Phosphorylation and Inactivation of the Pyruvate Dehydrogenase from the Anaerobic Parasitic Nematode, *Ascaris suum*

**STOICHIOMETRY AND AMINO ACID SEQUENCE AROUND THE PHOSPHORYLATION SITES**

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Tryptic digestion of the fully phosphorylated *Ascaris suum* pyruvate dehydrogenase complex yielded a single tetradecapeptide containing 2 phosphorylated serine residues. Its amino acid sequence was Tyr-Ser-Gly-His-Ser(P)-Met-Ser-Asp-Pro-Thr-Ser(P)-Tyr-Arg and was very similar to one of the tryptic phosphopeptides isolated from mammalian and yeast pyruvate dehydrogenases. At partial phosphorylation, three peptides were isolated which corresponded to the monophosphorylated (sites 1 and 2) and diphosphorylated tetradecapeptides. In contrast to results reported from mammalian complexes, phosphorylation of the ascarid complex paralleled inactivation, and no additional phosphorylation occurred after inactivation was complete. Complete inactivation of the complex was associated with the incorporation of 1.7–1.9 mol of phosphoryl groups/mol of α-pyruvate dehydrogenase subunit, and the strict preference of the pyruvate dehydrogenase kinase for site 1 was not observed. Whereas site 1 was initially phosphorylated more rapidly than site 2, at 50% inactivation, 41% of the incorporated phosphoryl groups were incorporated into site 2. In addition, substantial amounts of peptide monophosphorylated at site 2 also accumulated, suggesting that prior phosphorylation at site 1 was not necessary for phosphorylation at site 2. Phosphorylation also caused a marked decrease in the mobility of the α-pyruvate dehydrogenase subunit on sodium dodecyl sulfate-polyacrylamide gels and the apparent separation of mono- and diphosphorylated forms of the enzyme. The significance of these observations in the regulation of the unique anaerobic mitochondrial metabolism of *A. suum* is discussed.

Mitochondrial energy metabolism in body wall muscle of the adult parasitic nematode, *Ascaris suum*, is anaerobic and results in the accumulation of the organic acids acetate, propionate, succinate, 2-methyl butyrate, and 2-methyl valerate (1, 2). The tricarboxylic acid cycle is not functional, and electron transport is antimycin- and cyanide-insensitive (3). Instead of oxygen, unsaturated organic acids are used as terminal electron acceptors, and the NADH-dependent reductions of fumarate and 2-methyl branched-chain enoyl-CoAAs appear to be coupled to rotenone-sensitive, electron transport-associated ADP phosphorylations (4, 5).

In body wall muscle of adult *A. suum*, pyruvate is formed intramitochondrially by malic enzyme, and its subsequent oxidation by the pyruvate dehydrogenase complex (PDC) is an important regulatory site in the helminth’s unique anaerobic mitochondrial metabolism. Pyruvate oxidation generates not only the reducing power, but also the thioester linkages needed to drive branched-chain fatty acid synthesis through a reversal of β-oxidation (6, 7). The subunit composition of ascarid PDC is similar to complexes isolated from mammalian tissues, and its activity also is regulated by an intrinsic pyruvate dehydrogenase kinase and Mg2+-, Ca2+-dependent pyruvate dehydrogenase phosphatase, which catalyze the reversible phosphorylation of the α-pyruvate dehydrogenase subunit (E1α) (8, 9). However, many of the kinetic parameters of the ascarid complex differ markedly from mammalian PDCs and appear to be modified for the unique reducing environment present within the adult ascarid mitochondria (8, 14).

Recently, we have observed that phosphorylation of ascarid E1α is proportional to the inactivation of the complex, and no additional phosphorylation is observed after inactivation is complete. In addition, phosphorylation of ascarid E1α markedly reduces its mobility during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (9). These results contrast with those reported for mammalian PDCs, where three distinct phosphorylation sites have been identified, one of which is primarily responsible for inactivation and where phosphorylation does not appear to markedly alter the mobility of *E1α* during SDS-PAGE (10–12). This study identifies specific phosphorylation sites in ascarid *E1α* and characterizes the stoichiometry of site-specific phosphorylation and inactivation.

**EXPERIMENTAL PROCEDURES**

**Materials**

Pyruvate dehydrogenase complex (3–5 μmol of NADH produced per min/mg of protein) containing pyruvate dehydrogenase kinase activity was isolated from frozen *A. suum* muscle strips as described previously (9). Ascarids were obtained from Routh Packing (Sandleusky, OH). Partially purified bovine kidney pyruvate dehydrogenase phosphatase was a generous gift of Dr. T. Roche (Kansas State University, Manhattan, KS) or was prepared according to Pratt et al. (13). [1-32P]ATP was obtained from Du Pont-New England Nuclear. Trifluoroacetic acid was from Aldrich, and phenyl isothiocyanate was from Pierce Chemical Co. HPLC reagents were from Fisher. All other chemicals were of reagent-grade and were purchased from Sigma.

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1. The abbreviations used are: PDC, pyruvate dehydrogenase complex; *E1α*, α-pyruvate dehydrogenase subunit; MOPS, 3-(N-morpholino)propanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; PTH, phenylthiohydantoin.
Phosphorylation and Inactivation of PDC—Pyruvate dehydrogenase complex activity was measured spectrophotometrically by monitoring NADH formation at 340 nm as described previously (9, 14). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 1 mM MgCl2, 0.2 mM NAD+, 2 mM dithiothreitol, 0.1 mM thiamine pyrophosphate, 0.1 mM CoA, and 4 mM pyruvate. ATP-depending PDC inactivation was measured by following the decrease in PDC activity with time (9). PDC (1.5 mg/ml) was incubated in 50 mM MOPS (pH 7.0) or 50 mM Tris-HCl (pH 7.5) containing 3 mM dithiothreitol, 1 mM MgCl2, 25 mM NaF, and, where indicated, 60 mM KCl. After preincubation for 2 min at 30 °C, [γ-32P]ATP (1 mM final concentration; 100,000 cpm/μmol) was added, and incubation was continued in an aerobic atmosphere at 30 °C. For inactivation with [γ-32P]ATP as described above. Phosphorylated PDC was then precipitated with trichloroacetic acid (10%, w/v) and resuspended in 20 μl of 0.2 M NH4HCO3, and 1 mg of L-1tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was added. Following incubation for 4 h at 23 °C, an additional 1 mg of trypsin was added to achieve complete digestion. After 20 h, the incubation mixture was centrifuged (5000 g, 10 min) to remove insoluble material (~2% of the total radioactivity), lyophilized, and stored at −20 °C prior to phosphopeptide isolation.

The lyophilized tryptic peptides were resuspended in 2 M urea and filtered (0.45-μm pore size). Initially, the digest was chromatographed on a Sephadex G-50-50 column (16.8×89 cm) and eluted (15 ml/h) with 0.2 M NH4HCO3 (pH 8.2) to remove [γ-32P]ATP. Radioactive peptide fractions eluted in a broad peak (1.1 ml retention time of 10.5 h) and were pooled and lyophilized for further purification. Lyophilized peptide was resuspended in 0.2 M NH4HCO3, further purified on a Waters reverse-phase C18 Bondpak column (100×8 mm), and eluted (60 min, 1.5 ml/min) with a linear gradient of acetonitrile (0-60%) in 0.1% trifluoroacetic acid. Radioactive fractions eluted in a single peak were pooled and lyophilized. In subsequent peptide purifications, the Sephadex chromatography step was omitted. A small peak of [γ-32P]ATP was sometimes observed in the void volume during reverse-phase HPLC. Lyophilized phosphorylated PDC was recovered in cleavage peptide C4, whereas all of the radioactivity in peptide TA1 was recovered in cleavage peptide C1. However, they differed in the amount of covalently bound phosphoryl groups. Peptides TA1 and TA2 contained one phosphoryl group, whereas peptide TA12 contained two phosphoryl groups. The amino acid sequence analysis of peptide TA12 is presented in Table II. No PTH-derivatives were detected at cycles 5 and 12, which is consistent with the inability of phosphorylated serine residues to form PTH-derivatives. It appears that the ascarid tryptic phosphopeptide contains 2 phosphoryl groups and is very similar to a phosphorylated tetradecapeptide isolated from both yeast and mammalian pyruvate dehydrogenase complexes (10-12, 16).

Since all three of the phosphopeptides identified in this study had identical amino acid compositions and peptide TA12 contained twice as many phosphoryl groups as peptides TA1 and TA2, it appeared that phosphorylated PDC was more phosphorylated at different serine residues (5 or 12), as had been diluted previously with mammalian PDCs (10-12). To test this hypothesis, each phosphopeptide was cleaved with CNBr, and cleavage products were separated by HPLC (Fig. 2). Over 90% of the original radioactivity was recovered in four peptides (C1-C4). The amino acid compositions of these four peptides are presented in Table I. Peptides C1-C3 have similar amino acid compositions and appear to consist of residues 1–6, whereas peptide C4 consists of residues 7–14. The recovery of multiple peptides with amino acid compositions identical (C1 and C2) or similar (C3) to residues 1–6 is reproducible. Their origin is not completely clear, but they could result from a number of factors, such as an equilibrium of homoserine/homoserine lactone or microheterogeneity not detected previously. Interestingly, the substitution of a glycine residue for a serine residue observed in cleavage peptide C3 is observed at position 2 in the phosphopeptide derived from yeast PDC (Table II). All of the radioactivity in peptide TA2 was recovered in cleavage peptide C4, whereas all of the radioactivity in peptide TA1 was recovered in cleavage pep-
These results indicate that peptide TA12 is diphosphorylated peptide (TA12) which eluted at about 210 mM NH₄OAc (0-1 M ammonium acetate) during liquid chromatography. Elution of the peptides was performed as described under "Experimental Procedures" using a linear gradient of 75, 150, and 210 mM NH₄OAc, respectively. Fully phosphorylated PDC when phosphorylation was complete (8.9 ± 0.6 nmol for 12 different preparations of PDC). The initial rate of phosphorylation at both buffers. However, total ³²P incorporation and the linear relationship between phosphorylation and inactivation were the same under all incubation conditions (data not shown). Pyruvate dehydrogenase is a tetramer with a subunit composition of α₃β₃ (23). Based on an estimate of 20 pyruvate dehydrogenase tetramers/complex, about 3.5-3.8 mol of ³²P/mol of pyruvate dehydrogenase was incorporated. The role of phosphorylation at sites 1 and 2 of E₄₈ and the inactivation of the complex are not clear since the complete phosphorylation of both sites occurred during inactivation, and phosphorylation of neither site correlated directly with inactivation (Table IV and Fig. 4). Initially, phosphorylation at site 1 was more rapid than at site 2; and at 30% inactivation, 64% of the total ³²P incorporated was present in site 1. However, phosphorylation at site 2 was still substantial, and 20% of the total ³²P incorporated was present as monophosphorylated site 2, suggesting that prior phosphorylation at site 1 was not necessary for phosphorylation at site 2 to occur. At 50% inactivation, 50% of the total ³²P had been incorporated into the complex, and 59% was present in site 1 and 41% in site 2.

Phosphorylation and inactivation of PDC were accompanied by a marked decrease in the mobility of E₄₈ during SDS-PAGE (Fig. 5A). During inactivation, the complete conversion of E₄₈ from a higher to a lower mobility form was observed. The altered mobility of E₄₈ was reversible. Incubation of fully phosphorylated A. suum PDC with partially purified bovine kidney pyruvate dehydrogenase phosphatase, 10 mM MgCl₂ and 0.1 mM CaCl₂ for 60 min restored about 80% of the original PDC activity and converted most of the lower mobility form of E₄₈ to the higher mobility form. The bovine kidney pyruvate dehydrogenase phosphatase used in this study was about 20-fold less active with ascarid PDC than with bovine kidney PDC incubated under similar conditions (data not shown).

Autoradiography of PDC separated by SDS-PAGE during the time course of phosphorylation-inactivation revealed a band corresponding to the lower mobility form and, during the initial stages of phosphorylation, a second radioactive band intermediate in apparent molecular weight between the higher and lower mobility forms (Fig. 5B). At greater than 75% inactivation, this second radioactive band was not observed, even with different exposure times or the use of slower x-ray film (data not shown).

**DISCUSSION**

This study demonstrates that 2 serine residues in E₄₈ of the A. suum pyruvate dehydrogenase complex are phosphorylated by its endogenous pyruvate dehydrogenase kinase. The amino acid sequence of the tryptic phosphopeptide containing these phosphorylation sites has been established and is very similar to phosphorylation sites 1 and 2 identified in mammalian pyruvate dehydrogenase complexes (10-12). In mammalian pyruvate dehydrogenase complexes, three distinct phosphorylation sites have been identified, and inactivation is associated with the phosphorylation of site 1 (11). The ratio of site occupancy for up to 90% inactivation is 90:3:1 for sites 1-3, respectively; and phosphorylation of site 2 requires prior phosphorylation at site 1 (24). In the facultatively anaerobic yeast Saccharomyces cerevisiae, PDC does not appear to be phosphorylated in vivo, and pyruvate dehydrogenase kinase activity has never been detected (17, 25). However, when S. cerevisiae PDC is incubated with purified bovine kidney pyruvate dehydrogenase kinase, a single serine residue corresponding to site 1 is phosphorylated and results in the inactivation of the complex (17). In contrast, the role of the two peptide C4 and C1–C3. These results indicate that peptide TA12 is diphosphorylated and that peptides TA1 and TA2 were monophosphorylated at serine residues 5 and 12, respectively.

Inactivation and Site-specific Phosphorylation of A. suum PDC—When A. suum PDC was incubated with [γ-³²P]ATP, it was rapidly inactivated, and phosphorylation correlated directly with inactivation (Table IV and Fig. 3). No additional phosphorylation was observed after inactivation was complete, and about 9 nmol of ³²P/mg of protein was incorporated when phosphorylation was complete (8.9 ± 0.6 nmol for 12 different preparations of PDC). The initial rate of phosphorylation was more rapid (2–3-fold) in MOPS (pH 7.0) than in Tris-HCl (pH 7.5), and 60 mM KCl stimulated (2–3-fold) the initial rate of phosphorylation in both buffers.
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### Table I

**Amino acid composition of tryptic peptides and cyanogen bromide cleavage peptides derived from *A. suum* pyruvate dehydrogenase complex**

| Amino acid | TA1 | TA2 | TA12 | C1 | C2 | C3 | C4 |
|------------|-----|-----|------|----|----|----|----|
| Asp        | 1.09 (1) | 1.18 (1) | 1.13 (1) | 1.74 (2) | 1.84 (2) | 0.96 (1) | 1.17 (1) |
| Thr        | 0.99 (1) | 0.88 (1) | 0.99 (1) | 1.28 (1) | 1.48 (1) | 2.49 (2) | 1.06 (1) |
| Ser        | 3.39 (4) | 2.84 (4) | 2.94 (4) | 0.66 (1) | 0.84 (1) | 0.60 (1) | 1.63 (2) |
| Pro        | 0.99 (1) | 0.89 (1) | 1.03 (1) | 1.18 (2) | 0.92 (1) | 1.04 (1) | 0.94 (1) |
| Gly        | 0.96 (1) | 1.03 (1) | 0.90 (1) | 0.80 (1) | 0.84 (1) | 0.90 (1) | 0.90 (1) |
| Met        | 1.90 (2) | 1.85 (2) | 1.94 (2) | 1.18 (2) | 0.92 (1) | 1.04 (1) | 0.90 (1) |
| His        | 1.02 (1) | 1.01 (1) | 0.99 (1) | 0.90 (1) | 0.90 (1) | 0.85 (1) | 0.94 (1) |
| Arg        | 0.86 (1) | 1.00 (1) | 1.05 (1) | 0.90 (1) | 0.80 (1) | 0.85 (1) | 0.94 (1) |
| Total      | 14 | 14 | 14 | 6 | 6 | 6 | 8 |

*3P | 0.9 (1) | 1.0 (1) | 1.9 (2) | 1.0 (1) | 0.9 (1) | 1.1 (1) | 0.9 (1) |

*Impurities below 0.2 residue are omitted.

*Serine destruction was corrected for by extrapolation to zero time following hydrolysis for 24, 48, and 72 h.

*Methionine was determined as homoserine and homoserine lactone in cyanogen bromide fragments.

### Table II

**Comparison of amino acid sequence data for *A. suum*, mammalian, and yeast phosphopeptides**

| A. suum | Tyr-Ser-Gly-His-Ser(P)-Met-Ser-Asp-Pro-Gly-Thr-Thr-Tyr-Arg |
|---------|----------------------------------------------------------|
| Mammalian | Tyr-Gly-Gly-His-Ser(P)-Met-Ser-Asp-Pro-Gly-Thr-Thr-Tyr-Arg |
| Yeast | Tyr-His-Gly-His-Ser(P)-Met-Ser-Asp-Pro-Gly-Val-Ser(P)-Tyr-Arg |

*Described in Refs. 10-12.

*Described in Ref. 17.

### Table III

**Cyanogen bromide digestion of tryptic phosphopeptides derived from the *A. suum* pyruvate dehydrogenase complex**

The *A. suum* pyruvate dehydrogenase complex (15 mg of protein) was inactivated to about 50% activity with [γ-32P]ATP. Partially phosphorylated complex was then completely digested with trypsin, and tryptic phosphopeptides (TA1, TA2, and TA12) were isolated by high pressure liquid chromatography as described for Fig. 3. Purified phosphopeptides were then digested with cyanogen bromide, and cleavage peptides were separated by reverse-phase high pressure liquid chromatography as described for Fig. 4. Individual peaks (C1-C4) were collected and counted for radioactivity and analyzed for amino acid composition.

| Phosphopeptide | Radioactivity in tryptic peptides |
|----------------|----------------------------------|
| TA1 | TA2 | TA12 |
| Initial | 5.6 | 5.7 | 12.9 |
| Undigested | 0.4 | 1.3 | 3.5 |
| Cleavage peptides | | | |
| C1 | 1.4 | 0 | 0.7 |
| C2 | 2.9 | 4.7 | 0 | 2.1 | 4.0 |
| C3 | 0.4 | 0 | 1.2 |
| C4 | 0 | 3.9 | 3.8 |

% recovery 32P | 91 | 93 | 88

phosphorylation is observed after inactivation is complete.

No evidence for additional phosphorylation sites in the ascarid pyruvate dehydrogenase complex was ever observed. Either site 3 is not present in the ascarid complex, or, less likely, it is already phosphorylated in the purified complex.

During isolation, the ascarid complex is routinely incubated during the inactivation of the complex by phosphorylation, the complete conversion of *E*_<sub>60</sub> from a higher mobility to a lower mobility form was observed. This correlation has not been described previously for mammalian...
TABLE IV
Distribution of 32P in tryptic phosphopeptides
A. suum PDC was phosphorylated with pyruvate dehydrogenase kinase and \([\gamma-32P]\)ATP. At the indicated times, aliquots were removed and assayed for activity, total protein-bound phosphoryl groups, and distribution of radioactivity in the three tryptic phosphopeptides derived from E1a. Conditions are described under "Experimental Procedures."

| Time (min) | PDC activity | Total 32P | Radioactivity in peptides | Percentage inactivation | Maximum |
|-----------|--------------|----------|--------------------------|------------------------|---------|
|           | % inactivation | nmol/mg protein | TA1 | TA2 | TA12 | Site 1 | Site 2 |
| 0.5       | 16           | 1.63     | 61 | 24 | 15 | 24.9 | 11.5 |
| 1         | 31           | 5.00     | 49 | 20 | 31 | 43.2 | 29.8 |
| 2         | 54           | 5.01     | 30 | 12 | 58 | 66.0 | 45.8 |
| 3         | 74           | 6.55     | 25 | 11 | 64 | 83.3 | 62.9 |
| 5         | 87           | 7.64     | 12 | 8  | 80 | 88.7 | 81.9 |
| 30        | 98           | 8.92     | 1  | 3  | 96 | 96.0 | 98.0 |
| 60        | 99           | 8.96     | 1  | 2  | 97 | 97.0 | 99.0 |

Fig. 3. Phosphorylation and inactivation of A. suum pyruvate dehydrogenase complex. Purified complex (1.5 mg) was incubated at 30 °C in 50 mM Tris-HCl (pH 7.5) containing 2 mM MgCl₂, 3 mM dithiothreitol, 25 mM NaF, and 1 mM [\(\gamma-32P\)]ATP in a final volume of 1 ml. Aliquots were removed at the times indicated and either assayed spectrophotometrically for pyruvate dehydrogenase complex activity (O) or counted for protein-bound 32P (O) as described under "Experimental Procedures." Inset, relationship between percentage inactivation (y axis) and nmol of 32P incorporated per mg of protein (x axis) (O).

E1a or yeast E1α, phosphorylated with purified bovine kidney pyruvate dehydrogenase kinase, although some workers (17, 26-28) have described E1α doublets on autoradiographs of phosphorylated brain homogenates separated by SDS-PAGE. In contrast, phosphorylation of the mammalian branched-chain \(\alpha\)-ketoacid dehydrogenase does decrease its mobility during SDS-PAGE, and two radiolabeled bands have been identified by autoradiography which correspond to mono- and diphosphorylated dehydrogenase subunits (29). A similar situation appears to exist in ascarid PDC. Initially, two radiolabeled bands were observed which correspond to mono- and diphosphorylated E1a subunits. As phosphorylation proceeds, the formation of the diphosphorylated subunit is constant with time and correlates directly with the appearance of the lower mobility form of the enzyme observed on Coomassie Blue-stained gels. At full phosphorylation, only diphosphorylated E1a and the lower mobility form are present. These results suggest that both E1a subunits of the \(\alpha_2\beta_2\) tetramer are phosphorylated at complete inactivation, in agreement with the calculation of 3.5-3.8 mol of 32P incorporated per mol of pyruvate dehydrogenase.

The physiological significance of the differences in phosphorylation noted between ascarid and mammalian PDCs remains to be defined in studies using intact mitochondria, but the apparent lack of preference of the ascarid pyruvate dehydrogenase kinase for the inactivating phosphorylation site (Ser²) may relate to the potential problem of maintaining
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PDC activity under the reducing conditions present in adult A. suum mitochondria. Whereas third-stage larval ascaris are aerobic and possess a functional tricarboxylic acid cycle, adult ascaris, which reside in the microaerophilic lumen of the small intestine, are predominantly anaerobic and rely on PDC to fuel their unique mitochondrial metabolism (2, 30). The elevated NADH/NAD and acetyl-CoA/CoASH ratios present in mitochondria of the adult favor the inactivation of PDC, even though it appears that stimulation of the ascarid pyruvate dehydrogenase kinase is less sensitive to these elevated ratios than the corresponding mammalian kinase (9, 31). To maintain PDC activity under physiological conditions favoring inactivation, total PDC activity in adult ascarid body wall muscle is much greater than values reported from any other organism, in spite of the fact that the final specific activity of purified A. suum PDC is lower (<5 μmol of NADH formed per min/mg of protein) than values reported for yeast or mammals (10–20 μmol of NADH formed per min/mg of protein). Indeed, PDC in isolated A. suum muscle mitochondria is only 20% active, but because of its elevated amounts, substantial activity is still present even under conditions favoring pyruvate dehydrogenase kinase and substantial inactivation. The reason for the lower specific activity of the ascarid complex is unclear (9, 17, 32). It is recovered in high yield, and the results of this study suggest that it is fully dephosphorylated during activation.

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