times ovalbumin containing phosphates was synthesized. Experiments in which fish roe phosphoproteins were isolated at different levels of phosphorylation (21) also give evidence against cases 1 and 2. Since pepsinogen without a phosphoryl group has been isolated in trace amounts by Lee and Ryle (23), it is suggested that the given serine in pepsinogen is likely phosphorylated after the protein is synthesized.

Considering the extensive work done on the reoxidation of reduced proteins (24, 25) and the implications of the value of the primary structure of a protein in determining its final conformation, a study on the reoxidation (refolding) of reduced pepsinogen and dephosphopepsinogen was conducted to offer new supporting evidence to the possibility that pepsinogen is phosphorylated after it is synthesized and folded. These reoxidation studies also were used to delineate structural differences between dephosphopepsinogen and pepsinogen.

A combination of a procedure of Perlmann's for dephosphorylation of pepsinogen (19) and column separation techniques similar to those of Lee and Ryle (23) allowed considerable quantities of dephosphopepsinogen to be prepared. The work of Steiner et al. (26) was especially valuable to this reoxidation study, because they presented somewhat optimal conditions for the reduction and subsequent reoxidation of pepsinogen. The kinetics of the loss of the sulfhydryl groups of reduced
pepsinogen and dephosphopepsinogen with air reoxidation and the regain of potential pepsin activity were studied.

Assuming that pepsinogen is phosphorylated after it is synthesized there emerges the question of how and why one given serine—out of 46 serine residues of the protein (3, 27)—is phosphorylated. This question and case 3 could be satisfied if one were to assume a "super serine". A "super serine" is defined as a given serine that is preferentially phosphorylated over other serines in a protein by a protein kinase. The serine might have exceptionally high reactivity, with regard to phosphorylation, because it is especially accessible to the phosphorylating enzyme, or because it has an unusually high chemical reactivity.

In order to further understand the biosynthetic route of the phosphate of pepsinogen, a nonspecific protein kinase (28) was used to attempt the phosphorylation of dephosphopepsinogen.

The results of the reoxidation and phosphorylation of pepsinogen and dephosphopepsinogen gave some interesting indications as to the nature of the conformational process of re-folding and further substantiated a likely mechanism for the biosynthetic phosphorylation of pepsinogen.
EXPERIMENTAL

Materials

Crystalline porcine pepsinogen obtained from Worthington Biochemical Corporation, Freehold, New Jersey was used exclusively in the reported experiments. Potato acid phosphatase, ultra pure urea, and glycylglycine were supplied by Mann Research Laboratories, New York, New York. Bovine hemoglobin, β-mercaptoethanol, and piperazine-N,N'-bis(2-ethane sulfonic acid) monosodium monohydrate (PIPES) were obtained from Sigma Chemical Corporation, St. Louis, Missouri. N-ethylmorpholine and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from Aldrich Chemical Company, Milwaukee, Wisconsin. Carrier-free γ³²P-ATP was obtained from Amersham/Searle, Des Plaines, Illinois. The casein was kindly supplied by Dr. Emerson Bird. The protein kinase was a generous gift from Dr. Edwin Krebs. All other chemicals were of reagent grade and used without further purification.

Methods

Preparation of dephosphopepsinogen

Approximately 200 mg. of pepsinogen was dissolved in a pH 5.6 solution, containing 0.1 M acetate buffer and 0.05 M magnesium chloride. Incremental additions of a total of 4 mg. of potato acid phosphatase, dissolved in the same buffer, were added over a period of 24 hours to the pepsinogen solution.
incubating at 37°. Then, the reaction mixture was dialyzed for three to four hours against a pH 6.9, 0.005 M PIPES buffer containing 0.2 M NaCl. This PIPES buffer was also used as the DEAE cellulose column support buffer. After dialysis, the sample was applied to the 3.8 x 48.0 cm. DEAE cellulose column, and was eluted with a pH 6.9 linear salt gradient that varied from 0.2 M to 0.45 M and contained 0.005 M PIPES. The dephosphopepsinogen fractions, separated from the unattacked pepsinogen and potato acid phosphatase, were pooled and dialyzed extensively against a pH 7.2 dilute solution of a volatile buffer, N-ethylmorpholine, that was progressively decreased in concentration from 0.1 M to 0.001 M. Lyophilization of the dialyzed dephosphopepsinogen solution was the final step in the preparation.

**Microphosphorous assay**

Verification that the dephosphopepsinogen was isolated from pepsinogen was accomplished by the extremely sensitive and reproducible microphosphorous assay described by Cohen et al. (29). It consists of a 1 ml. sample, containing approximately 5 mg. of protein, to which is added, cautiously, 0.4 ml. of 95% sulfuric acid, 0.5 ml. 60% perchloric acid, and 0.5 ml. of a freshly made 8% ammonium molybdate solution. The mixture is heated to 170° in a micro Kjeldahl flask for approximately thirty minutes, and then the mixture is heated to 230° for one hour. After being cooled to room temperature, 4.4 ml.
of water is added to the flasks. Then, 0.2 ml. of a reducing solution, made from 100 ml. of 15% sodium pyrosulfite, 0.25 g. ANSA, and 1.0 g. of sodium sulfite, is added to the Kjeldahl flasks and the mixture boiled at 100° for 10 minutes. Finally, the mixture is cooled and quantitatively made up to 10 ml. volume with water, and its optical density is read at 820 nm.

Protein reduction and reoxidation

The protein reduction procedure is essentially that of Steiner et al. (26). The reduction mixture contained approximately 75 mg. of protein and 4% of β-mercaptoethanol in a solution of 0.02 M glycylglycine, 0.1 M KCl, and 8 M urea. This mixture was incubated at 37° for approximately four hours.

After the reduction was complete, the mixture was applied to a G-25 sephadex column equilibrated with pH 6.0, 0.1 M acetate buffer containing 0.1 M KCl. The separation of reduced protein from the urea and β-mercaptoethanol was carried out under 10 psi of nitrogen pressure to insure speedy separation under non-oxidizing conditions. An early fraction of reduced protein, kept chilled and under nitrogen, was typically diluted into the reoxidation mixture.

The protein reoxidations were carried out at conditions described by Steiner et al. (26) as being optimum with regard to pH, i.e. pH 7.6, 0.02 M glycylglycine, and 0.1 M KCl, and at a protein concentration high enough to allow the sulfhydryl content to be measured, yet low enough to prevent extensive
aggregation, i.e. around 0.1 mg./ml. The protein concentration of the reoxidation mixture was generally determined by the Lowry et al. protein concentration method (30) with pepsinogen as a standard reference solution, assuming a molar extinction coefficient at 280 nm. of 52,500 and a molecular weight of 42,000 (31). The 10° and 37° reoxidation temperatures were maintained to ±0.1° with a Forma water bath.

Measure of sulfhydryl loss during reoxidation

The monitoring of the sulfhydryl group loss during reoxidation was accomplished with DTNB. Routinely, a 2.0 ml. aliquot of the reoxidation mixture was added to 0.05 ml. of a 0.01 M DTNB solution, pH 8.0 and 0.2 M in glycylglycine. An extinction coefficient at 412 nm. of 13,600 per mole of sulfhydryl group was used to determine the number of sulfhydryl groups (32). In the case of the 10° reoxidation experiments, the reoxidation mixture - DTNB solution was incubated at 37° for 2 minutes to be certain that the color development was complete. The optical densities at 412 nm. were corrected for a DTNB blank as well as protein turbidity.

Measure of potential pepsin activity regained during reoxidation

The potential pepsin activity of the pepsinogen being reoxidized was determined by the standard assay of Anson (33) using hemoglobin as a substrate. Pepsinogen from the reoxidation mixture is diluted in 0.01 N HCl and incubated at 37° for
10 minutes, so that it becomes activated to pepsin. The amount of TCA soluble peptides that pepsin can produce in 10 minutes at 37° from an acidified 2% hemoglobin solution is a measure of its enzymatic activity. The optical density of the TCA soluble peptides is determined at 280 nm. and is corrected with a blank that contains an equal amount of TCA denatured pepsin. The pepsinogen and dephosphopepsinogen used in the reported experiments were typically 3600 units/mg. A unit is equal to an absorbancy at 280 nm. of 0.001 per minute for the TCA soluble hydrolysis products obtained under these assay conditions.

Phosphorylation of pepsinogen and dephosphopepsinogen

The phosphorylations of proteins with γ³²P-ATP using protein kinase were commonly done under the conditions described by Walsh et al. (28). Routinely, the 0.6 ml. reaction mixture contained about 4 mg./ml. of protein to be phosphorylated, 0.05 M acetate buffer, 1.6 mM ATP(γ³²P-ATP), 3.6 mM Mg(C₂H₃O₂)₂, 0.02 M NaF, 2.0 mM theophylline, 0.3 mM EDTA, 0.1 mM cyclic AMP, and approximately 0.3 mg./ml. of protein kinase. The reaction was initiated by adding the protein kinase, and was maintained at 30° for the course of the reaction.

In analyzing the amount of ³²P₁ incorporated into proteins, an assay modified from the assay of Mans and Novelli (34) and Thomas et al. (35) was used. Typically, a 50 \% aliquot, removed
from the reaction mixture, was spotted on the rough side of a 2 x 2 cm. piece of Whatmann 3 M chromatography paper. After the 50 λ aliquot soaked into the paper, approximately three seconds, it was then dropped into 20% TCA maintained at a temperature of 3°. This caused the protein to be precipitated into the matrix of the paper and stopped the reaction. This procedure was continued until all the points of the time course of ^32P incorporation were taken. Then the 2 x 2 cm. papers containing the precipitated protein were washed in the apparatus, illustrated in Figure 1, to remove the γ^32P-ATP. The five minute washings were at room temperature except where noted and were in the following order: 5% TCA; 5% TCA, 80°; 5% TCA; 5% TCA; 1:1 ethanol:acetone; and acetone. The papers were dried under a heat lamp overnight.

Each paper was next put into a scintillation bottle containing 17 ml. of scintillation fluid (5.0 g. PPO, 0.25 g POPOP per liter of toluene). An aliquot of the reaction mixture was diluted 400 times, and 50 λ, to be used as a standard, was spotted on a blank paper that had gone through the washing cycles. The samples were counted on a Packard Tri-Carb scintillation spectrophotometer, model 3310. Blanks were calculated from extrapolations to zero time incorporation and were used to correct for ^32P adsorbed to the protein. Casein was used to compare the activity of the protein kinase in these experiments to the activity reported earlier (28).
Figure 1. Washing apparatus for radiochemical assay

Paper support is #6 mesh wire cloth made of type 304 stainless steel.
STIRRING BAR

MAGNETIC STIRRER

400 ML BEAKER

PAPER SUPPORT

STIRRING BAR
RESULTS

Preparation of Dephosphopepsinogen

Isolation of dephosphopepsinogen

Figure 2 shows the separation of dephosphopepsinogen and pepsinogen. PIPES was used as a buffer for the column elution, rather than phosphate as reported by Lee and Ryles, since they also reported that phosphate in their elution buffer had a tendency to bind to the dephosphopepsinogen (23). Very early in the elution potato acid phosphatase comes off the DEAE column. Dephosphopepsinogen, lacking approximately 2 negative charges, binds less tightly to the positively charged DEAE cellulose and is eluted before pepsinogen. As indicated in Figure 2 special care was taken to conservatively choose the fractions for the source of dephosphopepsinogen.

A number of potential pepsin activity determinations on the isolated dephosphopepsinogen gave essentially the same activity as pepsinogen, i.e. approximately 3600 u/mg. This is in agreement with previous work (15).

Microphosphorous assay

In order to be certain that pepsinogen was dephosphorylated, the phosphorous content of the dephosphopepsinogen and pepsinogen, isolated from the DEAE column, was determined with the sensitive microphosphorous assay (29) previously mentioned. In Figure 3 the standard curve demonstrates the sensitivity
Figure 2. DEAE cellulose column separation of potato acid phosphatase, dephosphopepsinogen, and pepsinogen

Elution is accomplished with a linear gradient of 0.2 - 0.45 M NaCl in a pH 6.9, 0.01 M PIPES buffer. Each tube contained 7 ml. A conservative selection of test tubes containing dephosphopepsinogen was made and is indicated by arrows, i.e. tube number 285 through tube number 312, inclusive. The potato acid phosphatase fraction is represented by a dashed line to indicate that its location with regard to tube number is approximate.
DEPHOSPHOPEPSINOGEN
Figure 3. Standard curve for microphosphorous assay

The symbols X and O represent duplicate determinations at a given phosphorous concentration. The symbols ▲ and ▼ represent 4.77 mg. of dephosphopepsinogen and 4.32 mg. of pepsinogen, respectively. The experimental procedure is described in the methods section.
MICROGRAM PHOSPHOROUS / ML ALIQUOT
of this microphosphorous assay. The typical data points for pepsinogen and dephosphopepsinogen gave values of 1.03 moles of phosphorous per mole of pepsinogen and 0.01 mole of phosphorous per mole of dephosphopepsinogen.

Reoxidation Studies

Progress curves for % sulfhydryl group loss and % potential pepsin activity regain

In order to gain more insight about the phosphoryl group in pepsinogen, and some indication as to its effect on the folding of newly synthesized pepsinogen, the reoxidation of reduced pepsinogen and dephosphopepsinogen was studied. The results of the reoxidation of the reduced disulfide bridges of pepsinogen and dephosphopepsinogen are illustrated in Figure 4. Looking at the % sulfhydryl group loss and % potential pepsin activity regain as a function of time, two things are immediately obvious:

1) There is a strong temperature dependence in both % loss of sulfhydryl groups and % potential pepsin activity regain for pepsinogen. The rate of potential pepsin activity regain at 10° is slow compared to that at 37°.

2) The rate of reoxidation of sulfhydryl groups and rate of regain of potential pepsin activity in dephosphopepsinogen are dramatically faster than that of the rates with pepsinogen at a comparable temperature. With the phosphorylated zymogen at 37° a lag in the regain of potential pepsin activity with
Figure 4. Progress curves for % sulfhydryl group loss and % potential pepsin activity regain of reoxidized protein

The symbols □ and □ represent 0.105 mg./ml. of reduced pepsinogen being reoxidized at 10°. The symbols ▲ and ▼ represent 0.088 mg./ml. of reduced pepsinogen being reoxidized at 37°. The symbols ● and ○ represent 0.091 mg./ml. of reduced dephosphopepsinogen being reoxidized at 37°. The pH 7.6 reoxidation solution contained the appropriate amount of reduced protein and was 0.1 M in KCl and 0.02 M in glycglycine. The % sulfhydryl group loss and potential pepsin activity regain assays are described in the methods section.
% SH LOSS (CLOSED FIGURES)

TIME (HOURS)

DEPHOSPHOPEPSINOGEN

PEPSINOGEN

% POTENTIAL PEPisin ACTIVITY REGAIN (OPEN FIGURES)
time is apparent. This lag occurred reproducibly in all experiments at 37° with pepsinogen, and has been attributable to the initial formation of "wrong", i.e. not native, disulfide bonds (24). The apparent lack of a lag in the rate of regain of potential pepsin activity of dephosphopepsinogen may be due to the rapidity of the refolding process.

It should be noted that the approximately 70% regain of potential pepsin activity for the reoxidation of pepsinogen at 37° is rather high, when compared to previous work that showed approximately a 50% regain of potential pepsin activity for pepsinogen (26). The fact that a different temperature, 25°, was used for reoxidation in the previous work, and the likelihood of an improved commercial preparation of pepsinogen caused this difference not to be considered too significant.

Effect of temperature on pepsinogen reoxidation

It has been pointed out that the rate of reoxidation of reduced pepsinogen is strongly temperature dependent. Figure 5 is a first order plot of the loss of sulfhydryl groups upon reoxidation of reduced pepsinogen at 10°. This reoxidation through approximately 3 half-lives appears monophasic and gives a first order rate constant of 1.44 x 10^{-3} min^{-1}. A reoxidation of reduced pepsinogen, at a concentration similar to the 10° reoxidation, has been studied at 37°, and is illustrated in a first order plot in Figure 6, line 2. This first order plot of the reoxidation of reduced pepsinogen at 37° is
Figure 5. First order plot for sulfhydryl group loss during reoxidation of reduced pepsinogen at 10°

The pH 7.6 reoxidation solution contained 0.105 mg./ml. of reduced pepsinogen and was 0.1 M in KCl and 0.02 M in glycyglycine. The % sulfhydryl group loss assay is described in the methods section.
10°

- 0.105 mg/ml
Figure 6. First order plot for sulfhydryl group loss during the reoxidation of reduced pepsinogen at 37° and at a number of concentrations

The symbols □, ●, ■, ○ and △ represent reoxidation at 37° of 0.125, 0.119, 0.088, 0.054 and 0.036 mg./ml. of reduced pepsinogen, respectively. The pH 7.6 reoxidation solution contained the appropriate amount of reduced protein and was 0.1 M in KCl and 0.02 M in glycylglycine. The % sulfhydryl group loss assay is described in the methods section.
$37^\circ$

- $0.125 \text{ mg/ml}$
- $0.119 \text{ mg/ml}$
- $0.088 \text{ mg/ml}$
- $0.054 \text{ mg/ml}$
- $0.036 \text{ mg/ml}$
biphasic and gives first order rate constants of $1.69 \times 10^{-3}$ min$^{-1}$ and $3.46 \times 10^{-3}$ min$^{-1}$ for the first and second slopes, respectively. These observations, and the fact that a much higher potential pepsin activity was obtained from the reoxidation of reduced pepsinogen at 37°, prompted a study on the concentration dependence of reduced pepsinogen in its 37° reoxidation.

Effect of concentration on pepsinogen reoxidation

The reoxidation of reduced pepsinogen at five concentrations is seen in Figure 6. Lines 1, 2, 3, and 4 represent the first order plot of the reoxidation at concentrations of reduced pepsinogen from 0.125 mg/ml. to 0.054 mg/ml. These lines are clearly biphasic, and show the first phase, but apparently not the second phase, to be concentration dependent. Table 1 shows the half-times and rate constants as a function of concentration for the first and second phase. It is clear that the first phase is pseudo first order, because the half-times are concentration dependent, and the plot appears linear for a given concentration. It should be noted that the concentration dependence of the rate constants for the first phase is in the opposite direction of what one would intuitively expect, i.e. the rates increase, rather than decrease, with decreasing concentrations. Similar behavior has been observed with the reoxidation of Taka-amylase A (36).
Table 1. Half-times and rate constants for first order plot of the reoxidation of reduced pepsinogen at 37°

<table>
<thead>
<tr>
<th>Line</th>
<th>Concentration of pepsinogen (mg./ml.)</th>
<th>First Phase</th>
<th>Second Phase</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>0.125</td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;: 7 hrs. 5 min.</td>
<td>Rate constant: 1.63 x 10&lt;sup&gt;-3&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>0.119</td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;: 6 hrs. 50 min.</td>
<td>Rate constant: 1.69 x 10&lt;sup&gt;-3&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>0.088</td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;: 6 hrs. 20 min.</td>
<td>Rate constant: 1.82 x 10&lt;sup&gt;-3&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>0.054</td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;: 5 hrs. 25 min.</td>
<td>Rate constant: 2.13 x 10&lt;sup&gt;-3&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>0.036</td>
<td>t&lt;sub&gt;1/2&lt;/sub&gt;: 4 hrs.</td>
<td>Rate constant: 2.67 x 10&lt;sup&gt;-3&lt;/sup&gt; min&lt;sup&gt;-1&lt;/sup&gt;</td>
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Figure 6, line 6 (low concentration) shows an apparent lack of biphasic first order kinetics. This is probably because of a blending of similar rate constants for the two processes that are represented by the two phases.

To insure that cupric ions, which catalyze the reoxidation of sulfhydryl groups (36), were not present at very high levels in the reoxidation solutions, the following experiment was conducted. A reoxidation of pepsinogen with $10^{-6}$ M cupric ions was carried out at 37°. The results of this experiment showed that $10^{-6}$ M cupric ions had a significant effect on the reoxidation of reduced pepsinogen, causing a fourfold increase in the reoxidation rate of the first phase. Thus, it can safely be said that the level of contamination of cupric ions, that catalyze the reoxidation of sulfhydryl groups, is very much less than $10^{-6}$ M.

At pH 7.6 where pepsinogen is grossly, negatively charged, an experiment was conducted, in which the repulsive effect, between like-charged pepsinogen molecules, was minimized by high salt. The results of the reoxidation of reduced pepsinogen in 2.1 M KCl at 37° are illustrated as a first order plot in Figure 7. This reoxidation in high salt showed essentially one phase in the first order plot, demonstrated extensive aggregation, and resulted in essentially no potential pepsin activity. This slow rate for the reoxidation, $1.49 \times 10^{-3}$ min$^{-1}$, was similar to the rate of the first phase of the
Figure 7. First order plot for sulfhydryl group loss during the reoxidation of reduced pepsinogen in high salt at 37°.

The pH 7.6 reoxidation solution contained 0.123 mg./ml. of reduced pepsinogen and was 2.1 M in KCl and 0.02 M in glycyglycine. The sulfhydryl group loss assay is described in the methods section.
biphasic first order plot for the reoxidation of pepsinogen at 37° and with much lower concentrations of salt (Figure 6). As a result of this, it seems possible that the first phase of the reoxidation of pepsinogen at 37° and low salt could represent a somewhat nonspecific oxidation of sulfhydryl groups of pepsinogen.

A reaction order determination was carried out on the first phase of the log plot of the concentration dependent reoxidation of reduced pepsinogen, and is shown in Figure 8. By plotting the logarithms of the half-times of reoxidation versus the logarithms of the concentrations of pepsinogen, and determining the negative absolute value of the slope, and then adding one, the reaction order may be obtained (37). A reaction order of 0.68 was found. This reaction order, not being an integer, does much to emphasize the complexity of the first phase of reoxidation of reduced pepsinogen at 37°.

Having considered the reoxidation of pepsinogen, and having investigated the reoxidation rates somewhat in detail, the groundwork for an appreciation of the rates of reoxidation of reduced dephosphopepsinogen has been set down.

The reoxidation of reduced dephosphopepsinogen

The first order plot of the reoxidation of reduced dephosphopepsinogen at two concentrations is illustrated in Figure 9. A striking difference in the kinetics of the reoxidation of reduced dephosphopepsinogen and reduced pepsinogen is obvious
Figure 8. A $\log t_{1/2}$ vs. $\log$ pepsinogen concentration plot for determining reaction order

The half-times and concentrations of pepsinogen from the first phase of the biphasic first order plots of the loss of sulfhydryl groups in reduced pepsinogen at 37°, i.e. data from Table 1, are used as data points.
REACTION ORDER = 0.68

$(\text{mg PEPsiNOGEN/m1}) \times 10^{-2}$
Figure 9. First order plot for sulfhydryl group loss during the reoxidation of reduced dephosphopepsinogen

The symbols • and □ represent 0.091 and 0.039 mg. of reduced dephosphopepsinogen, respectively. The pH 7.6 reoxidation solution contained the appropriate amount of reduced dephosphopepsinogen and was 0.1 M in KCl and 0.02 M in glycylglycine. The % sulfhydryl group loss assay is described in the methods section.
- $0.091 \text{ mg/ml}$
- $0.039 \text{ mg/ml}$
when Figures 9 and 6 are compared. The first phases of the two plots at different concentrations of dephosphopepsinogen in Figure 9, if assumed to be first order, have rate constants of approximately $7.2 \times 10^{-3} \text{ min}^{-1}$ and $10.7 \times 10^{-3} \text{ min}^{-1}$ for the high and low concentrations, respectively. These rate constants are approximately four to five times the corresponding rate constants for similar pepsinogen solutions. Of course, if this first phase in Figure 9 is not assumed to be first order, it is nevertheless obvious that the initial rate of reoxidation of reduced dephosphopepsinogen is decidedly faster than the initial rate of reoxidation for reduced pepsinogen.

The second phases of the first order plots for the reoxidation of reduced dephosphopepsinogen at two concentrations have first order rate constants of $3.30 \times 10^{-3} \text{ min}^{-1}$ and $3.15 \times 10^{-3} \text{ min}^{-1}$. Interestingly, the second phase reoxidation of reduced dephosphopepsinogen has rates similar to the rates, approximately $3.5 \times 10^{-3} \text{ min}^{-1}$, for the second phase reoxidation of reduced pepsinogen, and, similarly, are essentially independent of concentration, i.e. truly first order.

In order to insure that no anomalies were introduced in the preparation and subsequent isolation of dephosphopepsinogen, a sample of pepsinogen, isolated from a dephosphopepsinogen preparation, was reduced and the reoxidation at 37° was studied. The results showed that pepsinogen, treated the same way as dephosphopepsinogen, behaved like the pepsinogen of Figure 6, i.e. a slow then fast first order rate.
A comparison of the rates of reoxidation of reduced dephosphopepsinogen and reduced pepsinogen will allow implications to be made regarding the nature of the folding of the pepsinogen chain after it is newly synthesized. This will be elaborated upon in the discussion.

Efficiency of reoxidation of reduced dephosphopepsinogen and pepsinogen

In order to have another comparison of dephosphopepsinogen and pepsinogen, the efficiency of the loss of sulfhydryl groups with respect to the regain of catalytic activity was studied. A plot of the loss of moles of sulfhydryl groups per mole of protein versus the percent regain of potential activity shows the efficiency of the reoxidation (38) and is illustrated in Figure 10. In this figure the time dependence of the reoxidation rate and of the potential pepsin activity regain is eliminated, and only the molecular efficiency is measured. Although, as shown in Figures 4, 6, and 9, the reduced dephosphopepsinogen reoxidation and regain of potential pepsin activity is a much faster process than that for pepsinogen, it appears that the pepsinogen reoxidation process is slightly more efficient. This can be accounted for if dephosphopepsinogen is less stable than pepsinogen at pH 7.6 and gradually loses some of its regained potential pepsin activity as the reoxidation takes place (4, 23).
Figure 10. The efficiency of sulfhydryl loss at 37° with regard to potential pepsin activity of pepsinogen and dephosphopepsinogen

The symbols • and □ represent 0.088 mg./ml. of pepsinogen and 0.091 mg./ml. of dephosphopepsinogen. The data points are taken from the appropriate smooth curves of Figure 4.
Moles SH / Mole Dephosphopepsinogen
%POTENTIAL PEPisin ACTIVITY REGAINED

- PEPSINOGEN
- DEPHOSPHPEPSINOGEN
From the data presented it seems that the absence of the phosphoryl group of pepsinogen has a very significant effect on the rates of reoxidation. Although not done in vivo, the fact that the dephosphorylated form reoxidizes and regains activity faster implies that it possibly could be the form which is synthesized, folded to a native-like conformation, and then finally phosphorylated.

Phosphorylation Studies

Phosphorylation of dephosphopepsinogen and pepsinogen with protein kinase

The above mentioned phosphorylation scheme would imply either one of the following possibilities:

a) A nonspecific protein kinase would phosphorylate a special serine, i.e. a "super serine".1

b) A specific kinase would recognize a given area on the dephosphopepsinogen molecule and phosphorylate the appropriate serine.

Though it is not possible to rule out a) or b), the following experiments suggest that the "super serine" of a) could exist.

1It should be noted that a "super serine" is defined as a serine group on a protein that is hyperactive with regard to phosphorylation by a protein kinase.
In order to be certain that the protein kinase was active and to test the radiochemical assay, casein was used as a substrate, as previously reported (28). Figure 11 illustrates the incorporation of phosphate into casein, and demonstrates the success of the radiochemical assay. Casein was phosphorylated at the rate of 0.21 μmoles of phosphate incorporated per milliliter of reaction mixture per hour, or, assuming that the protein kinase is saturated with substrate, 0.91 μmoles of phosphate incorporated per milligram of protein kinase per hour. The protein kinase being used as a phosphorylation catalyst in these experiments had approximately 15% of the phosphorylation activity, with casein as a substrate, of the protein kinase reported in the literature (28). In spite of its relatively low activity, the protein kinase was an effective phosphorylation catalyst.

Having demonstrated the activity of the protein kinase and the utility of the radiochemical assay, the phosphorylation of dephosphopepsinogen and pepsinogen was attempted. Looking at Figure 12, it is apparent that dephosphopepsinogen is phosphorylated at a substantially higher rate than pepsinogen. Calculations of the slopes of these time courses, assuming the protein kinase to be saturated with substrate, gave values of 0.46 μmoles and 0.09 μmoles of phosphorylation per milligram of protein kinase per hour for the rates of phosphorylation of dephosphopepsinogen and pepsinogen, respectively. The rate of
The pH 6.0 reaction mixture contained 6.0 mg./ml. of casein, 0.05 M acetate buffer, 1.6 mM ATP ($\gamma^{32}$P-ATP, $2.14 \times 10^8$ cpm/ml), 3.6 mM Mg($\text{C}_2\text{H}_3\text{O}_2$)$_2^-$, 0.02 M NaF, 2.0 mM theophylline, 0.3 mM EDTA, 0.1 mM Cyclic-AMP, and 0.23 mg./ml. of protein kinase. The procedure for the radiochemical assay is in the methods section.
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TIME (MINUTES)
0.0  1.0  0.2  0.3

CASEIN
Figure 12. Progress curve for the enzymatic phosphorylation of pepsinogen and dephosphopepsinogen

The pH 6.0 reaction mixtures contained 0.05 M acetate buffer, 3.6 mM Mg(C₂H₂O₂)₂, 0.02 M NaF, 2.0 mM theophylline, 0.3 mM EDTA, 0.1 mM Cyclic-AMP, and 0.38 mg./ml. of protein kinase. The reaction mixture for the phosphorylation of pepsinogen also contained 3.94 mg./ml. of pepsinogen and 1.8 mM ATP(γ³²P-ATP; 8.2 x 10⁷ cpm/ml). For the phosphorylation of dephosphopepsinogen the reaction mixture contained 3.57 mg./ml. of dephosphopepsinogen and 1.8 mM ATP(γ³²P-ATP; 5.3 x 10⁷ cpm/ml). The procedure for the radiochemical assay is described in the methods section.
phosphorylation of dephosphopepsinogen is at least five times the rate of phosphorylation of pepsinogen. A minimum value of five times is given because the possibility for self-incorporation of radioactive phosphate into the protein kinase was not examined.

When one considers that only approximately 2% of the serines, 1 out of 46, are phosphorylated in pepsinogen (3, 27), and that the rate of the phosphorylation of dephosphorylated pepsinogen, i.e. dephosphopepsinogen, is at least five times greater than the phosphorylation of pepsinogen, the differences between dephosphopepsinogen and pepsinogen are again emphasized.
In this study of the phosphoryl group of pepsinogen, the reoxidation of reduced pepsinogen and dephosphopepsinogen and the phosphorylation of the pepsinogen and dephosphopepsinogen were investigated. So that the studies on the reoxidation of reduced dephosphopepsinogen could be appreciated, the reoxidation of reduced pepsinogen was examined in detail.

When pepsinogen is reduced under the conditions of the experiments reported essentially all of the tertiary and most of the secondary structure of the protein is eliminated. This has been demonstrated in the fluorescence polarization work of Steiner et al. (26) and in the intrinsic viscosity measurements of Martin and Ames (39). It has been shown here that the reoxidation of this somewhat random, reduced pepsinogen is strongly temperature dependent. The extremely slow reoxidation at 10° may be accounted for by the nature of the large number of hydrophobic residues, approximately 50% (3), in pepsinogen. As pointed out by Kauzmann (40), the strength of hydrophobic bonds is directly related to temperature. At 10° the hydrophobic groups are better suited in an aqueous environment than at a higher temperature, and as a result, the contribution of the hydrophobic groups might well be the "key force" that tends to decrease the rate of reoxidation.
At 37° there is less tendency for reduced pepsinogen to resist reoxidation, and it has a significantly higher potential regain of activity. The first order plot of the 37° reoxidation of a comparative amount of pepsinogen is biphasic with a slow first phase that is inversely dependent on concentration, and apparently, a truly first order second phase. A reaction order of 0.68 for the first phase tends to emphasize its complexity. Though the entire reoxidation is complex, some qualitative remarks can be made about the reoxidation process, especially the inverse concentration dependence of the first phase.

It is suggested that the first phase at 37°, and probably much of the reoxidation at 10°, is mostly representative of nonspecific aggregation leading to a significant amount of intermolecular disulfide bonding. Evidence for such behavior in ribonuclease was given by sedimentation velocity data as a function of time for reoxidation (24). It was reported that a large amount of intermolecular disulfide bond formation was observed at early stages of reoxidation, and that, as time progressed, the amount of the aggregation decreased, concomitantly, with the regain of activity. This process is caused by disulfide interchange (24), i.e. a sulfhydryl displacing one-half of a disulfide bridge. One possible explanation for the inverse concentration dependence observed for the first phase would be concentration dependent competing reactions.
That is to say, at low concentrations less intermolecular and more intramolecular interactions occur. The intramolecular interactions statistically produce more native-like pepsinogen, and as a result, there is an increase in the first phase rate at 37° with a decreasing amount of reduced protein. At high concentrations of reduced protein, intermolecular formation of disulfide bonds is favored by mass action, and subsequently, there is a slower rate of reoxidation, since there is a smaller contribution to the rate by the faster intramolecular reoxidation process. A different explanation for an inverse rate dependence on concentration for reoxidation has been reported for the reoxidation of reduced Taka-amylase A (36). It was suggested that as the concentration of reduced protein is decreased the relative amount of solution contaminants, e.g. cupric ions, that catalyze the reoxidation of sulfhydryl group is increased, and therefore, the reoxidation has an inverse concentration dependence. The experiments described here cannot distinguish between these two possibilities.

The fourfold to fivefold increase in the first phase reoxidation rate for reduced dephosphopepsinogen, compared to reduced pepsinogen, convincingly demonstrates an unreported difference between dephosphopepsinogen and pepsinogen. This faster first phase, which could imply more intramolecular disulfide formation at an earlier time is in agreement with the protein chain being synthesized, folded, and then phosphorylated. The effect of a phosphate group on a refolding process,
where it normally isn't present, could be that it statistically makes the transition state more difficult to reach due to the repulsion of wrong combinations of juxtapositioned portions of the protein chain. Of course, these reoxidation experiments are not under in vivo conditions, but they do offer some supporting evidence to the idea that pepsinogen is phosphorylated after it is reoxidized to a native-like conformation. This idea is more acceptable in light of reports of other phosphoproteins, e.g. ovalbumin (22) and fish roe phosphoprotein (21), that are believed to be phosphorylated after synthesis.

Perlmann suggested the existence of a phosphodiester linkage in pepsinogen on the basis of the apparent inability of prostrate monophosphatase to liberate inorganic phosphate, and the fact that the pretreatment with a phosphodiesterase and subsequent attack by prostrate monophosphatase resulted in the release of the phosphate (19). Electrophoretic evidence supporting the idea of a phosphodiester linkage was also offered by Perlmann (19). Arguments to the absence of a phosphodiester linkage in pepsinogen are given by Clement et al. (18). These arguments include that no change in potential catalytic activity was observed with the dephosphorylated form of pepsinogen, that only one sequence containing a phosphoserine has been isolated (27), and that it was possible to remove the phosphate in pepsinogen with a potato phosphatase that apparently had no diesterase activity.
The likelihood of the existence of a phosphodiester linkage in pepsinogen is also questioned by the results of the re-oxidation experiments reported here. A phosphoryl group does not exist in at least the first 42 amino acids, 12% of the total number of residues (3), of the N-terminus of pepsinogen, since this section is lost on activation to pepsin. The sequence of at least the last 109 amino acids, 30% of the total number of residues, at the C-terminus of pepsinogen does not include the sequence of the phosphoserine peptide isolated by Stepanov et al. (27), (41). From this information it is obvious that the phosphoserine residue is away from the ends of the chain of reduced pepsinogen and located somewhere within the remaining 58% of the molecule. Assuming minimum conditions for phosphodiester formation in the remaining 58% of the reduced pepsinogen molecule, i.e. shortest possible distance between amino acid residues linked in between the phosphodiester bond, it is apparent that the randomized chain of reduced pepsinogen has to be restricted somewhat by the phosphodiester linkage. Since some additional order would be given to the molecule by the phosphodiester linkage, it seems that pepsinogen probably would fold faster, and because of a higher degree of randomness dephosphopepsinogen would fold slower. Exactly the opposite has been observed in the experiments reported here, suggesting the absence of a phosphodiester linkage in pepsinogen in view of this and previous results (18).
Assuming that the dephosphopepsinogen is phosphorylated after being folded to a native-like conformation, it remains to be explained how only one given serine is always phosphorylated. The existence of a large number of nonspecific kinases (42), that only one given serine out of 46 is phosphorylated in native pepsinogen (3, 27), that the phosphate of pepsinogen is easily removed (18), and that reduced dephosphopepsinogen reoxidizes much faster than reduced pepsinogen, fit together in the following mechanism for the phosphorylation of dephosphopepsinogen.

If one accepts that a "super serine" is formed and that conformational changes are slight when pepsinogen is dephosphorylated, it is easy to rationalize the observed behavior of protein kinase in phosphorylating dephosphopepsinogen. This rate of phosphorylation of dephosphopepsinogen is at least five times greater than the rate with pepsinogen, and yet only one serine out of 46 was changed to make dephosphopepsinogen. It should be noted that no major structural difference between these two protein species has been reported in the literature since the first dephosphorylation of pepsinogen in 1952 (15). This "super serine" could be positioned so that it extends away from the surface of the protein in easy contact with the protein kinase and ATP, or it could be the environment of a given area on the protein around the "super serine" that accounts for its phosphorylation by preferential binding of
the protein kinase. The phosphorylation of synthesized dephosphopepsinogen by a kinase, probably nonspecific, is suggested as a likely possibility in vivo by these and previous results (21, 22).

A possible reason for the phosphoryl group in pepsinogen, that is in accord with the proposed mechanism, is to stabilize the dephosphopepsinogen species so that it may be stored as pepsinogen in the zymogen granules.
SUMMARY

The reoxidation of reduced pepsinogen and dephosphopepsinogen and the rates of phosphorylation of pepsinogen and dephosphopepsinogen were studied to understand better the phosphoryl group in pepsinogen. Implications regarding the "when" and "how" of the biosynthesis of the phosphoryl group in pepsinogen were offered.

The reoxidation of pepsinogen was studied in detail so that a reference could be established for the comparison of dephosphopepsinogen. At 10° the reoxidation obeyed first order kinetics and had a rate constant of $1.44 \times 10^{-3} \text{ min}^{-1}$. The data of the 37° reoxidation at a similar concentration of reduced pepsinogen fit a biphasic first order plot, with a first phase rate of approximately $1.69 \times 10^{-3} \text{ min}^{-1}$, and a faster second phase reoxidation rate of approximately $3.46 \times 10^{-3} \text{ min}^{-1}$. The concentration dependence of the 37° reoxidation of reduced pepsinogen was also investigated. The first order plots for these data also seemed to be biphasic in nature with the first phase rate being inversely dependent on pepsinogen concentration and with faster second phases that appear to be truly first order. The initial phase, emphasized to be very complex by a reaction order of 0.68, was attributed primarily to intermolecular disulfide bond formation, while the last phase was likely due mainly to intramolecular disulfide bond formation. A study was also carried out on the reoxidation of...
reduced pepsinogen in a solution (high salt) which mimicked the case where aggregation, i.e. intermolecular disulfide bond formation, was predominant.

With reduced dephosphopepsinogen the reoxidation was studied at 37°. It demonstrated a striking difference from the reoxidation behavior of reduced pepsinogen. The first order plots of the reoxidation of reduced dephosphopepsinogen were also biphasic, but the first phase had a rate approximately four to five times the rate of reoxidation of a comparable concentration of reduced pepsinogen. The second phase of the log plot was similar to the second phase for pepsinogen. The striking difference in the first phase reoxidation rates of reduced pepsinogen and dephosphopepsinogen was taken to give evidence against the likelihood of a phosphodiester linkage in pepsinogen and to substantiate that perhaps the phosphoryl group is put on the pepsinogen molecule after it is synthesized and folded to a native-like conformation.

The phosphorylation of dephosphopepsinogen and pepsinogen by the nonspecific protein kinase was studied to gain insight into the mechanism of the biosynthesis of the phosphoryl group of pepsinogen. The result was that dephosphopepsinogen was phosphorylated at a rate of at least five times that of pepsinogen. A "super serine" was conceived to account for this behavior.

1"Super serine" is defined as a given serine that is preferentially phosphorylated over other serines in a protein by a protein kinase.
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ACKNOWLEDGEMENTS

The author is appreciative of the special friendship, enthusiasm, and example of his major professor, Donald Graves, inside and outside the lab.

Thanks go to Dr. James Thomas for his suggestions in the phosphorylation experiments and the use of his washing apparatus. That Dr. Malcolm Rougvie acted as a sounding board for some research ideas is appreciated.

The technical assistance of Don Mahuran on the pilot reoxidation experiments and Ed Anderson on the microphosphorous assays is appreciated.

Sincere gratitude is extended to the many friends in the graduate students and staff who have made life at Iowa State so pleasant.

The author acknowledges the assistance and encouragement of his mother and father who helped to make his education possible.

Finally, and most importantly, the author thanks his wife, Deanna, for their love.