III. THE PHOSPHORYLATED SITE IN HUMAN MUSCLE PHOSPHORYLASE a*

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The isolation and crystallization of both forms of glycogen phosphorylase from human skeletal muscle (1) have made possible a detailed study of the properties of these enzymes in comparison with those of rabbit skeletal muscle (2, 3). Phosphorylase b and a from both species have been shown to crystallize under similar conditions and to have similar pH optima, electrophoretic mobilities, sedimentation constants, and pyridoxal 5′-phosphate content (1). Phosphorylase b from human muscle, like phosphorylase b from rabbit muscle, can be converted to phosphorylase a by a purified preparation of phosphorylase b kinase isolated from rabbit muscle. The reaction requires Mg++ and adenosine triphosphate and is accompanied by the incorporation of 4 moles of phosphate per mole of phosphorylase u.l.

Recently it has been shown that rabbit and human muscle phosphorylases could be distinguished immunologically when injected into the goat (4). A previous study (1) indicated that no differences could be found when rooster antibodies to phosphorylase b were used. Since the structure of the specific site phosphorylated during the conversion of rabbit muscle phosphorylase b to a had previously been determined (5), it was of some interest to investigate the structure of the corresponding site of the human enzyme in order to determine the importance of this region of the two proteins in the immunological response. A preliminary account of the work has been presented (6).

EXPERIMENTAL PROCEDURE

Materials—Phosphorylase b was prepared from human skeletal muscle as described previously (1) to the crystallization step in the presence of AMP and Mg++. P32-labeled phosphorylase a was prepared from partially purified crystalline phosphorylase b (13,600 units per ml, 950 units per mg of protein) with rabbit muscle phosphorylase b kinase, prepared as described by Krebs, Graves, and Fischer (7), and with Mg++ and P32-labeled ATP (8, 9) in a manner identical with that described for the preparation of P32-labeled rabbit muscle phosphorylase a. The radioactive protein was precipitated from the conversion reaction mixture with ammonium sulfate, suspended in 0.02 M glycero-phosphate-0.012 M cysteine buffer, pH 6.8, and dialyzed against the same buffer overnight in the cold. The solution was centrifuged to remove a slight precipitate of cysteine, and the clear supernatant solution (48 ml, 21,580 units per ml, 1.8 X 10⁵ c.p.m. per mg of protein) was used in subsequent experiments.

Trypsin was obtained from Worthington Biochemical Corporation as a crystalline product containing approximately 50% MgSO₄. A solution of the enzyme was dialyzed in the cold against dilute HCl, pH 3.0, and neutralized with 10⁻³ M NaOH just before use. Carboxypeptidase B was prepared by the method of Folk and Gladner (10), and leucine aminopeptidase, by the procedure of Hill et al. (11).

RESULTS

Tryptic Attack on P32-labeled Phosphorylase a—It was previously shown that trypsin rapidly releases radioactive material from P32-labeled rabbit muscle phosphorylase a in a form which is soluble in cold trichloroacetic acid. After an examination of the optimal conditions needed for the release of P32-labeled peptides from rabbit muscle phosphorylase a, Fischer et al. (5) used a molar ratio of trypsin to phosphorylase a of unity and obtained over 90% of bound radioactivity soluble in trichloroacetic acid after 1 to 1½ hours. Over 95% of the protein was still precipitated in trichloroacetic acid. It appeared probable that smaller amounts of trypsin would quantitatively release bound radioactivity from P32-labeled phosphorylase a with correspondingly less concomitant degradation of the protein molecule, thereby diminishing the difficulties of isolating the P32-labeled peptides. A preliminary series of experiments was carried out, in which P32-labeled human skeletal muscle phosphorylase a was degraded with progressively smaller amounts of trypsin, and the release of radioactivity into a form soluble in trichloroacetic acid was followed. At the end of the reaction, the enzymatic reaction mixtures were precipitated with trichloroacetic acid, and soluble peptides were examined by using high-voltage paper electrophoresis in pyridine-acetate buffer, pH 6.5 (12), in one direction and paper chromatography in a butanol-acetic acid-water (4:1:5) solvent system in the other. When a molar ratio of trypsin to phosphorylase a of 1:10 was used, a quantitative release of radioactivity into a trichloroacetic acid-soluble form was found after approximately 20 minutes, and the "fingerprint" showed three radioactive peptides accompanied by very little other material staining with ninhydrin. By this means, purification of the P32-labeled peptides was possible directly by paper electrophoresis and paper chromatography, and the time-consuming fractionation step of column chromatography on an anion exchange...
In a large-scale experiment, \( \text{P}^{32} \)-labeled human muscle phosphorylase \( \alpha \) (90 ml, 8 mg per ml, 1.4 \( \mu \) moles) in 0.02 M glycero-phosphate-0.012 M cysteine, pH 6.8, was dialyzed overnight at 0°C against 0.001 M EDTA, pH 7.5. The enzyme was treated with Norit (500 mg) to remove AMP, which is known to interfere with the tryptic attack (5, 13). The solution was centrifuged, and trypsin (3.5 mg, 0.14 \( \mu \) mole, pH 8.1) was added and the volume brought to 110 ml with water. Aliquots (0.1 ml) were taken at intervals and added to 1.9 ml of 5% trichloroacetic acid. The precipitated protein was removed by centrifugation after 15 to 30 minutes at 0°C, and the supernatant solutions were counted for radioactivity. Aliquots (0.01 ml) of the trypsin-phosphorylase \( \alpha \) reaction mixture were also removed and analyzed for phosphorylase activity, determined in the presence and absence of AMP (3). A rapid release of radioactivity into a trichloroacetic acid-soluble form was found; the release was paralleled by a simultaneous conversion of phosphorylase \( \alpha \) to the form previously designated as \( b' \) (5) and requiring AMP for activity (Fig. 1). After 20 minutes of incubation, cold 50% trichloroacetic acid (12.2 ml) was added to the whole reaction mixture to stop further tryptic attack. After standing at 0°C overnight, the suspension was centrifuged, and the precipitate was washed with two 5-ml portions of cold 5% trichloroacetic acid. The supernatant solution and washings were combined, extracted five times with equal volumes of ether, and freeze-dried. The freeze-dried material was dissolved in water for chromatography.

**Purification of the \( \text{P}^{32} \)-labeled \( \beta \)-peptides**—Large-scale purification of the three \( \text{P}^{32} \)-labeled peptides was carried out directly by streaking the material on sheets of Whatman No. 3 paper and by using the high-voltage paper electrophoretic system described above. After the separation, the radioactive bands were located by radioautography with Eastman Kodak "no screen" x-ray film and then were cut out and eluted downward with water. The eluates were freeze-dried and stored at 0°C. The dry material was dissolved in water and further purified by paper chromatography in butanol-acetic acid-water (4:1:5). One major and two minor peptides were isolated; the relative positions of the peptides after high-voltage electrophoresis in pyridine-acetate buffer, pH 6.5, were similar to those of the corresponding peptides isolated from rabbit muscle phosphorylase \( \alpha \) (5). The terminology of the previous work is followed, and the three radioactive peptides are called, respectively, C, B, and A. Further electrophoretic and paper chromatographic examination of each \( \text{P}^{32} \)-labeled peptide under various conditions showed only one radioactive spot in each case and no other radioactive or ninhydrin-staining material. The recoveries of the radioactive peptides are summarized in Table I. The low recovery after electrophoretic and chromatographic purification was tolerated, since high purity of peptides was considered more desirable than high yield.

**Amino Acid Analysis and Sequence Studies of \( \text{P}^{32} \)-labeled Peptides**—For analysis, the peptides (0.10 to 0.25 \( \mu \) mole) were hydrolyzed in sealed tubes at 110°C in twice-redistilled 5.7 N HCl (0.2 to 0.5 ml) for 16 to 36 hours, and the hydrolysates were dried in a vacuum over NaOH pellets. Paper chromatography was carried out in the butanol-acetic acid-water (4:1:5) solvent and, for Peptide C, the amino acids were quantitatively eluted and estimated by the method of Connell, Dixon, and Hanes (14). The amino acid analysis of Peptide C indicated the average values: lysine, 1.00; glutamic acid, 0.87; isoleucine, 0.93; serine, 0.87; valine, 0.96; and arginine, 0.98. The amino acid composition of the three radioactive peptides is shown in Table II together with the chromatographic properties of the peptides. The composition of these peptides is identical with that of the analogous ones isolated from rabbit muscle phosphorylase \( \alpha \) (5).

The exact identity in amino acid sequence of the radioactive hexapeptide obtained from phosphorylase \( \alpha \) from both species was ascertained by the following series of experiments. Peptide C (0.2 \( \mu \) mole) was treated with 2,4-dinitrofluorobenzene as described by Fraenkel-Conrat, Harris, and Levy (15). The only dinitrophenylated derivative was shown by paper chromatography to be di-dinitrophenyl lysine. Tryptic attack of Peptide C (0.2 \( \mu \) mole) was carried out for 80 hours at pH 8.0. The enzymatic mixture was inactivated by heating and examined by high-voltage electrophoresis and paper chromatography. A radioactive band migrating in the position of Peptide B was

### Table I

**Recoveries of \( \text{P}^{32} \)-labeled peptides after tryptic attack of human muscle phosphorylase \( \alpha \)**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total recovery of radioactive material in each fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original ( \text{P}^{32} )-labeled phosphorylase ( \alpha )</td>
<td>12</td>
</tr>
<tr>
<td>Trichloroacetic acid supernatant solution after tryptic attack</td>
<td>10</td>
</tr>
<tr>
<td>Combined fractions eluted after high voltage electrophoresis and paper chromatography</td>
<td>4.5</td>
</tr>
</tbody>
</table>

* Corrected for radioactive decay.
Further degradation by leucine aminopeptidase was slow, but lysine and glutamine from Peptide C (0.2 pmole), giving as a major product a radioactive tripeptide that could be identified with the isolated from rabbit muscle phosphorylase a. As in the latter case, it would appear that the cleaving of the Lys-Glu-NH₂ bond of Peptide C by trypsin produced Peptide B with an amino-terminal glutaminyl residue. The ability of such an amino-terminal glutaminyl residue to undergo cyclization to the pyrrolidone derivative on heating is well known (16, 17).

In the present case, as in the analogous case of Peptide B from rabbit muscle phosphorylase a, such cyclization occurs, producing Peptide A, which is nonreactive with ninhydrin but, on total acid hydrolysis, releases the same amino acids as Peptide B.

**DISCUSSION**

The comparative studies of glycogen phosphorylases from rabbit and human skeletal muscle reported here and elsewhere (1) establish the close similarity of the proteins from the two species. The interest in the identity of, for example, pyridoxal 5'-phosphate content (1) and the structures of the phosphorylated sites is heightened by the finding of a definite immunological difference between the enzymes isolated from the two sources (4). The antigenic portion of the phosphorylase molecules must, therefore, be looked for elsewhere in the structure of the proteins. The establishment of the identity of the phosphorylated sites of rabbit and human muscle phosphorylase a is, of course, no indication of the identity of the two molecules. Indeed, it is possible that phosphorylation of phosphorylase b from any animal source by rabbit muscle kinase is specific for the amino acid sequence described above, irrespective of the structure of the remainder of the peptide chain. This conjecture, together with the finding that phosphorylase b kinase isolated from rabbit skeletal muscle is able to phosphorylate phosphorylase b from a wide variety of mammalian tissues and species (18, 19), may point to the identity of the phosphorylated regions of the phosphorylase a molecules from the various sources.

The action of trypsin on phosphorylase has been extensively studied over the years, and the effect of the reaction is well established (13, 21). The most striking feature of the reaction is the conversion of phosphorylase a, which is active in the total absence of AMP, to an AMP-requiring form of phosphorylase, phosphorylase b'. Phosphorylase b' is analogous to phosphorylase b, which is also AMP-requiring, and which is produced from phosphorylase a by the specific phosphorylase phosphatase, PrP enzyme (21, 22). The enzymatic properties of phosphorylase b' and b appear to be very similar, a finding which is perhaps surprising since phosphorylase b' is formed solely by the removal of phosphate groups from serine residues of phosphorylase a, whereas phosphorylase b results from a more general proteolytic attack of the phosphorylase a molecule (5). In this connection, it is well known that phosphorylase b' cannot be reconverted to phosphorylase a by phosphorylase b kinase. The present paper, however, shows that the trypic conversion of phosphorylase a to an AMP-requiring form may be more specific than hitherto considered. The virtual absence of ninhydrin-reactive material

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**Table II**

<table>
<thead>
<tr>
<th>Properties</th>
<th>Peptide A</th>
<th>Peptide B</th>
<th>Peptide C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Electrophoretic migration on paper, pH 6.5, at 2000 volts in 1 hour (in cm)</td>
<td>+5</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Approximate Rf values in butanol-acetic acid-water (4:1:5)</td>
<td>0.5</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Ninhydrin reaction</td>
<td>None</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Amino acids present after total acid hydrolysis</td>
<td>Glu, Ileu, Ser, Val, Arg</td>
<td>Glu, Ileu, Ser, Val, Arg</td>
<td>Lys, Glu, Ileu, Ser, Val, Arg</td>
</tr>
</tbody>
</table>

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**Table III**

<table>
<thead>
<tr>
<th>Enzymatic attack</th>
<th>Material attacked</th>
<th>Peptide formed</th>
<th>Amino acids in radioactive peptide formed</th>
<th>Amino acid released</th>
<th>NH₂-terminal amino acid by DFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>Peptide C</td>
<td>Lys, Glu-NH₂, Ileu, P-Ser, Val, Arg</td>
<td>Lys</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>Peptide B</td>
<td>Glu-NH₂, Ileu, P-Ser, Val, Arg</td>
<td>Glu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine aminopeptidase</td>
<td>Peptide C</td>
<td>Ileu, P-Ser, Val, Arg</td>
<td>Lys, Glu-NH₂, Ileu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxypeptidase B</td>
<td>Peptide C</td>
<td>Lys, Glu-NH₂, Ileu</td>
<td>Arg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

produced with a simultaneous release of lysine as the sole free amino acid. The radioactive band was isolated from the paper, and the amino acid composition was found to be identical with that of Peptide B, with an amino-terminal glutamic acid on DFB analysis. Leucine aminopeptidase, after 30 hours, released lysine and glutamine from Peptide C (0.2 µmole), giving as a major product a radioactive tetrapeptide containing iso-lysine as the amino-terminal residue, as determined by DFB analysis; serine, valine, and arginine were identified in the aqueous phase. Further degradation by leucine aminopeptidase was slow, but extensive attack yielded a radioactive tripeptide that could be isolated. DFB analysis of the tripeptide showed the presence of O-phosphoserine as the amino-terminal residue, together with the free amino acids, valine, and arginine, present in the aqueous phase. Carboxypeptidase B attack of Peptide C (0.2 µmole) released only arginine after 24 hours' reaction; this result is consistent with the fact that Peptide C originated from a trypic attack of the protein.

The above results, summarized in Table III, clearly indicate that the hexapeptide isolated from human muscle phosphorylase a has the structure: Lys-Glu-NH₂-Ileu-P-Ser-Val-Arg identical with that isolated from rabbit muscle phosphorylase a. As in the latter case, it would appear that the cleaving of the Lys-Glu-NH₂ bond of Peptide C by trypsin produced Peptide B with an amino-terminal glutaminyl residue. The ability of such an amino-terminal glutaminyl residue to undergo cyclization to the pyrrolidone derivative on heating is well known (16, 17). In the present case, as in the analogous case of Peptide B from rabbit muscle phosphorylase a, such cyclization occurs, producing Peptide A, which is nonreactive with ninhydrin but, on total acid hydrolysis, releases the same amino acids as Peptide B.

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*The abbreviation used is: DFB, 2,4-dinitrofluorobenzene.*
other than the three radioactive peptides described above is a remarkable indication of the reactivity to trypsin of a limited number of the peptide bonds in the phosphorylase a molecule which are normally available to the proteolytic enzyme. Theoretically, there are approximately 150 susceptible bonds per phosphorylase subunit. It is presumed that the introduction of phosphate groups into human skeletal muscle phosphorylase b leads to a doubling of the molecular weight of the protein, as has been demonstrated for the rabbit muscle enzyme. It has been suggested that the observed increase in molecular weight is a consequence of the partial neutralization by the phosphate groups of a highly basic site of the polypeptide chains of phosphorylase b which otherwise would tend to keep the chains apart by electrostatic repulsion. An additional explanation might be found if the introduction of phosphate groups into phosphorylase b resulted in a conformational change in the region of the protein undergoing phosphorylation such that the probability of chain interactions is increased. The accessibility of the phosphorylated site to trypsin, chymotrypsin, papain, and a Bacillus subtilis protease (Nagarse) may well indicate that this portion of the protein is structurally less highly organized than the rest of the molecule and also that it occupies a more "exposed" position on the surface of the enzyme.

**SUMMARY**

1. The phosphorylation of human skeletal muscle phosphorylase b by the specific phosphorylase b kinase of rabbit skeletal muscle in the presence of Mg++ and P32-labeled adenosine triphosphate is described.

2. Trypsin releases over 90% of the bound radioactivity of P32-labeled phosphorylase a within 20 minutes in a form not precipitable by 5% trichloroacetic acid.

3. Purification of the radioactive peptides by high-voltage electrophoresis and paper chromatography is described, and the structure of the major peptide is shown to be: Lys–Glu–NH2–Ileu–P–Ser–Val–Arg. The structures of two minor peptides were also determined and shown to be derived from the hexapeptide.

4. The significance of the results, together with the known structure of the phosphorylated site of rabbit skeletal muscle phosphorylase a, is discussed.

**REFERENCES**

Comparative Studies on Glycogen Phosphorylase: III. THE PHOSPHORYLATED SITE IN HUMAN MUSCLE PHOSPHORYLASE a
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