Succinyl Coenzyme A Synthetase of *Escherichia coli*. Sequence of a Peptide Containing the Active-Site Phosphohistidine Residue†

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ABSTRACT: Succinyl coenzyme A synthetase of *Escherichia coli* is known to involve the intermediate participation of a 3-phosphohistidine residue in its catalytic mechanism. By use of ion-exchange chromatographic methods, we have isolated a dodecapeptide, containing the active-site phosphohistidine residue, obtained by digestion of the phosphorylated enzyme with trypsin. Its amino acid sequence has been determined to be: Met-Gly-His(PO_3^{2-})-Ala-Gly-Ala-Ile-Ile-Ala-Gly-Gly-Lys. We describe a method for the selective purification of this peptide which may have utility in studies of other phosphoproteins.

It appears that there exists a small group of phosphotransferases which probably involve the intermediate participation of a phosphohistidine residue in their catalytic mechanisms. This group may include succinyl-CoA synthetase from both bacterial and mammalian sources (Kreil and Boyer, 1964; Mitchell *et al.*, 1964), nucleoside diphosphokinase (Norman *et al.*, 1965), glucose 6-phosphatase (Feldman and Butler, 1969), phosphoglyceromutase (Rose, 1970), an acid phosphatase (Igarashi *et al.*, 1970), a bacterial sugar transport protein (Kundig *et al.*, 1964), and possibly ATP citrate lyase (Cottam and Srere, 1969; Mårdh *et al.*, 1971). In all of the above cases, phosphohistidine has been isolated from digests of phosphoprotein prepared by incubation of the enzyme with radioactive substrate. In one case, that of *Escherichia coli* succinyl-CoA synthetase, measurements have been made of the kinetics of formation and turnover of the phosphohistidine residue which confirm its catalytic function as an intermediate (Bridger *et al.*, 1968). In addition to the phosphohistidine residue, evidence has been presented in support of the intermediate participation of enzyme-bound succinyl phosphate in the succinyl-CoA synthetase reaction (Nishimura and Meister, 1965; Nishimura, 1967; Hildebrand and Spector, 1969).

E. coli succinyl-CoA synthetase is believed to have a molecular weight of approximately 140,000 (Ramaley *et al.*, 1967) and appears to be constituted of two subunit types, with the smaller of the two subunits bearing the potential phosphohistidine residue (Bridger, 1971). In this communication, we report the isolation and amino acid sequence determination of a dodecapeptide containing the active-site phosphohistidine residue of this enzyme.

Materials and Methods

Succinyl-CoA synthetase was prepared from succinate-grown *E. coli* (Crooke's strain) essentially according to the method of Leitzmann *et al.* (1970). We deviate from their method by substituting QAE-Sephadex in place of DEAE-Sephadex for ion-exchange chromatography, and by including a final purification step, passage through G-100 Sephadex, in most preparations. The enzyme used throughout this study was shown to be chromatographically and electrophoretically homogeneous, and had a specific activity in excess of 40 units/mg.

Special Reagents and Materials. [γ-^{32}P]ATP was prepared according to the method of Ramaley *et al.* (1967), using [^{32}P]-orthophosphate obtained from New England Nuclear. Polyamide thin-layer chromatography sheets were obtained from the Cheng Chin Trading Co. by way of Allied Scientific Co. TPCK-trypsin[1] (trypsin which had been treated with TPCK to inactivate chymotrypsin) was the product of the Worthington Biochemical Corporation. We are indebted to Drs. D. R. Whitaker and L. B. Smillie for a sample of α-lytic protease from *Sorangium* sp. (Whitaker 1965).

Preparation and Digestion of Phosphoenzyme. In a typical protocol, 120 mg of succinyl-CoA synthetase was incubated in a final volume of 6 ml with 0.2 mM [γ-^{32}P]ATP (5×10^8 cpm/μmole), 10 mM $MgCl_2$, 0.05 M Tris-Cl (pH 7.2), and 0.05 M KCl for 15 min at 25°. The mixture was then passed through a 1 × 60 cm column of G-50 Sephadex, equilibrated with 0.05 M Tris-Cl–0.05 M KCl (pH 8.1). The labeled protein peak was collected and was found to contain 0.9 phosphoryl group/140,000 daltons of protein, in harmony with earlier observations (Ramaley *et al.*, 1967) that saturating concentrations of ATP yield only about one phosphohistidine residue per enzyme molecule. To the pooled phosphoenzyme (15 ml) was then added 1.2 mg of TPCK-trypsin dissolved in 0.6 ml of the same buffer. Digestion was allowed to proceed for 5 hr at 37°, after which the peptides were separated according to methods outlined in Results.

Desalting of Phosphohistidine-Containing Peptides. Because of the acid lability of the N–P bond of the phosphohistidine residue, we have found it unsatisfactory to carry out chromatography of the peptides in volatile buffers followed by lyophilization to accomplish desalting—substantial hydrolysis of the phosphohistidine residue takes place during such lyophilization. A suitable alternative procedure which we have adopted throughout this study involves the passage of the pooled solution of peptide, obtained from ion-exchange chromatography, through a 2.5 × 100 cm column of G-25 Sephadex equilibrated with 0.05 N ammonium hydroxide. Subsequent lyophilization of the peptide may then be accomplished with little or no attendant hydrolysis.

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[1] Abbreviations used are: TPCK, L-(tosylamido-2-phenyl)ethyl chloromethyl ketone; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl.

TABLE I: Amino Acid Composition and Sequence of Histidine-Containing Peptides.

Peptide	Amino Acid Composition and Sequence
PH-1	Met- Gly - His - Ala - Gly - Ala - Ile - Ile - Ala - Gly - Gly - Lys
	1.02 1.04 1.00 1.03 1.04 1.03 0.60 0.60 1.03 1.04 1.04 1.10
PH-2	Met- Gly - His - Ala - Gly - Ala - Ile - Ile - Ala
	0.75 1.05 1.00 1.00 1.05 1.00 0.69 0.69 1.00
H-1	Met- Gly - His - Ala - Gly - Ala - Ile - Ile - Ala - Gly - Gly - Lys
	1.00 1.01 1.00 1.04 1.01 1.04 0.60 0.60 1.04 1.01 1.01 1.02
H-3	Val - Ala - Glx - Glx - Thr - His - His - Leu - Ile - Lys
	1.04 1.00 0.92 0.92 0.90 0.95 0.95 0.88 0.84 1.00

Amino Acid Analysis. Prior to anaylsis, purified peptides were lyophilized and taken up in 0.05 N *N*-ethylmorpholine (pH 8.0) and passed through a 1 × 45 cm column of G-15 Sephadex equilibrated with that buffer. The peptide solution was again lyophilized and dissolved in a small volume of *N*-ethylmorpholine buffer. Samples were subjected to hydrolysis (constant-boiling HCl, 110°, 20 hr) and analysis was carried out on a Beckman–Spinco Model 121 amino acid analyzer.

Sequence Determination. The amino acid sequences of the peptides were determined using the dansyl-Edman procedure (Gray, 1967). The dansylated amino acids were identified by thin-layer chromatography on polyamide layers (Woods and Wang, 1967; Hartley, 1970).

Results

Chromatographic Isolation of [^{32}P]Phosphopeptide. The acid lability of the phosphohistidine residue precludes the possibility of using high-voltage electrophoresis at pH 6.5 or less for resolution of the mixture of peptides obtained by tryptic digestion of phosphorylated succinyl-CoA synthetase. We have, however, devised an empirical method using ion-exchange chromatography at alkaline pH for the purification of the [^{32}P]phosphopeptide. The mixture of peptides obtained by proteolysis of 120 mg of [^{32}P]phosphoenzyme was chromatographed on QAE-Sephadex A-25, and the results are given on Figure 1. There are two peaks of radioactivity; the smaller peak (tubes 168–174) was identified as [^{32}P]orthophosphate. The larger peak containing the desired peptide was pooled and desalted according to the procedure described under Materials and Methods. This mixture was then applied to a column of Dowex 1, and the resulting chromatographic pattern is shown on Figure 2. As before, the smaller peak of radioactivity was identified as [^{32}P]orthophosphate. The larger peak was pooled and desalted, and a sample was subjected to high-voltage electrophoresis (pH 6.5, 3 kV, 45 min). Only one peptide was observed (peptide PH-1). The amino acid composition was determined and is shown in Table I. N-Terminal amino acid analysis showed only dansylmethionine. Sequence determination was therefore undertaken and the results are also reported in Table I. (The low analysis for isoleucine noted in Table I is undoubtedly the result of the unusual stability of the Ile–Ile sequence.) Since identification of α-dansylhistidine is difficult in peptides containing lysine because of the similar migration of α-dansylhistidine and ε-*N*-dansyllysine, we attempted further proteolysis of peptide PH-1 in the hope of obtaining a shorter peptide containing histidine but having no lysine residue. This was achieved by digestion of a sample of the purified [^{32}P]phosphopeptide PH-1 with α-lytic protease (molar ratio 200:1) for 2 hr at 25°, followed by chromatography on a 1 × 40 cm column of Dowex-2X equilibrated with 0.05 M ammonium acetate (pH 8.1). Following elution by a linear gradient of ammonium acetate to a final concentration of 0.5 M, a single peak of radioactivity (peptide PH-2) was detected, pooled, and desalted. The composition and amino acid sequence of this peptide are also given in Table I. These results confirm

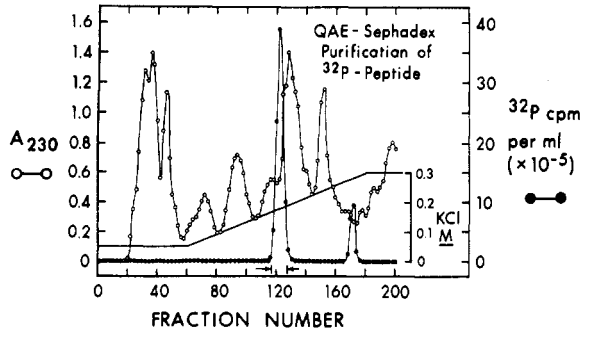

FIGURE 1: Separation of peptides obtained by digestion of 120 mg of [^{32}P]succinyl-CoA synthetase with trypsin. Chromatography was carried out on a 1.5 × 112 cm column of QAE-Sephadex A-25, equilibrated with 0.05 M Tris-Cl–0.05 M KCl (pH 8.1). The gradient of increasing KCl concentration is indicated. Tubes 117–127 were pooled and subjected to further analysis.

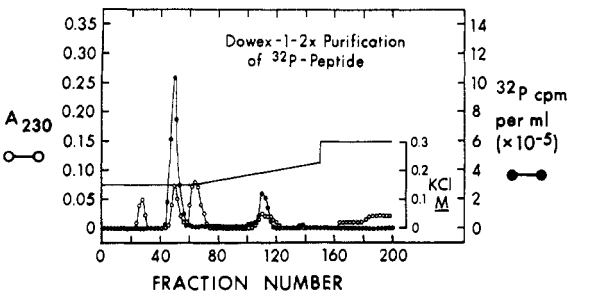

FIGURE 2: Chromatography of mixture of peptides obtained from experiment of Figure 1. A 1.5 × 112 cm column of Dowex 1-2X was equilibrated with 0.05 M Tris-Cl–0.15 M KCl (pH 8.1). Elution was accomplished with a gradient of increasing KCl concentration as shown.

the sequence in the vicinity of the phosphohistidine residue found from peptide PH-1, and indicate that α-lytic protease rather specifically catalyzed the hydrolysis of the Ala–Gly bond, resulting in the release of the C-terminal tripeptide from peptide PH-1.

Selective Purification of the Phosphohistidine-Containing Peptide. To further confirm the amino acid composition and sequence of the active-site peptide, we have developed an alternative procedure for the selective purification of peptides containing phosphohistidine residues. The method takes advantage of the acid lability of such residues, and is related in principle to diagonal electrophoretic methods developed by Hartley and coworkers (see Hartley, 1970) and to a method devised for isolation of pyridoxal 5′-phosphate containing peptides (Strausbauch and Fischer, 1970). The mixture of peptides obtained by tryptic hydrolysis of [^{32}P]phosphorylated succinyl-CoA synthetase was first subjected to ion-exchange chromatography on QAE-Sephadex (Figure 3, panel A) under conditions the same as those used in the experiment represented by Figure 1. The peak containing the [^{32}P]phosphopeptide was pooled, desalted, lyophilized, and then reapplied to an identical QAE-Sephadex column. Elution conditions were the same as before, and the resulting chromatograph is shown on Figure 3, panel B. The fractions containing the [^{32}P]phosphopeptide were again pooled, desalted, and lyophilized, but this time the powder was taken up in 5 ml of 0.1 N HCl and incubated at 25° for 12 hr to accomplish hydrolysis of the N–P bond of the phosphohistidine residue. The solution was then diluted with 50 ml of water, lyophilized, and reapplied to a third identical QAE-Sephadex column. Following elution as before (Figure 3, panel C), a new peak at tubes 31–35 appeared which was presumed to be the desired peptide now lacking the negatively charged phosphoryl group, hence being bound less strongly to the anion exchanger. The peak (peptide H–I) was pooled and desalted as before. A sample was subjected to high-voltage electrophoresis (pH 1.8, 3 kV, 45 min); only one ninhydrin-positive spot was apparent. The composition and sequence of peptide H-1 were determined and found to be identical with those reported for peptide PH-1 (Table I).

Sequence of Another Histidine-Containing Peptide. Ancillary to these studies, we have purified and determined the amino acid sequence of a second histidine-containing peptide derived from succinyl-CoA synthetase. This peptide (peptide H-3) was found to migrate together with peptide PH-1 on the QAE-Sephadex columns under the conditions reported herein, but the two peptides may be separated by further chromatography on Dowex 1 as shown on Figure 2. The peak of absorbance at tubes 60–70 contains peptide H-3, which was further purified by electrophoresis (pH 6.5, 3 kV, 45 min). Studies of the composition and sequence of peptide H-3 are given in Table I. The occurrence of two adjacent histidine residues was noted with interest. It should be emphasized that neither of the histidine residues of peptide H-3 was derived from phosphohistidine.

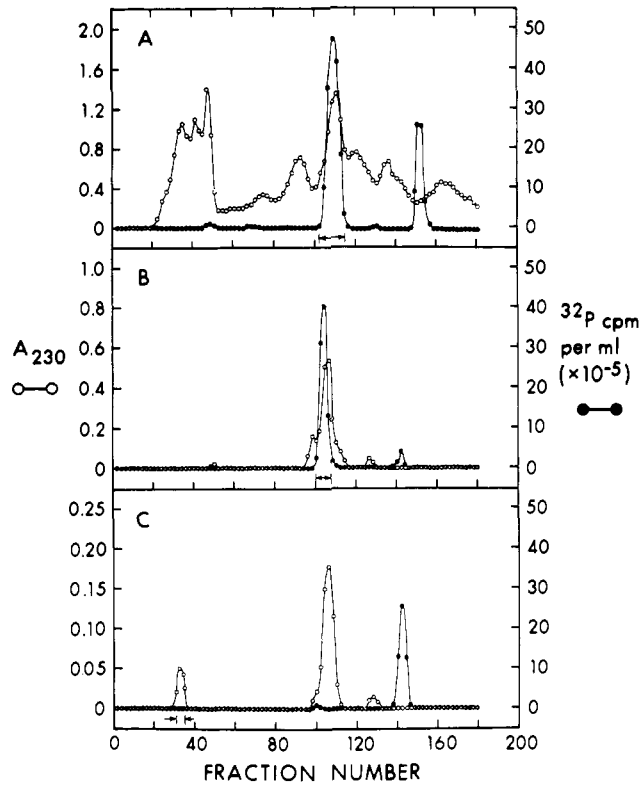

FIGURE 3: Selective purification of peptide containing potential phosphohistidine residue. In all cases, chromatography was carried out on columns (2.5 × 100 cm) of QAE-Spehadex A-25, previously equilibrated with 0.05 M Tris-Cl–0.05 M KCl (pH 8.1). Elution was accomplished by a linear gradient of KCl from 0.05 to 0.30 M. Panel A: separation of peptides obtained by tryptic digestion of 120 mg of [^{32}P]succinyl-CoA synthetase. The indicated fractions were pooled, desalted (by passage through G-25 Sephadex equilibrated with 0.05 M NH_4OH and lyophilization), and were applied to a second column. The chromatogram is shown in panel B. Pooled fractions were then desalted as before, subjected to mild acid treatment (see text), and rechromatographed a third time (panel C). Fractions containing the desired peptide were pooled as indicated. See text for further details.

Discussion

The sequence which we have determined for peptide PH-1 represents the first known active-site sequence for a phosphohistidine-containing enzyme. The sequence contains no features that are readily interpreted in terms of further understanding of the catalytic mechanism. Nevertheless, it will be of great interest to determine whether sequence homology exists between succinyl-CoA synthetase and any of the other enzymes noted above which are also thought to involve intermediate participation of phosphohistidine in their catalysis. The possibility of such homology would appear particularly strong in the case of ATP citrate lyase, which shows close parallels to succinyl-CoA synthetase in the nature of both the overall reaction and the proposed citryl phosphate and citryl-CoA intermediates (Inoue *et al.*, 1967, 1968; Walsh and Spector, 1969). In the case of that enzyme, however, the possibility exists that the formation of the phosphohistidine residue may be an artifact produced during alkaline hydrolysis of the phosphorylated enzyme (Suzuki *et al.*, 1969).

We believe that our procedure for selective purification of the phosphohistidine-containing peptide may be widely applicable to other phosphorylated proteins. To achieve removal of the phosphoryl residue and the concomitant decrease in net negative charge in peptides containing phosphorylated histidine, lysine, or carboxyl groups, the mild acid treatment we have used should generally suffice. In peptides containing phosphate esters such as phosphoserine, phosphate removal could be accomplished by treatment with alkaline phosphatase. In fact, this approach has been used successfully in the selective purification of a peptide containing the reduced pyridoxal phosphate derivative of *E. coli* glutamate decarboxylase (Strausbauch and Fischer, 1970).

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Haloacetol Phosphates. Selective Alkylation of Sulfhydryl Groups of Rabbit Muscle Aldolase by Chloroacetol Phosphate†

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ABSTRACT: Chloroacetol phosphate (3-chloro-1-hydroxy-2-propanone phosphate), a reagent structurally similar to the substrate dihydroxyacetone phosphate, has been examined as a potential active-site-specific reagent of rabbit muscle aldolase. At neutral pH, the reagent is a competitive inhibitor of the enzyme (K_I = 0.6 mM). Based on its ability to quench aldolase fluorescence and to protect the enzyme against trypsin inactivation, chloroacetol phosphate appears to bind at the dihydroxyacetone phosphate site. However, Schiff-base formation between reagent and enzyme (also, indicative of interaction with the dihydroxyacetone phosphate binding site) was not detected by the criterion of inactivation with borohydride. At pH 10.0, the reagent irreversibly inactivates the enzyme. Several observations suggest that chloroacetol phosphate inactivates aldolase by the preferential alkylation of one sulfhydryl group per catalytic subunit in the vicinity of the active site. (1) The degree of inactivation is directly proportional to the extents of sulfhydryl alkylation and reagent incorporation. (2) Other protein side chains are not modified, since the number of sulfhydryl groups modified equals the number of reagent molecules linked to the enzyme. (3) The substrates, Fru-1,6-P_2 and dihydroxyacetone phosphate, as well as the competitive inhibitor, P_i, protect the enzyme from inactivation. (4) Autoradiograms of peptide maps of aldolase inactivated by chloroacetol [^{32}P]phosphate reveal one major radioactive peptide.

Haloacetol phosphates (3-halo-1-hydroxy-2-propanone phosphates) were synthesized as potential active-site-specific reagents for enzymes that catalyze reactions involving dihydroxyacetone phosphate (Hartman, 1968a, 1970a). With triose phosphate isomerase, these reagents show absolute specificity for the active site and thereby permitted the identification of a glutamyl γ-carboxylate as a probable catalytically functional group (Hartman, 1968b, 1971; Coulson *et al.*, 1970a,b).

We hoped that haloacetol phosphates would be useful in a comparative study of the active sites of class I and class

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