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Streptococcal Phosphoenolpyruvate–Sugar Phosphotransferase System: Amino Acid Sequence and Site of ATP-Dependent Phosphorylation of HPr†

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ABSTRACT: The amino acid sequence of histidine-containing protein (HPr) from *Streptococcus faecalis* has been determined by direct Edman degradation of intact HPr and by amino acid sequence analysis of tryptic peptides, V8 proteolytic peptides, thermolytic peptides, and cyanogen bromide cleavage products. HPr from *S. faecalis* was found to contain 89 amino acid residues, corresponding to a molecular weight of 9438. The amino acid sequence of HPr from *S. faecalis* shows extended homology to the primary structure of HPr proteins from other bacteria. Besides the phosphoenolpyruvate-dependent phosphorylation of a histidyl residue in HPr, catalyzed by enzyme I of the bacterial phosphotransferase system, HPr was also found to be phosphorylated at a seryl residue in an ATP-dependent protein kinase catalyzed reaction [Deutscher, J., & Saier, M. H., Jr. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6790–6794]. The site of ATP-dependent phosphorylation in HPr of *S. faecalis* has now been determined. [^{32}P]P-Ser-HPr was digested with three different proteases, and in each case, a single labeled peptide was isolated. Following digestion with subtilisin, we obtained a peptide with the sequence -(P)Ser-Ile-Met-. Using chymotrypsin, we isolated a peptide with the sequence -Ser-Val-Asn-Leu-Lys-(P)Ser-Ile-Met-Gly-Val-Met-. The longest labeled peptide was obtained with V8 staphylococcal protease. According to amino acid analysis, this peptide contained 36 out of the 89 amino acid residues of HPr. The following sequence of 12 amino acid residues of the V8 peptide was determined: -Tyr-Lys-Gly-Lys-Ser-Val-Asn-Leu-Lys-(P)Ser-Ile-Met-. Thus, the site of ATP-dependent phosphorylation was determined to be Ser-46 within the primary structure of HPr.

Histidine-containing protein (HPr)[1] functions as a phosphate carrier protein in the phosphoenolpyruvate (PEP)–sugar phosphotransferase system (PTS) mediated uptake reaction of carbohydrates in most anaerobic and facultatively anaerobic bacteria (Hengstenberg, 1977; Robillard, 1982; Meadow et al., 1984). HPr becomes phosphorylated at the N-1 position of His-15 in a PEP-dependent reaction catalyzed by enzyme I of the PTS (Simoni et al., 1973; Beyreuther et al., 1977;

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[1] Abbreviations: HPr, histidine-containing protein; P-His-HPr, HPr phosphorylated at histidyl residue 15; P-Ser-HPr, HPr phosphorylated at seryl residue 46; PTS, phosphoenolpyruvate–sugar phosphotransferase system; PEP, phosphoenolpyruvate; III, factor III or enzyme III; P-III, phosphorylated III protein; TFA, trifluoroacetic acid; Pth, phenylthiohydantoin derivatives of amino acids; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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Weigel et al., 1982b). The intermediary P-enzyme I carries the phosphoryl group bound to the N-3 position of a single histidyl residue (Weigel et al., 1982a; Alpert et al., 1985a,b). P-His-HPr transfers the phosphoryl group to different III proteins (also called factor III or enzyme III). P-III proteins carry the phosphoryl group bound to the N-3 position of a single histidyl residue (Kalbitzer et al., 1981; Deutscher et al., 1982; Dörschug et al., 1984; Meadow & Roseman, 1982; Waygood et al., 1984). Each P-III protein interacts with a certain sugar-specific enzyme II, the membrane-bound component of the PTS. Enzyme II catalyzes the transfer of the phosphoryl group from P-III to the sugar and its concomitant uptake. A phosphorylated enzyme II intermediate has been documented (Peri et al., 1984; Waygood et al., 1984). A second phosphorylation of HPr from *Streptococcus pyogenes* was found to occur at a seryl residue, which was phosphorylated by an ATP-dependent protein kinase (Deutscher & Saier, 1983). The protein kinase was stimulated by fructose 1,6-diphosphate and inhibited by P_i (Deutscher & Engelmann, 1984; Reizer et al., 1984). In vivo formation of P-Ser-HPr was dependent on the uptake and metabolism of certain carbohydrates (Deutscher & Saier, 1983), suggesting a regulatory function for P-Ser-HPr. Indeed, P-Ser-HPr was found to be a poor substrate in the PEP-dependent phosphorylation reaction catalyzed by enzyme I (Deutscher et al., 1984). However, III proteins were found to stimulate the PEP-dependent phosphorylation of P-Ser-HPr, giving rise to a doubly phosphorylated (P-Ser, P-His) HPr (Deutscher et al., 1984). In this paper, we describe the primary structure of HPr from *Streptococcus faecalis* and identify the site of ATP-dependent phosphorylation. This is the second report about the site of phosphorylation for a bacterial ATP-dependent protein kinase. Only for isocitrate dehydrogenase from *Escherichia coli* has the sequence round the site of phosphorylation been determined (Borthwick et al., 1984; Malloy et al., 1984).

Materials and Methods

Cell Cultivation and Disruption. A 100-L fermenter with sterilized medium [1% yeast extract (Ohly, Hamburg), 0.2% tryptone (Difco), 1% glucose, and 0.25% Na_2HPO_4] was inoculated with a 3-L overnight culture of *Streptococcus faecalis* strain 26487 (Streptococcen Zentrale, Kiel). During growth, the pH was kept at 7 by addition of 10% KOH. After 5–6 h of growth (OD_{580} = 10–11), bacteria were harvested with a continuous-flow centrifuge (Westfalia). The yield was about 1 kg of cells (wet weight). Cells (300 g) were suspended in 500 mL of standard buffer (0.05 M Tris-HCl, pH 7.2, 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, and 0.1 mM sodium azide) and broken in a Dynomill KDL (Bachofen), operating at continuous flow using glass beads with a diameter of 0.5 mm. Cell debris was removed by centrifugation at 25000*g* for 30 min at 8 °C.

Purification of HPr. Cell-free extract was used to purify HPr as described by Kalbitzer et al. (1982).

Preparation of P-Ser-HPr and [^{32}P]P-Ser-HPr. P-Ser-HPr was prepared by using partially purified ATP-dependent HPr kinase. HPr (10 mg) was incubated at 37 °C together with 20 mM $MgCl_2$, 5 mM fructose 1,6-diphosphate, 20 mM ATP, and partially purified HPr kinase [150 μL of the concentrate after the DEAE-cellulose chromatography step (Deutscher & Engelmann, 1984)] in the presence of 25 mM Tris-HCl buffer, pH 7.5. After 1-h incubation, the reaction mixture with a total volume of 2 mL was loaded on a DEAE-cellulose column (Whatman DE-52, 2.5 × 10 cm). HPr and P-Ser-HPr were separated by applying a 500-mL linear gradient of 0–0.2 M NaCl in 50 mM Tris-HCl (pH 7.2). Both HPr- and P-Ser-HPr-containing fractions were pooled, desalted on a Sephadex G-25 column (3 × 25 cm), and lyophilized. The final yield was 6–7 mg of P-Ser-HPr and 2–3 mg of HPr. For the preparation of [^{32}P]P-Ser-HPr, 1 mg of HPr was incubated at 37 °C together with partially purified HPr kinase [15 μL of the concentrate after the DEAE-cellulose chromatography step (Deutscher & Engelmann, 1984)], 20 mM $MgCl_2$, 5 mM fructose 1,6-diphosphate, 25 mM Tris-HCl, and [γ-^{32}P]ATP (50 μCi, 3000 Ci/mmol). After 30-min incubation, unlabeled ATP was added to give a final concentration of 0.5 mM. After a further 30-min incubation at 37 °C, the assay mixture with a total volume of 200 μL was separated by HPLC on a TSK IEX-545 DEAE column (150 × 6 mm, Bio-Rad) by applying a linear gradient of 0–0.4 M NaCl in 10 mM Tris-HCl, pH 6.8, for 50 min. The temperature was 30 °C and the flow rate 0.8 mL/min. Radioactivity and ultraviolet absorbance (230 nm) of the effluent were monitored by a radioactivity detector (Berthold LB 504) connected to an Apple IIe computer and by an ultraviolet monitor (Jasco Uvidec 100 III). Under the above reaction conditions, about 60% of the radioactivity was incorporated into HPr to form [^{32}P]P-Ser-HPr. However, [^{32}P]P-Ser-HPr and [γ-^{32}P]ATP were not completely separated, and, thus, [γ-^{32}P]ATP was removed by desalting on a Sephadex G-25 column (0.8 × 10 cm). [^{32}P]P-Ser-HPr was lyophilized in a vacuum centrifuge (Savant).

Isolation of Labeled and Unlabeled Peptides. To 1 mg of P-Ser-HPr dissolved in 200 μL of 50 mM NH_4HCO_3, pH 8.0, was added [^{32}P]P-Ser-HPr (10^5–10^6 cpm). To this solution was added 20 μg of different proteases (either subtilisin, chymotrypsin, or V8 staphylococcal protease). After 3-h incubation at 37 °C together with subtilisin, the digest was loaded on a Servachrome Si 100 Polyol DEAE column (10 μm, 4.6 × 250 mm; Serva). Peptides were separated by HPLC using a linear gradient of 0–0.3 M NaCl in 10 mM Tris-HCl, pH 6.8, for 40 min at a flow rate of 0.7 mL/min. The fraction containing the single labeled peptide was loaded on a Nucleosil C8 reversed-phase column (10 μm, 4.6 × 250 mm; Bischoff) and eluted with a linear gradient of 0–50% solvent B for 40 min at a flow rate of 1 mL/min (solvent A was 0.1% TFA, pH 2.0; solvent B was 50% acetonitrile in H_2O). If chymotrypsin or V8 staphylococcal protease was used, the digest was loaded on a Nucleosil C8 column (10 μm, 4.6 × 250 mm; Bischoff). Peptides were separated by applying different gradients of acetonitrile in 0.1% TFA. Labeled peptides were rechromatographed on the same column using flattened gradients of acetonitrile in 0.1% TFA in the range where the labeled peptide eluted. For the determination of the amino acid sequence of HPr, tryptic, thermolytic, and cyanogen bromide cleavage reactions were performed as described by Beyreuther et al. (1977). V8 proteolytic cleavage reactions were performed according to Stüber et al. (1985). Tryptic and V8 peptides were separated by HPLC on a Nucleosil C8 column (Bischoff; 10/μm, 4.6 × 250 mm). However, the tryptic fragment covering amino acid residues 46–83 could not be eluted from the C8 column even at 95% acetonitrile. It was isolated by ion-exchange chromatography of 2 mg of tryptic-digested HPr on a DEAE-cellulose column (Whatman DE-52, 2.5 × 6 cm) by applying a linear gradient of 5–300 mM NH_4HCO_3. Peptides obtained by other proteolytic or chemical cleavage reactions were separated by the fingerprint technique as described by Beyreuther et al. (1977). The fluorescent spots were eluted with 1 M ammonia at 5 °C. The eluted material was sequenced manually or was dansylated for N-terminal analysis and hydrolyzed with 0.05 mL of 50% 12 M HCl in propionic acid at 150 °C for 15 min (Westall

& Hesser, 1974) for subsequent amino acid analysis.

Amino Acid Analysis of Peptides. Individual peptides were hydrolyzed in 6 M HCl in vacuo at 110 °C for 24 h. Amino acid analysis was performed by using a Bio Cal BC 200 amino acid analyzer equipped with a 0.4 × 23 cm Durrum DC 6A column. Elution of the column was performed with a modified lithium citrate buffer system (Benson et al., 1967).

Manual Sequencing. Manual sequence analysis of labeled peptides was performed according to Tarr (1975), with fuming hydrochloric acid. The anilinothiazolidine amino acids were extracted at two different pH values, as described by Tarr (1982). The Pth-amino acid derivatives were identified by using a gradient HPLC system with a Beckman Ultrosphere ODS column (4.6 × 250 mm). Solvent A was 63 mM acetic acid, adjusted to pH 4.5 with ammonia; solvent B was acetonitrile. Separation was achieved at 54 °C and at a flow rate of 0.5 mL/min using the following gradient: 0–5 min, 20% solvent B (isocratic); 5–19 min, 20–48% solvent B; 19–35 min, 48% solvent B (isocratic). Peptides (1–10 nmol) isolated from fluorescamine-stained fingerprints according to Beyreuther et al. (1977) were sequenced manually with the dansyl–Edman method of Bruton and Hartley (1970).

Automatic Sequencing. The N-terminal 39 residues of intact HPr (100 nmol) were determined by automated Edman degradation using a spinning cup sequencer (updated Beckmann Model 890B) as described by Zaiss and Beyreuther (1983). Peptides (0.5–1.5 nmol) were subjected to automated gas–liquid solid-phase Edman degradation (Applied Biosystems Model 470A or Model 470A equipped with an on-line Model 120A Pth analyzer). Samples were dissolved in 30 μL of formic acid and dried on the glass fiber disks preloaded with 1.5 mg of preconditioned polybrene (Aldrich). Sequencing was performed as described by Hewick et al. (1981). The Pth samples were identified by reversed-phase HPLC using a C18 column (2.1 × 220 mm) mounted in an Applied Biosystems 120A Pth analyzer or by reversed-phase HPLC using a Du Pont cyanopropyl column (Zorbax CN, 4.6 × 250 mm) attached to an LKB HPLC system equipped with an HPLC 2150 pump and a 2151 variable wavelength detector operating at 269 nm. The latter system was combined with a Waters WISP Model 710A for automatic sample injection and employed for Pth analysis of samples from the Beckman sequencer and the Applied Biosystems gas-phase sequencer which is not attached to an HPLC for on-line detection. Chromatograms were plotted on a Shimadzu C-R3A or a Trilab Model II integrator, the latter being used for integration of the output of the on-line Pth analyzer. Complete separation of the Pth-amino acid derivatives on the cyanopropyl column was achieved by using 0.1–0.2 M sodium acetate, pH 5.2, containing 23.65% tetrahydrofuran, 6.6% acetonitrile, and 2.84% methanol as eluent (Beyreuther et al., 1983). The molarity of the eluent was 0.1 M for new columns and resulted in elution of Pth-His between Pth-Gly and Pth-Ala and of Pth-Arg after Pth-Trp. To maintain the optimal separation of Pth-His and Pth-Arg after repeated analyses, an increase of the sodium acetate concentration in steps of 0.01 M was necessary after each 300 injections. A sufficient separation for up to 3000 injections could thus be obtained. The flow rate for the cyanopropyl column was 125 mL/min, and the column operated at 30 °C. The Pth-amino acid derivatives were prepared for injection by evaporating the solvent in a Speed-Vac (Savant Instruments). Samples were redissolved in 25 μL of methanol followed by 25 μL of water and placed in the automatic sample injector (off-line detection). The on-line system employed gradient elution using 0.11 M sodium acetate, pH 4.0, and 5% tetrahydrofuran as solvent A and acetonitrile as solvent B (Users Manual, Pth analyzer Model 120 A, Applied Biosystems, version 1.0, June 1985). The column was equilibrated with 16% solvent B and developed by linear gradients from 16% to 21% solvent B in 5.5 min and from 21% to 41% solvent B in 12.5 min, and finally eluted with 41% solvent B for another 5 min and regenerated with 60% solvent B for 9 min before the next cycle was started. The flow rate was 200 μL/min, and the temperature was 55 °C.

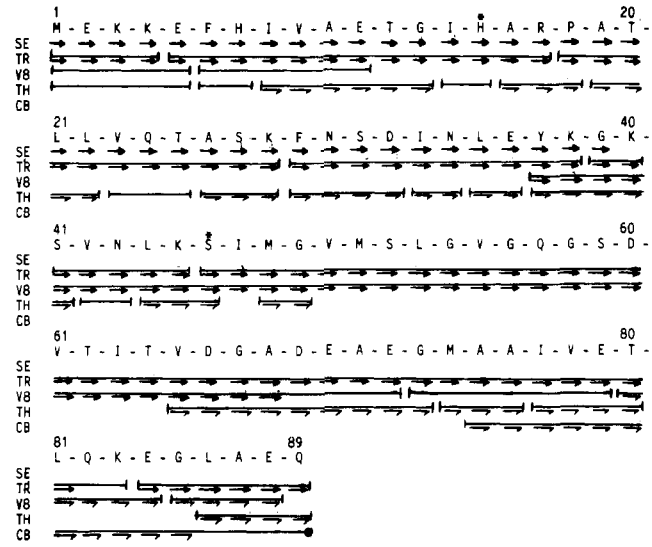


FIGURE 1: Amino acid sequence (one-letter code) of HPr from *S. faecalis*. Amino acid residues were determined by automated sequence analysis (→) or by manual sequencing (dansyl method, —). When no arrows are present, only the N-terminal amino acid and the amino acid composition of the peptide have been determined. SE means direct Edman degradation of intact HPr, TR means tryptic peptides, V8 means V8 proteolytic peptides, TH means thermolytic peptides, and CB means cyanogen bromide cleavage products. The asterisks indicate the sites of PEP-dependent and ATP-dependent phosphorylation in HPr.

RESULTS

Amino Acid Sequence Determination. Automated Edman degradation of intact HPr, performed as described under Materials and Methods, gave unambiguous information of the sequence spanning residues 1–39 (Figure 1). Following tryptic digestion of HPr, eight peptides could be isolated covering the whole amino acid sequence. However, only seven peptides were separated by HPLC on a Nucleosil C8 reversed-phase column run with a linear gradient of 0–60% acetonitrile in 0.1% TFA, pH 2.0, for 50 min at a flow rate of 1 mL/min (Figure 2). The tryptic peptide spanning residues 46–83 could not be eluted from the reversed-phase column but was isolated by ion-exchange chromatography on a DEAE-cellulose column as described under Materials and Methods. Out of the 38 amino acid residues of this tryptic peptide, the sequence of only 36 could be determined (Figure 1). Obviously, trypsin did not cleave the peptide bond in the Lys-Lys sequence at positions 3 and 4 to a significant extent. The C-terminal glutamine of the peptide spanning residues 84–89 was detected only during three out of six independent sequence determinations. Following cleavage with V8 staphylococcal protease, 7 of the 10 expected peptides were isolated by HPLC on a reversed-phase column (Figure 6B). The Glu-Lys sequence in positions 2 and 3 and the Glu-Ala sequence in positions 70 and 71 were obviously not cleaved by the V8 staphylococcal protease. Only 32 out of the 36 amino acid residues of the peptide spanning residues 37–72 could be determined. The peptides spanning

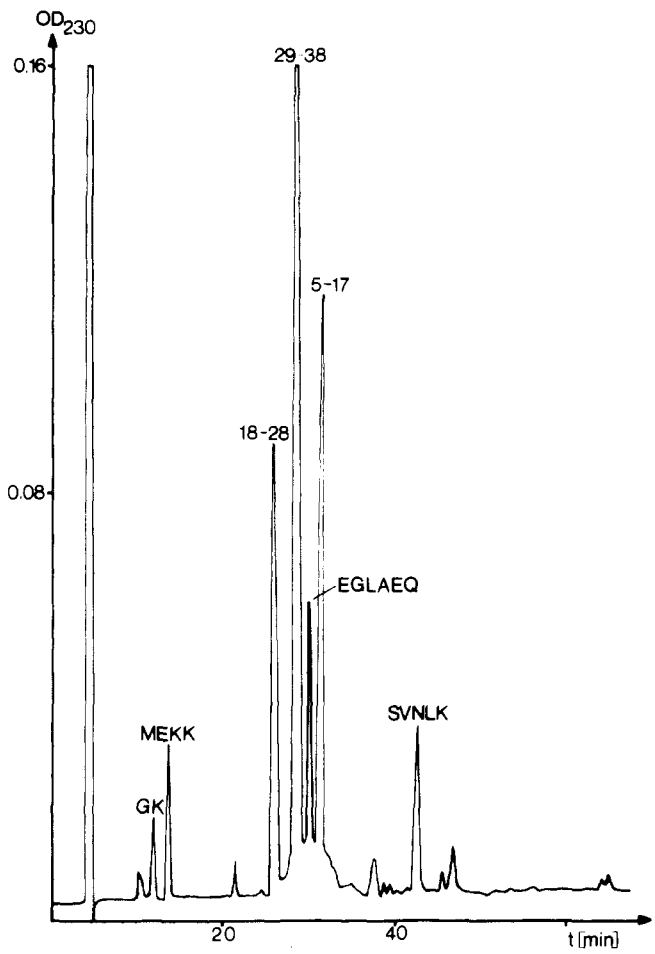


FIGURE 2: Separation of tryptic cleavage products derived from 1 mg of HPr by HPLC on a reversed-phase column (C8). Solvent A, 0.1% TFA, pH 2.0; solvent B, 85% acetonitrile. Gradient, 0–80% solvent B in solvent A for 65 min; temperature, 21 °C; flow rate, 1 mL/min.

residues 6–11 and 73–79 eluted at the same position from the reversed-phase column. The two peptides were separated by the fingerprint technique, and the N-terminal amino acid and also the amino acid composition of both peptides have been determined. For the V8 proteolytic peptide covering residues 12–36, only the amino acid composition has been determined which was found to be in full agreement with the sequence data derived from automated Edman degradation of intact HPr and from tryptic peptides. After cleavage of HPr with thermolysin and separation of the cleavage products by the fingerprint technique, 19 peptides were isolated. For 14 thermolytic peptides, the amino acid sequence has been determined by manual sequencing, whereas for 5 peptides only the N-terminal amino acid and the amino acid composition have been determined. All of them could be attributed to certain regions in the amino acid sequence of HPr. Only one of the five expected cyanogen bromide fragments was isolated by the fingerprint technique. The peptide was sequenced manually and was found to cover residues 75–89. Out of the 15 amino acid residues of this peptide, only 11 have been determined. However, this cyanogen bromide fragment was important for the alignment of the tryptic peptide covering residues 84–89 and the V8 proteolytic peptide covering residues 85–88. Taken together with the sequence information derived from sequence analysis of intact HPr and of the peptides, obtained by different cleavage methods, an unambiguous amino acid sequence of HPr from *S. faecalis* can be presented (Figure 1).

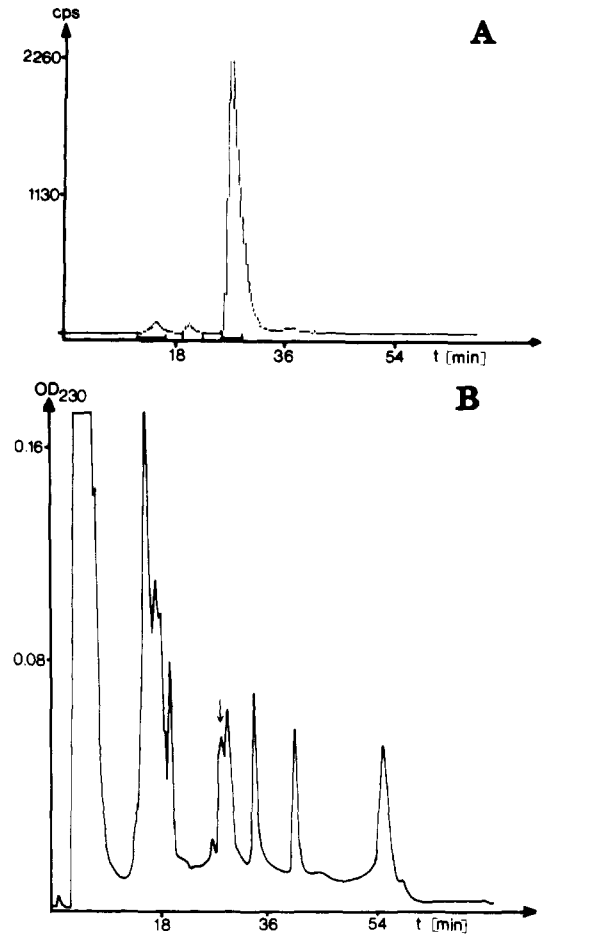


FIGURE 3: Separation of peptides derived from 1 mg of [^{32}P]P-Ser-HPr by cleavage with subtilisin by HPLC on a DEAE column (Si 100 Polyol, Serva). Peptides were separated by applying a linear gradient of 0–0.3 M NaCl in 10 mM Tris-HCl, pH 6.8, for 40 min at 40 °C. (A) Distribution of radioactivity in the effluent. (B) Absorbance at 230 nm of the effluent. The peptide, comigrating with the main radioactive peak, is marked with an arrow.

Isolation of a Labeled Peptide from a Subtilisin Digest. [^{32}P]P-Ser-HPr (1 mg) was digested with subtilisin, and the resulting mixture of peptides was separated on a Si 100 Polyol DEAE column as described under Materials and Methods. The absorbance at 230 nm and the radioactivity of the effluent were measured, and only one major radioactive peptide was detected (Figure 3A). The first minor radioactive peak is most likely due to P_i hydrolyzed from P-Ser-HPr during the proteolytic cleavage reaction. In Figure 3B, the arrow indicates the elution position of the radioactive peptide. According to UV absorbance, the radioactive peptide clearly overlaps with another peptide. The radioactive peptide was, therefore, collected and further purified on a Nucleosil C8 reversed-phase column (10 μm, 4.6 × 250 mm; Bischoff). A linear gradient of 0–25% acetonitrile in 0.1% TFA, pH 2.0, was run for 40 min at 40 °C at a flow rate of 1 mL/min. The elution profiles (Figure 4) show that the overlapping peptides obtained (Figure 3) were separated (Figure 4B) with the radioactive peptide being the first to elute (Figure 4A). This radioactive peptide appeared from the UV absorbance (Figure 4B) to be itself comprised of two very closely eluting peptides. Both parts of the double peak were collected separately and lyophilized. However, amino acid analysis revealed that both parts contained the three amino acids Ser, Met, and Ile in a 1:1:1 ratio (Table I). We assume that methionine was partially oxidized in the tripeptide and that we separated the peptide containing the oxidized from the peptide containing the nonoxidized

Table I: Comparison of Amino Acid Analysis and Sequence Data of the Three Labeled Peptides Obtained after Cleavage with either Subtilisin, Chymotrypsin, or V8 Staphylococcal Protease

	subtilisin peptide		chymotryptic peptide		V8 peptide	
amino acid	amino acid analysis	sequence	amino acid analysis	sequence	amino acid analysis	sequence
Asp			1.09	1	3.71	4
Thr					1.95	2
Ser	0.83	1	1.68	2	3.57	4
Glu			0.15		2.57	3
Gly			1.24	1	6.38	6
Ala					2.00	2
Val			2.00	2	4.90	5
Met	1.01	1	1.80	2	1.76	2
Ile	1.00	1	1.00	1	1.90	2
Leu			1.06	1	2.24	2
Tyr					0.90	1
Lys			1.00	1	3.19	3

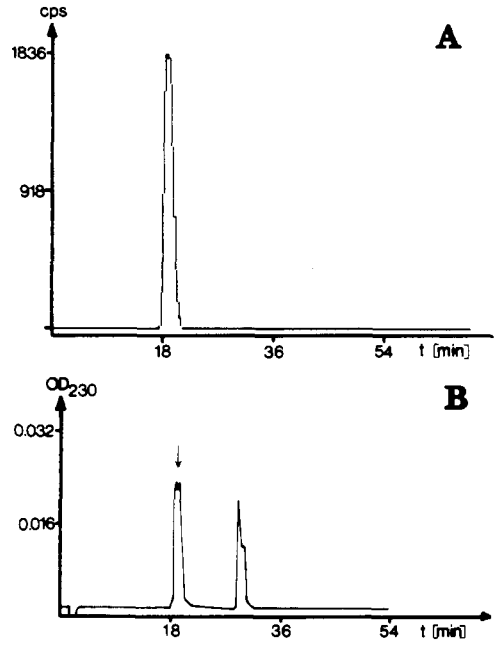


FIGURE 4: Rechromatography of the labeled subtilisin peptide on a reversed-phase column (C8, Bischoff). Solvent A, 0.1% TFA, pH 2; solvent B, 50% acetonitrile. Gradient, 0–50% solvent B in solvent A for 40 min; temperature, 40 °C; flow rate, 1 mL/min. (A) Distribution of radioactivity in the effluent. (B) Absorbance at 230 nm of the effluent.

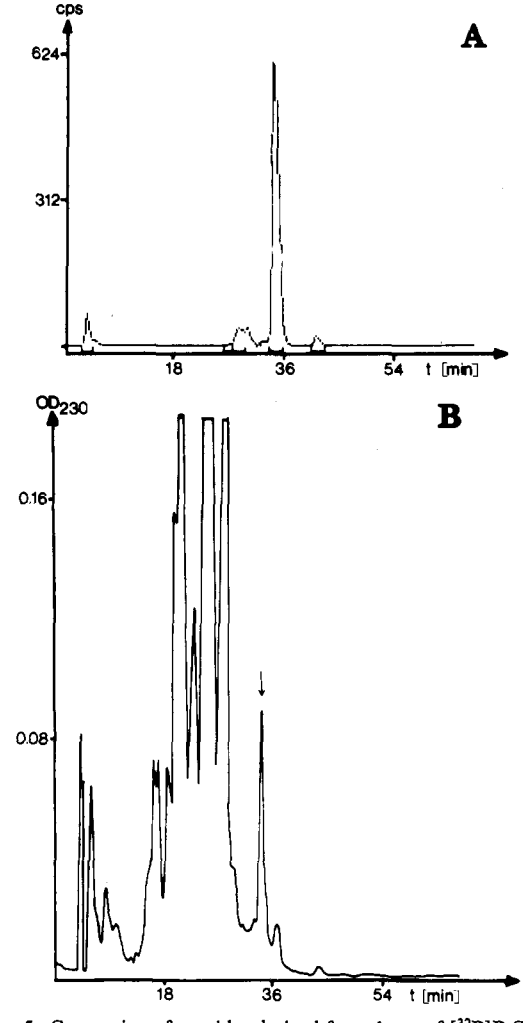


FIGURE 5: Separation of peptides derived from 1 mg of [^{32}P]P-Ser-HPr by cleavage with chymotrypsin on a reversed-phase column (C8, Bischoff). Solvent A, 0.1% TFA, pH 2.0; solvent B, 85% acetonitrile. Gradient, 0–30% solvent B in solvent A for 25 min and 30–70% solvent B in solvent A for 40 min; temperature, 40 °C; flow rate, 1 mL/min. (A) Distribution of radioactivity in the effluent. (B) Absorbance at 230 nm of the effluent. The arrow indicates the labeled peptide.

methionine. Manual sequence analysis revealed the order -Ser-Ile-Met- in this tripeptide (Table I). ATP-dependent phosphorylation of HPr was shown to occur at a seryl residue (Deutscher & Saier, 1983). The seryl residue in position 1 of the above tripeptide must, therefore, carry the phosphoryl group. This was further supported by measuring the radioactivity present in an aliquot of the extraction buffer following each degradation step. The highest amount of radioactivity was found after the first degradation step with 1352 cpm. The amount of radioactivity dropped to 259 cpm after the second and to 107 cpm after the third degradation step. After the first degradation step, Pth-serine could not be detected at 269 nm. Instead, a strong peak was observed at 315 nm. This further supports that the seryl residue in position 1 carries the phosphoryl group, because Pth-phosphoserine breaks down readily to give Pth-dehydroserine. Pth-dehydroserine does not absorb significantly at 269 nm but absorbs at 315 nm (Borthwick et al., 1984).

Isolation of a Labeled Chymotryptic Peptide. The incubation mixture of 1 mg of [^{32}P]P-Ser-HPr and 20 μg of chymotrypsin was loaded on a C8 reversed-phase column run with an acetonitrile gradient. According to Figure 5A, we obtained one major radioactive peak. The radioactive peptide eluted as a single major peak (Figure 5A) and corresponded to the single peptide peak indicated by the arrow in Figure 5B. The radioactive peptide was collected and rechromatographed on the same column by applying a nonlinear gradient of 0–35% solvent B for 10 min and 35–60% solvent B for 50 min. Following the second chromatography, we obtained only one radioactive peak corresponding to the main peak observed at 230 nm. The peptide was collected and lyophilized. Amino

acid analysis revealed that it consisted of 11 amino acids: 1 Asx, 2 Ser, 1 Gly, 2 Val, 2 Met, 1 Ile, 1 Leu, and 1 Lys (Table I).

The amino acid sequence in this peptide was determined to be -Ser-Val-Asn-Leu-Lys-Ser-Ile-Met-Gly-Val-Met-. The chymotryptic peptide contained the subtilisin peptide -Ser-Ile-Met- in positions 6–8. Again, no Pth-amino acid could be detected at 269 nm following the sixth degradation step. Absorption was only observed at 315 nm. On the other hand, the seryl residue in position 1 of the chymotryptic peptide could be detected at both 169 and 315 nm. The highest amount of radioactivity was extracted after the sixth degradation step, indicating that the seryl residue in position 6 of the above peptide carries the phosphoryl group.

An unusual finding was that chymotrypsin cleaves the Lys–Ser bond between positions 40 and 41 of HPr. Lys–Ser is not a substrate site for chymotrypsin. Contamination of chymotrypsin with trypsin cannot be excluded. However, the single radioactive peptide produced by cleavage with chymotrypsin argues against trypsin being responsible for the cleavage of the Lys–Ser bond, as trypsin should also attack the Lys–Ser bond between positions 45 and 46, producing a much shorter labeled peptide.

Isolation of a Labeled Peptide from a V8 Protease Digest. One milligram of [^{32}P]P-Ser-HPr was incubated together with 20 μg of V8 staphylococcal protease. Peptides were loaded on a C8 reversed-phase column and eluted by applying a linear gradient of 0–60% acetonitrile in 0.1% TFA, pH 2.0, over 50 min at 40 °C. Figure 6A shows one major peak of radioactivity in the effluent besides several minor peaks. Figure 6B shows the absorbance at 230 nm. The main peak of radioactivity comigrates with the major peak of Figure 6B, as indicated by the arrow. The peptide was collected and rechromatographed on a C8 reversed-phase column using a gradient of 0–25% solvent B for 5 min and 25–60% solvent B for 50 min. Buffer A was 0.1% TFA, pH 2.0; buffer B was 85% acetonitrile. We again obtained a single radioactive peak, which was collected and lyophilized. According to amino acid analysis, it consisted of 36 amino acid residues (Table I). The sequence of the first 12 amino acid residues was determined to be -Tyr-Lys-Gly-Lys-Ser-Val-Asn-Leu-Lys-Ser-Ile-Met-.

Attempts To Phosphorylate the V8 Peptide. According to amino acid analysis, the labeled V8 peptide consists of 36 amino acid residues and comprises most of the C-terminal half of HPr. ^{1}H NMR measurements revealed that HPr of *S. faecalis* contains a single tyrosyl residue (Kalbitzer et al., 1982), which is located in position 37 according to the sequence data (Figure 1). The V8 peptide, therefore, spans residues 37–72. Because of its large size, we assumed that the peptide might be phosphorylated by the ATP-dependent HPr kinase. Two milligrams of HPr was digested with V8 staphylococcal protease as described for [^{32}P]P-Ser-HPr. The single peptide, absorbing at 280 nm, was purified by using the same procedure as described for the labeled V8 peptide. Amino acid analysis revealed that the peptide is identical with the labeled V8 peptide. After lyophilization, a phosphorylation experiment was carried out by using the same conditions as described for the synthesis of [^{32}P]P-Ser-HPr. After incubation, the reaction mixture was loaded on a C8 reversed-phase column run with a linear gradient of 0–60% acetonitrile in 0.1% TFA. No radioactivity eluted together with the V8 peptide, indicating that the peptide purified according to the above procedure is not a substrate of the ATP-dependent HPr kinase, even though the peptide comprises almost half of the protein including the site of ATP-dependent phosphorylation. The V8 peptide also

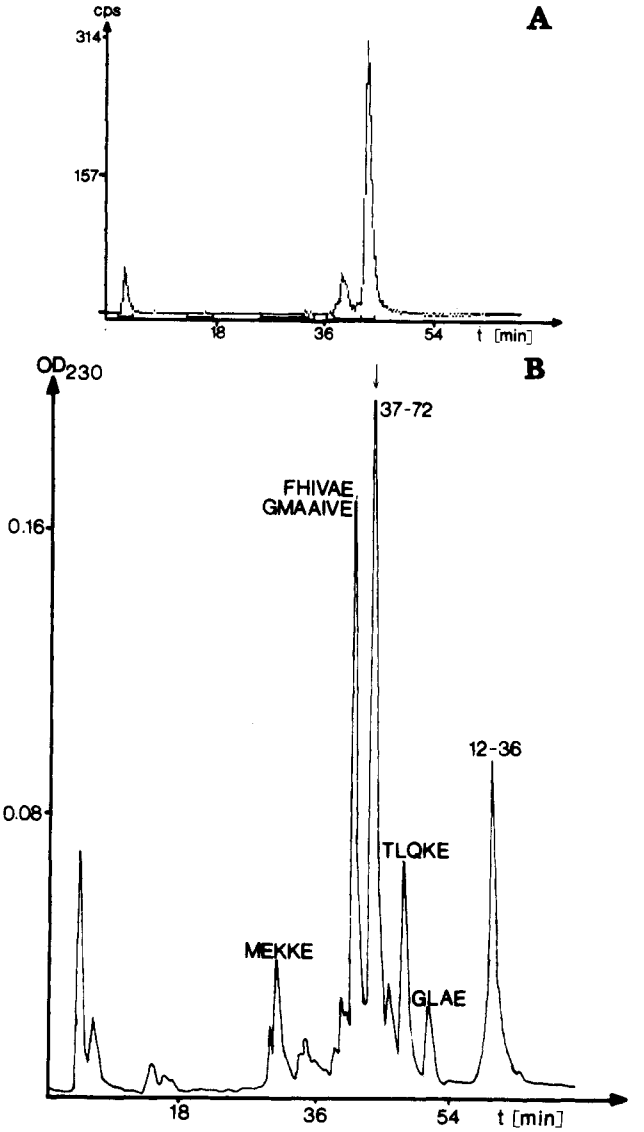


FIGURE 6: Separation of peptides derived from 1 mg of [^{32}P]P-Ser-HPr by cleavage with V8 staphylococcal protease on a reversed-phase column (C8, Bischoff). Solvents, temperature, and flow rate were as described in Figure 5. Gradient, 0–60% solvent B in solvent A for 50 min. (A) Distribution of radioactivity in the effluent. (B) Absorbance at 230 nm of the effluent. The arrow indicates the labeled peptide.

did not inhibit ATP-dependent phosphorylation of HPr. When 20 μg of HPr was phosphorylated by the ATP-dependent HPr kinase in the absence or presence of 40 μg of the V8 peptide, phosphorylation of HPr was not affected. As expected, the V8 peptide was also not phosphorylated by PEP and enzyme I.

Discussion

Multiple phosphorylation of proteins is not a rare event (Rubin & Rosen, 1975; Krebs & Beavo, 1979). Protein kinases sometimes phosphorylate proteins at several, in most cases identical, amino acid residues. On the other hand, rat liver ATP–citrate lyase was found to be phosphorylated in an ATP-dependent protein kinase catalyzed reaction at a seryl residue (regulatory site) and to form an intermediary 3-phosphohistidine during catalysis (active site) (Williams et al., 1985). Similarly, HPr of Gram-positive bacteria can be phosphorylated at a seryl residue (regulatory site) and at a histidyl residue (active site). However, the two different phosphorylation reactions in HPr are catalyzed by two dif-

Table II: Comparison of the ATP-Dependent Site of Phosphorylation in HPr of *Streptococcus faecalis* with a Homologous Region in HPr of *Staphylococcus aureus* and *Escherichia coli* and the Site around the Phosphorylatable Ser-19 of Troponin I from Rabbit Skeletal Muscle

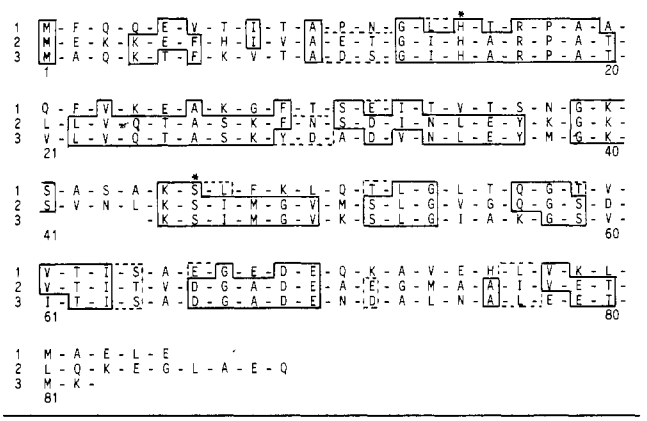
S. faecalis	-Lys[40]-Ser-Val-Asn-Leu-Lys-Ser-Ile-Met-Gly-Val[50]-
S. aureus	-Asp[38]-Ser-Met-Gln-Leu-Lys-Ser-Leu-Xxx-Gly-Val[47]-
E. coli	-Lys[40]-Ser-Ala-Ser-Ala-Lys-Ser-Leu-Phe-Lys-Leu[50]-
troponin I	-Arg[13]-Arg-Gln-His-Leu-Lys-Ser-Val-Met-Leu-Gln[23]-

ferent protein kinases, each using its own phosphoryl donor. PEP-dependent phosphorylation of HPr, catalyzed by enzyme I, is part of the PTS-mediated sugar uptake reaction. P-enzyme I phosphorylates the N-1 position of histidyl residue 15 in HPr (Beyreuther et al., 1977; Gassner et al., 1977; Weigel et al., 1982b). The possible involvement of protein phosphorylation in a regulatory phenomenon, termed inducer expulsion (Reizer & Panos, 1980), and an earlier observation that, after growth of streptococcal cells on glucose-containing medium, two different HPr species with a different migration behavior on native polyacrylamide gels could be isolated (H. P. Muss, W. Hengstenberg, and J. Deutscher, unpublished results) led to the discovery of ATP-dependent phosphorylation of HPr in streptococcal cells (Deutscher & Saier, 1983). ATP-dependent phosphorylation of HPr requires a protein kinase and occurs at a seryl residue (Deutscher & Saier, 1983). Activity of the protein kinase was stimulated by fructose 1,6-diphosphate and inhibited by P_i (Deutscher & Engelmann, 1984; Reizer et al., 1984).

In the last few years, ATP-dependent protein phosphorylation has been established in bacterial cells (Cozzone, 1984). The first well-characterized example was ATP-dependent phosphorylation of isocitrate dehydrogenase from *Escherichia coli* (Garnak & Reeves, 1979; Wang & Koshland, 1981). Recently, the site of ATP-dependent phosphorylation in isocitrate dehydrogenase has been reported (Borthwick et al., 1984; Malloy et al., 1984). The sequence around the phosphorylatable seryl residue was found to be -Ile-Arg-Ser(P)-Leu-Asn-. This sequence shows some similarity to the site of ATP-dependent phosphorylation in HPr of *S. faecalis* with the sequence -Leu-Lys-Ser(P)-Ile-Met-.

The amino acid sequences of three labeled peptides isolated from [^{32}P]P-Ser-HPr using three different proteases fit into the sequence of HPr from *Streptococcus faecalis* (Figure 1). It is the seryl residue in position 46 of HPr which carries the phosphoryl group following ATP-dependent phosphorylation. However, the sequences of the labeled peptides did not fit into the primary structure of HPr from *S. faecalis* previously published by Stüber (1982). Around Ser-46, the sequence was not correct, and reinvestigation revealed that a piece of 19 amino acid residues was missing in the former sequence. This area is now covered by the V8 peptide spanning residues 37–72 and the tryptic peptide spanning residues 46–83. The ATP-dependent HPr kinase isolated from *S. faecalis* was found to phosphorylate HPr's isolated from other Gram-positive bacteria like *Streptococcus lactis*, *S. pyogenes*, *Staphylococcus aureus*, *Staphylococcus carnosus*, *Lactobacillus casei*, and *Bacillus subtilis* (Deutscher & Engelmann, 1984; J. Deutscher and W. Hengstenberg, unpublished results). ATP-dependent HPr phosphorylation was, therefore, assumed to occur at a preserved region in the amino acid sequence of HPr. Among the Gram-positive bacteria, only the sequence of HPr from *S. aureus* has so far been reported (Beyreuther et al., 1977). There is indeed a homologous region in the amino acid sequence of HPr from *S. aureus* including seryl residue 44 as a possible target of the ATP-dependent HPr kinase (Table II). However, HPr isolated from *Escherichia coli* was not found

Table III: Comparison of Amino Acid Sequences of HPr from (1) *S. typhimurium* (Powers & Roseman, 1984) and (2) *S. faecalis* and (3) the First 40 Amino Acid Residues and 3 Tryptic Fragments of HPr from *B. subtilis* (Stüber, 1982)[a]



[a] The tryptic peptides were arranged according to their homology with HPr from *S. faecalis*. They thus cover positions 46–82 in HPr of *B. subtilis*.

to be a substrate of the ATP-dependent HPr kinase isolated from either *S. faecalis* (Deutscher & Engelmann, 1984) or *S. pyogenes* (Reizer et al., 1984). Moreover, ATP-dependent phosphorylation of HPr in *E. coli* or other Gram-negative bacteria has so far not been clearly demonstrated. The amino acid sequence of HPr from *Salmonella typhimurium* and *E. coli* (based on the nucleotide sequence of the corresponding ptsH gene for the latter) has been determined and was found to be identical for the two proteins (T. Doering and D. Saffen, unpublished results; Powers & Roseman, 1984; De Reuse et al., 1985). Thus, both proteins have the sequence -Lys-Ser-Leu- spanning residues 45–47. This is similar to -Lys-Ser-Ile- in HPr from *S. faecalis* and identical with -Lys-Ser-Leu- in HPr from *S. aureus* (Table II). However, homology is restricted to these three amino acid residues which might explain that HPr from *E. coli* is not a substrate of the ATP-dependent HPr kinase isolated from either *S. faecalis* or *S. pyogenes*. On the other hand, the presence of Ser-46 suggests that ATP-dependent HPr phosphorylation may also occur in Gram-negative bacteria, although this has so far not been demonstrated.

The site of ATP-dependent phosphorylation of HPr also resembles the reactive center of lima bean trypsin inhibitor (Deutscher, 1985) and one of the phosphorylatable sites of troponin I of rabbit skeletal muscle (Huang et al., 1974) (Table II). A cAMP-dependent protein kinase isolated from rabbit cardiac muscle phosphorylates two seryl residues in troponin I, one of which is located in position 19 (Wilkinson & Grand, 1975). The sequence Leu-Lys-Ser-Val-Met around seryl residue 19 of troponin I is almost identical with the site of ATP-dependent phosphorylation in HPr (Table II). Two arginyl residues are located in positions 13 and 14 of troponin I. Similarly, a lysyl residue is present in position 40 of HPr which according to preliminary X-ray analysis of HPr from *E. coli* is located next to seryl residue 46 (O. El-Kabbani and L. Delbaere, unpublished results) and which may be important for the ATP-dependent phosphorylation.

So far, the amino acid sequence of HPr from *S. aureus* (Beyreuther et al., 1977) and from *S. typhimurium* (Powers & Roseman, 1984) and the DNA sequence of the ptsH gene of *E. coli* (De Reuse et al., 1985; T. Doering and D. Saffen, unpublished results) as well as part of the amino acid sequence of HPr from *B. subtilis* (Stüber, 1982) have been determined. Table III compares the sequence of HPr from *S. faecalis* with

the sequence of HPr from *S. typhimurium* and the sequence of the first 40 amino acid residues of HPr from *B. subtilis*. HPr from *S. faecalis* and HPr from *B. subtilis* show strong homology, whereas homology between HPr from *S. faecalis* and *S. typhimurium* is restricted mainly to the regions around the phosphorylatable amino acid residues His-15 and Ser-46. Homology can also be found in the C-terminal half of these two proteins. From preliminary X-ray analysis of *E. coli* HPr, which is identical with HPr from *S. typhimurium* (Powers & Roseman, 1984), it was predicted that Glu-66 forms an ion pair with His-15 (Waygood et al., 1985). In *S. faecalis* HPr, the glutamyl residue is exchanged for an aspartyl residue. Around position 66, there are several clusters of homologous amino acids, comparing HPr from *S. faecalis* with HPr from *S. typhimurium* (Table III). Moreover, most of the differences between the two proteins are due to conservative changes. The hydropathy profiles of the two proteins [according to Kyte & Doolittle (1982)] are very similar.

Besides the tryptic peptides covering the first 40 amino acid residues in HPr from *B. subtilis*, 3 additional tryptic peptides were isolated and sequenced (Stüber, 1982). Each of these peptides shows strong homology to a certain region of the C-terminal half of HPr from *S. faecalis*. They were, therefore, arranged according to their homology to *S. faecalis* HPr as shown in Table III. The three tryptic peptides cover amino acid residues 46–82 in the sequence of HPr from *B. subtilis*. The sequence of HPr from *S. aureus* was not included in Table III. A comparison with the sequence of HPr from *S. faecalis* revealed that the two proteins are very similar up to residue 37. Preliminary results suggest that the previously presented sequence of HPr from *S. aureus* (Beyreuther et al., 1977) contains several errors beyond residue 37. Mainly, the tryptic peptide containing Tyr-56 in the former sequence was found to be composed of more than 11 amino acids. Careful amino acid analysis of the intact protein suggests that HPr from *S. aureus* is composed of 85 instead of 70 amino acid residues. A correction of the sequence will be presented.

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Erythrocruorin from the Aquatic Snail *Helisoma trivolvis*. Quaternary Structure and Arrangement of Subunits

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ABSTRACT: The subunit structure of erythrocruorin from the planorbid snail *Helisoma trivolvis* was studied. The native protein was found to have a sedimentation coefficient of 34.7S and a molecular weight, as determined by sedimentation equilibrium, of 2.25×10^6. Iron and heme determinations gave 0.270 and 3.21%, corresponding to minimal molecular weights of 20 700 and 19 200, respectively. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence and absence of 2-mercaptoethanol gave single bands with mobilities corresponding to molecular weights of 1.9×10^5 and $\sim 4 \times 10^5$, respectively. Sedimentation equilibrium in 6 M guanidine hydrochloride in the presence and absence of 2-mercaptoethanol gave molecular weights of 1.87×10^5 and 3.82×10^5, respectively. We conclude that a *Helisoma* erythrocruorin molecule is composed of 12 single polypeptide chain subunits, each carrying 10 hemes. In the molecule, the subunits are grouped in pairs, the members of each pair being held together by disulfide bonds. We propose a model for the molecule composed of 12 spherical subunits arranged in a shell structure with tetrahedral symmetry. Projections of the model are consistent with 10-membered ring and rhombic profiles observed in the electron microscopy of negatively stained *H. trivolvis* erythrocruorin [Terwilliger, N. B., Terwilliger, R. C., & Schabtach, E. (1976) *Biochim. Biophys. Acta 453*, 101–110] and with hexagonal ring structures seen in the electron micrographs of *Planorbis corneus* erythrocruorin [Wood, E. J., & Mosby, L. J. (1975) *Biochem. J. 149*, 437–445].

Erythrocruorin is the name given to extracellular hemoglobins that occur mainly among species from the phyla Annelida, Arthropoda, and Mollusca (Chung & Ellerton, 1979; Wood, 1980; Terwilliger, 1980; Vinogradov, 1985). In the molluscs, erythrocruorin is found in some marine bivalves and in a family of pulmonate snails, the Planorbidae (Read, 1966; Bonaventura & Bonaventura, 1983; Terwilliger & Terwilliger, 1985). The first structural study of a planorbid erythrocruorin was carried out by Svedberg and Eriksson-Quensel (1934). Recent studies include those on erythrocruorin from *Biomphalaria glabrata* (Figueiredo et al., 1973; Almeida & Neves, 1974), *Planorbis* sp. (Waxman, 1975), *Planorbis corneus* (Wood & Mosby, 1975), and *Helisoma trivolvis* (Terwilliger et al., 1976). The results show similarity in the sedimentation coefficient (33.5–35.2 S) and the molecular weight of the native molecule [$(1.65–1.75) \times 10^6$], the molecular weight of the polypeptide chain [$(1.75–2.20) \times 10^5$], and the minimal weight per heme (18 100–22 300) [see the review by Chung and Ellerton (1979)].

Planorbid erythrocruorin has also been studied by electron microscopy. Electron micrographs of *P. corneus* erythrocruorin reveal hexagonal ring structures (Wood & Mosby, 1975). Projections of negatively stained *H. trivolvis* erythrocruorin show 10-membered rings with a central structure (Terwilliger et al., 1976). This apparent difference in the electron micrographs may suggest, as pointed out by Chung and Ellerton (1979), differences in the quaternary structures of *Planorbis* and *Helisoma* erythrocruorins. In view of the taxonomical closeness of *Planorbis* and *Helisoma*, and the similarities in their erythrocruorins mentioned above, this idea seems rather improbable.

The present paper is concerned with a structural study of erythrocruorin from *H. trivolvis*. Our results lead us to propose a model for the quaternary structure of planorbid erythrocruorin. This model provides an explanation for the differences in the electron micrographs of erythrocruorins from *Planorbis* and *Helisoma*.

Materials and Methods

Preparation of Erythrocruorin. Snails identified as *H. trivolvis* were collected from a freshwater pond, located at the botanical gardens of Tel-Aviv University. Erythrocruorin was extracted in the following manner. About 50 animals were washed and dried and then placed in 5-mL syringes. They were then crushed and the resulting hemolymph collected in a small quantity of 0.1 M phosphate buffer, pH 6.8. The hemolymph was twice centrifuged at 15000*g* for 20 min in order to remove particulate matter. The resulting supernatant

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