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Staphylococcal Phosphoenolpyruvate-Dependent Phosphotransferase System: Purification and Characterization of a Defective Lactose-Specific Factor III Protein†

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ABSTRACT: Factor III protein specific for lactose ($FIII^{Lac}$) is part of the lactose-specific phosphotransferase system of *Staphylococcus aureus*. It is phosphorylated by the phosphorylated histidine-containing phospho-carrier protein (P-HPr). Phosphorylated $FIII^{Lac}$ (P-$FIII^{Lac}$) transfers the phosphoryl group via the enzyme II^{Lac} to the sugar during its uptake. The defective $FIII^{Lac}$ ($FIII^{Lac*}$) described here, isolated from *Staphylococcus aureus* strain S714G, showed most of the properties found for active $FIII^{Lac}$. It could still be phosphorylated by phosphoenolpyruvate, enzyme I, and HPr. As reported for the active protein, phosphorylation caused a dramatic structural change leading to increased hydrophobicity of the phosphorylated protein. As a consequence, P-$FIII^{Lac*}$, like P-$FIII^{Lac}$, bound to detergent micelles. But still, P-$FIII^{Lac*}$ was not able to transfer its phosphoryl group to lactose, indicating that the interaction with enzyme II^{Lac} is prohibited. To assay $FIII^{Lac*}$ during the purification procedure, we therefore used Ouchterlony tests with antibodies raised against $FIII^{Lac}$. The amino acid sequence of $FIII^{Lac}$ and of the first 56 amino acids of $FIII^{Lac*}$ revealed a difference for only one position: glycine in position 18 of $FIII^{Lac}$ is changed to glutamic acid in $FIII^{Lac*}$. This result, the exchange of an amino acid in the N-terminus of $FIII^{Lac}$, is in agreement with our previous findings that the N-terminal part of $FIII^{Lac}$ provides the binding domain for enzyme II.

The uptake of lactose in staphylococcal cells is achieved by the phosphoenolpyruvate (PEP)[1]-dependent phosphotransferase system (Hengstenberg et al., 1967; Simoni et al., 1973; Simoni & Roseman, 1973). This system consists of four proteins. Enzyme I and HPr are the two common nonspecific proteins, whereas FIII and enzyme II carry the sugar specificity. The following phosphoryl transfer reactions are involved in the uptake of lactose (Kalbitzer et al., 1981):

$$\text{PEP} + \text{enzyme I} \xrightleftharpoons{Mg^{2+}} \text{P-enzyme I} + \text{pyruvate}$$

$$\text{P-enzyme I} + \text{HPr} \rightleftharpoons \text{P-HPr} + \text{enzyme I}$$

$$\text{P-HPr} + FIII^{Lac} \rightleftharpoons \text{P-}FIII^{Lac} + \text{HPr}$$

$$\text{P-}FIII^{Lac} + \text{lactose}_{out} \xrightarrow{\text{enzyme } II^{Lac},\ Mg^{2+}} FIII^{Lac} + \text{lactose-6-P}_{in}$$

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The PEP-dependent phosphorylation of $FIII^{Lac}$ via enzyme I and HPr causes a drastic structural change as can be seen by CD spectroscopy (Deutscher et al., 1982) and NMR measurements (Kalbitzer et al., 1981). As a consequence of the structural change, $P\text{-}FIII^{Lac}$ gains a strong hydrophobic character. It has been proposed that the very hydrophobic N-terminal part of the protein, which also carries the enzyme II binding site, is turned to the surface of $FIII^{Lac}$ upon phosphorylation by PEP, enzyme I, and HPr (Deutscher et al., 1982). It has also been shown that $P\text{-}FIII^{Lac}$ in contact with nonionic detergents dissociates into its subunits (Hengstenberg, 1977).

Here we report the purification of a defective $FIII^{Lac}$ protein which we have isolated from the mutant strain S714G. The position of the mutation in the amino acid sequence and also the functional consequences of the defect are identified.

Materials and Methods

Bacterial Strains. Staphylococcus aureus strains S714G and S305A are both Lac constitutive. S714G carries in addition a mutation in the gene coding for $FIII^{Lac}$ (Hengstenberg et al., 1968). The cell still produces the defective protein. Cells of both strains were grown in a 100-L Chemap fermenter at 37 °C to late log phase. The complex growth medium contained in 100 L 1000 g of yeast extract (Ohly, Hamburg), 200 g of tryptone (Difco), and 20 g of $MgSO_4 \cdot 2H_2O$.

Factor III^{Lac}. Factor III^{Lac} was purified according to K. Stüber et al. (unpublished results).

Factor III^{Lac*}. Factor III^{Lac*} was isolated from strain S714G; 100 g of cells were suspended in 1.5 mL of standard buffer and broken in a Dynomill (Fa. Bachofen). The crude extract was applied to a DEAE column (DE-23, 12 × 30 cm) run with a 6-L linear gradient of 0–0.8 M NaCl in standard buffer (50 mM Tris-HCl, pH 7.5, 10^{-4} M DTT, 10^{-4} M PMSF, and 10^{-4} M EDTA). $FIII^{Lac*}$ eluted at 0.35 M NaCl. $FIII^{Lac*}$-containing fractions were pooled and fractionated by ammonium sulfate precipitation (43–65%)

The pellet of the final ammonium sulfate precipitation was dissolved in 100 mL of standard buffer and brought to pH 5.0 with acetic acid. After centrifugation, the pellet contained $FIII^{Lac*}$. It was dissolved in 40 mL of standard buffer and chromatographed on a Sephadex G-150 column (9 × 100 cm). $FIII^{Lac*}$-containing fractions were pooled and applied to a DEAE-cellulose column (DE-52, 1.6 × 27 cm) run with a 500-mL linear gradient of 0–0.6 M NaCl in standard buffer. $FIII^{Lac*}$ eluted at 0.2 M NaCl. The $FIII^{Lac*}$ pool (70 mL) was concentrated to 5 mL by pressure dialysis using an Amicon UM-2 membrane and was then subjected to high-pressure size-exclusion chromatography on a TSK G 2000 SW column (LKB) in 0.5-mL portions. The column was run with 100 mM phosphate buffer, pH 6.8, at a flow rate of 0.7 mL/min and 21 °C. $FIII^{Lac*}$-containing fractions were collected, desalted on a Sephadex G-25 column (4 × 25 cm), and lyophilized. We finally got 8–10 mg of electrophoretically pure $FIII^{Lac*}$. To assay the $FIII^{Lac*}$ protein during purification, we used antibodies raised against wild-type $FIII^{Lac}$ in an Ouchterlony double-diffusion test. The assay was performed as described by Deutscher et al. (1982), using a 1% agarose solution. After 6 h in a humidity chamber at 37 °C, the precipitates were visualized by indirect illumination, which allowed rough quantitative evaluation.

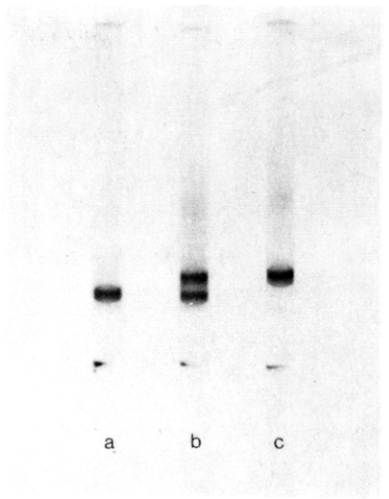

FIGURE 1: Native polyacrylamide gel electrophoresis. In lane a, 20 μg of $FIII^{Lac*}$ was loaded on the gel. Lane b shows a mixture of 20 μg of $FIII^{Lac}$ and 20 μg of $FIII^{Lac*}$ and lane c 20 μg of $FIII^{Lac}$. It is obvious that $FIII^{Lac*}$ migrates faster than $FIII^{Lac}$ on native polyacrylamide gels. The anode was at the bottom of the figure.

HPr. HPr of *S. aureus* was purified according to Beyreuther et al. (1977).

Enzyme I. Enzyme I of *Streptococcus faecalis* was purified according to C. A. Alpert et al. (unpublished results).

Enzyme II^{Lac}. Enzyme II^{Lac} was purified according to Schäfer et al. (1981).

6-Phospho-β-galactosidase. 6-Phospho-β-galactosidase was purified from *S. aureus* strain S305A by using a modified method of Hengstenberg & Morse (1975). The following steps were included: chromatography on DEAE-cellulose (DE-23), fractionated ammonium sulfate precipitation (55–75% saturation), chromatography on Sephadex G-150, and chromatography on DEAE-cellulose (DE-52).

Polyacrylamide Gel Electrophoresis. The tube gels contained 7.5% acrylamide, 0.13% methylenebis(acrylamide), and 0.4 M Tris–glycine, pH 9.3. Gels were stained with Coomassie Brilliant Blue. The destaining solution contained 7.5% acetic acid and 5% methanol (v/v) in water.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was performed according to Laemmli (1970). The gels contained 15% acrylamide.

^{32}P-Labeled Phosphoenolpyruvate. [^{32}P]PEP was synthesized and purified according to C. A. Alpert et al. (unpublished results).

Photometric Assay of $FIII^{Lac}$ and $FIII^{Lac*}$ Phosphorylation. During PEP-dependent phosphorylation of $FIII^{Lac}$, pyruvate is formed which can be reduced to lactate by using LDH (hog muscle, Boehringer) and NADH. The reaction mixture was placed in a semimicrocuvette and contained in a total volume of 200 μL 300 pmol of enzyme I (*S. faecalis*), 8.6 nmol of $FIII^{Lac}$ or $FIII^{Lac*}$, 400 pmol of LDH, 2.5 mM $MgCl_2$, 2.5 mM PEP, 0.4 mM NADH, and 50 mM Tris-HCl, pH 7.5. The assay mixture was preincubated 5 min at 30 °C before the reaction was started by adding HPr (*S. aureus*). The

[1] Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; PEP, phosphoenolpyruvate; HPr, histidine-containing phospho-carrier protein; P-HPr, phosphorylated HPr; $FIII^{Lac}$, factor III protein specific for lactose; $P\text{-}FIII^{Lac}$, phosphorylated $FIII^{Lac}$; $FIII^{Lac*}$, defective $FIII^{Lac}$ isolated from *Staphylococcus aureus* strain S714G; $FIII^{Glc}$, factor III protein specific for glucose; NMR, nuclear magnetic resonance; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography; CD, circular dichroism; PTH, phenylthiohydantoin; ONPG, *o*-nitrophenyl β-D-galactopyranoside; LDH, lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; P-enzyme I, phosphorylated enzyme I; P-His-HPr, HPr phosphorylated at a histidyl residue.

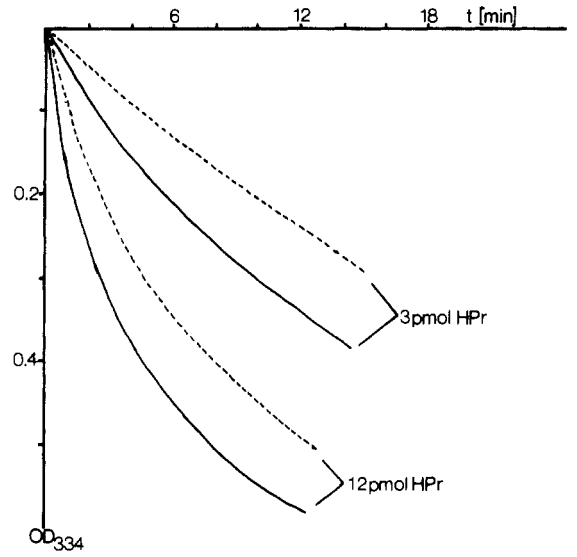

FIGURE 2: Photometric assay of FIII^{Lac} and FIII^{Lac*} phosphorylation. The reaction mixture was placed in a semimicrocuvette and contained in a total volume of 200 μL 300 pmol of enzyme I (*S. faecalis*), 8.6 nmol of FIII^{Lac} or FIII^{Lac*}, 400 pmol of LDH (hog muscle, Boehringer), 2.5 mM $MgCl_2$, 2.5 mM PEP, 0.4 mM NADH, and 50 mM Tris-HCl, pH 7.5. After a 5-min incubation at 30 °C, the reaction was started by adding different amounts of HPr (*S. aureus*, 12 or 3 pmol). The decrease of NADH was followed at 334 nm. FIII^{Lac}, solid line; FIII^{Lac*}, broken line.

decrease of NADH was followed at 334 nm.

Results

A defective FIII^{Lac} protein was isolated from *S. aureus* strain S714G by using a large-scale preparation described under Materials and Methods. Ouchterlony double-diffusion tests with antibodies raised against active FIII^{Lac} were used to assay FIII^{Lac*} during the purification procedure, which yielded homogeneous protein (Figure 1, lane a). Figure 1 also shows that FIII^{Lac*} migrates faster than FIII^{Lac} on native polyacrylamide gels (lanes b and c). We now tried to localize the biochemical defect of FIII^{Lac*} as there were several possibilities for functional disorder. First we attempted to phosphorylate FIII^{Lac*} with PEP, enzyme I, and HPr: 100 μg of FIII^{Lac*} was incubated together with 10 μg of enzyme I of *S. faecalis* and 10 μg of HPr of *S. aureus*, 1 mM $MgCl_2$, and [^{32}P]PEP (carrier free) for 3 min at 37 °C. Cold PEP was subsequently added to a final concentration of 1 mM, and after a further 3-min incubation at 37 °C, the assay mixture was applied to a TSK G 2000 SW column. Under the above reaction conditions, approximately one-third of the [^{32}P]PEP was used to phosphorylate FIII^{Lac*}. As the same result was obtained when active FIII^{Lac} was used, it was obvious that the interaction with P-HPr was not abolished (data not shown). For a more quantitative description of FIII^{Lac} and FIII^{Lac*} phosphorylation, we used a photometric assay. During the PEP-dependent phosphorylation of FIII^{Lac} or FIII^{Lac*}, pyruviate is formed, which was reduced to lactate with LDH and NADH. The decrease of NADH was followed at 334 nm. From Figure 2, it can be seen that FIII^{Lac*} is more slowly phosphorylated than FIII^{Lac} (almost 2-fold). However, during in vitro sugar phosphorylation, FIII^{Lac*} was found to be at least 100-fold less active than FIII^{Lac}. The slower phosphorylation of FIII^{Lac*} by P-His-HPr can therefore not be responsible alone for its low activity.

The next step which might have been prevented in FIII^{Lac*} was the conformational change caused by phosphorylation of FIII^{Lac}. This change not only could be followed by CD spectroscopy but also could induce a strong hydrophobic character of the phosphorylated protein (Deutscher et al., 1982). P-FIII^{Lac} was shown to bind to Triton X-100 micelles whereas FIII^{Lac} did not. We therefore loaded a mixture of FIII^{Lac*} (1 mg) and ^{32}P-FIII^{Lac*} (0.2 mg) on a Sephadex G-100 column (1.6 × 70 cm) equilibrated with 1% Triton X-100 in 50 mM NH_4HCO_3, pH 7.8. The result is shown in Figure 3.

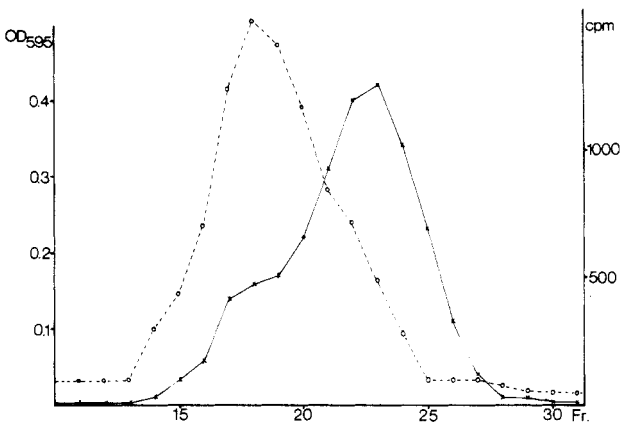

FIGURE 3: Chromatography of FIII^{Lac*} and ^{32}P-FIII^{Lac*} on a Sephadex G-100 column in the presence of 0.1% Triton X-100. A mixture of 1 mg of FIII^{Lac*} and 0.2 mg of ^{32}P-FIII^{Lac*} was loaded on a Sephadex G-100 column (1.6 × 70 mm) and then eluted with 0.1% Triton X-100 in 50 mM NH_4HCO_3, pH 7.8. The amount of protein in the fractions (solid line) was determined according to Bradford (1976). Aliquots were used to measure radioactivity (broken line).

The migration position of FIII^{Lac*} in the absence of Triton X-100 had been previously determined. In Figure 3, FIII^{Lac*} migrated to the same position in the presence of Triton X-100. The detergent had no influence on the migration behavior of FIII^{Lac*} whereas ^{32}P-FIII^{Lac*} migrated much faster as can be seen from the distribution of radioactivity. Phosphorylation of FIII^{Lac*} therefore must cause the same conformational change as was found for the phosphorylation of FIII^{Lac}.

The last reaction step, which could be affected by the FIII^{Lac*} mutation, was the interaction with membrane-bound enzyme II^{Lac} and the transfer of the phosphoryl group to the sugar. To test this possibility, we incubated FIII^{Lac*} with [^{32}P]PEP, enzyme I, HPr, 6-phospho-β-galactosidase, and ONPG for 3 min at 37 °C. FIII^{Lac} and FIII^{Lac*} were used in a 2-fold excess over [^{32}P]PEP (with regard to a subunit size of 12 000 daltons for FIII). Under these conditions, almost all [^{32}P]PEP was used to phosphorylate FIII^{Lac} or FIII^{Lac*} (Figure 4A, lanes c and h). We then added enzyme II^{Lac}. If FIII^{Lac*} could not transfer the phosphoryl group to the sugar, then the soluble PTS proteins would be expected to remain phosphorylated much longer than in the case of FIII^{Lac}. In the presence of ^{32}P-FIII^{Lac}, ONPG was phosphorylated to ONPG-6-P, which was hydrolyzed by 6-phospho-β-galactosidase. The reaction mixture therefore turned slightly yellow, whereas in the presence of ^{32}P-FIII^{Lac*} it remained colorless. Aliquots of the reaction mixture were brought to 1% $NaDodSO_4$, and the samples were then separated by $NaDodSO_4$–polyacrylamide gel electrophoresis. Phosphorylation of the proteins was determined by autoradiography. Figure 4A shows that in the presence of FIII^{Lac*} the PTS proteins remained phosphorylated much longer than in the presence of FIII^{Lac}, indicating that in FIII^{Lac*} the interaction with enzyme II^{Lac} is prevented. If this conclusion is correct, we anticipated that the mutation in FIII^{Lac*} might lie within the first 38 amino acids of the N-terminal part of the protein. A peptide, comprising these 38 amino acids, had previously been isolated and was found to compete with FIII^{Lac} for the

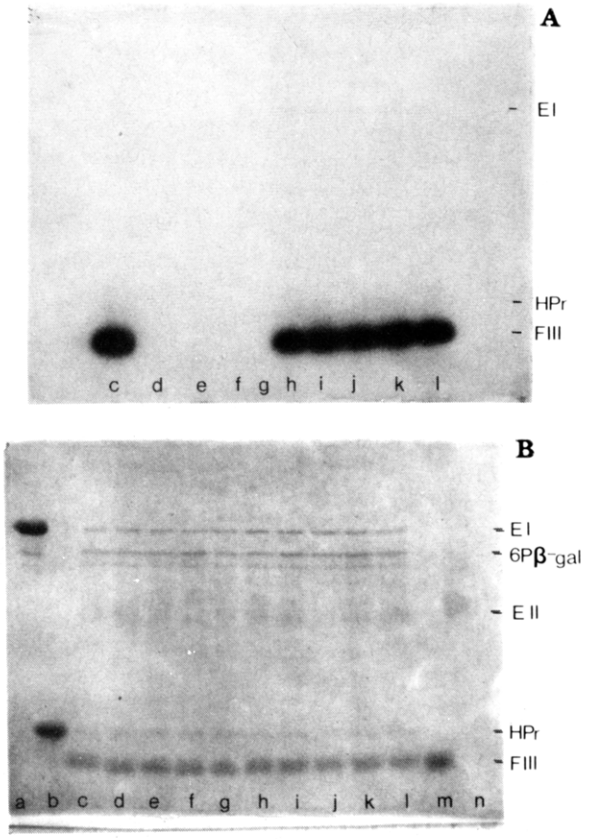

FIGURE 4: Autoradiogram after $NaDodSO_4$ gel electrophoresis. 100 μg of $FIII^{Lac}$ (lanes c–g) or 100 μg of $FIII^{Lac*}$ (lanes h–l) was incubated in 50 mM Tris-HCl, pH 7.5, together with 30 μM [^{32}P]PEP (200 μCi/μmol), 25 μg of enzyme I, 25 μg of HPr, 25 μg of 6-phospho-β-galactosidase, 3.5 mM $MgCl_2$, and 3.5 mM ONPG for 3 min at 37 °C. Then enzyme II^{Lac}, solubilized in 0.1% Triton X-100, was added, and the reaction mixture (total volume of 120 μL) was further incubated at 37 °C. After 10 s and 3, 6, 13, and 25 min, 20-μL aliquots were taken and brought to 1% $NaDodSO_4$ in 50 mM Tris-HCl, pH 9.3. The samples were loaded on $NaDodSO_4$ gels. Phosphorylation of the proteins was detected after autoradiography for 6 h. In the presence of $FIII^{Lac}$, the soluble PTS proteins were dephosphorylated after 3 min (lane d), whereas in the presence of $FIII^{Lac*}$ the proteins remained phosphorylated even after 25 min (lanes h–l). (B) The same $NaDodSO_4$ gel as described in panel A after being stained and destained. Lane a shows 20 μg of enzyme I (*S. faecalis*), lane b 20 μg of HPr (*S. faecalis*), lane m 20 μg of $FIII^{Lac*}$, and lane n 5 μg of enzyme II^{Lac}. In the presence of Triton X-100, enzyme II^{Lac} does not migrate according to its molecular weight of 55 000 on $NaDodSO_4$ gels.

binding site of enzyme II. This part of the protein was therefore presumed to represent the binding domain of $FIII^{Lac}$ for enzyme II^{Lac} (Deutscher et al., 1982). The first evidence for a different amino acid composition of $FIII^{Lac}$ and $FIII^{Lac*}$ came from peptide mapping of a digest obtained with V-8 staphylococcal protease. Peptides were separated by HPLC on a Zorbax C8 reversed-phase column as described by K. Stüber et al. (unpublished results). The results are shown in Figure 5A,B. Figure 5A shows the peptide separation obtained for $FIII^{Lac}$ and Figure 5B that for $FIII^{Lac*}$. It is obvious that peptide F of Figure 5A is not present in Figure 5B but that two additional peaks, denoted F_1 and F_2, are present. The peptides C and D are known to change their positions with slight differences in pH and column performance. Peptide F had previously been isolated and sequenced and had been shown to belong to the N-terminal part of $FIII^{Lac}$, comprising the amino acids of positions 13–26 (K. Stüber et al., unpublished results).

We therefore determined the amino acid sequence of the N-terminal part of $FIII^{Lac*}$ by automated Edman degradation

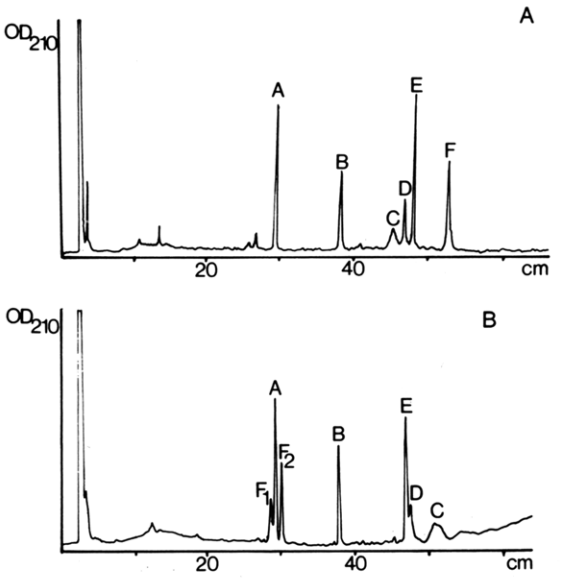

FIGURE 5: (A) HPLC of $FIII^{Lac}$, digested with V-8 staphylococcal protease, on a Zorbax C8 column. Five nanomoles of the digested protein was loaded on the column, which was eluted with a linear gradient of 0–70% buffer B. (Buffer A was 5 mM NH_4HCO_3, pH 6.0; buffer B was 60% acetonitrile in 50 mM NH_4HCO_3, pH 6.0). (B) HPLC of $FIII^{Lac*}$, digested with V-8 staphylococcal protease, on a Zorbax C8 column. The elution conditions were the same as those for panel A.

according to Beyreuther (1977). By this method, the amino acid sequence of $FIII^{Lac*}$ was obtained up to position 55: Met-Asn-Arg-Glu-Glu-Val-Gln-Leu-Leu-Gly-Phe-Glu-Ile-Val-Ala-Phe-Ala-*Glu*-Asp-Ala-Arg-Ser-Lys-Phe-Leu-Glu-Ala-Leu-Thr-Ala-Ala-Gln-Ala-Gly-Asp-Phe-Ala-Lys-Ala-Asp-Ala-Leu-Ile-Glu-Glu-Gly-Asn-Asn-Cys-Ile-Ala-Gln-Ala-His-Lys-.

A comparison with the amino acid sequence of $FIII^{Lac}$ (K. Stüber et al., unpublished results) revealed only one difference: glycine in position 18 of $FIII^{Lac}$ was exchanged for glutamic acid in $FIII^{Lac*}$. The result is in agreement with peptide mapping of the V-8 digest. V-8 staphylococcal protease cleaves peptide bonds after glutamic acid. Through exchange of glycine for glutamic acid in position 18, peptide F is split into two pieces by V-8 staphylococcal protease. The change of glycine to glutamic acid in $FIII^{Lac*}$ was confirmed by the experiments shown in Figure 1. By virtue of the additional negative charge, $FIII^{Lac*}$ migrated faster on native polyacrylamide gels than did $FIII^{Lac}$.

Discussion

$FIII^{Lac}$ of the staphylococcal phosphotransferase system has been called a phase transfer catalyst as it is phosphorylated in the cytoplasm by PEP, enzyme I, and HPr and transfers the phosphoryl group via the membrane-bound enzyme II to the sugar being transported. This function of $FIII^{Lac}$ can be split into several steps. The unphosphorylated proteins HPr and $FIII^{Lac}$ show a strong interaction as was documented by NMR measurements (Kalbitzer et al., 1981). Evidence for binding between HPr and $FIII^{Glc}$ of *Escherichia coli* has also been reported by Jablonski et al. (1983). HPr, phosphorylated by PEP and enzyme I, transfers the phosphoryl group to a single histidyl residue of $FIII^{Lac}$ (His-82 in the amino acid sequence) (Deutscher et al., 1982; K. Stüber et al., unpublished results). The phosphorylation of $FIII^{Lac}$ causes a change in the peptide folding: a hydrophobic region is turned to the surface of the protein. This allows P-$FIII^{Lac}$ to interact with the membrane. The binding to enzyme II^{Lac}, which is followed by the transfer of the phosphoryl group to the transported

sugar, is achieved by the N-terminal part of $FIII^{Lac}$, comprising the first 38 amino acids (Deutscher et al., 1982). Other labs also reported that after modification of the N-terminal part of $FIII^{Glc}$ from *Salmonella typhimurium* the protein could still be phosphorylated but was no longer able to transfer the phosphoryl group to the sugar. This is the case when the first seven amino acids of the N-terminal part are split off by a protease (Meadow et al., 1982) or when the NH_2-terminal amine was modified with fluorescein (Jablonski et al., 1983).

The defect in $FIII^{Lac*}$, isolated from *S. aureus* strain S714G, could be attributed to the last step, the binding of $FIII^{Lac*}$ to the membrane-bound enzyme II^{Lac}, even though the transfer of the phosphoryl group from P-His-HPr to $FIII^{Lac*}$ is also slightly slowed down. A conformational change of $FIII^{Lac*}$, caused by phosphorylation, could still be demonstrated. However, the transfer of the phosphoryl group from P-$FIII^{Lac*}$ to the sugar was drastically slowed down. The genetic defect could also be localized in the sequence of $FIII^{Lac*}$. Glycine in position 18 of active $FIII^{Lac}$ is exchanged for glutamic acid in $FIII^{Lac*}$. The locus of the genetic defect is in agreement with the biochemical results as the N-terminal part of $FIII^{Lac}$ of *S. aureus* and $FIII^{Glc}$ of *E. coli* is believed to represent the binding domain for the corresponding enzyme II (Deutscher et al., 1982; Meadow et al., 1982; Jablonski et al., 1983). It must be determined if all FIII protein carry their recognition site for enzyme II at the N-terminus while the histidine of the active center is in the C-terminal part.

The phosphorylation of $FIII^{Lac}$ with the concomitant conformational change, observed also for $FIII^{Lac*}$, could be of general interest. Through this conformational change, P-$FIII^{Lac}$ gains a strong hydrophobic character, which allows its attachment to the membrane. The same mechanism could also be suggested for protein transport across the membrane. In the case of cytochrome *c* of *Neurospora crassa*, it has been shown that only the holoenzyme can be transported from the cytoplasm through the outer mitochondrial membrane (Hennig & Neupert, 1981). The covalent binding of the protohemin via a thioether bond, which leads to the holoenzyme, causes effects on the protein very similar to those found for $FIII^{Lac}$ after phosphorylation. Through a structural change, antibodies raised against the apoenzyme did not precipitate the holoenzyme. The holoenzyme showed a strong hydrophobic character whereas the apoenzyme did not (Zimmermann et al., 1979). There is also evidence that phosphorylation itself may be necessary for the protein transport across the membrane. The transport of the small subunit of ribulosebisphosphate carboxylase/oxygenase of spinach chloroplasts was reported to be dependent on a protein kinase of the outer envelope membrane (Soll & Buchanan, 1983). It is known that most secreted proteins in both prokaryotic and eukaryotic cells carry a "signal sequence" at the N-terminal part of the protein (Kreil, 1981). The signal sequence comprises 18–30 amino acids. In $FIII^{Lac}$, we found that the N-terminal part of the protein is probably buried on the inside of the protein but becomes exposed to the surface when $FIII^{Lac}$ is phosphorylated by P-HPr. P-$FIII^{Lac}$ shows a strong hydrophobic character and can bind to membrane vesicles, whereas $FIII^{Lac}$ does not. A similar activation mechanism by phosphorylation or other protein modifications may be necessary for the transport of some proteins across the membrane, for example, for the above-mentioned ribulosebisphosphate carboxylase/oxygenase.

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