The Amino Acid Sequence Around the "Reactive" Sulfhydryl Groups in Adenosine Triphosphocreatine Phosphotransferase *

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ABSTRACT: A number of sulfhydryl reagents react in stoichiometric proportions to inactivate adenosine triphosphocreatine (ATP-creatine) phosphotransferase. Two sulfhydryl groups of the six available on the enzyme are preferentially attacked by these reagents. Two equivalents of 2,4-dinitrofluorobenzene have been used to label the two reactive sulfhydryl groups on ATP-creatine phosphotransferase.

After pepsin digestion at 2°, an octapeptide containing *S*-dinitrophenylcysteine residue was obtained in over 50% yield. This indicates that both reactive sulfhydryl groups have the same amino acid sequence throughout these eight amino acids. The sequence about these two reactive sulfhydryl groups is Val-Leu-Thr-CySH-Pro-Ser-AspNH_2-Leu. These data leave no doubt that the potent inhibition of the enzymatic activity of ATP-creatine phosphotransferase caused by the "Sanger's reagent" (2,4-dinitrofluorobenzene) is owing to its reaction with two sulfhydryl groups.

After reaction of the two reactive sulfhydryl groups in ATP-creatine phosphotransferase with FDNB[1] (Sanger's reagent), iodoacetic acid, or *p*-mercuribenzenesulfonate (Kuby and Mahowald, 1959; Mahowald and Kuby, 1960; Mahowald *et al.*, 1962b; Watts *et al.*, 1961), enzymatic activity is completely lost. This inactivation was most effective with FDNB which, when allowed to react at 0°, caused all enzymatic activity to disappear within a few minutes.

In this study FDNB was used to label the two reactive sulfhydryl groups for the purpose of isolating a peptide containing the *S*-DNP-cysteine residue and of determining the sequence of amino acids about these reactive cysteine residues. Thomson *et al.* (1964) have recently reported a 25-amino acid peptide obtained from this enzyme with trypsin as the hydrolyzing enzyme and ^{14}C-labeled iodoacetic acid as the labeling group. The octapeptide obtained in this study with pepsin as the hydrolyzing enzyme corresponds to the portion around the cysteine residue of the peptide isolated by him.

Experimental Procedure

ATP-creatine phosphotransferase was isolated from rabbit muscle by procedure B of Kuby *et al.* (1954), and twice recrystallized as previously reported (Mahowald *et al.*, 1962b), except that frozen tissue was used (obtained from Pel Freeze Biologicals, Inc., Rogers, Ark.). The meat was thawed during the previous 24 hours, first at room temperature and then at 5°, and used immediately for the preparation. The enzyme preparation used in this study had a specific activity between 65 and 75 units/mg as determined by the acid molybdate–labile phosphate method of assaying the enzyme (Kuby *et al.*, 1954). The enzyme concentration was determined by the biuret method (Gornall *et al.*, 1949), and by the enzyme's absorption at 280 mμ as reported by Kuby *et al.* (1962).

The dinitrophenylamino acids, FDNB, and carboxypeptidase were obtained from Mann Research Laboratories, Inc. All solvents and chemicals were of analytical grade and the solvents were distilled before use. Pepsin was a thrice-crystallized preparation from California Corp. for Biochemical Research. Nagarse, a crystalline bacterial proteinase, was obtained from Enzyme Development Corp., New York City.

Dowex 50W-X2, 200–400 mesh, was roughly sized by the hydrolytic backwashing method of Hamilton (1958) in such a manner as to remove 10–15% of the resin as "fines" and 25–30% as heavy or large particles. The middle-sized resin was cycled with 1 N NaOH and 3 N HCl, the resin being washed with distilled water between the NaOH and the HCl treatment and after the final HCl treatment. The Dowex resin was stored wet until needed. Several days before a column was needed, sufficient wet resin was settled several times with 0.2 N pyridine buffered with acetic acid to a *p*H of 3.1, and the column was packed by pouring in a slurry of resin suspended in the buffer. Each column was flushed for 10–20 hours with 0.2 N pyridine-acetate buffer, *p*H 3.1, before use. The column was jacketed and maintained at 30° except where noted. Eluates off the column were collected in a fraction collector

* From the Department of Biochemistry, Cornell University Medical College, New York City. *Received December 9, 1964.* This work was supported in part by a grant (GM-10460) from the National Institute of General Medical Sciences, U.S. Public Health Service.

[1] The abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; *S*-DNP-cysteine, *S*-2,4-dinitrophenylcysteine.

and the absorption at 330 mμ[2] was determined with a Beckman DB spectrophotometer connected to a transferator (Gilson Medical Electronics, Model AT6) and a recorder (Sargent, Model SRL). This combination allowed rapid analysis of the contents of the tubes from the fraction collector and also permitted identification of the colored material by a rapid scanning of the ultraviolet spectrum.

Sephadex G-25, 100–200 mesh, was prepared by washing the material first with 0.5 N NaOH and then with distilled water until the *p*H was 8 or less. The material was then washed with 0.5 N HCl at 5° followed by distilled water until the *p*H was no lower than 4. The Sephadex G-25 was used in either a 1 × 70–cm column or a 2 × 10–cm column. The material was poured into the column as a slurry in 0.2 N acetic acid. Columns containing Sephadex G-25 were used to separate peptides by partition chromatography and reused as described by Yamashiro (1964). Table I lists four solvent systems employed in this study. The contents of the tubes from the fraction collector from the Sephadex G-25 partition columns were analyzed as described with the Dowex columns, except that approximately 1 ml of methanol was added to each test tube to avoid difficulty in reading the optical density when two phases were present. The volume in each tube was not adjusted before the optical density was read, and therefore only the position and not the amount of the eluted material was determined.

TABLE I: Solvent Systems used in Partition Chromatography of Peptides on Sephadex G-25 Columns.[a]

Solvent System	Component	Ratio
A	1-Butanol–1-propanol–3% pyridine, 3% acetic acid in water	2:1:3
B	1-Butanol–1-propanol–benzene–3% pyridine, 3% acetic acid in water	4:1:1:6
C	1-Butanol–1-propanol–benzene–3% pyridine, 3% acetic acid in water	8:1:3:12
D	1-Butanol–benzene–3% pyridine, 3% acetic acid in water	1:1:2

[a] Columns were used and regenerated as described by Yamashiro (1964).

[2] The maximum absorption of the purified *S*-DNP-cysteine octapeptide occurs at 339 mμ (see Figure 7). A value of 330 mμ rather than the 339 mμ was used to follow the label (*S*-DNP-Cys) because in the crude fractions (with organic solvents present) the peak absorption of the labeled peptides was usually found in this region of the spectrum and *S*-DNP-cysteine absorbs with a maximum at 330 mμ.

The eluates were combined for further study by removal of the sample from those tubes containing the material of interest, but rejecting the contents of the first tube or tubes and the last tube or tubes depending upon the sharpness of the peak. The combined fractions were evaporated on a rotary flash evaporator with a dry-ice trap and a vacuum pump assembly. To hasten evaporation a water bath maintained at 30° was used to warm the contents of the flask. Before the contents of the flask was brought to dryness, distilled water was added at several intervals to drive off the organic solvents first.

To determine the concentration of any peptide, a sample was removed and hydrolyzed for 20 hours in an evacuated sealed tube as previously described (Mahowald *et al.*, 1962a), except that analytical grade hydrochloric acid from Malinckrodt Chemical Works was used and diluted 1:1, instead of the constant-boiling hydrochloric acid.

Peptides were considered purified and identifiable when contaminating amino acids or peptides represented less than 10% of the identified peptide and when the amino acids of the peptide in question were in mole/mole ratios. Higher contamination has been reported. In most cases the peptides were sufficiently pure to make it difficult to locate the position of amino acids other than those found in the peptide on the chromatogram.

Amino acid analyses were carried out according to the procedure of Moore *et al.* (1958) with the use of a Beckman/Spinco Model 120 B amino acid analyzer and of a 1 × 50–cm column for the acidic and neutral amino acids and a 10 × 0.6–cm column for the basic amino acids. To determine the *S*-DNP-cysteine a 0.5 M, *p*H 5.8 citrate buffer was used instead of the 0.35 M, *p*H 5.8 citrate buffer recommended by the manufacturer (Spackman, 1962). This modification results in elution of *S*-DNP-cysteine between ammonia and arginine, whereas without this change it appears with the ammonia peak. The ninhydrin reaction with *S*-DNP-cysteine results in about equal optical density at both 440 and 570 mμ wavelengths. The *C* value (height in OD × width, in number of dots) is approximately 110% of the *C* value for proline, both calculated from the 440 mμ wavelength curve. After 20 hours of acid hydrolysis, one-third of the *S*-DNP-cysteine is destroyed.

For the labeling of ATP-creatine phosphotransferase with the dinitrophenyl group, FDNB was diluted in isopropyl alcohol so that accurately measured samples could be obtained. Twenty μl of FDNB in 5 ml of isopropyl alcohol was prepared fresh just before it was needed. Such a solution contains 32.6 μmoles/ml of FDNB. To the enzyme at 0° was added 2 equivalents of FDNB plus a 5% excess, and the solution was mixed and allowed to stand for 15 minutes. After this period, which allowed more than sufficient time according to previous studies (Mahowald *et al.*, 1962b), the enzyme was considered labeled and the *p*H was adjusted for the digestion of the enzyme.

The digestions of the proteins or peptides were

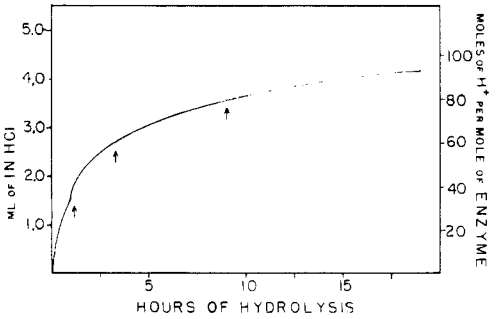

FIGURE 1: Hydrolysis of 45.2 μmoles of ATP-creatine phosphotransferase by pepsin at 30°, *p*H 2. Pepsin (10 mg) in 1 ml of 0.01 N HCl was added at the times indicated by the arrows. On the lefthand ordinate are plotted the ml of 1 N HCl necessary to maintain *p*H 2 and on the righthand ordinate the moles of HCl per mole of acid, which approximates the number of peptide bonds split. On the abscissa is plotted the time in hours.

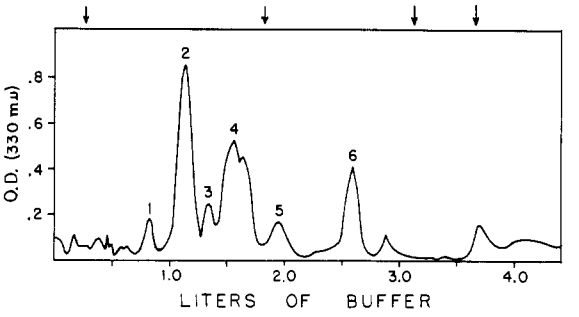

FIGURE 2: Chromatographic separation of peptides released by pepsin digestion at 30° for 19 hours of 45.2 μmoles of ATP-creatine phosphotransferase. The separation was performed on a 2 × 150-cm Dowex 50W-X2 column with the temperature initially at 30° and a flow rate of 120 ml/hour. The eluting buffer for the first 240 ml was 0.2 N pyridine-acetate buffered at *p*H 3.1, after which a gradient with 0.5 N pyridine-acetate buffer, *p*H 4.1, was initiated using a 3-liter mixing flask. When a total of 1.8 liters of buffer had passed onto the column, a 2.0 N, *p*H 5.1 pyridine-acetate buffer was used to develop the gradient. After a total volume of 3.1 liters of buffer had passed onto the column, the 2.0 N pyridine-acetate buffer, *p*H 5.1, was used directly as the eluting buffer. Finally, after a total of 3.65 liters of buffer had passed onto the column, the temperature of the column was raised to 50° until the end of the run. The test tubes containing the peptides in the zones labeled 1 through 6 were subjected to further purification to identify the nature of the material.

conducted in a thermostated beaker in which were immersed the electrodes of the Radiometer TTT-1 and SBU titrigraph assembly as well as the syringe drive, SBU-1. The beaker was sealed with a Teflon plug, and a nitrogen atmosphere was maintained above the solution. The rate of the reaction (hydrolysis) was followed by the rate of addition of acid or base to maintain a given *p*H. Pepsin digestions were carried out at *p*H 2 and pepsin was added as a suspension in 0.01 N HCl. The digestion of peptides by Nagarse was carried out at *p*H 7.8 and at 30°. Carboxypeptidase digestion was carried out at *p*H 8 and at 40°.

Cleavage of *S*-DNP-cysteine peptides at the cysteine residue was performed as described by Sokolovsky and Patchornik (1964). The *S*-DNP-cysteine peptide (approximately 4 μmoles) was allowed to react with 10 ml of 0.01 N sodium methoxide at room temperature for 6 hours. The solution was diluted with 20 ml of water and acidified with 0.1 N HCl. The reaction mixture was evaporated to dryness. The residue was dissolved in 30 ml of water and acidified to *p*H 2 with HCl, and the acid solution was refluxed for 3 hours. The reaction mixture was evaporated to dryness *in vacuo* and chromatographed on a Sephadex G-25 column, 1 × 70 cm, with the A solvent system. The eluates were analyzed for peptide material by the Folin-Lowry method (Lowry *et al.*, 1951) for peptide material and fractions were combined and analyzed for amino acid composition according to the separation of material. The zone containing the peptide derived from the COOH-terminal portion of the original peptide was treated with 5 ml of 0.1 N KOH to which 0.5 ml of 30% H_2O_2 was added. After 2 hours at room temperature, the reaction mixture was acidified with 0.2 N acetic acid and evaporated to dryness. The NH_2-terminal amino acid was determined by the procedure of Fraenkel-Conrat *et al.* (1955).

Determination of the COOH-terminal amino acid on peptides by hydrazinolysis was performed according to the procedure described by Greenstein and Winitz (1961).

Results

Digestion of ATP-creatine Phosphotransferase with Pepsin at 30°. The amount of 3.67 g (45.2 μmoles) of ATP-creatine phosphotransferase in 61 ml of solution at approximately *p*H 8, 0°, was labeled with 93 μmoles of FDNB reagent. After 15 minutes the labeled enzyme was transferred to a jacketed reaction vessel and then adjusted to *p*H 2. The temperature was maintained at 30° by pumping water through the jacketed beaker from a constant-temperature bath. The hydrolysis of the enzyme was initiated by the addition of 10 mg of pepsin, and more pepsin was added as indicated in Figure 1. The data in Figure 1 show that the rate of hydrolysis is initially very rapid, but then decreases. Assuming that pepsin does not expose any "buried" basic groups not previously titrated by the acid, then it can be assumed that after 19 hours approximately 90 peptide linkages have been hydrolyzed.

Immediately following the pepsin digestion, the

solution was applied to the top of a Dowex 50W-X2 column 2 × 150 cm. The results of this chromatogram are shown in Figure 2. The test tubes containing the 330 mμ absorbing material in each zone indicated in the figure were combined and subjected to additional purification to identify peptides containing the *S*-DNP-cysteine label group.

Purification and Identification of S-DNP-cysteine Peptides. The zones obtained from the Dowex columns were further purified by at least two solvent systems on Sephadex G-25 columns. The peptide containing *S*-DNP-cysteine residue was followed in the eluates from the columns by absorption at 330 mμ.

ZONE 1. This zone contained a small amount of *S*-DNP-cysteine as determined by amino acid analysis of a purified component of the zone. However, no peptide was purified to a degree sufficient for identification.

ZONE 2. The chromatography of this fraction on a 2 × 120-cm column of Sephadex G-25 in solvent system A (*vide supra*) resulted in two major peaks and a fast-eluting mixture of peaks. The results of this chromatogram are shown in Figure 3A. The chromatography of the fast-eluting mixture of peaks with the B solvent system resulted in recovery of 1 μmole of *S*-DNP-cysteine peptide, too impure to ascertain its composition. Chromatography of the combined eluates which contain the material with an R_F of 0.45 in the B solvent system on a 2 × 120-cm column are shown in Figure 3B. The *S*-DNP-cysteine peptide had an R_F of 0.14 and a sample removed for amino acid analysis showed that it contained 18 μmoles of an impure labeled peptide. The peptide was again placed on the same column with the same solvent system, but amino acid analysis of hydrolysates of different areas of the peak showed that the later part of the peak contained a heptapeptide, and the early portion the heptapeptide plus contaminating peptides. The composition of the peptide was Val (Leu, Thr, *S*-DNP-Cys, Pro, Ser, $AspNH_2$), and the NH_2-terminal group was valine, as determined by the dinitrophenylation method of Fraenkel-Conrat *et al.* (1955).

Chromatography of the combined eluates which contained the material with an R_F of 0.32 (see Figure 3A) on a Dowex 50W-X2 column 1 × 50 cm with 0.2 N pyridine-acetate buffer resulted in a single 330 mμ absorbing peak. On amino acid analysis of a sample, this peak was found to contain Leu (Thr, *S*-DNP-Cys, Pro, Ser, $AspNH_2$), and the N-terminal group was leucine. This hexapeptide was recovered as 8.4 μmoles or a 9.3% yield.

ZONE 3. This zone was chromatographed on a 1 × 70-cm column with the B solvent system and the results are shown in Figure 3C. The tubes containing the major peak with an R_F of 0.45 were combined and chromatographed on Sephadex G-25 in the D solvent system. A peak of 330 mμ absorbing material with an R_F of 0.20 was found. Amino acid analysis showed it to be a tetrapeptide with an amino acid composition of (Val, Leu, Thr, *S*-DNP-Cys) but it contained alanine and leucine at one-half the concentration as contaminants. The amount of the tetrapeptide was 0.61 μmole. No other peptides were identified.

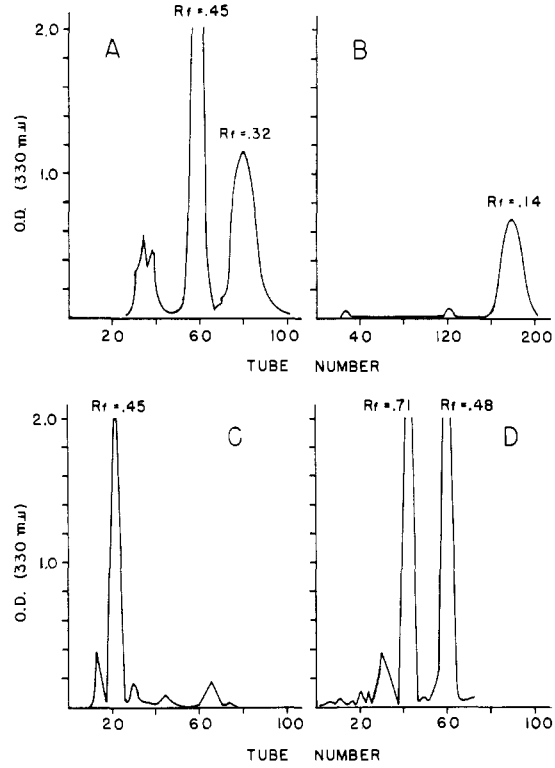

FIGURE 3: Chromatographic purification of peptides on Sephadex G-25 partition columns of the zones of 330 mμ absorbing material from the Dowex column. On the ordinate is the approximate OD of each tube and on the abscissa the tube number. (A) Chromatographic purification of peptides on Sephadex G-25 partition column using the A solvent system; volume of fractions is 5.1 ml, hold-up volume is 133 ml. (B) Chromatographic purification of the *S*-DNP-cysteine peptide shown in (A) with an R_F of 0.45 on a 2 × 120-cm Sephadex G-25 column with the B solvent system; volume of fractions is 5.0 ml, hold-up volume is 135 ml. (C) Chromatographic separation of peptides from zone 3 of the Dowex column (see Figure 2) on a 1 × 70-cm Sephadex G-25 partition column with the B solvent system; volume of fractions is 2.5 ml, hold-up volume is 24 ml. (D) Chromatographic separation of the two peptides from zone 4 of the Dowex column (see Figure 2) on a 2 × 120-cm partition column with the B solvent system; volume of the fraction is 4.6 ml. hold-up volume is 130 ml.

ZONE 4. This zone was first chromatographed on a 2 × 120-cm column of Sephadex G-25 with the A solvent system. The double peak indicated on the original Dowex 50 column (see Figure 2) was also seen in this chromatogram, with the separation incomplete. The eluates containing both substances were combined and chromatographed on a 2 × 120-cm Sephadex G-25 column with the B solvent system.

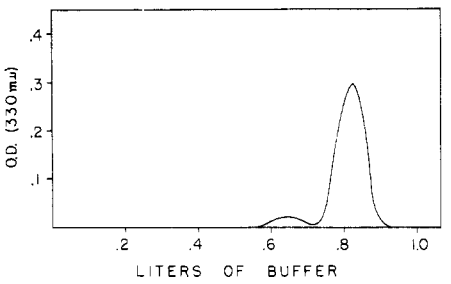

FIGURE 4: Chromatographic analysis of 2.05 μmoles of ATP-creatine phosphotransferase digested by 10 mg of pepsin at 2° for 6 hours on a column containing Dowex 50W-X2 resin, 1 × 150 cm. Eluting buffer throughout the run was 0.2 N, *p*H 3.1 pyridine-acetate buffer and the temperature of the column was controlled at 30°. The buffer flow rate was 60 ml/hour. The data were collected using a constant-flow pump of the amino acid analyzer, Beckman/Spinco, Model 120 B, and eluate from the column was passed through a microflow cell in a Beckman DB spectrophotometer adjusted to read at 330 mμ wavelength. The recording of the data was accomplished by connecting the spectrophotometer to the recorder supplied with the amino acid analyzer so that all three recording pens printed on the same curve. The eluates were collected in a fraction collector.

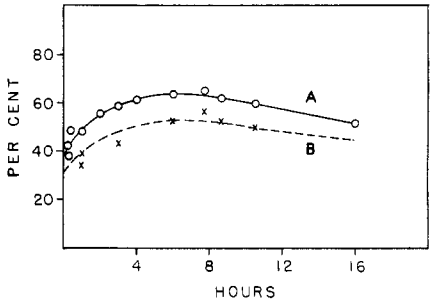

FIGURE 5: Recovery of octapeptide from 2.05 μmoles of ATP-creatine phosphotransferase hydrolyzed with 10 mg of pepsin at 2°. Assuming two sites equal to 100% recovery, curve A (———) is the recovery of the labeled octapeptide when calculated as described in the text and legend to Figure 4, for the data obtained from different time intervals. Curve B (– – – –) is the data of the actual recovery of octapeptide by amino acid analysis after two additional chromatographic purification steps of Sephadex G-25 (see Figure 6). The amount recovered for the 6-, 8-, 9-, and 11-hour hydrolysis periods was 2.20, 2.28, 2.24, and 2.12 μmoles, respectively, more equivalents than the 2.05 μmoles of enzyme used.

The results of this chromatogram are shown in Figure 3D. The first large band of 330 mμ absorbing material with an R_F of 0.71 was an octapeptide with an amino acid composition of Val (Leu, Thr, *S*-DNP-Cys, Pro, Ser) $AspNH_2$-Leu, with valine NH_2-terminal. Carboxypeptidase liberated leucine and a small amount of asparagine. This octapeptide was recovered in 11.2 μmoles, a yield of 12.4% of the original label. The second large fraction of eluted material (see Figure 3D) which had an R_F of 0.48 had an amino acid composition of Leu (Thr, *S*-DNP-Cys, Pro, Ser, $AspNH_2$, Leu) and leucine at the NH_2-terminal position. This peptide was recovered as 8.8 μmoles or a 9.8% yield.

ZONE 5. No identifiable peptides could be purified by the techniques used.

ZONE 6. Although this zone represents about 10% of the labeled material, the substance appeared to be unstable and turned a deep yellow on lyophilization. Gel filtration on Sephadex G-25 with 0.2 N acetic acid as the eluting solvent gave a peak in low yield with an amino acid composition of (*S*-DNP-Cys, Pro, Ser, $AspNH_2$, Leu) plus contaminating peptides. The instability may be owing to the *S*-DNP-Cys residue's being NH_2-terminal on this peptide (*vide infra*).

Rate of Pepsin Hydrolysis at 2°. Preliminary studies on the hydrolysis of ATP-creatine phosphotransferase by pepsin at 2° showed the rate of hydrolysis to be similar to the rate of 30° (see Figure 1), as demonstrated by the rate of addition of acid to maintain the *p*H. However, on chromatography of this hydrolysate on Dowex 50 resin only a single 330 mμ absorbing peak was noted. Therefore the rate of liberation of this single peptide was studied by stopping the pepsin digestion at different time intervals, and analyzing for the peptide quantitatively.

A stock solution of ATP-creatine phosphotransferase containing 2.05 μmoles/2 ml was used in this study. To a 2-ml aliquot of the enzyme at 2° was added 4.2 μmoles of a fresh solution of FDNB. After 15 minutes the *p*H was adjusted to 2 and maintained at that point with the Radiometer *p*H-stat using 0.1 N HCl. At zero time 10 mg of pepsin was added to the solution. At a given interval of time, varying from 15 minutes to 16 hours, the *p*H was adjusted to 7 to inactivate the pepsin. After 30 minutes the *p*H was adjusted to 2 and the sample was placed on a Dowex 50W-X2 column, 1 × 150 cm, prepared as before. The result of the chromatogram for the sample hydrolyzed for 6 hours is shown on Figure 4. The quantity of material in the peak was determined from the area under the curve and standardized by chromatography of a known amount of crystalline octapeptide (*vide infra*). The data collected from a series of experiments at different time intervals are shown in Figure 5, curve A. Hydrolysis of the enzyme occurs initially at a very rapid rate until one of the two sites to be liberated is freed. The rate decreases until about 6 hours, after which the breakdown of the 330 mμ absorbing material is going at a faster rate than is its liberation from the enzyme. The single large band of 330 mμ absorbing material which was eluted off the Dowex resin (see Figure 4) was combined and subjected to purification on a Sephadex G-25 column, 1 × 70 cm, with the A solvent system. The results of this chromatogram are shown in Figure 6A. The tubes containing the large

band of 330 mμ absorbing material eluted were combined and the material was placed on another Sephadex G-25 column, 1 × 70 cm, with the C solvent system; the results of this chromatogram are shown in Figure 6B. The eluates from the tubes containing the large band of 330 mμ absorbing material were combined and analyzed for amino acid composition. The amino acid composition was (Val, Leu, Thr, *S*-DNP-Cys, Pro, Ser, $AspNH_2$, Leu). The results of the plot of the actual recovery of the octapeptide are shown in Figure 5, curve B. Between 6 and 12 hours of pepsin hydrolysis results in a recovery of octapeptide of more equivalents than the starting amount of enzyme.[3] This is sufficient evidence that both reactive cysteine residues have the same amino acid sequence around the reactive cysteines at least through these eight amino acids.

Preparation of Larger Amounts of Octapeptide by Pepsin Digestion at 2°. ATP-creatine phosphotransferase (4.69 g; 58 μmoles) in 62 ml of solution was allowed to react with 130 μmoles of FDNB for 15 minutes. The *p*H was adjusted to approximately 2, and 250 mg of pepsin suspended in 5 ml of 0.01 N HCl was added. At this concentration of ATP-creatine phosphotransferase, a gel formed when the *p*H was lowered; however after addition of the pepsin the solution quickly became more liquid and the *p*H could be controlled. The pepsin was allowed to react for 6 hours, after which time the sample was immediately placed on a 2 × 150-cm, Dowex 50W-X2 column. The eluting buffer solution was the 0.2 N pyridine-acetate buffer, *p*H 3.1, used before. The flow rate was 120 ml/hour and the eluates were collected in a fraction collector. Analysis of the fractions for material absorbing at 330 mμ resulted in a chromatogram similar to that shown in Figure 4, except that the volumes needed to elute the peak material were about two times those recorded in Figure 4. Ignoring the fast-eluting small peak, the fractions containing the 330 mμ absorbing material were combined and chromatographed on a Sephadex G-25 column, 2 × 120 cm, with the A solvent system. The results of the chromatogram were similar to the data shown in Figure 6A. The eluates in the tube from the large peak of 330 mμ absorbing material were combined and chromatographed on Sephadex G-25, on a 2 × 120-cm column with the C solvent system. Crystals in large balls with a fuzzy surface formed on the walls of the test tubes in the fraction collector containing the yellow color of the *S*-DNP-cysteine peptide. The tubes were agitated and the fine needle crystals were noted with a 10× microscope eyepiece. These tubes were allowed to stand for several days and the crystals collected. These crystals[4] had a mp of 217.5–219.5° (corr) with softening and decomposition at 211°. The ultraviolet and visible spectra of the solvent-free peptide are shown in Figure 7 and compared with that for *S*-DNP-cysteine. The peptide has a molar extinction coefficient of 1.13×10^4 at 339 mμ when taken in 0.2 N acetic acid solution. *S*-DNP-cysteine has a maximum absorption at 330 mμ, whereas the octapeptide has its maximum at 339 mμ.

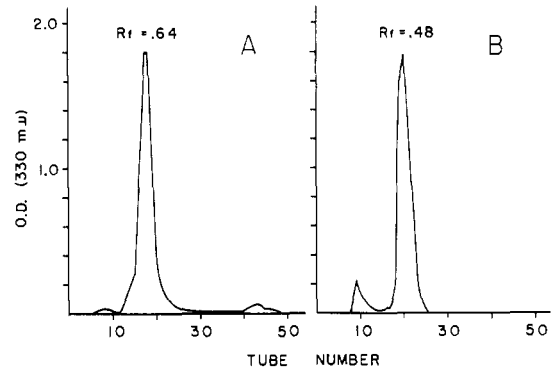

FIGURE 6: Chromatographic purification of *S*-DNP-cysteine octapeptide (Val, Leu, Thr, *S*-DNP-Cys, Pro, Ser, $AspNH_2$, Leu) on Sephadex G-25 partition columns 1 × 70 cm. On the ordinate is the approximate OD of each tube and on the abscissa is the tube number. (A) Chromatography of the combined tubes from the Dowex resin column of the large band of 330 mμ absorbing material with the A solvent system; volume of fraction is 2.4 ml, hold-up volume is 27.0 ml. (B) Chromatographic purification of the peak from the chromatogram in Figure 6A with the C solvent system; volume of fractions is 2.4 ml, hold-up volume is 24 ml.

Digestion of the Octapeptide by Nagarse. The octapeptide (10.4 μmoles) was subjected to 0.5 mg of Nagarse for 2 hours at 30° and *p*H 7.8. The solution was acidified with a small amount of dilute acetic acid and evaporated to dryness on a flash evaporator. The material was then placed on Sephadex G-25, 1 × 70 cm, and eluted with the A solvent system. The results

[3] The amount of enzyme in μmoles was determined through the use of the biuret reagent and by using 81,000 for the mol wt of the enzyme. The amount of octapeptide in μmoles was determined by amino acid analyses of the hydrolyzed peptide after three chromatographic procedures. There are inherent experimental errors in the biuret determination, mol wt determination, and in the amino acid analysis, which may raise a question as to the significance of the increased yield of the octapeptide above the amount of starting enzyme. However, it should be emphasized that the octapeptide was obtained in as high as 110% yield from the enzyme after three chromatographic procedures where quantitative recovery is usually not expected. In addition the sum of the errors introduced through the use of 81,000 as the mol wt and through the biuret determination of the amount of enzyme, as well as through the hydrolyses and amino acid analyses of the octapeptide, would not be expected to exceed 10%.

[4] The ultraviolet spectrum of these crystals in 0.2 N acetic acid showed a high absorption at around 250–260 mμ, somewhat characteristic of pyridine. This absorption was not present after the crystals were dissolved in dilute acetic acid and lyophilized. The amount of absorption would indicate approximately 2.7 moles of pyridine per mole of peptide. Presumably it is the pyridine salt as well as a complex with the dinitrophenyl group which permits the easy crystallization.

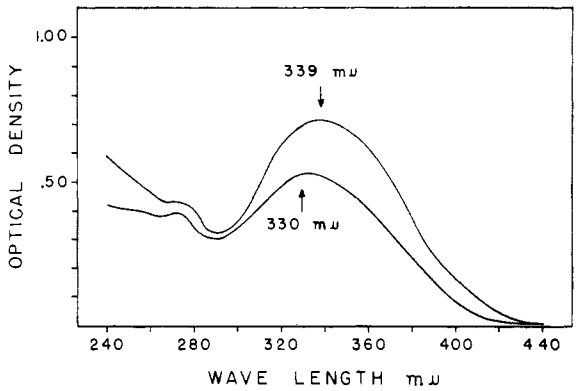

FIGURE 7: The absorption spectrum of crystalline octapeptide (Val, Leu, Thr, *S*-DNP-Cys, Pro, Ser, $AspNH_2$, Leu) on curve A in 0.2 N acetic acid, $c = 0.0528$ μmole/ml with molar absorbancy at 340 mμ of 1.13×10^4 M^{-1} cm^{-1}, and of *S*-DNP-cysteine (Mann Research Specialty Co.) on curve B in 0.2 M acetic acid, $c = 0.0544$ μmole/ml with a molar absorbancy at 330 mμ of 0.96×10^4 M^{-1} cm^{-1}.

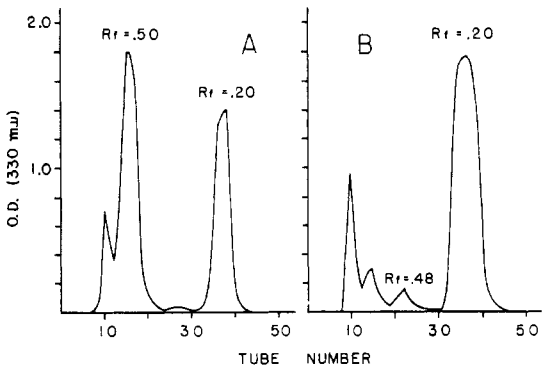

FIGURE 8: Chromatographic purification of *S*-DNP-peptides and of the R_F 0.50 peak. (A) Purification of *S*-DNP peptides obtained from a Nagarse digestion of the octapeptide on a Sephadex G-25 partition column 1×70 cm with the A solvent system. The ordinate gives the approximate OD of each tube, and on the abscissa is the tube number. Volume of fractions is 3.3 ml, hold-up volume is 24 ml. (B) Chromatographic purification of the peak with an R_F of 0.50 from the chromatogram in (A) with the C solvent system. Volume of fractions is 3.4 ml; hold-up volume is 24 ml.

are shown in Figure 8A. The material eluted with the 330 mμ absorption in a band with an R_F of 0.20 contained a tetrapeptide with an amino acid composition of Thr (*S*-DNP-Cys, Pro, Ser), and threonine as the NH_2-terminal amino acid. The yield of this peptide was 2.8 μmoles or 27%. The fast-eluting material was combined and chromatographed on a Sephadex G-25 column, 1×70 cm, with the C solvent system. The results are shown in Figure 8B. The large band with an R_F of 0.20 was found to be a hexapeptide with an amino acid composition of Val (Leu, Thr, *S*-DNP-Cys, Pro, Ser) and with valine as the NH_2-terminal amino acid. It was recovered in a 59% yield (6.2 μmoles). It can be seen that the starting material with an R_F of 0.48 (see Figure 7B) has been completely digested by the Nagarse enzyme preparation.

Cleavage of the Octapeptide at the S-DNP-cysteine Residue. Peptides containing cysteine can be split by formation of dehydroalanine and hydrolysis of the dehydroalanine residue. The method as outlined by Sokolovsky and Patchornik (1964) was followed. This method involves the dinitrophenylation of the peptide and removal of dinitrothiophenol by treatment with sodium methoxide. The resulting dehydroalanine peptide is hydrolyzed by mild acid hydrolysis. This results in the original cysteine residue forming, on one side, an amide and, on the other, a pyruvic acid linked to the neighboring amino acid by a peptide bond. The pyruvic acid residue can be removed, thus exposing a new NH_2-terminal position which can be identified in the usual way.

This procedure was used several times to study the octapeptide. The yield of the two peptide fractions was about 30%, with most of the material appearing as unsplit octapeptide. The COOH-terminal portion of the octapeptide was chromatographed on Sephadex G-25 in the A solvent system with an R_F of 0.18 and amino acid composition of (Pro, Ser, Asp, Leu). The NH_2-terminal portion was recovered in the wash, with both portions of the original peptide being contaminated by unsplit octapeptide. The COOH-terminal portion (Pro, Ser, Asp, Leu) was treated with alkaline hydrogen peroxide to remove the pyruvic acid residue, and after acidification and evaporation to dryness was tested for its NH_2-terminal group. The DNP-peptide was hydrolyzed for 2 hours with concd HCl or for 6 hours with 6 N HCl. In neither instance was a DNP-amino acid found on paper chromatography of the acid-ether extract. Following hydrolysis with 12 N HCl and analysis on the amino acid analyzer, a 40% loss of proline was found but no loss of serine, aspartic acid, or leucine. These data are not inconsistent if proline is at the NH_2-terminal position.

COOH-*terminal Amino Acid of the Hexapeptide Val (Leu, Thr, S-DNP-Cys, Pro, Ser).* Studies with carboxypeptidase indicated that this peptide was resistant to the action of this enzyme. Therefore other methods were explored. Hydrazinolysis of the peptide followed by dinitrophenylation was used to identify the COOH-terminal residue. Under these conditions only DNP-serine was found.

Table II lists the various peptides purified from ATP-creatine phosphotransferase, with the resulting conclusion that the octapeptide sequence in the native enzyme is Val-Leu-Thr-CySH-Pro-Ser-$AspNH_2$-Leu.

Discussion

The octapeptide containing *S*-DNP-cysteine was recovered in a yield of over 50% (see Figure 5), suffi-

TABLE II: Peptides Obtained from ATP-creatine Phosphotransferase and Method Used to Liberate the Peptide.[a]

Peptide	Obtained by
Val (Leu, Thr, *S*-DNP-Cys, Pro, Ser, $AspNH_2$)	Pepsin at 30°
Leu (Thr, *S*-DNP-Cys, Pro, Ser, $AspNH_2$)	Pepsin at 30°
(Val, Leu, Thr, *S*-DNP-Cys)	Pepsin at 30°
Val (Leu, Thr, *S*-DNP-Cys, Pro, Ser, $AspNH_2$, Leu)	Pepsin at 30° and 2°
Leu (Thr, *S*-DNP-Cys, Pro, Ser) $AspNH_2$-Leu	Pepsin at 30°
Val (Leu, Thr, *S*-DNP-Cys, Pro) Ser	Nagarse on octapeptide
Thr (*S*-DNP-Cys, Pro, Ser)	Nagarse on octapeptide
(Val, Leu, $ThrNH_2$) Pro? (Ser, $AspNH_2$, Leu)	From octapeptide by cleavage

[a] As a result of the partial sequence of these eight peptides the sequence of the octapeptide as it is present in the enzyme is Val-Leu-Thr-CySH-Pro-Ser-$AspNH_2$-Leu.

cient to leave no doubt that both reactive sulfhydryl groups have the same amino acid sequence throughout these eight amino acids. The octapeptide was found to contain valine as the NH_2-terminal amino acid. This peptide was also cleaved by removal of 2,4-dinitrothiophenol with sodium methoxide and hydrolysis of the dehydroalanine residue formed by this reaction. In addition, hydrolysis of the octapeptide with the bacterial enzyme preparation Nagarse, to give a tetrapeptide and hexapeptide, also aided in the determination of the sequence. Table II lists all the peptides identified and the proposed sequence of amino acids in the octapeptide. No peptides were found with the *S*-DNP-cysteine residue which do not fit into the proposed sequence.

The digestion of ATP-creatine phosphotransferase by pepsin was found to take a different course at 2° than at 30°. Numerous labeled peptides, which apparently are further digestion products of the labeled octapeptide, were found under these circumstances; however, at 2° mainly the octapeptide could be obtained and in good yield. At 2° pepsin releases the octapeptide from one of the two sites at a much faster rate than it releases the octapeptide from the remaining site (see Figure 5). It is possible that the two sites may differ at one or both ends of this common sequence of eight amino acids, or that, after removal of one site, the tertiary structure of the enzyme may change so as to prevent the attack of pepsin. The data obtained and presented here could not be used to differentiate between these two or even other possibilities.

It can be pointed out that the labeling of reactive sulfhydryl groups with the dinitrophenyl group serves as a useful tool for the study of the sequence about reactive sulfhydryl groups. With little or no modification of the methods presented here, other enzymes with other reactive sulfhydryl groups could also be studied in a similar manner. However, the only other enzyme so far reported to have a similar sensitivity to the "Sanger's reagent" is ATP-AMP phosphotransferase from rabbit muscle.

The use of partition chromatography with Sephadex G-25 as the supporting phase has been particularly helpful in the purification of the labeled peptides. It might be pointed out that ATP-creatine phosphotransferase is a moderately large protein with a molecular weight of 81,000 (Noda *et al.*, 1954), and that, after hydrolysis with pepsin and three chromatograms on columns, a single octapeptide was obtained in over 50% yield and sufficiently pure to crystallize in the collecting tubes.

The presence of proline next to the reactive sulfhydryl is of particular interest. As the evidence previously published indicates that these two reactive sites are part of the reactive sites of the enzyme, it appears from the presence of proline neighboring the cysteine residue that this portion of the molecule may not be in the form of an α helix. Threonine and serine have been found close to reactive sulfhydryl groups in other enzymes in which the amino acid sequence has been studied (Harris *et al.*, 1963; Li and Vallee, 1964).

Acknowledgments

The author wishes to thank Mrs. Mary Wickham and Mrs. Caroline Holzhauser for their skillful and conscientious technical assistance. The author is particularly grateful to Dr. Donald Yamashiro for permission to use his procedure on Sephadex G-25 partition chromatography before publication. The author also wishes to express his gratitude to Professor du Vigneaud for his continued interest and support.

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Purification and Properties of Uridine Diphosphate Glucose–Glycogen Glucosyltransferase from Rat Liver*

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ABSTRACT: Soluble uridine diphosphate glucose–glycogen glucosyltransferase has been purified approximately 1500-fold from rat liver by means of a procedure which utilizes reversible thermal inactivation of the enzyme as a means of removing it from the particulate glycogen to which it is bound. The most highly purified preparations were found to be heterogeneous mixtures of protein containing small amounts of phosphorylase, branching enzyme, and glycogen. Further studies of reversible inactivation indicated that it involves either a change in conformation of the enzyme protein or dissociation of the enzyme molecule into smaller, less active (or inactive) units which, in either case, do not bind effectively to glycogen. Glucosyltransferase from muscle did not undergo such a transformation on heating at 37° and differed in other regards from the hepatic enzyme. The structural requirements for stimulation of hepatic glucosyltransferase by hexose phosphate have been defined. Studies of the glucose-6-phosphate stimulation of glucosyltransferase have shown that glucose-6-phosphate decreases the inhibitory effect of uridine diphosphate in a partially competitive manner. From these and other data a more specific hypothesis regarding the mechanism of glucose-6-phosphate stimulation has been formulated.

Leloir and Goldemberg (1960) localized UDP-glucose–glycogen glucosyltransferase (EC 2.4.1.11; Leloir and Cardini, 1957) to the particulate glycogen fraction of liver homogenates, and utilized the association with glycogen as a means for partially purifying the enzyme. They reported that such preparations of glucosyltransferase were stimulated severalfold, and also protected from thermal inactivation, by glucose-6-P. Activity could be eluted from particulate glycogen with glycogen of low molecular weight, lending support to the view of Luck (1961) that the enzyme is bound directly to glycogen molecules rather than to fragments of endoplasmic reticulum. In studies of thermal inactivation of liver glucosyltransferase Steiner (1961) found that partially inactivated preparations could be restored to full activity by incubating them with glucose-6-P and certain salts. Moreover, the less active form of the enzyme was not bound to glycogen particles and could be separated from them by high-speed centrifugation. These observations provided a means for preparing soluble hepatic glucosyltransferase almost free of polysaccharide. In this paper the purification procedure is described. Evidence is presented which indicates that reversible inactivation leads to dissociation of the

* From the Department of Biochemistry, University of Chicago, Chicago, Ill. *Received October 6, 1964; revised January 13, 1965.* Supported by a grant (AM-04931) from the National Institute of Arthritis and Metabolic Diseases, U.S. Public Health Service.

† Holder of a Research Career Development Award of the U.S. Public Health Service.