A Tryptic Peptide Containing a Unique Serine Phosphate Residue in Rabbit Phosphoglucomutase

By CELIA. P. MILSTEIN AND C. MILSTEIN

*Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge, and
Medical Research Council Laboratory of Molecular Biology, Cambridge*

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³²P-labelled phosphoglucomutase was digested with trypsin after denaturation and two peptides were isolated that contained the bulk of the radioactivity bound to peptides. Both peptides appeared to derive from an identical section of the molecule. Peptic and subtilisin digests of the tryptic peptides were prepared. The resulting radioactive peptides were purified and their sequences studied. The presence of a single serine [³²P]phosphate residue was clearly established. Difficulties in purification and low yields, especially of the tryptic peptide, prevented exhaustive sequence studies, but a tentative sequence is proposed as:

Ala-Ile-Gly-Gly-Ile-Ile-Leu-Thr-Ala-SerP-His-Asx-Pro-Gly-Gly-Pro-(Asx₂,Gly)-
Phe-Gly-Ile-Lys

(where SerP represents serine phosphate and Asx represents aspartic acid or asparagine). The results do not support the presence of two serine phosphate residues in the denatured enzyme, but confirm previous results of a unique sequence around a single serine phosphate residue.

Phosphoglucomutase (D-glucose 1,6-diphosphate-D-glucose 1-phosphate phosphotransferase, EC 2.7.5.1) is an enzyme that catalyses the transfer of phosphate from C-1 of α -glucose 1-phosphate to C-6 of glucose 6-phosphate. Glucose 1,6-diphosphate is required as a cofactor of the reaction (Cardini, Paladini, Caputto, Leloir & Trucco, 1949). After denaturation and hydrolysis of the rabbit muscle enzyme a phosphate residue remains attached to a serine residue (Anderson & Jollès, 1957; Kennedy & Koshland, 1957). This was described as a unique serine residue present in a sequence Thr-Ala-SerP-His-Asx* by Milstein & Sanger (1961). However, the presence of two phosphorylated sites was proposed by Harshman & Najjar (1965), who also indicated that the two sites occurred in the vicinity of the peptide chain. Some of these results appeared unsatisfactory (see the Discussion section), and the fact that they were incompatible with the results previously reported (Milstein & Sanger, 1961) prompted us to re-investigate the problem. In a preliminary communication Milstein (1966b) showed that the sequence around a unique reactive serine residue was in agreement with the one reported by Milstein & Sanger (1961) for the rabbit muscle enzyme and

also with the preliminary report of Hashimoto, Del Rio & Handler (1966) for the flounder enzyme. We now present further sequence studies of a tryptic peptide containing 23 residues and a single serine residue present as serine phosphate.

MATERIALS AND METHODS

Rabbit muscle phosphoglucomutase was isolated by the method of Najjar (1962). Trypsin and pepsin were twice-crystallized salt-free preparations (batch TR3F6401 and batch PM702 respectively) from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Subtilisin was from Novo Terapeutisk Laboratorium, Copenhagen, Denmark. Carboxypeptidase A was a three-times-crystallized di-isopropyl phosphorofluoridate-treated preparation (A-DFP), suspended in water, from Worthington Biochemical Corp.

Labelling of the enzyme. Rabbit muscle phosphoglucomutase was labelled by means of substrate made by enzymic phosphorylation of starch by using potato juice in the presence of H₃³²PO₄ (Milstein & Sanger, 1961). The method was modified (Milstein, 1966b) by substituting 0.2 M-tris-HCl buffer, pH 7.4, for phosphate buffer and by omitting histidine and MgCl₂ from the incubation mixture. The labelled enzyme after being dialysed for 1 day was passed through a Sephadex G-25 column (1.4 cm. $\times$ 100 cm.) in 0.1 M-ammonium acetate buffer, pH 6.0, at a flow rate of 36 ml./hr.

Denaturation of the enzyme. Four methods were used to denature the enzyme before proteolysis.

* Abbreviations (in amino acid sequences): SerP, serine phosphate; Asx, aspartic acid or asparagine.

(1) The enzyme (20–30 mg./ml.) contained in a dialysis bag was dialysed against freshly made 8M-urea for 8–12 hr. at room temperature. The urea solution was changed every 4 hr. and after 12 hr. dialysis against urea the enzyme was dialysed against water until urea-free.

(2) The enzyme was heated in a boiling-water bath for 30 min. and the suspension dried in a desiccator over P_2O_5 .

(3) The enzyme was denatured with 10 vol. of acid acetone (1 vol. of 0.5N-HCl with 4 vol. of acetone) at room temperature for 15 min. and the suspension dried in a desiccator over NaOH pellets.

(4) N-HCl (2 vol.) was added to 1 vol. of enzyme solution (5 mg./ml.), the mixture left at room temperature for 15 min. and the suspension dried in a desiccator over NaOH pellets.

Digestion of protein and peptides with proteolytic enzymes.

(a) Trypsin. The protein was suspended in 1% (w/v) NH_4HCO_3 , pH 8.6. The enzyme was added as a solution in the ratio 1:100 (by weight), the final concentration of protein being 5 mg./ml. The mixture was incubated at 37° for 18–20 hr.

(b) Pepsin. The peptide (50–100 μ moles) was dissolved in 0.2 ml. of 0.01N-HCl, pH 2.1, and 5 μ l. of a freshly prepared aqueous solution of pepsin (10 mg./ml.) was added. The mixture was incubated for 10 hr. at 37°.

(c) Subtilisin. The peptide (50–100 μ moles) was dissolved in 0.5 ml. of 1% (w/v) $(NH_4)_2CO_3$, pH 9.0, and 20 μ l. of a freshly prepared aqueous solution (1 mg./ml.) of subtilisin was added. The mixture was incubated at 37° for 18 hr.

High-voltage paper electrophoresis. This was carried out in an apparatus similar to that described by Michl (1951). The buffer systems and coolants at pH 6.5, 3.5 and 2.0 were as described by Ambler (1963). The current used was 53 v/cm. In all the runs a parallel separation of a mixture composed of lysine, arginine, histidine, alanylglycine, glycine, valine, ϵ -DNP-lysine, glutamic acid, aspartic acid, taurine, cysteic acid, Xylene Cyanol FF and a spot of red Pentel pen was done on the same paper.

Preparation and isolation of tryptic peptides. A small amount (5–10 mg.) of labelled phosphoglucomutase (2×10^5 – 5×10^5 counts/min./mg.) was mixed with a larger amount (0.6–0.7 g.) of unlabelled enzyme, denatured by one of the methods described above and digested with trypsin. The tryptic digest was run through a Sephadex G-25 column (2 cm. $\times$ 122 cm.) in *n*-acetic acid at a flow rate of 24–30 ml./hr. (Fig. 1). The radioactive tryptic peptides contained in the first fraction were separated by paper electrophoresis (Whatman 3MM paper) at pH 6.5 for 2–3 hr. and at pH 3.5 for 3 hr.

Identification and location of peptides on paper. The radioactive peptides were located by radioautography. The non-radioactive peptides were located by dipping the paper in ninhydrin-cadmium reagent (Heilmann, Barrolier & Watzke, 1957). The specific colour test for histidine (Dent, 1947) was also used. Mobilities of peptides at pH 6.5 were measured relative to aspartic acid, the basic peptides being indicated by a negative sign. Mobilities of peptides at pH 3.5 and 2.0 were expressed relative to the mobility of lysine, valine or alanylglycine (as stated in each case) in the same run and taking as zero mobility the midpoint of a spot of taurine.

Partial acid hydrolyses. These were carried out by the first method described by Milstein & Sanger (1961).

Fig. 1. Tryptic digest of ^{32}P -labelled phosphoglucomutase run through Sephadex G-25 in *n*-acetic acid at a flow rate of 24–30 ml./hr. The radioactive peaks are, from left to right, tryptic peptides, glucose 1,6-diphosphate, glucose 6-phosphate and P_i .

Total acid hydrolysis. Peptide samples (5–20 μ moles) were hydrolysed in 0.2 ml. of constant-boiling HCl in evacuated sealed tubes at 105° for 18 hr., unless otherwise stated.

Amino acid analysis and N-terminal analysis. These were carried out as described by Milstein (1966a).

Edman degradation. The procedure described by Gray (1967) was carried out on the dephosphorylated peptide. The peptide (30–40 μ moles) was dissolved in 0.05 ml. of 0.2M- $(NH_4)_2CO_3$, pH 9.1, and 2 μ l. of a freshly prepared aqueous solution (1 mg./ml.) of highly purified *Escherichia coli* alkaline phosphatase was added. The mixture was incubated for 24 hr. at room temperature. The bacterial alkaline phosphatase was prepared by Pigretti & Milstein (1965).

Determination of C-terminal residues. The peptide (10–20 μ moles) was dissolved in 0.2 ml. of 0.1M- NH_4HCO_3 , pH 8.8, and 10 μ l. of a carboxypeptidase solution (3 mg./ml.) was added. The mixture was incubated at 37° for 5 hr., then dried under vacuum and examined in the amino acid analyser. The enzyme solution was prepared according to the method of Fraenkel-Conrat, Harris & Levy (1955) and diluted to 3 mg./ml. with the NH_4HCO_3 buffer.

RESULTS

The phosphoglucomutase was denatured before being digested with trypsin. In early experiments the enzyme was first denatured by dialysing against 8M-urea and then against water until the urea was removed (Milstein & Sanger, 1961). Other methods of denaturation were tried and compared quantitatively. The tryptic digests of the labelled enzyme previously denatured were examined by paper electrophoresis at pH 6.5 at 53 v/cm. for 1½–2 hr. on

Table 1. Comparison of different methods of denaturation of ^{32}P -labelled phosphoglucumutase

The denatured labelled enzyme was treated with trypsin and examined by paper electrophoresis (Whatman no. 52 paper) at pH 6.5 at 53 v/cm. for 1½–2 hr. Conditions: (a) 216000 counts/min./mg. (labelling in the presence of cysteine and Mg^{2+}), 0.4 mg. of enzyme, 50 µg. of trypsin, 17 hr., 37°; (b) 435000 counts/min./mg. (labelling in the absence of cysteine and Mg^{2+}), 0.06 mg. of enzyme, 10 µg. of trypsin, 17 hr., 37°.

Method of denaturation	% of total radioactivity recovered					
	Heat		Acid acetone		Acid	
	(a)	(b)	(a)	(b)	(a)	(b)
Band no.						
1	2.7	1.2	2.0	3.4	1.7	4.5
2	10.5	9.9	10.1	13.1	8.3	13.3
3	2.8	1.1	1.8	1.4	2.2	1.4
3'	2.2	0.6	0.9	0.4	1.0	0.5
4 (Glucose 1-phosphate)	5.7	5.6	—	—	—	—
5 (Glucose 6-phosphate)	3.0	36.2	44.0	38.8	48.5	44.1
6 (Glucose 1,6-diphosphate)	46.2	10.2	11.9	13.9	7.2	1.7
7 (P_i)	27.0	36.2	29.1	29.1	31.0	34.4
Total	100.1	101.0	99.8	100.1	99.9	99.9

Whatman no. 52 paper. The paper was radioautographed and spots were cut out and counted in a gas-flow counter (Table 1 and Fig. 2). In spite of the fact that the ^{32}P -labelled enzyme was purified through Sephadex G-25, considerable amounts of glucose phosphate, glucose 1,6-diphosphate and P_i were found in the paper when the denatured enzyme, digested with trypsin, was examined by electrophoresis. The yield of labelled tryptic peptides (Table 1) seemed to be only marginally affected by the method of denaturation, and the acid acetone or acid treatment was used in later experiments. Bands 4, 5, 6 and 7 of Fig. 2 were demonstrated to be glucose 1-phosphate, glucose 6-phosphate, glucose 1,6-diphosphate and P_i . This was done by incubating a portion of the eluted spots with phosphoglucumutase in the presence of Mg^{2+} and cysteine at pH 7.5 at 30° for 15 min. The reaction was stopped by heating the solution in a boiling-water bath for 10 min. The dried samples were examined by paper electrophoresis at pH 6.5 at 53 v/cm. for 2 hr. and radioautographed. Bands 4, 5 and 6 were substrates, since they all produced ^{32}P -labelled phosphoglucumutase. Band 5 was not acid-labile and had the same mobility as the glucose 6-phosphate marker. Unhydrolysed band 6 had the mobility of glucose 1,6-diphosphate marker and after partial acid hydrolysis gave a radioactive spot with the mobility of unlabelled glucose 6-phosphate marker. Bands 5 and 6 gave similar results when compared with markers by electrophoresis at pH 3.5. Band 4, not present in acid-denatured enzyme, is the acid-labile glucose 1-phosphate. We could not compare quantitatively the denaturation methods used with the one using urea, since P_i , glucose phosphate (1 or 6) or glucose 1,6-diphos-

phate released by the enzyme during denaturation diffuse away when the sample is dialysed to remove the urea.

Bands 1 and 2 contained about 90% of the radioactivity attached to peptides. Both peptides, after partial acid hydrolysis and electrophoresis at pH 3.5, gave the same pattern of radioactive peptides, which was identical with that reported by Milstein & Sanger (1961). The experiment therefore showed that a single phosphorylated serine residue found after the use of three different methods of denaturation of the protein accounts for most and possibly all of the [^{32}P]phosphate covalently bound to the protein.

The two bands, 1 and 2, corresponded to bands B and A respectively in the work of Milstein & Sanger (1961). These two bands were analysed after purification by paper electrophoresis at pH 3.5 and 2.0 (Table 2). The two peptides T1 and T2 seemed to contain 22–23 residues, assuming one histidine residue and one lysine residue. Peptide T1 contained alanine as *N*-terminus. The *N*-terminal sequence determined by the 'dansyl'-Edman technique suggested the following sequence:

This result was obtained in two different experiments.

The difference in mobility of the two tryptic peptides of apparently identical composition was surprising. In fact the mobility of the peptide was affected by subsequent treatment. Electrophoresis at pH 6.5 followed by electrophoresis at pH 3.5 affected the charge of the peptide, since on repetition of the electrophoresis at pH 6.5 an increase in the mobility of both peptides was observed (Table 3).

Spots with increased mobility were also observed after a single radioactive spot was incubated in an atmosphere saturated with pyridine-acetate buffer, pH 6.5, used in the electrophoretic runs (Milstein,

1964) and then again subjected to electrophoresis at pH 6.5 (Fig. 3 and Table 3). This type of mobility change has been observed previously in peptides containing aspartic acid (Naughton, Sanger, Hartley & Shaw, 1960). Deamidation of asparagine residues and destruction of tryptophan residues as alternative possibilities were tested as follows. Bands 1 and 2 were wetted with formic acid and kept wet for 3 hr. at room temperature. After being dried, they were subjected to paper electrophoresis at pH 6.5; about 20% of band 1 was transformed into band 2. A similar result was obtained when bands 1 and 2 were kept under performic acid vapour and subjected to electrophoresis at pH 6.5. Thus acid treatment or oxidizing conditions have less effect on the peptides than the treatment with pH 6.5 buffer.

The yield of the tryptic peptides was quite small, always below 10% in moles/mole of starting protein, and losses due to adsorption on the paper, and possibly on glass, were large. Thus, after paper electrophoresis in one dimension and elution of the spot, the recovery was only 30% of the amount put on the paper (Whatman no. 1). Low yields of phosphopeptides may have been due also to the ratio of phospho- and dephospho-enzyme present. Two different conditions of labelling did not seem to affect this ratio significantly (Table 1). An attempt was made to use purified phosphoenzyme by fractionating phosphoglucomutase on a Whatman DE 52 cellulose column. With this preparation, however, the recovery of radioactive phosphopeptides was negligible. It seemed possible that during the column fractionation part of the glucose phosphates became detached, resulting in lower yields of covalently bound phosphate.

Peptides T1 and T2 were digested with pepsin and each one gave rise to a new radioactive peptide of increased mobility. In spite of the difference in mobility, both peptic peptides gave amino acid analyses that suggested that they had identical compositions (Table 4). Peptide T2P was subjected to 'dansyl'-Edman degradation, which indicated the sequence:

Thr-Ala-Ser-His-Asx-Pro-Gly-Gly-Pro

Fig. 2. Radioactive pattern obtained when a tryptic digest of denatured ^{32}P -labelled phosphoglucomutase was examined by paper electrophoresis at pH 6.5 at 53 v/cm. for 2 hr. The enzyme was denatured with (a) acid, (b) acid acetone and (c) heat. The positions of bands 4 and 5 were very sensitive to the pH of the buffer and in some runs band 4 was in front of band 5. The positions occupied by the blue (b) and the red (r) markers at the end of the run are ringed.

Table 2. *Radioactive tryptic peptides from ^{32}P -labelled phosphoglucomutase*

Peptides T1 and T2 were purified by paper electrophoresis at pH 6.5, 3.5 and 2.0. The amino acid composition of peptide T2 is the average of results obtained in two different experiments. Mobilities of peptides at pH 3.5 were calculated by taking the mobility of Ala-Gly during the same run as 1, and for those at pH 2.0 free valine was used as reference.

Peptide	Mobility			Amino acid composition (residues/mol.)												
	pH6.5	pH3.5	pH2.0	Lys	His	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Phe
T1	0.14	0.52	0.50	0.9	1.1	2.8	1.2	1.3	0.7	2.5	5.1	2.2	0.5	2.7	1.1	1.0
T2	0.22	0.24	0.60	1.0	1.1	2.9	0.9	1.1	0.8	2.1	5.1	2.2	0.3	2.2	1.2	0.9

Table 3. *Interconversion of peptides (bands 1 and 2)*

The radioactive material from bands 1 and 2 was subjected separately to paper electrophoresis at pH 6.5 and the resulting radioactive spots were re-run at pH 3.5 (stage 1). The two bands thus obtained were run at pH 6.5 (stage 2). The two resulting bands were incubated at pH 6.5 for 6 hr. at 37° and re-run at pH 6.5 (stage 3).

Electrophoretic mobility at pH 6.5	Presence of bands 1 and 2				
	0.14	0.23	0.33	0.39	0.54
Stage 1					
Band 1	+++				
Band 2		+++			
Stage 2					
Band 1		+++			
Band 2				+++	
Stage 3					
Band 1		+++	Traces	+	
Band 2			Traces	++	Traces

Fig. 3. Effect of incubation at pH 6.5 on the mobility of the radioactive tryptic peptides obtained from ^{32}P -labelled phosphoglucomutase. Bands 1 and 2 (Fig. 2) were fractionated by electrophoresis at pH 3.5 (not shown) giving bands 1a and 1b and bands 2a and 2b respectively. These were subjected to paper electrophoresis again at pH 6.5 at 53 v/cm. for 2 hr., after incubation at pH 6.5 for 6 hr. at 37°. The arrow indicates the position of the blue marker at the end of the run.

Three non-radioactive ninhydrin-positive spots, derived from peptides T1 and T2, were also obtained and were the same for both digests. One of them was purified and analysed (Table 4). 'Dansyl'-Edman degradation gave the sequence:

Gly-Ile-Lys

The tryptic peptides were also digested with subtilisin. Several radioactive peptides were obtained. The main one, peptide T2S2 (Table 5), had the same amino acid composition and *N*-terminus as the radioactive peptides T1P and T2P. Carboxypeptidase released phenylalanine from it. Peptide T2S0 contained, in addition to the residues of peptides T1P or T2P, the three residues of the non-radioactive peptide T1-2P3, suggesting that this peptide follows peptide T1P since they derive from a tryptic peptide containing only one basic residue. Several minor subtilisin peptides were also obtained but not analysed.

These results suggested the amino acid sequence around the radioactive serine phosphate residue shown in Fig. 4.

DISCUSSION

The purification of the labelled peptides in sufficient quantities to allow sequence work was found to be difficult. The major problem, probably, was adsorption of the peptide on the paper (and possible on the glassware). The Sephadex columns, on the other hand, gave almost quantitative recoveries. It should be emphasized that phosphoglucomutase is a protein of molecular weight about 74000 (Taylor, Lowry & Keller, 1956) and therefore the purification of its degradation products was expected to be difficult. This difficulty is in fact larger than its size would suggest because the phosphorylation does not involve all the

Table 4. *Peptides derived from peptic digestion of peptides T1 and T2*

Peptides T1P and T2P were purified by paper electrophoresis at pH 6.5 and 2.0. Peptide T1-2P3 was purified by paper electrophoresis at pH 6.5 and 3.5. Mobilities of peptides at pH 3.5 and 2.0 were calculated by taking the mobility of free lysine during the same run as 1.

Peptide	Mobility			Amino acid composition (residues/mol.)												
	pH 6.5	pH 3.5	pH 2.0	Lys	His	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Phe
Radioactive																
T1P	0.45	—	0.20	0.3	0.8	3.0	1.0	0.9	0.4	2.1	2.7	1.1	0.2	0.2	0.3	0.8
T2P	0.58	—	0.18	—	0.9	2.8	0.9	1.0	—	1.9	2.8	1.0	—	—	—	1.0
Non-radioactive																
T1-2P3	-0.44	0.81	—	0.9	—	—	—	—	—	—	1.0	—	—	1.1	—	—

Table 5. *Peptides obtained by subtilisin digestion of peptide T2*

Peptides T2S0 and T2S2 were purified by paper electrophoresis at pH 6.5 and their mobilities were 0.36 and 0.59 respectively.

Peptide	Amino acid composition (residues/mol.)												
	Lys	His	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Phe
T2S0	0.7	1.1	2.9	0.8	1.0	0.4	1.7	4.2	1.2	0.1	0.7	0.3	1.0
T2S2	0.2	1.1	2.9	0.9	0.9	0.2	1.7	3.0	1.1	—	0.1	0.1	1.0

Fig. 4. Proposed sequence of the tryptic peptide containing the ^{32}P -labelled serine phosphate.

molecules (Yankeelov, Horton & Koshland, 1964). The isolated tryptic peptides gave an amino acid analysis that suggested some contamination. Throughout, the most conspicuous contaminant appeared to be glutamic acid. In the tryptic peptides considerable amounts of valine and glutamic acid were present but were not compatible with the sequence results. The number of isoleucine residues given in the amino acid analysis is low, 2.7, whereas four are shown in the sequence (Fig. 4). The Ile-Ile bond is, however, highly resistant to acid hydrolysis. The glycine content according to the amino acid composition should be five residues, which is again low since the sequence displayed in Fig. 4 presents six glycine residues. Several attempts to clarify these points failed because of the difficulties encountered in the purification of the tryptic peptides.

Two tryptic peptides that account for almost all the labelled peptides are of very similar composition and give the same pattern of radioactive peptides after partial acid hydrolysis, indicating that they represent the same section of the molecule. The

peptic digest of the two peptides gave rise to radioactive peptides in which the electrophoretic mobility difference was preserved. The amino acid compositions of these peptides were again identical in spite of the difference in mobility, and the patterns of the non-radioactive peptides obtained by peptic digestion were identical.

Different mobilities of the same aspartic acid-containing peptides have been described previously (Naughton *et al.* 1960), these being due to the conversion of the α -aspartyl residue in the peptide into the $\alpha\beta$ -ring form (lacking the negative charge) and subsequently into the β -form. These inter-conversions were observed to occur at pH 6.5. Peptides T1 and T2 have three aspartyl residues (Table 2), which might be converted from the normal α -aspartyl into the $\alpha\beta$ -ring form and then into the β -form. However, the charge difference between α - and β -aspartyl derivatives is not shown at pH 6.5 and the results obtained would imply that two or three aspartic acid residues are partly in the uncharged $\alpha\beta$ -ring form after digestion with the proteolytic enzyme. The presence of a second

phosphorylated site as an alternative explanation seems unlikely. Only one serine residue is present in the peptide, and threonine phosphate was absent from partial acid hydrolysates. Histidine phosphate (unstable in acid) cannot be excluded, since phosphate might be bonded to both histidine and serine present in the same peptide.

The proposed sequence of the peptic peptides agrees with the one presented by Hashimoto *et al.* (1966) for flounder phosphoglucomutase, namely:

Thr-Ala-Ser-His-Asx-Pro-Gly-Gly-Pro-Asx-Asx-Gly-Phe

The proposed sequence, however, does not agree with the sequence reported by Harshman & Najjar (1965) for the rabbit muscle enzyme and further does not allow for a second phosphorylated serine residue in the same peptide. These authors reported the following sequence:

Thr-Ala-Ser-His-Asx-Gly-Glu-Ser-Ala-Gly-Leu-Asx-Leu

in which a second serine residue is separated from the one belonging to the sequence Thr-Ala-Ser-His-Asx by four residues towards the C-terminus. Our sequence extends further to the C-terminus, not only in the tryptic peptides that contain lysine but also in the peptic and subtilisin peptides derived from them. Only one serine residue is in fact present in the peptides isolated in the present work. On the other hand, proline and phenylalanine, found to be present in our peptides, are absent from the Harshman & Najjar (1965) sequence. It is difficult to understand these discrepancies. They might be partly due to the use, by these authors, of partial acid hydrolysis. This degradation method is not suitable for studying long sequences; it is unspecific and produces artifacts, e.g. inverted dipeptides like SerP-Gly (Naughton *et al.* 1960; Fölsh, Mellander & Strid, 1960) and Ala-SerP (Milstein, 1964). The mobilities of some peptides described by Harshman & Najjar (1965) are in fact difficult to reconcile with the relation between mobility of peptides and their charge and size (Offord, 1966).

It has been previously shown that the sequences around the serine residue of the active centre in phosphoglucomutase are probably identical in yeast, rat and rabbit (Milstein, 1961). The identity of the sequence of the rabbit muscle enzyme reported in this paper and that of flounder enzyme (Hashimoto *et al.* 1966) emphasizes its importance in the activity of the enzyme.

REFERENCES

- Ambler, R. P. (1963). *Biochem. J.* **89**, 349.
 Anderson, L. & Jollès, G. R. (1957). *Arch. Biochem. Biophys.* **70**, 121.
 Cardini, C. E., Paladini, A. C., Caputto, R., Leloir, L. F. & Trucco, R. E. (1949). *Arch. Biochem.* **22**, 87.
 Dent, C. E. (1947). *Biochem. J.* **41**, 240.
 Fölsh, G., Mellander, O. & Strid, L. (1960). *Acta chem. scand.* **14**, 625.
 Fraenkel-Conrat, H., Harris, J. I. & Levy, A. L. (1955). *Meth. biochem. Anal.* **2**, 359.
 Gray, W. R. (1967). *Methods in Enzymology*, vol. 11, p. 139. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
 Harshman, S. & Najjar, V. A. (1965). *Biochemistry*, **4**, 2526.
 Hashimoto, T., Del Rio, C. & Handler, P. (1966). *Fed. Proc.* **25**, 408.
 Heilmann, J., Barrolier, J. & Watzke, E. (1957). *Hoppe-Seyl. Z.* **309**, 219.
 Kennedy, E. P. & Koshland, D. E. (1957). *J. biol. Chem.* **228**, 419.
 Michl, H. (1951). *Mh. Chem.* **82**, 489.
 Milstein, C. (1961). *Biochem. J.* **79**, 26 r.
 Milstein, C. (1964). *Biochem. J.* **92**, 410.
 Milstein, C. (1966a). *Biochem. J.* **101**, 338.
 Milstein, C. P. (1966b). *Biochem. J.* **100**, 40 c.
 Milstein, C. & Sanger, F. (1961). *Biochem. J.* **79**, 456.
 Najjar, V. A. (1962). In *The Enzymes*, 2nd ed., vol. 6, p. 161. Ed. by Boyer, P. D., Lardy, H. A. & Myrbäck, K. New York: Academic Press Inc.
 Naughton, M. A., Sanger, F., Hartley, B. S. & Shaw, D. C. (1960). *Biochem. J.* **77**, 149.
 Offord, R. E. (1966). *Nature, Lond.*, **211**, 591.
 Pigretti, M. M. & Milstein, C. (1965). *Biochem. J.* **94**, 106.
 Taylor, J. R., Lowry, C. & Keller, P. (1956). *Biochim. biophys. Acta*, **20**, 115.
 Yankeelov, J. A., jun., Horton, H. R. & Koshland, D. E., jun. (1964). *Biochemistry*, **3**, 349.