Staphylococcal Phosphoenolpyruvate-Dependent Phosphotransferase System: Purification and Characterization of the Mannitol-Specific Enzyme III^{mtl} of *Staphylococcus aureus* and *Staphylococcus carnosus* and Homology with the Enzyme II^{mtl} of *Escherichia coli*†

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ABSTRACT: Enzyme III^{mtl} is part of the mannitol phosphotransferase system of *Staphylococcus aureus* and *Staphylococcus carnosus* and is phosphorylated by phosphoenolpyruvate in a reaction sequence requiring enzyme I (phosphoenolpyruvate–protein phosphotransferase) and the histidine-containing protein HPr. In this paper, we report the isolation of III^{mtl} from both *S. aureus* and *S. carnosus* and the characterization of the active center. After phosphorylation of III^{mtl} with [^{32}P]PEP, enzyme I, and HPr, the phosphorylated protein was cleaved with endoproteinase Glu(C). The amino acid sequence of the *S. aureus* peptide carrying the phosphoryl group was found to be Gln-Val-Val-Ser-Thr-Phe-Met-Gly-Asn-Gly-Leu-Ala-Ile-Pro-His-Gly-Thr-Asp-Asp. The corresponding peptide from *S. carnosus* shows an equal sequence except that the first residue is Ala instead of Gln. These peptides both contain a single histidyl residue which we assume to carry the phosphoryl group. All proteins of the PTS so far investigated indeed carry the phosphoryl group attached to a histidyl residue. According to sodium dodecyl sulfate gels, the molecular weight of the III^{mtl} proteins was found to be 15 000. We have also determined the N-terminal sequence of both proteins. Comparison of the III^{mtl} peptide sequences and the C-terminal part of the enzyme II^{mtl} of *Escherichia coli* reveals considerable sequence homology, which supports the suggestion that II^{mtl} of *E. coli* is a fusion protein of a soluble III protein with a membrane-bound enzyme II. In particular, the homology of the active-center peptide of III^{mtl} of *S. aureus* and *S. carnosus* with the enzyme II^{mtl} of *E. coli* allows one to predict the N-3 histidine phosphorylation site within the *E. coli* enzyme.

The uptake of mannitol in staphylococcal cells is achieved by the phosphoenolpyruvate (PEP)[1]-dependent phosphotransferase system (Simoni et al., 1968; Friedman & Hays, 1977). This system is described by the following reaction scheme:

$$\text{PEP} + \text{enzyme I} \overset{Mg^{2+}}{\rightleftharpoons} \text{P-enzyme I} + \text{pyruvate} \quad (1)$$

$$\text{P-enzyme I} + \text{HPr} \rightleftharpoons \text{P-HPr} + \text{enzyme I} \quad (2)$$

$$\text{P-HPr} + \text{enzyme III}^{mtl} \rightleftharpoons \text{P-enzyme III}^{mtl} + \text{HPr} \quad (3)$$

$$\text{P-enzyme III}^{mtl} + \text{mannitol} \xrightarrow[\text{enzyme II}^{mtl}]{Mg^{2+}} \text{mannitol-P} + \text{enzyme III}^{mtl} \quad (4)$$

Enzyme I and HPr are the two common nonspecific and constitutively expressed proteins of the system. Enzyme III and the membrane-bound enzyme II will be expressed only in the presence of the substrate. Each of the proteins carries the phosphoryl group attached to a histidyl residue, either at the N-3 (EI and EIII) or at the N-1 position (HPr and EII) (Saier et al., 1985). The above reaction scheme differs from the phosphorylation sequence in *Escherichia coli*, which lacks an enzyme III^{mtl}. In this microorganism, the phosphoryl group is transferred directly from phospho-HPr to mannitol by the enzyme II^{mtl}. In 1983, Lee and Saier published the nucleotide sequence of the mtlA gene of *E. coli*, which codes for the enzyme II^{mtl}. A hydropathy plot of the predicted amino acid sequence shows a hydrophobic N- and a hydrophilic C-terminus. It was speculated that this hydrophilic region of enzyme II^{mtl} serves as the III component.

Materials and Methods

Enzyme I. Enzyme I was purified according to Alpert et al. (1985).

HPr Protein. HPr was purified according to Beyreuther et al. (1977).

Purification of Enzyme III^{mtl}. Enzyme III^{mtl} was isolated from *Staphylococcus aureus* strain 5601 (Simoni & Roseman, 1973) and from *Staphylococcus carnosus* strain TM300 (Keller et al., 1983). Cells were grown in a 100-L Chemap fermenter to late log phase. Cells (300 g) were disrupted in a Dynomill (Fa. Bachofen). The crude extract was centrifuged for 1 h at 22000*g*. The supernatant was applied to a DEAE-cellulose column (DE-23, 12 × 30 cm, Whatman). The column was first washed with standard buffer (0.05 M Tris-HCl, 10^{-4} M DTT, 10^{-4} M PMSF, and 10^{-4} M EDTA,

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[1] Abbreviations: PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate-dependent phosphotransferase system; HPr, histidine-containing phosphocarrier protein; P-HPr, phosphorylated HPr; E III^{mtl}, enzyme III specific for mannitol; P-E III^{mtl}, phosphorylated E III^{mtl}; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; NaDodSO$_4$ or SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; BSA, bovine serum albumin; OVA, ovalbumin; HPLC, high-performance/pressure liquid chromatography; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene.

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pH 7.5) to collect membranes. Soluble proteins were then eluted with a two-step gradient of (1) 0–0.4 M NaCl (6 L) and (2) 0.32–0.9 M NaCl (6 L) in standard buffer. III^{mtl}-containing fractions were pooled and adjusted to 60% saturation with ammonium sulfate. After centrifugation, III^{mtl} remained in the supernatant. The supernatant was applied to a Butyl-TSK column (2.5 × 15 cm; Merck, Darmstadt, West Germany) equilibrated with 55% ammonium sulfate in standard buffer and eluted with a gradient of 55–0% ammonium sulfate (2.4 L) in standard buffer. III^{mtl} fractions were pooled and concentrated by pressure dialysis (Amicon, UM-2 membrane, 76 mm) and then applied to a Sephadex G-75 column (5 × 90 cm). The column was eluted with standard buffer, and III^{mtl}-containing fractions were pooled, applied to a Q-Sepharose column (2.5 × 10 cm; Pharmacia, Upsala), and eluted with a gradient of 0–0.5 M NaCl (0.8 L) in standard buffer. The resulting III^{mtl} pool was desalted on a Sephadex G-25 column (4 × 25 cm) and lyophilized. We obtained 10–15 mg of electrophoretically pure protein.

Enzyme II^{mtl}. The membrane fractions from the DE23 wash step (see above) were sedimented for 3 h at 50000*g*. One gram of the pellet was resuspended in 15 mL of standard buffer. This suspension was used in the assay for III^{mtl} as the II^{mtl} component.

Assay for III^{mtl}. The assay mixture contained 10 μg of enzyme I, 10 μg of HPr, 5 μL of enzyme II suspension (see above), 20–50 μL of sample, and 100 μL of "assay mix". The "assay mix" contained the following components (concentrations in parentheses were the final concentrations in the complete test mixture): NaF (25 mM), $MgCl_2$ (12.5 mM), DTT (2.5 mM), PEP (5 mM), mannitol (10 mM), [^{14}C]mannitol (40000 cpm), phosphate buffer pH 7.7 (50 mM). The total volume of the reaction mixture was 145–175 μL depending on the volume of sample. Incubation time was 30 min at 37 °C. Reaction was stopped with 1 mL of water. The mixture was then applied to a Dowex 1-X2 column (1 × 2.5 cm). After the column was washed with water to remove unphosphorylated mannitol, mannitol-1-P was eluted with 4 mL of LiCl (1 M). The eluate was mixed with 10 mL of scintillation cocktail, and cpm were messured in a Packard Tri-Carb scintillation counter.

Scintillation Cocktail. The scintillation cocktail contained the following components: 1250 mL of toluene, 700 mL of Triton X-100, 6.25 g of PPO, and 0.125 g of POPOP.

High-Performance Liquid Chromatography. For chromatography on the reversed-phase columns, we used the following equipment: low-pressure gradient former M 250 B (Gynkothek, Munich), Jasco HPLC pump (Biotronik), Rheodyne 1725 syringe sample injector, and Jasco Uvidec 100 III UV monitor with variable wavelength.

Size-Exclusion Chromatography. Enzyme III^{mtl} was phosphorylated with [^{32}P]PEP. The reaction mixture contained 50 μg of enzyme III^{mtl}, 5 μg of HPr, 5 μg of enzyme I, 5 μL of 50 mM $MgCl_2$, and 0.5 μCi of [^{32}P]PEP (carrier free). The final volume was 65 μL (in 50 mM NH_4HCO_3), and the incubation time was 10 min at 37 °C. The mixture was loaded onto a BioSil TSK 125 column (300 × 7.5 mm Bio-Rad) and eluted with buffer (20 mM Na_2HPO_4, pH 6.8, and 100 mM Na_2SO_4). The flow rate was 0.5 mL/min. Cerenkov radiation was measured by using a Berthold LB 504 HPLC radioactivity monitor connected to an Apple IIe computer.

Isolation of the Active-Center Peptides. To isolate the active center of E III^{mtl}, the protein was phosphorylated with [^{32}P]PEP. The reaction mixture contained 250 μg of III^{mtl} (*S. aureus* or *S. carnosus*), dissolved in 0.1 mL of 50 mM NH_4HCO_3, 10 μg of HPr, 10 μg of enzyme I, 5 μL of 50 mM $MgCl_2$, and 0.5 μCi of [^{32}P]PEP carrier free). The incubation time was 15 min at 37 °C. Subsequently, 250 nmol of "cold" PEP was added, and the mixture was incubated for another 15 min at 37 °C to achieve complete phosphorylation of enzyme III. After this period, *S. aureus* protease V8 (Miles) was added to the reaction mixture [ratio of protease to protein 1:25 (w/w)] and incubated for 4 h at 37 °C. Peptides were separated on an RP 8 10-μm column (Serva) or on a RP 7 300-μm column (Gynkothek, Munich) using an acetonitrile gradient: solvent A, 0.1% TFA; solvent B, 100% acetonitrile, HPLC grade E (Rathburn). The column was eluted with a linear gradient from 0% to 60% solvent B in 60 min at a flow rate of 1 mL/min. Radioactivity and ultraviolet absorbance (230 nm) of the effluent were monitored by a radioactivity detector (Berthold LB 504) and by an ultraviolet monitor (Jasco Uvidec 100 III). The peaks containing radioactivity were collected manually and evaporated in a vacuum centrifuge.

Synthesis of [^{32}P]PEP. [^{32}P]PEP was synthesized according to Parra (1982).

Amino Acid Analysis. Salt-free peptides were hydrolyzed in vacuo for 24 h in 6 N HCL at 110 °C and analyzed on a BC 200 amino acid analyzer (Biocal) using DC 6A resin (Durrum).

Sequence Determination. The peptides were sequenzed on a gas-phase sequencer according to Hewick et al. (1981).

$NaDodSO_4$–Polyacrylamide Gels. $NaDodSO_4$ gels were prepared according to Schägger and von Jagow (1987) and contained 10% acrylamide.

Tris–Glycine–Polyacrylamide Gels. Tris–glycine tube gels contained 7.5% acrylamide and 0.4 M Tris–glycine, pH 9.3. The gels were stained with Coomassie Brilliant Blue. The destaining solution contained 7.5% acetic acid and 5% methanol (v/v) in water (Hjerten et al., 1965).

Production of Anti-$EIII^{mtl}$ Antibodies. Five hundred micrograms of III^{mtl} from *S. aureus* dissolved in 0.5 mL of 50 mM potassium phosphate buffer, pH 7.5, was homogenized with 0.5 mL of complete Freund's adjuvant and injected intramuscularly into a rabbit. Thirty days later, an intramuscular booster injection with the same amount of protein in Freund's adjuvant was made. The rabbit was drugged with ether at day 42 and exsanguinated, which yielded 250 mL of blood.

Dot Blotting. The dot-blotting procedure was performed according to the Bio-Rad instructions: The protein sample (1 μL, containing 1 μg or less of protein) was applied to a nitrocellulose membrane and allowed to dry completely. The membrane was blocked in gelatin–TBS for 30 min, transferred to the antibody solution, and incubated for 2 h. To remove free and nonspecific bound antibody, the membrane was washed with TBS and subsequently transferred to the protein A–HRP solution (1 h). Again, the membrane was washed with TBS and immersed into the development solution. A purple dot indicated positive reaction. The following buffers were used: TBS, 50 mM Tris and 500 mM NaCl, pH 7.5; gelatin–TBS, 1% gelatin dissolved in TBS; antibody solution, 1:100 dilution of antibody in gelatin–TBS; protein A–HRP solution, 1:2000 dilution of protein A–horseradish peroxidase conjugate in gelatin–TBS; development solution, 60 mg of 4-chloro-1-naphthol dissolved in 20 mL of methanol and mixed with 100 mL of TBS and 60 μL of 30% hydrogen peroxide.

RESULTS

Enzyme III^{mtl} was isolated from *S. aureus* and *S. carnosus*

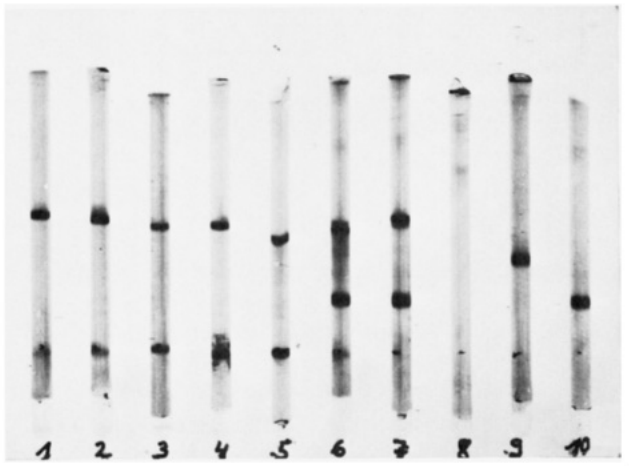

FIGURE 1: Native polyacrylamide gel electrophoresis of purified III^{mtl} of *S. carnosus*. Salt effect and phosphorylated III^{mtl}. The protein was dissolved in 50 mM NH_4HCO_3 in the presence of different amounts of NaCl. The phosphorylation mixtures contained 5 μg of III^{mtl}, 5 μg of HPr, 2 μg of E I, 5 μL of 50 mM $MgCl_2$, and 20 μL of 50 mM PEP. Incubation time was 10 min at 37 °C. Samples containing 5 μg of protein were applied on the gels and electrophoresed. Lanes 1–5, III^{mtl} + 0, 0.3, 0.6, 0.9, and 1 M NaCl, respectively. Lane 6, P-III^{mtl} + 0.9 M NaCl (upper band) and P-HPr (lower band). Lane 7, P-III^{mtl} without salt (upper band) and P-HPr (lower band). Lane 8, 2 μg of enzyme I only. Lane 9, 5 μg of HPr (*Streptococcus faecalis*) only. Lane 10, P-HPr.

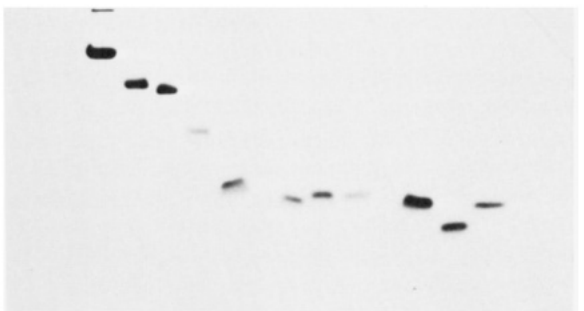

FIGURE 2: $NaDodSO_4$–polyacrylamide gel electrophoresis of purified enzyme III^{mtl} of *S. aureus* and *S. carnosus* and marker proteins (5–10 μg). From left to right: BSA (68 000 daltons), OVA (45 000 daltons), aldolase subunit (40 000 daltons), chymotrypsinogen (24 500 daltons), myoglobin (17 000 daltons), 5 μg of III^{mtl} *S. aureus*, 5 μg of III^{mtl} *S. carnosus*, 2 μg of III^{mtl} *S. carnosus*, cytochrome *c* (13 000 daltons), III^{lac} *S. aureus*, and HPr (*S. faecalis*).

by using a large-scale preparation described under Materials and Methods. The proteins were pure according to native polyacrylamide gel electrophoresis and SDS–polyacrylamide gels. Subjecting a sample of pure desalted protein dissolved in 50 mM NH_4HCO_3 to native polyacrylamide electrophoresis, we were first surprised that the protein banded at a position distant from that we had observed with samples from column fractions containing III^{mtl}. Protein from salt-containing fractions migrated with the bromophenol blue marker whereas the desalted protein was shifted toward the cathode. Figure 1 shows the effect of salt on the migration behavior of the *S. carnosus* protein. The *S. aureus* protein behaved similarly (not shown). NaCl was added to the protein solution (1 mg in 1 mL of 50 mM NH_4HCO_3) to achieve the desired salt concentration. Then samples were applied to the gels. As shown in Figure 1 (lanes 1–5), the amount of fast-moving protein increases when the salt concentration is increased. Migration of the phosphorylated protein is not affected by addition of salt (Figure 1, lanes 6 and 7). The molecular weight of III^{mtl} was estimated to be 15 000. On polyacrylamide–SDS gels, the protein banded between the cytochrome *c* (13 000 daltons) and the myoglobin (17 000 daltons) markers (Figure 2). Data derived from gel filtration experiments with the phosphorylated *S. aureus* protein indicated a molecular weight of approximately 35 000 in the native state (not shown). Antibodies raised against enzyme III^{mtl} of *S. aureus* did not precipitate the two III^{mtl} proteins in the Ouchterlony double-diffusion test, whereas the dot-blotting procedure with protein A–horseradish peroxidase conjugate as the indicator for antibody–protein complexes was positive. The reaction was more sensitive with the *S. aureus* protein, indicating some structural differences between the III^{mtl} proteins of the two species. The high purity of the enzyme preparations allowed us to determine the amino acid compositions (Table I) and the N-terminal sequences of the proteins. In Figure 3, both sequences are compared with the matches marked with an asterisk. The isolation of the active-center peptides was performed as described under Materials and Methods. Figure 4 shows the peptide map of ^{32}P-labeled enzyme III^{mtl} of *S. aureus* after digestion with endoproteinase Glu(C) (also named *S. aureus* protease V8). The only major labeled peptide is designated as V8-A. The peptide map of the *S. carnosus* protein is shown in Figure 5. Again, V8-A corresponds to the labeled peptide. From this chromatography run, we have picked two other peaks (V8-1 and V8-2) and subjected them also to amino acid analysis and gas-phase sequencing. The results from analytical work are presented in Table II. The differences between analysis and sequencing data are probably due to incomplete sequencing by the loss of short peptides. Figure 6 compares the sequences of the active-center peptides (V8-A). Both are nearly identical and contain only a single histidyl residue which could serve as the phosphorylation site. From peptides V8-1 and V8-2, we obtained the following amino acid sequences: KAGQALVD and DYIQAMKDR, respectively. For reasons outlined under Discussion, we have compared all of the sequence pieces so far derived from the

Table I: Amino Acid Compositions of Enzymes III^{mtl} from *S. aureus* and *S. carnosus*[a]

amino acid	*S. aureus* quant	*S. aureus* rnd[b]	*S. carnosus* quant	*S. carnosus* rnd[b]
Asx	20.2	20	18.4	18
Thr	6.1	6	6.9	7
Ser	8.0	8	7.5	8
Glx	21.9	22	24.2	24
Pro	2.0	2	2.0	2
Gly	11.5	12	10.8	11
Ala	13.8	14	14.6	15
Val	13.4	13	13.6	14
Cys	nd[c]		nd[c]	
Met	2.1	2	1.8	2
Ile	10.1	10	10.0	10
Leu	10.1	10	10.8	11
Tyr	1.2	1	1.0	1
Phe	4.9	5	5.9	6
Trp	nd[c]		nd[c]	
Lys	9.1	9	8.1	8
His	2.8	3	1.9	2
Arg	1.9	2	2.0	2
no. of residues		139		141

[a] Data were calculated from 24- and 96-h hydrolysis (*S. carnosus*); only 24-h hydrolysis of the *S. aureus* protein. [b] Rounded values. [c] nd means not determined.

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H2N-SELFSNDNIFLNVNVNSQNEAIEKAGKALVDSGAVTDAYI   EIII^mtl S.aureus
     ***** *****
H2N-TELFSNENIFLNQSFED                          EIII^mtl S.carnosus
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FIGURE 3: Comparison of the N-terminal amino acid sequences of the III^{mtl} proteins.

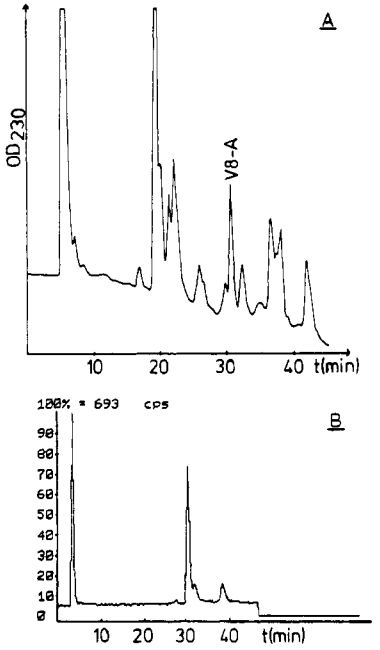

FIGURE 4: (A) Peptide map of ^{32}P-III^{mtl} (*S. aureus*) on a reversed-phase column (RP 8, Serva) after cleavage with protease V8. Solvent A, 0.1% TFA; solvent B, 100% CH_3CN. Gradient, 0–60% solvent B in solvent A over 60 min. Absorbance at 230 nm of the effluent. The labeled peptide, carrying the phosphoryl group, is designated V8-A. (B) Distribution of radioactivity.

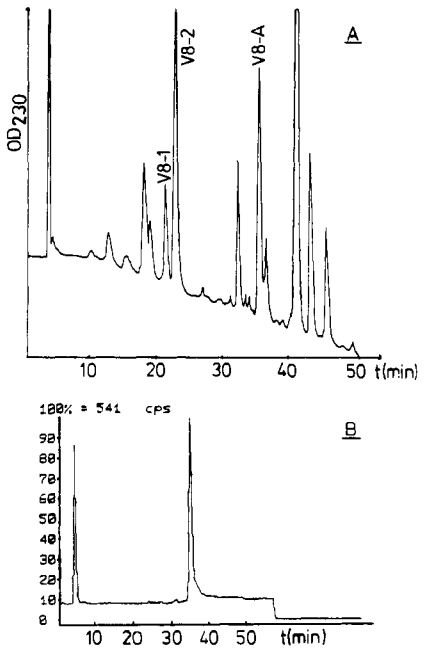

FIGURE 5: (A) Peptide map of ^{32}P-III^{mtl} (*S. carnosus*) on a reversed phase column (RP 7, Gynkothek) after cleavage with protease V8. Solvent A, 0.1% TFA; solvent B, 100% CH_3CN. Gradient, 0–60% solvent B in solvent A over 60 min. Absorbance at 230 nm of the effluent. The labeled peptide, carrying the phosphoryl group, is designated V8-A. For the other marked peaks, see text. (B) Distribution of radioactivity.

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H2N-QVVSTFMGNGLAIPHGTDD    EIII^mtl S.aureus
     **********
H2N-AVVSTFMGNGLAIPHGTD     EIII^mtl S.carnosus
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FIGURE 6: Comparison of the active-center peptides of the III^{mtl} proteins.

III^{mtl} proteins with the C-terminal part of the enzyme II^{mtl} of *E. coli* (Figure 7). Each of the peptides shows significant homology with the C-terminal enzyme II part. The suggested active site is especially well conserved in the *E. coli* protein.

Table II: Amino Acid Analysis and Sequencing Data Derived from III^{mtl} Peptides[a]

amino acid	*S. aureus* V8-A	*S. carnosus* V8-A	V8-1	V8-2
Asx	2.7 (3)	2.4 (2)	1.0 (1)	2.0 (2)
Thr	1.9 (2)	2.1 (2)	1.0 (0)	
Ser	1.0 (1)	1.4 (1)		
Glx	2.4 (1)	2.1 (0)	1.8 (1)	2.1 (1)
Pro	0.9 (1)	0.9 (1)		
Gly	3.2 (3)	2.7 (3)	1.7 (1)	
Ala	1.1 (1)	1.9 (2)	3.2 (2)	1.0 (1)
Val	2.1 (2)	1.4 (2)	2.1 (1)	
Cys				
Met	0.8 (1)	0.8 (1)		0.9 (1)
Ile	1.2 (1)	1.3 (1)		1.0 (1)
Leu	1.2 (1)	1.3 (1)	1.0 (1)	
Tyr				0.8 (1)
Phe	1.0 (1)	1.3 (1)		
Lys			1.1 (1)	1.0 (1)
His	1.0 (1)	0.9 (1)		
Arg				1.0 (1)
no. of residues	19	18	8	9

[a] Amino acid analysis data were calculated from 24-h hydrolysis. The number of amino acid residues derived from peptide sequencing is given in parentheses.

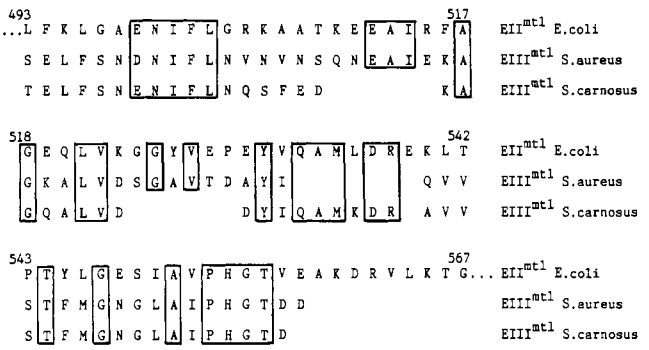

FIGURE 7: Comparison of all peptides so far derived from the III^{mtl} proteins with the C-terminal part of the enzyme II^{mtl} of *E. coli*. Regions of strict homology are boxed. The amino acid position is indicated for enzyme II^{mtl} beginning with 493.

DISCUSSION

The enzymes III^{mtl} of *S. aureus* and *S. carnosus* both have a molecular weight of approximately 15 000 according to SDS gels. Friedman et al. (1977) reported the molecular weight of the III^{mtl} protein of *S. aureus* to be 18 000 (±1000) in the native state. As this value agrees fairly well with that of the denaturated protein, we conclude the III^{mtl} proteins to be monomer. Furthermore, the above authors reported a reversible aggregation of the enzyme which was more pronounced at lower salt and higher protein concentrations. We assume that aggregation is also responsible for the salt-dependent migration behavior of the proteins on native gels as reported in this paper. Therefore, the upper band on the gel (Figure 1, lane 1) should correspond to the aggregated protein. It is very interesting that migration of the phosphorylated protein is not affected by salt. P-III^{mtl} always banded at positions that correspond to the aggregated material. Possibly, the III^{mtl} proteins become stabilized to an aggregate upon phosphorylation. P-III^{mtl} eluted from the gel filtration column at a retention time that corresponds to a molecular weight of approximately 35 000 (results not shown). Therefore, the phosphorylated protein most likely is a dimer or trimer. The amino acid sequencing data presented here reveal the homology between the III^{mtl} proteins of *S. aureus* and *S. carnosus*. In addition, antibodies raised against III^{mtl} of *S. aureus* also react with III^{mtl} of *S. carnosus*, although reaction was

somewhat weaker. The most important observation was the obvious homology between the III^{mtl} peptides and the C-terminal part of the enzyme II^{mtl} of *E. coli*. This protein belongs to a phosphotransferase system which lacks a soluble III protein. Saier et al. (1985) have introduced a theory on the evolutionary relatedness of the proteins of the bacterial PEP-dependent sugar PTS. The theory was mainly based on comparisons of molecular weights between enzyme III/enzyme II pairs and enzymes II of systems lacking a III protein. According to this theory, the enzyme II^{mtl} of *E. coli* is a fusion product of a hydrophilic III protein with a membrane-bound E II. Our data support this suggestion on a more significant structural basis, as all peptides of the III^{mtl} proteins so far sequenced show good homology with the C-terminal part of the *E. coli* enzyme II^{mtl} (Figure 7). Enzyme II^{mtl} of *E. coli* consists of 637 amino acids (Lee & Saier, 1983). According to our results, the enzyme III analogous region of enzyme II^{mtl} of *E. coli* starts within the hydrophilic C-terminus at residue 493. From this position to the end, there are 145 amino acids. We have calculated a similar number of residues from the amino acid compositions of the III^{mtl} proteins (Table I). The homology of the active-center peptides with enzyme II (positions 553–556) is convincing evidence for the enzyme III type 3-P-His phosphorylation site in the *E. coli* protein. We strongly suggest that His-554 represents this site. It is remarkable that enzyme II^{bgl} of *E. coli* also contains the sequence PHGT within its C-terminus at positions 608–611 (Bramley & Kornberg, 1987a). This protein catalyzes the uptake and concomitant phosphorylation of β-glucosides and receives the phosphoryl group directly from HPr; no enzyme III^{bgl} is involved in β-glucoside transport. His-609 of II^{bgl} therefore seems to be the III-type phosphorylation site. However, there is a much greater similarity between II^{bgl} and III^{glc} of *E. coli* which favors His-547 of II^{bgl} to act as the 3-P-His phosphorylation site (Bramley & Kornberg, 1987b). Because of these findings, the real phosphorylation site of II^{bgl} remains unclear.

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Registry No. $EIII^{mtl}$, 97162-87-3; EII^{mtl}, 37278-09-4; L-His, 71-00-1.

REFERENCES

Alpert, C.-A., Frank, R., Stüber, K., Deutscher, J., & Hengstenberg, W. (1985) *Biochemistry 24*, 959.

Beyreuther, K., Raufuss, H., Schrecker, O., & Hengstenberg, W. (1977) *Eur. J. Biochem. 75*, 275–286.

Bramely, H. F., & Kornberg, H. L. (1987a) *J. Gen. Microbiol. 133*, 563–573.

Bramley, H. F., & Kornberg, H. L. (1987b) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4777–4780.

Friedman, S. A., & Hays, J. B. (1977) *J. Bacteriol. 130*, 991–999.

Friedman, S. A., Cooper, R. M., & Hays, J. B. (1977) *FEMS Microbiol. Lett. 1*, 311–315.

Hewick, R. M., Hunkapillar, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256*, 7990–7997.

Hjerten, S., Jerstedt, S., & Tiselius, A. (1965) *Anal. Biochem. 11*, 219.

Jacobson, G. R., Lee, C. A., & Saier, M. H., Jr. (1979) *J. Biol. Chem. 254*, 249–252.

Keller, G., Schleifer, K. H. & Götz, F. (1983) *Plasmid 10*, 270–278.

Kyte, J., & Doolittle, R. F. (1982) *J. Mol. Biol. 157*, 105–132.

Lee, C. A., & Saier, M. H., Jr. (1983) *J. Biol. Chem. 258*, 10761–10767.

Parra, F. (1982) *Biochem. J. 205*, 643–645.

Saier, M. H., Jr., Grenier, F. C., Lee, C. A., & Waygood, E. B. (1985) *J. Cell. Biochem. 27*, 43–56.

Schägger, H., & von Jagow, G. (1987) *Anal. Biochem. 166*, 368–379.

Simoni, R. D., & Roseman, S. (1973) *J. Biol. Chem. 248*, 966–976.

Simoni, R., Smith, M., & Roseman, S. (1968) *Biochem. Biophys. Res. Commun. 31*, 804–811.

Stüber, K., Deutscher, J., Sobek, H. M., Hengstenberg, W., & Beyreuther, K. (1985) *Biochemistry 24*, 1164–1168.