Rabbit Muscle Phosphofructokinase: Studies of the Subunit Molecular Weight and Structure

ISOLATION OF CARBOXYMETHYLATED Cysteinyl PEPTIDES AND SEDIMENTATION EQUILIBRIUM STUDIES*

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SUMMARY

The molecular weight of the subunit of rabbit muscle phosphofructokinase has been investigated by techniques which include sodium dodecyl sulfate gel electrophoresis, sedimentation equilibrium experiments, and the isolation of carboxymethylcysteine-containing tryptic peptides. Sedimentation equilibrium experiments in high concentrations of guanidine, in 0.5 M acetic or propionic acid, yield values of 75,000 to 85,000 for the subunit molecular weight. Sodium dodecyl sulfate gel electrophoresis resulted in values usually about 75,000 with the protein migrating as a single band. On the other hand, only eight to nine peptides containing carboxymethylcysteine were isolated from the trypsin digestion mixture, which would indicate a molecular weight of about 42,500 to 47,500. These peptides comprise about 40 % of the amino acid residues for this molecular weight.

From the data presented here, we have concluded that the subunit molecular weight of phosphofructokinase is 75,000 to 85,000. Not only are these subunits identical, but each subunit seems to consist of 2 similar units. Furthermore, these units appear to be linked in such a way as to be resistant to denaturing conditions.

Despite a wealth of information about the properties of rabbit muscle phosphofructokinase, there has been some disagreement as to the subunit molecular size and little information as to whether the subunits which comprise the active monomeric form of the enzyme are or are not identical. It has been shown that the molecular properties of this enzyme are complex, with the active enzyme, having a molecular weight of about 320,000 to 360,000, being composed of several subunits (1). At mildly acidic pH values (pH ~ 6), the native enzyme has been observed to dissociate into an inactive species of about 160,000 to 190,000 (1, 2). Paetkau and Lardy, on the basis of sedimentation studies in sodium dodecyl sulfate at alkaline pH values, concluded that this material was comprised of 2 identical subunits of 93,000 (1). In a later paper, however, Paetkau et al. obtained sedimentation equilibrium data which indicated that the molecular weight of subunit was 47,000 in 5.5 M guanidine hydrochloride, but that it decreased to 25,000 in 6 M guanidine hydrochloride (3). On the basis of these data and tryptic peptide mapping experiments, they proposed the existence of nonidentical subunits of 25,000. Higher values of the subunit molecular weight have been obtained by others. Scopes and Penny, using sodium dodecyl sulfate polyacrylamide gels, reported a value of 73,000 (4); this value is similar to that of 80,000 reported by Uyeda, which was based on sedimentation equilibrium studies of the enzyme dissociated by extensive maleylation (5). Recently, Leonard and Walker reported a molecular weight of 75,000 to 85,000 in 6 M guanidine hydrochloride as measured by sedimentation velocity and sedimentation equilibrium experiments (6). These values are consistent with the observation that there appears to be a single binding site (per 93,000) for several ligands, including fructose 6-phosphate, AMP, ADP, and cyclic AMP (7). Similarly, modification with 5,5'-dithiobis (2-nitrobenzoi acid) indicated 1 very reactive residue per 93,000 g of protein (8).

Because of the discrepancies between various workers concerning both the molecular size and the question of identity of the subunits and because we wished to examine the physical and kinetic properties in greater detail, we undertook further characterization of the subunit. As will be shown, the values we obtain in sodium dodecyl sulfate gels or by sedimentation equilibrium studies in guanidine or acid are in the range of 75,000 to 85,000 and thus agree with several other reports (3–5). On the other hand, the minimum molecular weight obtained on the basis of isolation of tryptic peptides in which the cysteine residues had been labeled with [14C]iodoacetic acid is about one-half of this...
We thus conclude that, although the subunit molecular weight is about 75,000 to 85,000, this subunit may be made up of 2 similar units linked in such a way as to be resistant to the usual denaturing conditions.

**MATERIALS AND METHODS**

Crystalline rabbit muscle phosphofructokinase was obtained as an ammonium sulfate suspension from Sigma Chemical Co. Enzymatic activity was determined spectrophotometrically in a coupled assay at 23° as described previously (2, 9). The enzyme used in these studies had a specific activity of 160 to 190 units per mg and was judged to be of high purity based on similar values in the literature and its behavior in the ultracentrifuge (8).

Trypsin, treated with N-acrylamide-2-phenylethyl chloromethyl ketone, was purchased from Worthington Biochemical Corp. Thermolysin was obtained from Daiwa Kasei K. K., Osaka, Japan.

Crystalline [1-14C]iodoacetic acid with a specific activity of 13.6 mC per mmole was obtained from New England Nuclear Corp. Unlabeled iodoacetic acid was recrystallized from cold petroleum ether (b.p. 30-60°) before use. Pyridine was redistilled from solid ninydrin before use.

Sodium dodecyl sulfate was obtained from Pierce Chemical Company. Guanidine hydrochloride was purchased from Mann.

**Sedimentation Equilibrium Experiments—High speed equilibrium sedimentation (10) was performed in a Spinco model E analytical ultracentrifuge equipped with an absorption scanner attachment and an RTIC unit.** Absorbance values (at 280 nm) versus cell position were obtained by using the absorption scanner attachment and analyzed by using a computer method (11). This method involves tracing the scanner recording with an analog-to-digital device from which the weight-average molecular weights over any section of the cell can be calculated. In the figures used in this paper, absorbance and radial positions were obtained from the computer print-out of the results.

For equilibrium sedimentation experiments in 0.5 M acetic or propionic acid, the enzyme was prepared at pH 8, freed of ATP by charcoal treatment (2), incubated with an excess of dithiothreitol, and then passed over a Sephadex G-25 column equilibrated with the acid in the presence of 1 mM EDTA and 0.2 mM dithiothreitol.

For equilibrium sedimentation experiments in guanidine hydrochloride, charcoal-treated enzyme (2) was precipitated by the addition of saturated ammonium sulfate to 0.5 saturation, and the precipitate was dissolved in the guanidine hydrochloride which contained either 0.05 or 0.1 M sodium phosphate buffer (pH 8), 1 mM EDTA, and 0.2 mM dithiothreitol. Carbonamidomethylation was carried out by incubating this enzyme with a 20-fold excess of iodoacetamide at room temperature for 10 to 30 min and stopping the reaction by addition of excess 2 mercaptoethanol. The enzyme was prepared for sedimentation equilibrium either by dialysis against the guanidine or by passing the enzyme over a Sephadex G-25 column equilibrated with guanidine. Results obtained from various experiments did not seem to depend on whether the enzyme had been reacted with iodoacetamide or whether the enzyme was dialyzed against guanidine or passed over the Sephadex column.

**Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis—** Electrophoresis in cylindrical polyacrylamide gels in the presence of sodium dodecyl sulfate was carried out at neutral pH, essentially as described by Weber and Osborn (12). The gels contained 5% acrylamide (4.85% acrylamide, 0.15% bisacrylamide), 0.1% N,N',N,N'-tetramethylethlenediamine, 0.1 M sodium phosphate (pH 7.2), and 0.1% sodium dodecyl sulfate and were polymerized by the addition of 0.03% ammonium persulfate. The electrode buffers were 0.1 M sodium phosphate (pH 7.2). The cathode buffer also contained 0.1% sodium dodecyl sulfate.

**Preparation and Isolation of Labeled Cysteine Peptides—** Radioactive S-carboxymethylated protein was prepared in the following manner. The enzyme (500 mg) was dissolved in 20 ml of 6 M guanidine hydrochloride (pH 9.3) and reduced in a nitrogen atmosphere in the presence of 7.5 mM dithiothreitol for 12 hours. After adjusting the pH to 8.0, a neutral solution containing 200 μmoles of radioactive iodoacetic acid was added. Alkylation was allowed to proceed for 5 min at room temperature. The reaction was stopped by addition of a 10-fold molar excess of 2-mercaptoethanol. After dialysis against four changes of de-ionized water for 24 hours, the radioactive S-carboxymethylated protein was recovered by lyophilization.

Trypsin digestion was performed in a pH-stat at pH 8.9. Lyophilized protein prepared as indicated above was suspended in water to give a concentration of 10 mg per ml. Trypsin (10 mg per ml in 0.001 N HCl) was added to give a final concentration of 2% (w/w), and digestion was allowed to proceed for 12 hours at 37°. At this time, a second aliquot of trypsin was added, and digestion was continued for 6 hours. The reaction was stopped by the addition of 6 N HCl to pH 2.0. The acid-insoluble material was separated from the acid-soluble material by centrifugation.

The acid soluble supernatant was fractionated on a column (2.0 × 20 cm) of Dowex 50-X8 (Spinco amino acid analyzer resin, AA 15) at 55°. The column was equilibrated with 0.05 mM pyridine acetate (pH 2.5) previous to addition of the samples. Elution was carried out at a flow rate of 80 ml per hour by using a double linear gradient (13). The first gradient was composed of 500 ml each of 0.05 mM pyridine acetate (pH 2.5) and 0.5 mM pyridine acetate (pH 3.75) and was followed by a second gradient consisting of 500 ml each of 0.5 mM pyridine acetate (pH 3.75) and 2.0 mM pyridine acetate (pH 5.0). The column was stripped with 100 ml of 6 N pyridine acetate (pH 5.0). The eluent was collected in 6.0-ml fractions. The over-all peptide separation was monitored automatically by ninhydrin color at 570 nm after alkaline hydrolysis, and the peptides containing S-[14C]carboxymethylcysteine were located by removing aliquots from alternate fractions and measuring radioactivity in a Packard model 3370 liquid scintillation spectrometer. Subfractions of the pools containing radioactivity were carried out on columns of either Dowex 1-X2 or Dowex 50-X2. In some cases, further subfractions were performed using Sephadex G-25 columns. Dowex 1-X2 columns (0.9 × 150 cm), pre-equilibrated with 3% pyridine, were eluted at 35° by using a flow rate of 30 ml per hour with a four step gradient consisting of 200 ml each of 3% pyridine, 0.5% pyridine acetate (pH 5.5), 1.0% pyridine acetate (pH 5.0), and 2.0% pyridine acetate (pH 5.0). Dowex 50-X2 columns (0.9 × 50 cm), pre-equilibrated with 0.2% pyridine acetate (pH 3.1), were developed at 30 ml per hour with a linear gradient composed of 250 ml each of 0.2% pyridine acetate (pH 3.1) and 2.0% pyridine acetate (pH 5.0) at 35°. Sephadex G-25 columns (0.9 × 150 cm) were eluted at room temperature with 0.1% acetic acid at a flow rate of approximately 10 ml per hour.

The acid-insoluble portion of the tryptic digestion mixture was dried by lyophilization and solubilized in 3% pyridine by the addition of 2 N NaOH to pH 9.0. Fractionation was achieved on a column (0.9 × 150 cm) of Dowex 1-X2 by using the continuous gradient system described above. The column was...
stripped with 100 ml of 6 N pyridine acetate (pH 5.0) and then
100 ml of glacial acetic acid. Subfractionations of the radioac-
tive pools were carried out on columns (0.9 x 20 cm) of Dowex
50-X8. A four step gradient composed of 200 ml each of 0.05 N
pyridine acetate (pH 2.5), 0.2 N pyridine acetate (pH 3.1), 0.5 N
pyridine acetate (pH 3.75), and 2.0 N pyridine acetate (pH 5.0)
was used to develop the column at a flow rate of 30 ml per hour
and at 55°.
Aliquots of the pools containing radioactivity were hydrolyzed
in evacuated tubes with 6 N HCl for 24 hours at 110°. Amino
acid analyses were performed on an automatic amino acid
analyzer.

**RESULTS**

**Extent of Carboxymethylation**—Several amino acid analyses of
the carboxymethylated protein yielded an average value of 0.067
µmoles of carboxymethylcysteine per 362.9 µg of protein. This
corresponds to 1 cysteine residue per 5310 molecular weight and
agrees with the cysteine content determined independently as
cysteic acid after performic acid oxidation. This value for the
cysteine content is essentially identical with that obtained by
others (1, 14). Thus, all of the cysteine residues appear to have
been carboxymethylated.

**Separation of Acid-soluble Tryptic Peptides**—The mixture of
soluble peptides obtained after acidification of the tryptic diges-
tion mixture was fractionated on a column of Dowex 50-X8.

The elution profiles of both ninhydrin color after alkaline hy-
drolysis and radioactivity are shown in Fig. 1. All pools con-
taining radioactivity were analyzed both in the initial and subse-
quent fractionations. As indicated by the radioactivity, the
acid-soluble cysteinyl peptides were contained in the six pools
designated A to F.

Amino acid analyses revealed that none of these pools con-
tained a single peptide and in order to purify further the radio-
active peptides, subsequent fractionations of each pool were per-
formed. The method used involved fractionation on columns of
either Dowex 1 or Dowex 50, and the procedure for each pool is
indicated in Table I. Pools E and F of Fig. 1, upon subfrac-
tionation, were each found to contain a single [14C]carboxymethyl-
cysteinyl peptide as judged by both the profile of the radioactivity
and the amino acid composition. On the other hand, Pools A
and C each contained two unique radioactive peptides which
have been designated as A-1, A-2, C-1, and C-2. Two additional
low yield radioactive peptides were found in Pool C and had
amino acid compositions identical to those of A-2 and C-1.

Subfractionation of each of the Pools B and D resulted in isoi-
ation of a purified Cm-cysteinyl peptide although of low yield.
In addition, an impure radioactive fraction was obtained from
Pool B having a carboxymethylcysteine content, which, if localized
in a single peptide would have been equivalent to a yield of 6.5%.
However, when submitted to further purification, we were unable
to obtain any material in sufficient amount or purity to be able
to determine whether or not it was a unique peptide.

The amino acid composition of the isolated carboxymethyl-
cysteinyl peptides with the total number of residues in each and
the yields are given in Table I. The percentage of yield for each
peptide is calculated from the actual yield in micromoles of car-
boxymethylcysteine after subfractionation relative to the micro-
moles of protein originally digested assuming (as discussed later)
a molecular weight of 44,000.

There is good evidence that these compositions represent highly
purified peptide material. For six of the peptides, the ratio of
carboxymethylcysteine to either arginine or lysine is approxi-
mately unity. The likelihood that any of these peptides repre-
sents a mixture of two or more peptides both containing a Cm-
cysteinyl residue and lysine (or arginine) seems remote since
purification involved two different types of ion exchange columns.

Further, paper electrophoresis of each of the purified peptides
indicated only a single major radioactive spot occurring at the
position of the major amount of peptide as indicated by ninhydrin
color. This was true for all of the peptides, except A-2, which
appears to contain a non-radioactive peptide and the radioactive
one. This peptide contains neither arginine nor lysine and could
be either the COOH-terminal peptide of the protein or an acid
cleavage product (for example at an aspartyl-prolyl bond) of a
larger peptide. Since there was no lysine or arginine on which
to base the cysteine content, this peptide was digested with ther-
molysin, and the digest was subjected to paper electrophoresis.

Only one radioactive spot was observed, indicating that the

**FIG. 1.** Elution profile of the acid-
soluble tryptic peptides on a column
(2.0 x 20 cm) of Dowex 50-X8. The
solid line is the ninhydrin absorbance
measured at 570 nm, whereas the
dashed line is the radioactivity. The
column was developed with a pyrid-
ine-acetate gradient at 55° as de-
scribed in the text.
original peptide probably contained only a single residue of cysteine. Peptide F contains both arginine and lysine and probably represents a lysyl-arginine or arginyl-lysine sequence at the COOH terminus. Further indication of the purity of four of the peptides shown in Table I is the fact that they have also been isolated from the acid-insoluble material (see below).

Separation of Acid-insoluble Tryptic Peptides—The acid-insoluble precipitate from the initial trypsin digest was solubilized in 3% pyridine and fractionated on a column of Dowex 1-X2. The elution profile is shown in Fig. 2. All pools containing radioactivity were analyzed both in the initial and subsequent fractionations. The amount of radioactivity in Pools 5 to 8 and material stripped from the column were hydrolyzed in 60% acid and evaporated, most (70%) of the radioactivity recovered. However, most of these counts were not incorporated into S-[14C]carboxymethylcysteine. Peptide A contains a small amount of S-[14C]carboxymethylcysteine. Peptide E contains in terminating the alkylation reaction, as well as oxidation products produced during acid hydrolysis, both of which are extensively occluded in the acid precipitate (15). Thus, when Pools 5 to 8 and material stripped from the column comprised approximately 60% of the radioactivity recovered. However, most of these counts were not incorporated into S-[14C]carboxymethylcysteine. Although the nature of this material was not investigated, it probably represents S-[14C]carboxymethylmaleimidoethanol which is formed in terminating the alkylation reaction, as well as oxidation products produced during acid hydrolysis, both of which are extensively occluded in the acid precipitate (15). Thus, when Pools 5 to 8 and material stripped from the column were hydrolyzed in 6 N HCl and evaporated, most (70%) of the radioactivity was observed to be volatile. These pools together did appear to contain a small amount of S-[14C]carboxymethylcysteine. However, when each of these pools was individually subfractionated, only Pool 5 was found to contain a peptide in any appreciable yield (see below). Furthermore, the yield of this peptide was such that it could account for almost all the Cm-cysteine observed in the sum of the pools combined (Pools 5 to 8 and stripped material).

Pools 1 to 5 all contained [14C]-labeled peptides but were contaminated with some unlabeled peptides. Further fractionation of each pool was achieved on columns (0.9 x 20 cm) of Dowex 50-X8. The compositions of isolated peptides from Pools 2 to 5 are given in Table II. Examination of these compositions indicates that four of the peptides correspond to a peptide found in the acid-soluble portion of the tryptic digestion mixture. By comparing the data in Table II with that of Table I, it is clear that peptides from Pools 2, 4, and 5 (Table II) have compositions identical with C-1, B, and A-2 (Table I), respectively. Amino acid analysis (not shown) of the refractionated material of Pool 1 indicated a peptide similar to Peptide F of Table I but in very low yield and somewhat impure. Pool 3 appears to contain two peptides, one of which (3-A) is identical in composition to Peptide C-2 of Table I. The second, lower yield peptide (3-B) appears to be a larger form of the Peptide A-2 (Table I). Peptide A-2 has no arginine or lysine and, as indicated earlier, either could represent the COOH-terminal peptide or could be a product of acid-catalyzed hydrolysis of an aspartyl-proline bond (16). Since 3-B contains arginine and appears to have 1 more prolyl residue than A-2, it seems likely that this peptide (A-2) is indeed a product of acid cleavage rather than the COOH-terminal peptide. Thus we conclude that 3-B and A-2 are related and should not be considered as two separate peptides.

The data of Tables I and II indicate therefore that at least six unique peptides have been isolated in relatively good yield, whereas two peptides (D and E) are present in somewhat lower yield. There is a possibility that one more peptide might exist which has not been detected by the isolation procedure. We conclude, therefore, that there are eight to nine Cm-cysteine-containing tryptic peptides. The significance of this number will be discussed later.

**Sedimentation Equilibrium Studies in Denaturing Solvents**

Sedimentation equilibrium experiments have been performed in 0.5 M acetic acid, 0.5 M propionic acid, and guanidine hydrochloride solutions at several different molarities between 3.5 and 7 M. Fig. 3 shows plots of ln absorbancy versus (radial distance)² for several such runs. In general, all such plots are linear, indicating homogeneity with respect to the molecular weight dis-

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**Table I**

| Amino acid            | A-1 | A-2 | B   | C-1 | C-2 | D   | E   | F   |
|-----------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Lysine                | 0.91| 0.01| 0.01| 0.01| 0.01| 0.01| 0.01| 0.01|
| Histidine             | 1.00| 1.00| 1.00| 1.00| 1.00| 1.00| 1.00| 1.00|
| Arginine              | 0.05| 0.05| 0.05| 0.05| 0.05| 0.05| 0.05| 0.05|
| S-Carboxymethylcysteine| 0.93| 0.93| 0.93| 0.93| 0.93| 0.93| 0.93| 0.93|
| Aspartic acid         | 1.00| 1.00| 1.00| 1.00| 1.00| 1.00| 1.00| 1.00|
| Threonine             | 4.09| 4.09| 4.09| 4.09| 4.09| 4.09| 4.09| 4.09|
| Serine                | 2.37| 2.37| 2.37| 2.37| 2.37| 2.37| 2.37| 2.37|
| Glutamic acid         | 5.47| 5.47| 5.47| 5.47| 5.47| 5.47| 5.47| 5.47|
| Proline               | 2.91| 2.91| 2.91| 2.91| 2.91| 2.91| 2.91| 2.91|
| Glycine               | 5.72| 5.72| 5.72| 5.72| 5.72| 5.72| 5.72| 5.72|
| Alanine               | 7.54| 7.54| 7.54| 7.54| 7.54| 7.54| 7.54| 7.54|
| Valine                | 4.90| 4.90| 4.90| 4.90| 4.90| 4.90| 4.90| 4.90|
| Methionine            | 2.76| 2.76| 2.76| 2.76| 2.76| 2.76| 2.76| 2.76|
| Isoleucine            | 0.70| 0.70| 0.70| 0.70| 0.70| 0.70| 0.70| 0.70|
| Leucine               | 5.04| 5.04| 5.04| 5.04| 5.04| 5.04| 5.04| 5.04|
| Tyrrosine             | 1.06| 1.06| 1.06| 1.06| 1.06| 1.06| 1.06| 1.06|
| Phenylalanine         | 1.00| 1.00| 1.00| 1.00| 1.00| 1.00| 1.00| 1.00|
| Yield (%)*            | 8   | 11  | 4   | 24  | 15  | 3   | 4   | 15  |

**Purification procedure**

Dowex 1-X Dowex 1-X Dowex 1-X Dowex 1-X Dowex 1-X Dowex 1-X Dowex 50-X Dowex 50-X

* Actual yield after subfractionation.
TABLE II
Amino acid composition of acid-insoluble tryptic peptides of phosphofructokinase

| Amino acid                        | 2(=C-1) | 3A (=C-2) | 3-B | 4(=B) | 5(=A-2) |
|-----------------------------------|----------|-----------|------|-------|----------|
| Lysine                            | 1.00     |           |      |       |          |
| Histidine                         |          |           |      |       |          |
| Arginine                          | 0.70     | 1.00      | 1.13 |       |          |
| β-Carboxymethylcysteine           | 1.02     | 0.92      | 1.09 | 1.00  | 1.00     |
| Aspartic acid                     | 2.09     | 2.95      | 4.25 | 3.20  |          |
| Threonine                         | 1.09     | 1.28      | 1.59 | 4.17  | 1.80     |
| Serine                            | 1.04     | 1.63      | 1.85 | 1.29  |          |
| Glutamic acid                     | 2.15     | 0.88      | 0.73 | 0.86  |          |
| Proline                           | 0.71     | 0.86      | 1.5  | 0.76  |          |
| Glycine                           | 1.87     | 3.16      | 6.58 | 1.09  |          |
| Alanine                           | 1.09     | 1.7       | 1.92 | 1.35  |          |
| Valine                            | 2.00     | 0.90      | 1.66 | 0.71  | 1.02     |
| Methionine                        | 0.81     | 0.83      | 1.07 |       |          |
| Isoleucine                        | 1.04     | 1.81      | 0.67 |       |          |
| Leucine                           | 1.91     | 0.89      | 1.71 | 2.73  | 1.00     |
| Tyrosine                          |          |           |      |       |          |
| Phenylalanine                     |          |           |      |       |          |
| Yield (%)                         | 5        | 4         | 2    | 5     | 3        |

* Actual yield after subfractionation.

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75,000 was obtained in good agreement with that of Scopes and Penny (3), who used a slightly different technique. However, occasionally the phosphofructokinase band migrated more slowly, indicating molecular weight values in the range of 80,000 to 100,000. This variability was not observed for any of the protein standards. Neither incubation of the enzyme at 100° for 2 to 5 min in the presence of 1% sodium dodecyl sulfate nor carbamidomethylation of the enzyme seemed to affect this variability in the rate of migration. It was noted that under all conditions only a single band was observed in the gels.

**DISCUSSION**

Although there is some variability in molecular weight values obtained in sedimentation equilibrium and sodium dodecyl sulfate gel experiments, the results are consistent with a value of 75,000 to 85,000. This result for the molecular weight of the subunit is similar to that reported by Uyeda for enzyme dissociated by extensive maleylation (5), by Leonard and Walker by sedimentation experiments in guanidine (6), by Scopes and Penny using gels (4), and by Pavelich and Hammes using sodium dodecyl sulfate gels or chromatography in guanidine hydrochloride. On the basis of the cysteine content of the protein, one may calculate that such a single nonrepeating polypeptide chain of this molecular weight should yield 14 to 16 radioactive peptides. However, the number of tryptic peptides containing [14C]carboxymethylcysteine observed is well below that. Tables I and II show that we have obtained in yields of 8 to 20% six tryptic peptides, each of which is unique with respect to amino acid composition and each of which contains a single Cm-cysteine residue. Two other peptides (D and E) have been isolated although in relatively lower yields but they appear to represent unique peptides. All other fractions containing radioactivity were hydrolyzed and analyzed; either they contained no Cm-cysteine or were present in such low yields that it was impossible to obtain further separation or analysis. It is possible that within these other fractions, one more unique peptide might be present, but we were unable to detect it. Such very low yield material could represent some contamination of the enzyme with other proteins or with isozymes. In this regard it should be noted that any appreciable impurity in the enzyme preparation would be indicated by a large number of low yield Cm-cysteine-containing tryptic peptides, and this also was not observed. It is noteworthy that most of the peptides isolated from the acid-insoluble fraction were the same as those observed in the acid-insoluble material. In preliminary studies, performed at a 4-fold lower protein concentration, no acid-insoluble material was observed. Thus, in retrospect, it would appear that the acid-insoluble material arises primarily from aggregation of the tryptic peptides at high concentration. In summary, therefore, we conclude that, within experimental error, there are eight to nine unique Cm-cysteine-containing tryptic peptides in rabbit muscle phosphofructokinase and that the enzyme preparation was relatively homogeneous.

Based on the number of cysteine residues observed in the amino acid composition, eight to nine Cm-cysteine-containing peptides would correspond to a molecular weight of about 42,500 to 47,500, with the isolated peptides comprising about 40% of such a unit. This calculation not only indicates that the subunits of 75,000 to 85,000 are probably identical with respect to amino acid sequence, but that each of these subunits consists of 2 similar, if not identical, units. Thus, the subunit molecular weight values obtained by sedimentation equilibrium and sodium dodecyl sulfate gel electrophoresis are approximately twice as large as that calculated on the basis of the peptide isolation procedure. If the subunit form produced in the presence of sodium dodecyl sulfate, acid, or guanidine is comprised of 2 similar (perhaps identical) units, they appear to be linked in such a way as to be resistant to the usual denaturing conditions. Since carbamidomethylation of cysteine residues does not appreciably affect the molecular weight values obtained in guanidine, the possibility of disulfide bond formation to link 2 units together appears to be ruled out.

There appears to be little or no carbohydrate or phosphate covalently associated with the protein (18) and therefore no possibility of stable linkages through such moieties. It is possible, however, that 2 such units are linked through a peptide bond since treatment of native enzyme with trypsin yields a subunit of approximately 40,000 instead of 75,000 when the subunit molecular weight is determined by using sodium dodecyl sulfate gels.2

It seems likely that only a complete amino acid sequence of the protein would resolve the question of the structure of the subunit. However, if the structure proposed above is correct, it would be of interest with respect to ligand binding studies. Thus fructose-6-P, AMP, ADP, and cyclic AMP bind to the extent of 1 mole per 90,000 (7). On the other hand, ATP binding studies indicate 3 moles bound per 90,000 molecular weight (7). Such binding may reflect a characteristic of some enzymes which becomes evident within recent years, that is, half-site reactivity. For example, Levitzki et al. (19) have shown that the affinity label 6-iodo-5-oxonorleucine reacts with only one-half of the glutamine sites of Escherichia coli CTP synthetase although the subunits are identical. It has been pointed out that a number of enzymes show half-site reactivity (19, 20). Thus it is possible that because of, for example, strong negative cooperativity, fructose-6-phosphate, AMP, ADP, and cyclic AMP might bind to only 1 of 2 available units of 44,000. On the other hand, since ATP is both a substrate and inhibitor, it is possible that one of these functions is reflected in half-site reactivity.

It has also been indicated that there is only one rapidly reacting sulhydryl group per 90,000 molecular weight (8). Again there is a precedent for this. Bovine liver glutamate dehydrogenase contains six identical peptide chains (21). However, we have shown that only three of the six available lysine residues at position 428 (21) in glutamate dehydrogenase are reactive towards trinitrobenzene sulfonate (22). Furthermore, modification of these residues results in marked changes in kinetic and molecular properties (24).

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