# PHOSPHORYLATION OF A SUGAR-SPECIFIC PROTEIN COMPONENT OF THE LACTOSE TRANSPORT SYSTEM IN *STAPHYLOCOCCUS AUREUS*[*]

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Summary

A sugar-specific component of the lactose transport system in *Staphylococcus aureus*, Factor $III^{lac}$, is phosphorylated as an intermediate in the over-all transfer of a phosphoryl group from PEP to lactose. P-$III^{lac}$ is isolated and shown to be a substrate for the final phosphoryl transfer reaction to sugar, catalyzed by Enzyme $II^{lac}$.

Many bacterial species contain a phosphotransferase system (PTS) which mediates the transport of a variety of carbohydrates across the cell membrane (1). The reaction catalyzed by the PTS is the transfer of phosphate from phosphoenolpyruvate (PEP) to sugar. Initial studies (2) with the PTS showed that this over-all reaction resulted from at least two distinct steps:

$$\text{[Enzyme I]}\quad \text{PEP} + \text{HPr} \longleftrightarrow \text{P-HPr} + \text{Pyruvate} \qquad (1)$$

$$\text{[Enzyme II]}\quad \text{P-HPr} + \text{Sugar} \longrightarrow \text{Sugar-P} + \text{HPr} \qquad (2)$$

Enzyme I and HPr are common to all sugars phosphorylated by the PTS and are found primarily in the soluble fraction of cell lysates. The molecular weights of the HPr proteins from three different organisms are in the range of 9,000 to 10,000. As will be reported elsewhere, the intermediate in Reaction I is phospho-Enzyme I.

The transfer of the phosphoryl group from P-HPr to sugar requires the

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sugar-specific components of the PTS, most of which are inducible.[1] In all such systems thus far analyzed in this laboratory, two proteins are required, at least one of which is membrane-bound. For example, the *E. coli* constitutive Enzyme II has been dissociated into two proteins, as well as lipid and divalent cation (3).

In earlier studies with *S. aureus*, which apparently transports and utilizes all sugars via the PTS (4), we reported (5) that lactose utilization requires the induction of a membrane protein (Enzyme $II^{lac}$) and a cytoplasmic protein (Factor $III^{lac}$). The present studies provide evidence that the transfer of the phosphoryl group from P-HPr to lactose (and its analogues) involves the following series of reactions:

$$2P\text{-}HPr + III^{lac} \longleftrightarrow P_2\text{-}III^{lac} + 2HPr \quad (3)$$

$$[\text{Enzyme } II^{lac}]\, P_2\text{-}III^{lac} + 2 \text{ lactose} \longrightarrow 2 \text{ lactose-P} + III^{lac} \quad (4)$$

## EXPERIMENTAL

*S. aureus* 5601 cells were grown in media containing 1% galactose and 1% bactopeptone which induces the lactose system (5). Cells were grown on large scale by Dr. Thomas Stoudt of Merck and Co., without whose generous assistance these experiments would not have been possible. $^{32}$P-enolpyruvate was prepared according to the procedure previously reported (3). All other materials were obtained from commercial sources.

Crude extracts were prepared from 0.5 to 1 kg of cells by homogenization with glass beads in an Eppenbach Micro-mill. The purification procedures, to be reported elsewhere in detail, separated the PTS components from each other. Enzyme $II^{lac}$ was purified by extracting washed membranes with 1% sodium deoxycholate followed by 0.005 M NaOH. The crude supernatant fluid containing

[1] In accord with recommended nomenclature (3,5), the abbreviations Enzyme $II^{lac}$ and Factor $III^{lac}$ designate the sugar-specific (lactose) components of the PTS obtained when cells are grown under inducing conditions. TMG = methyl thio - β - D - galactopyranoside.

Enzyme I, $III^{lac}$, and HPr was treated with streptomycin and subsequently adjusted to pH 4.9; HPr remained in the supernatant fluid and was purified by DEAE-cellulose chromatography and ammonium sulfate precipitation (65 to 100%) followed by column chromatography on Sephadex G-75, DEAE-cellulose (0 to 0.2 M KCl), and hydroxylapatite (0.001 to 0.02 M potassium phosphate). The pH 4.9 precipitate was dissolved and treated with ammonium sulfate (40 to 80%); Enzyme I and $III^{lac}$ were separated by Sephadex G-75 chromatography. Enzyme I was purified by chromatography on DEAE-cellulose (0.2 to 0.35 M KCl), while $III^{lac}$ was isolated by chromatography on hydroxylapatite (0.15 M potassium phosphate) followed by DEAE-cellulose (0.12 to 0.20 M KCl).

The following purification factors were attained: Enzyme $II^{lac}$ about 10-fold over washed membranes; Enzyme I about 20-fold; HPr, about 200-fold; and $III^{lac}$ about 400-fold. The HPr appeared essentially homogeneous (disc-gel electrophoresis at pH 9.5 and 4.5 and equilibrium ultracentrifugation), and contained 1 histidine residue per mole of protein; three methods for determining molecular weight gave values of about 9,000. Factor $III^{lac}$ appeared homogeneous (disc-gel electrophoresis and equilibrium ultracentrifugation) and showed a molecular weight of about 37,000. However, when $III^{lac}$ was analyzed by sodium dodecyl sulfate-gel electrophoresis, and by ultracentrifugation in 4 M guanidine, the molecular weight appeared to be between 9,000 and 12,000, indicating that it is composed of 3 to 4 subunits.

## RESULTS

### Transfer of the Phosphoryl Group from PEP to Factor $III^{lac}$

Previous work established that participation of $III^{lac}$ in the over-all reaction was subsequent to P-HPr formation. The data presented in Table I show that $III^{lac}$ was phosphorylated in the presence of catalytic amounts of Enzyme I and HPr. Omission of either I or HPr resulted in no $P\text{-}III^{lac}$ formation (data not shown). It is also apparent that two phosphoryl groups were incorporated per mole of $III^{lac}$. $^{32}P_2\text{-}III^{lac}$ has been isolated from large

Table I

*Transfer of Phosphate from $^{32}$PEP to Factor III$^{lac}$*

| Incubations | Time | $^{32}$P-III | Ratio of $^{32}$P to III$^{lac}$ |
|---|---|---|---|
| | *min.* | *cpm* | |
| 1. III$^{lac}$ + $^{32}$PEP (1 mμmole) | 15 | 8,800 | 1.64 |
| 2. III$^{lac}$ + $^{32}$PEP (1 mμmole) | 30 | 7,950 | 1.52 |
| 3. III$^{lac}$ + $^{32}$PEP (10 mμmoles) | 10 | 10,500 | 1.94 |
| 4. III$^{lac}$ + $^{32}$PEP (10 mμmoles) | 20 | 9,750 | 1.88 |
| 5. — + $^{32}$PEP (1 mμmole) | 30 | 260 | — |

Incubations were conducted at 37° and contained III$^{lac}$ (10 μg, 0.27 mμmole); Enzyme I, 8.2 μg for incubations 1, 2, and 5, and 16.4 μg for 3 and 4; HPr, 0.2 μg; potassium phosphate buffer, pH 8.3, 0.5 μmole; $MgCl_2$, 50 mμmoles; and $^{32}$PEP, 1.94 x $10^7$ cpm/μmole as indicated. The total volumes of incubations 1, 2, and 5 were 35 μl and 3 and 4, 55 μl. EDTA (1 μmole) was added to stop the reaction. Aliquots of each incubation were spotted on strips of Whatman DE 81 (DEAE) paper (2 x 10 cm) and subjected to ascending chromatography in 30% ethanol-0.05 M KCl-1 mM EDTA-10 mM Tris-HCl buffer, pH 8. Under these conditions $^{32}$P-III$^{lac}$ remained at the origin and $^{32}$PEP and $^{32}$Pi migrate near the solvent front. The chromatograms were then cut into 1 cm strips and counted in toluene liquid scintillation fluid.

scale incubations by chromatography on Sephadex G-75 and the pH stability of the phosphoryl linkage examined. At pH 3.6 and 46° the rate of hydrolysis was similar to that obtained for P-HPr from *E. coli*, thus suggesting that the linkage in P-III$^{lac}$ is to a histidine residue (as has been demonstrated for P-HPr).

Transfer of the Phosphoryl Group from P-HPr to III$^{lac}$ (Reaction 3)

The direct transfer of the phosphoryl group from P-HPr to III$^{lac}$ is demonstrated in Figure 1. $^{32}$P-HPr was isolated by chromatography on Sephadex G-75 and was free of $^{32}$PEP and extraneous proteins. $^{32}$P-HPr and III$^{lac}$ were then incubated under the conditions shown in Figure 1 and $^{32}$P transfer was assayed by separating the two proteins by G-75 chromatography. Since both protein preparations were pure, we assume that no additional protein component is required for the transfer. The reaction showed no metal requirement (no inhibition by EDTA), and phosphoryl transfer was very rapid: incubation at 0°

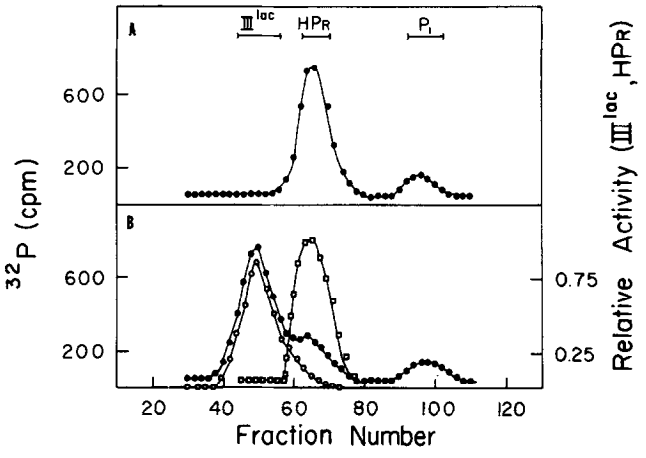


Fig. 1. *Transfer of the Phosphate from $^{32}$P-HPr to III$^{lac}$.* Incubation mixtures were as follows: $^{32}$P-HPr, 0.6 mμmole, 42,000 cpm (this $^{32}$P-HPr preparation also contained 0.54 mμmole of HPr since it was not completely phosphorylated in these experiments); $MgCl_2$, 1 μmole; in the absence (A) or presence (B) of 4.15 mμmoles of III$^{lac}$ in a total volume of 500 μl. Incubation was carried out at 37° for 2 min, 500 μl of water were added and the total reaction mixture was applied to a Sephadex G-75 column (1.8 x 40 cm) which was equilibrated with 1 mM Tris-Cl buffer, 50 mM KCl, pH 9. Fractions were collected and aliquots removed for $^{32}$P determination (●), enzymatic assay of HPr (□) and III$^{lac}$ (○). In addition results obtained by 1) substituting 10 μmoles of EDTA for $MgCl_2$ or 2) incubation for 0 min at 0° instead of for 2 min at 37°, were the same as those shown in B. The enzymatic assays for HPr and III$^{lac}$ were done by measuring $^{14}$C-thiomethyl galactoside-P formation as previously reported (5). The small $^{32}$P peak at the end of the chromatogram is $^{32}$Pi which was present in the $^{32}$P-HPr preparation. All samples were counted in Triton-toluene liquid scintillation solution.

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followed by rapid separation at 4° gave essentially the same results as incubation at 37°. The reaction is freely reversible and transfer of the phosphoryl group from $^{32}$P-III$^{lac}$ to HPr was easily demonstrable (data not shown). Although an accurate equilibrium constant has not yet been determined, preliminary results show that it is approximately 1.

While the results shown in Figure 1 indicate that $^{32}$P was transferred from $^{32}$P-HPr to III$^{lac}$, essentially the same results would have been obtained if $^{32}$P-HPr were tightly bound to III$^{lac}$ or if it exchanged with one of the subunits of this protein. To eliminate these possibilities, HPr was chemically acetylated with $^{14}$C-acetic anhydride (2 moles of $^{14}$C per mole of HPr), the labeled HPr was phosphorylated (0.8 mole of $^{32}$P per mole of $^{14}$C-HPr), and

the doubly labeled product was incubated with $III^{lac}$ as described in Figure 1. Virtually complete transfer of $^{32}P$ was obtained, while all of the $^{14}C$ remained in the HPr peak. Thus we conclude that Reaction 3 (see Introduction) is established.

Transfer of the Phosphoryl Group from P-$III^{lac}$ to TMG (Reaction 4)

In order to ensure that the P-$III^{lac}$ formed in previous reactions was indeed an active intermediate in the over-all reaction, $^{32}$P-$III^{lac}$ was isolated by chromatography on Sephadex G-75 from a large-scale incubation mixture similar to that shown in Table I. The isolated $^{32}$P-$III^{lac}$ was then incubated with Enzyme $II^{lac}$ and TMG; TMG-$^{32}P$ was only formed in the presence of Enzyme $II^{lac}$ (Table II).

Interaction of P-$III^{lac}$ and TMG with Enzyme $II^{lac}$

The data presented above and reported previously (5) show that $III^{lac}$ is specific for lactose and its analogues, and that it acts as an intermediate

Table II

*Transfer of phosphate from $^{32}P$-$III^{lac}$ to TMG*

| Incubation | $^{32}P$ -III Remaining | $^{32}P$-TMG Formed |
|---|---|---|
| | *cpm* | *cpm* |
| 1. Complete | 634 | 3200 |
| 2. Minus Enzyme $II^{lac}$ | 3627 | 0 |
| 3. Minus TMG | 3428 | 0 |
| 4. Minus Enzyme $II^{lac}$ and TMG | 3745 | 0 |

The complete incubation contained dithiothreitol, 0.5 µmole; Tris-HCl buffer, pH 9, 2.5 µmoles; Enzyme $II^{lac}$, 33 µg; $MgCl_2$, 0.05 µmole; $^{32}$P-$III^{lac}$, 0.137 mµ-mole, 4800 cpm; in a total volume of 85 µl. Incubation was conducted at 37° for 2 min. Aliquots of each incubation were spotted on Whatman No. 3MM paper and subjected to high voltage electrophoresis in 1% sodium tetraborate for 20 min at 4000 volts. Under these conditions $^{32}$P-III remains at the origin and $^{32}$P-TMG moves toward the anode. The paper strips were cut into 2 cm strips and counted in toluene scintillation fluid.

phosphate carrier between P-HPr and the sugar. Both P-III$^{lac}$ and sugar are substrates for Enzyme II$^{lac}$. Therefore, some preliminary kinetic studies were carried out in an attempt to clarify the relationship of the three components (II$^{lac}$, P-III$^{lac}$ and TMG); since Enzyme II is particulate and was not extensively purified, the results should be interpreted with caution.

Figure 2 shows the initial rates of TMG-P synthesis, using a generating system for P-III$^{lac}$ under conditions where all the III$^{lac}$ added to the system was phosphorylated. The apparent $K_m$ for TMG increased with increasing concentration of P-III$^{lac}$; the estimated values for this $K_m$ at 0.0 M P-III$^{lac}$ and at infinite concentration of P-III$^{lac}$ were 0.03 mM and 1.0 mM, respectively. The data indicate an ordered mechanism involving formation of a ternary complex between P-III$^{lac}$, TMG, and Enzyme II$^{lac}$ and excludes a "ping-pong" mechanism involving intermediate transfer of P from P-III$^{lac}$ to Enzyme II$^{lac}$. Other studies including product inhibition patterns and direct binding measurements are currently in progress to define the mechanism of the last reaction.

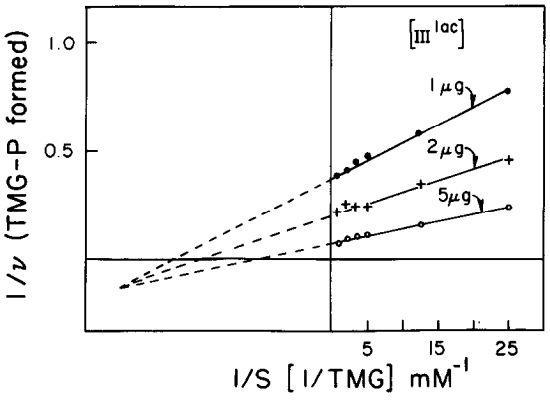


Fig. 2. *Kinetics of the Enzyme II$^{lac}$ Reaction.* Incubations contained potassium phosphate buffer, 20 µmoles, pH 7.5; phosphoenolpyruvate, 2 µmoles; KF, 0.5 µmole; dithiothreitol, 1 µmole; Enzyme II$^{lac}$ 33 µg; Enzyme I, 35 µg; HPr, 15 µg; $MgCl_2$, 1 µmole; III$^{lac}$, 1, 2, 5 µg as indicated (the III$^{lac}$ used in these experiments was not completely pure and therefore no molar values are given); $^{14}$C-thiomethyl β-D-galactoside, concentrations as shown. Final volume was 240 µl. Incubation was at 37° for 30 min and the TMG-P was detected as previously reported (5).

## DISCUSSION

The data presented in this paper established the reaction sequence for

the lactose phosphotransferase system in *S. aureus*. The phosphorylation of $III^{lac}$ represents the first demonstration of a reversible covalent alteration of an isolated solute-specific transport protein. It is our contention that $III^{lac}$ and $II^{lac}$ together form the functional carrier complex for the transport of lactose and its analogues in this organism. The data demonstrate that P-$III^{lac}$ is the direct energy-coupling protein between P-HPr and $II^{lac}$. Since $III^{lac}$ seems to be comprised of 3 to 4 subunits, and only 2 phosphoryl groups can be incorporated per molecule, it is attractive to speculate that the remaining subunit(s) are involved in the interactions with $II^{lac}$. The specificity of $III^{lac}$ apparently results from its interaction with the specific Enzyme $II^{lac}$ rather than a direct interaction with sugar.

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