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# IRREVERSIBLE INACTIVATION OF THE MEMBRANE-BOUND ENZYME $II^{lac}$ OF THE LACTOSE PHOSPHOTRANSFERASE SYSTEM OF *STAPHYLOCOCCUS AUREUS* BY TRITON X-100 AND PROTECTION BY SUBSTRATES

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## Summary

Enzyme $II^{lac}$, the membrane-bound component of the lactose phosphotransferase system of *Staphylococcus aureus*, catalyzes the phosphorylation-transport reaction below:

$$\text{phospho-Factor III}^{lac} + \text{sugar} \xrightarrow{\text{Enzyme II}^{lac}} \text{Factor III}^{lac} + \text{sugar phosphate}$$

(The sugar can be lactose or one of its analogs.) The effects of the non-ionic detergents Triton X-100, Brij 35, and Tween 40 on the activity of Enzyme $II^{lac}$ were studied. Especially striking effects were observed using Triton X-100, a detergent previously used to solubilize and isolate this enzyme. A systematic study of Triton effects over a range of concentrations and temperatures demonstrated three aspects of Triton-membrane interaction. At 0.1% Triton and 25°C Enzyme $II^{lac}$ is activated, but remains particulate. At 0.5% Triton and 25°C, it is almost completely solubilized, with good retention of activity. At 0.5% Triton and 37°C, it is rapidly and irreversibly inactivated. Sugar substrates and inhibitory sugar analogs protect Enzyme $II^{lac}$ against inactivation; the effect is specific for β-galactosides. The other substrate of Enzyme $II^{lac}$, phospho-Factor $III^{lac}$, does not affect Triton inactivation, and the product analog galactose 6-phosphate slightly enhances the inactivation rate.

## Introduction

Detergents are commonly employed to disperse or "solubilize" membrane-bound enzymes, thus facilitating their isolation [1]. Non-ionic detergents,

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especially Triton X-100, have proved particularly useful. The latter has previously been used to solubilize Enzyme $II^{lac}$ [2,3], the membrane-bound component of the phosphoenolpyruvate-dependent lactose phosphotransferase system of the gram-positive bacterium *Staphylococcus aureus*. This multienzyme system catalyzes the concomitant phosphorylation and membrane transport of sugars in that organism [4]. In addition to Enzyme $II^{lac}$, the system consists of three soluble components, Enzyme I, histidine protein and Factor $III^{lac}$. Both the last protein and Enzyme $II^{lac}$ are specific for β-galactosides. The sugar phosphorylation is accomplished by a series of phosphoryl transfers [5–7]:

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

$$P\text{-Enzyme I} + \text{histidine protein} \rightleftharpoons P\text{-histidine protein} + \text{Enzyme I} \quad (2)$$

$$P\text{-histidine protein} + 1/3\ \text{Factor III}^{lac} \rightleftharpoons 1/3\ (P_3\text{-Factor III}^{lac}) + \text{histidine protein} \quad (3)$$

$$1/3\ (P_3\text{-Factor III}^{lac}) + \beta\text{-galactoside} \xrightarrow{\text{Enzyme II}^{lac}} \beta\text{-galactoside-6-}P + 1/3\ \text{Factor III}^{lac} \quad (4)$$

Apparently translocation of sugar across the membrane is concomitant with reaction 4, so that Enzyme $II^{lac}$ is directly implicated in the transport process. We have studied the interaction of Triton X-100 with Enzyme $II^{lac}$ over a range of temperatures and detergent concentrations, and the effects of other components (substrates, products, cofactors) of the phosphotransferase reaction on those interactions. The results can be described in terms of three aspects of detergent-membrane interaction: activation, solubilization and inactivation. The influence of reaction components on the inactivation process can be determined quantitatively and provides a convenient assay for their interaction with Enzyme $II^{lac}$.

## Materials and Methods

Materials and analytical procedures were as previously described [8,9], except where indicated. Triton X-100 was obtained from Sigma. Brij 35 and Tween 40 were gifts of Dr. Charles Waechter, and Dr. Nessly Craig, respectively. Phosphate dilution buffer contains 10 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA and 1 mM dithiothreitol. Tris dulution buffer contains 10 mM Tris · HCl buffer, pH 7.4, instead of potassium phosphate and the same concentrations of EDTA and dithiothreitol. Detergent concentrations are stated as % (v/v).

*Preparation of staphylococcal lactose phosphotransferase system components*

The preparation of purified fractions of Enzyme $II^{lac}$, Enzyme I, histidine protein and Factor $III^{lac}$ from mechanically disrupted cells of *S. aureus* strain C 22 (constitutive for lactose phosphotransferase proteins [4]) has been described in detail [5,8]. In the final step of the described procedure for Factor $III^{lac}$, pooling and concentration of the active fractions obtained by

DEAE-cellulose chromatography yielded a preparation which was about 75% pure, as estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. When required, homogeneous Factor $III^{lac}$ was prepared as described by Hays et al. [5], with the following change. The DEAE-cellulose pool (about 9 ml) was dialyzed overnight against 1 mM sodium phosphate buffer, pH 6.8, containing 0.5 mM dithiothreitol, applied to a 1.3 × 7.5 cm column (about 8 ml) of hydroxyapatite (Bio-Rad), and washed with 50 ml of the same buffer. The column was eluted with a 300 ml linear gradient of sodium phosphate buffer (pH 6.8, 2—40 mM) containing 0.5 mM dithiothreitol, at about 0.3 ml per min. The peak fractions were pooled, concentrated by lyophilization and dialyzed overnight against Tris dilution buffer. Polyacrylamide gel electrophoresis of this material in the presence of sodium dodecyl sulfate yielded a single band. We consider appearance of a single band (molecular weight about 11 000) under these conditions to be strong evidence of homogeneity, since the molecular weight of the preparation under non-denaturing conditions is 35 000 [5].

For Enzyme $II^{lac}$, washed membranes were prepared from cell-free extracts by two cycles of alternate ultrasonic irradiation and low-speed and high-speed centrifugation [8], and used without further treatment. Although the resulting preparation was quite heterogeneous and did not represent any significant purification of Enzyme $II^{lac}$ with respect to the other membrane components, it seemed desirable for this study not to subject the membranes to further purification procedures (e.g. detergent treatments) which might alter the very protein-lipid interactions under investigation. The mechanical treatment was quite effective in removing peripheral proteins, however; for instance the background level of Factor $III^{lac}$ in washed membranes corresponded to less than 1 pmol of Factor $III^{lac}$ per 100 pmol of Enzyme $II^{lac}$. The level of the latter enzyme was estimated to be about 500 pmol per mg membrane protein by a [$^{14}$C]lactose binding assay [6]. When viewed in the electron microscope, the preparations contain membranous vesicles of various sizes, cell wall fragments, and some uncharacterized debris. The lipid : protein ratio corresponded to about 380 nmol of lipid phosphorus per mg of membrane protein.

*Assay of phosphorylation of $^{14}$C-labeled β-galactosides in vitro*

Details of the assay have been described previously [3,8]. Briefly, ice-cold reaction mixtures were prepared from stock solutions so that the final volumes (usually about 300 μl) contained the following components: phosphoenolpyruvate (pH 7.3, 3 mM); potassium phosphate buffer (pH 7.4, 10 mM); KF (3 mM); dithiothreitol (2 mM): all four lactose phosphotransferase protein components (Enzyme I, histidine protein, Factor $III^{lac}$, Enzyme $II^{lac}$); $MgCl_2$ (4 mM); appropriate concentrations of either $^{14}$C-labeled methylthio β-galactoside (New England Nuclear) or $^{14}$C-labeled isopropylthio β-galactoside (Calatomic). The stock phosphotransferase protein solutions were all made using phosphate dilution buffer. Excess amounts of Enzyme I and histidine protein and rate-limiting amounts of Factor $III^{lac}$ were used, unless otherwise indicated. When appropriate, detergents were added to the mixtures after the other assay components. Complete assay mixtures were mixed briefly and incubated at 25° C, usually for 1 h. After the reaction was stopped by chilling

on ice, the samples were assayed for the presence of $^{14}$C-labeled sugar phosphate by ion-exchange chromatography as previously described [10]. In all experiments the rate of $^{14}$C-labeled sugar phosphate formation was directly proportional to the amount of Enzyme II$^{lac}$ used and was constant for at least 60 min. (Since Enzyme II$^{lac}$ is the catalyst for Reaction 4 and $P_3$-Factor III$^{lac}$ is its substrate, both components are rate limiting when Enzyme I and histidine protein are in excess and the Factor III$^{lac}$ concentration is well below its Michaelis constant.)

*Detergent inactivation experiments*

Detergents were tested for irreversible inactivation of phosphotransferase enzymes by a two-step procedure: treatment with detergent, then subsequent assay for sugar phosphorylation activity under non-inactivating conditions.

Inactivation treatments were initiated by mixing detergents with buffered solutions containing phosphotransferase proteins (final volumes usually 50—100 μl), and stopped by chilling the mixtures on ice and diluting them (to final volumes of 300 μl) with potassium dilution buffer plus the additional components required to reconstitute a complete sugar phosphorylation assay mixture. These samples were then incubated and assayed for $^{14}$C-labeled sugar phosphate as described above. Detergent concentrations in all treated samples and in untreated controls were adjusted to the same final value prior to the sugar phosphorylation incubations. The final detergent concentrations always corresponded to conditions previously shown to result in a level of sugar phosphorylation activity equal to or greater than that in control assay mixtures without detergent (see Fig. 1). Apparent first-order rate constants for inactivation, $k$, were determined from the slopes of plots of log (fraction activity remaining) versus time. Substrates, products and inhibitor of sugar phosphorylation were tested for their effects on the rate of inactivation of Enzyme II$^{lac}$ by Triton as follows: Inactivation experiments were performed at 37° C as described above, using 0.5% Triton and various concentrations of the (unlabeled) compounds tested. Subsequent assay of residual sugar phosphorylation activity was accomplished by incubation of the mixture for 60 min at 25° C in the presence of 0.1% Triton and radioactive β-galactosides. The concentrations of the latter were sufficient to completely eliminate competitive inhibition of the assay by the unlabeled substrate or substrate analog which had been present during the prior inactivation treatment. (At the low Triton concentration in the subsequent assay (0.1%) no inactivation occurs at 25° C. Furthermore, the saturating concentrations of substrate present in the assay mixture protect the enzyme against activation which might occur under more severe conditions. Thus there is no further inactivation during the assay.)

## Results

*Inactivation of Enzyme II$^{lac}$ preparations by Triton treatment*

When the rate of phosphorylation of methylthio β-galactoside was measured at 25° C at various concentrations (0—0.5%, v/v) of Triton X-100, the activity versus concentration curve of Fig. 1 (circles) was obtained. Maximal stimulation (about 2-fold in these experiments) occurred at 0.1% Triton. In another

set of experiments in 0.1% Triton (data not shown) the apparent Michaelis constant for sugar was found to be 0.27 ± 0.03 mM, compared to 0.13 ± 0.03 mM in the absence of Triton, whereas the maximum velocity was elevated 3-fold in Triton. This stimulatory effect on the sugar phosphorylation assay at 25°C is in marked contrast to an apparently irreversible inactivation of Enzyme $II^{lac}$ during prior treatment with Triton at 37°C in the absence of the other assay components (Fig. 1, squares). The kinetics of this inactivation were first order to about 10% residual Enzyme $II^{lac}$ activity (Fig. 2). Apparent inactivation rate constants, $k$, were obtained from the plots of Fig. 2, and the marked temperature dependence was analyzed. The points on a plot of ln $k$ versus (absolute temperature)$^{-1}$ gave a good fit to a single straight line whose slope corresponded to an apparent activation enthalpy of 48 kcal/mol. The rate of inactivation increases sharply with Triton concentration in the range 0.05—0.2%. The increase is most dramatic at 37°C (Fig. 3).

In order to determine whether the inactivation could be reversed by separation of the membrane from Triton, the following experiments were performed. (Triton is not easily removed from these membranes by dialysis.) A suspension of Enzyme $II^{lac}$ in 0.5% Triton at 25°C was diluted 20-fold with phosphate dilution buffer and centrifuged at 4°C for 90 min at 250 000 × $g$. Evidentally

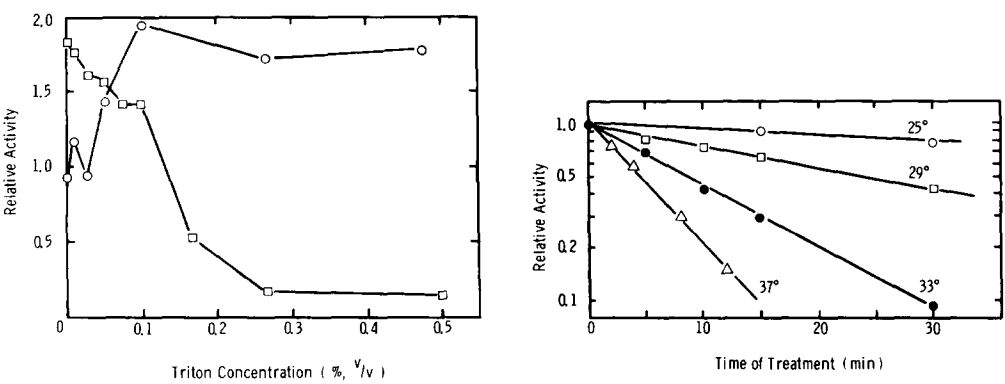


**Fig. 1. Effect of Triton X-100 on the rate of β-galactoside phosphorylation. (A) Effect of detergent present during phosphorylation assays (○). Phosphorylation assays were performed as described under Materials and Methods, using 200 μg of Enzyme $II^{lac}$ preparation, 3 μg of Factor $III^{lac}$, excess Enzyme I and histidine protein, 3.3 mM $^{14}$C-labeled methylthio β-galactoside (specific activity, $1.5 \cdot 10^5$ cpm per μmol), and the detergent concentrations indicated. Total assay volume was 300 μl. A relative activity of 1.0 corresponds to 0.5 nmol per min. Data points represent the average of duplicate determinations. (B) Effect of prior treatment of Enzyme $II^{lac}$ with Triton X-100 (□). Inactivation experiments were performed as described under Materials and Methods, using 200 μg of Enzyme $II^{lac}$ and the indicated Triton concentrations. Prior treatment was at 37°C for 30 min in a volume of 60 μl; subsequent phosphorylation assay was as described under A above, except that final Triton concentration in all assays was 0.1%.**

**Fig. 2. Time course of inactivation of Enzyme $II^{lac}$ by 0.5% Triton at various temperatures. Inactivation treatments were performed and residual Enzyme $II^{lac}$ determined by the sugar phosphorylation assay as described under Materials and Methods. Inactivation samples (volume, 60 μl) contained 50 μg Enzyme $II^{lac}$ preparation and Triton X-100 to a final concentration of 0.5%. Treatments were at the temperatures indicated: ○, 25°C; □, 29°C; ●, 33°C; △, 37°C. (In similar inactivation experiments at 37°C using 200 μg of Enzyme $II^{lac}$, the inactivation rates were the same within experimental error.) Assays of residual Enzyme $II^{lac}$ activity by the sugar phosphorylation assay at 25°C for 60 min in 0.1% Triton were as described under Materials and Methods, using 4 μg Factor $III^{lac}$, and 1 mM $^{14}$C-labeled isopropylthio β-galactose (specific activity, $10^5$ cpm per μmol).**

very little Triton adheres to the pellets obtained from this detergent-removal procedure, since a membrane sample mixed with Triton (but not heated at 37° C) before centrifugation and a control sample mixed only with aqueous buffer yielded pellets which had identical Enzyme $II^{lac}$ activities, even after subsequent resuspension in 1/20 volume and incubation for 30 min at 37° C. (This latter treatment should have inactivated any resuspended pellet in which the residual Triton concentration was greater than about 0.1%.) However, when a membrane sample was first inactivated by treatment with 0.5% Triton at 37° C for 30 min, and then diluted, sedimented, and resuspended, less than 10% of the activity of the control sample was recovered in the pellet. This is the same loss (90%) which results if the resuspended pellet from a control sample is subsequently made 0.5% in Triton and heated at 37° C for 30 min. Thus we conclude that once these membranes have been inactivated by Triton, activity cannot be restored by their physical removal from the detergent.

Since Triton and other detergents have previously been used to "solubilize" Enzyme $II^{lac}$ [2,3], it was important to determine whether the enzyme remained particulate during the detergent inactivation treatment. Membrane samples in phosphate dilution buffer containing no detergent, or containing 0.1 or 0.5% Triton X-100, were sedimented at 25° C for 90 min at 250 000 × *g*, and the Enzyme $II^{lac}$ activity remaining in the supernatant was compared to that of a control sample not subjected to centrifugation. When detergent was not present during sedimentation only 13% of the activity remained in the supernatant. In the presence of 0.1% Triton the supernatant activity was 21% of the control value, and in 0.5% Triton it was 92%. It is thus likely that the conditions of the irreversible inactivation experiments (37° C, 0.5% Triton) were such that the Enzyme $II^{lac}$ was in a non-sedimentable ("solubilized") form. The Triton concentrations used for inactivation are well above the critical micelle concentration (about 0.02% [1]).

*Protection of Enzyme $II^{lac}$ by sugar substrates and substrate analogs against inactivation by Triton*

Substrates and inhibitory substrate analogs were tested for their effect upon the rate of inactivation by Triton, using the procedure described under Materials and Methods. The lactose analog methylthio β-galactoside effectively protected Enzyme $II^{lac}$ against inactivation by 0.5% Triton at 37° C. The degree of protection, as measured by the decrease in the rate of inactivation, was a function of the sugar concentration (Fig. 4A). A strong protective effect was exhibited by the substrate analog methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside (Fig. 4B), previously shown to be a competitive inhibitor of β-galactoside phosphorylation in the presence of Triton [9]. The specificity of this protective effect is indicated by the failure of the anomer of the inhibitor, methyl 6-*O*-(*p*-toluenesulfonyl) α-galactoside, to protect. This compound is not an inhibitor of β-galactoside phosphorylation [8].

A quantitative estimate of the protective efficiency of these compounds was obtained in the following way. If it is assumed that the rate of inactivation of Enzyme $II^{lac}$ complexed with protective agent is negligible compared to that of free enzyme, then the dependence of the apparent inactivation rate $k$ upon protective agent concentration $[A]$ should follow a hyperbolic binding iso-

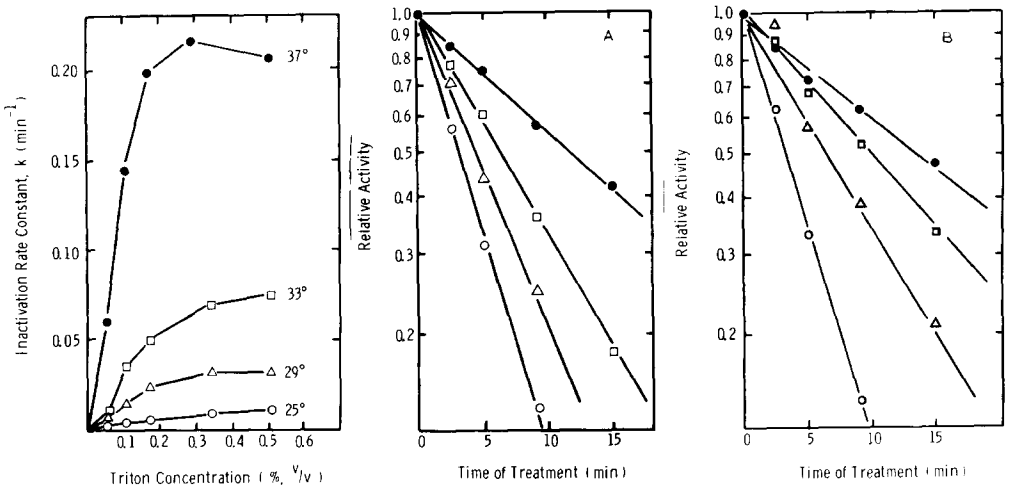


Fig. 3. Dependance of inactivation rate on concentration and temperature. Inactivation time courses similar to those of Fig. 2 were used to determine an apparent first-order inactivation rate constant, $k$, at each of the temperatures and Triton concentrations indicated. All treatment samples (volume, 60 $\mu$l) contained 50 $\mu$g of Enzyme $II^{lac}$ preparation. The treatment and subsequent assay procedures were as described under the legend for Fig. 2. ○, treatment at 25°C; △, 29°C; □, 33°C; ●, 37°C.

Fig. 4. (A) Protection of Enzyme $II^{lac}$ by substrate sugar against inactivation by Triton X-100. Samples (total volume, 60 $\mu$l) containing 150 $\mu$g Enzyme $II^{lac}$ preparation, Triton X-100 (final concentration, 0.5%), and unlabeled thiomethyl $\beta$-galactoside, at the concentrations indicated below, were treated at 37°C. Subsequent assay of residual activity (after 5-fold dilution of treatment mixtures) was as described under the legend for Fig. 2, except that the $^{14}$C-labeled isopropylthio $\beta$-galactoside was 6.7 mM. (The Michaelis constant for this substrate is about 1/10 of that for thiomethyl $\beta$-galactoside.) ○, no thiomethyl $\beta$-galactoside present during inactivation treatment; △, 0.7 mM thiomethyl $\beta$-galactoside present; □, 2.2 mM thiomethyl $\beta$-galactoside present; ●, 8.2 mM thiomethyl $\beta$-galactoside present. (B) Protection of Enzyme II by substrate sugar analog against inactivation by Triton X-100. Samples (final volume, 60 $\mu$l) containing 150 $\mu$g Enzyme $II^{lac}$ preparation. Triton X-100 (final concentration, 0.5%) and the indicated concentrations of methyl-6-*O*-(*p*-toluenesulfonyl) $\beta$-galactoside were treated at 37°C, then diluted and assayed as described under the legend for A. ○, no methyl-6-*O*-(*p*-toluenesulfonyl) $\beta$-galactoside present during inactivation treatment; △, 0.63 mM methyl-6-*O*-(*p*-toluenesulfonyl) $\beta$-galactoside present; □, 1.25 mM methyl-6-*O*-(*p*-toluenesulfonyl) $\beta$-galactoside present; ●, 2.5 mM methyl-6-*O*-(*p*-toluenesulfonyl) $\beta$-galactoside present.

therm,

$$k = k_0 K_{pr}/([A] + K_{pr}) \qquad (5)$$

Here $k_o$ is the inactivation rate for free enzyme and $K_{pr}$ is the concentration of protective agent which results in half-maximal protection. This latter parameter can thus be determined from plots of $1/k$ versus $[A]$.

In Table I, the $K_{pr}$ determined thus for the substrate methylthio $\beta$-galactoside is compared to the Michaelis constants ($K_m$ values) for this compound under various conditions. Similarly, the $K_{pr}$ for the inhibitor methyl 6-*O*-(*p*-toluenesulfonyl) $\beta$-galactoside is compared to its inhibition constants ($K_i$ values). For the tosyl galactoside inhibitor, the agreement between $K_{pr}$ and $K_i$ is fairly good. For the substrate sugar, however, the $K_{pr}$ value is much higher than the $K_m$. It may be that the structure of the tosyl sugar is such that it protects Enzyme $II^{lac}$ more efficiently when bound to the membrane. However, the comparison here is of $K_{pr}$ values, which reflect static binding of sugar

TABLE I

COMPARISON OF KINETIC PARAMETERS WITH PROTECTION PARAMETERS

Micahelis constants ($K_m$ values) for methylthio β-galactoside at the conditions indicated were obtained from double reciprocal plots of the data from sugar phosphorylation experiments performed as described under Methods and Materials. Samples contained 50 μg of Enzyme $II^{lac}$ preparation, and the final concentration of Factor $III^{lac}$ was 0.1 μM. (The $K_m$ for sugar is not strongly dependent on Factor $III^{lac}$ concentration; see ref. 9.) Enzyme I and histidine protein were present in excess. The inhibition constants ($K_i$ values) for methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside are those determined previously under similar conditions. The parameter $K_{pr}$ is the concentration necessary for half-maximal protection of Enzyme $II^{lac}$. Values of $K_{pr}$ were obtained from the data of Fig. 3 as described in the text.

| Parameter | Temperature | Triton concentration (v/v) | Data for methylthio β-galactoside (mM) | Data for methyl 6.-*O*-(*p*-toluenesulfonyl) β-galactoside (mM) |
|---|---|---|---|---|
| $K_m$ | 37 | None | 0.15 | — |
| $K_m$ | 25 | 0.1% | 0.37 | — |
| $K_m$ | 25 | None | 0.13 | — |
| $K_i$ | 37 | None | — | 0.34 |
| $K_i$ | 25 | 0.1% | — | 0.22 |
| $K_{pr}$ | 37 | 0.5% | 3.42 | 0.78 |

and inhibitor to Enzyme $II^{lac}$ in 0.5% Triton at 37°C, with $K_m$ and $K_i$ values, which reflect the steady-state level of binding during the phosphorylation proc process in 0.1% Triton at 25°C. Thus the reason for the greater protection by the tosyl sugar cannot be unequivocally determined.

A further experiment was performed to determine whether the protective effect was exerted only at the beginning of the inactivation (e.g. protection against some hypothetical event by which the detergent "primes" the membranes for inactivation) or whether protection could be exerted at any time

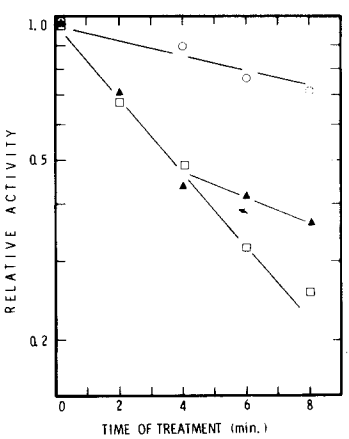


Fig. 5. Effect of time of addition on protection of Enzyme $II^{lac}$ by a sugar substrate analog. Tubes containing identical samples (volume, 0.45 μl) of Enzyme $II^{lac}$ (50 μg) dissolved in 0.66% Triton X-100, and the indicated additions, were treated at 37°C. At 2-min intervals, one tube from each set was withdrawn and chilled rapidly to 0°C. All tubes were subsequently diluted and assayed for residual sugar phosphorylation activity as described in the legend for Fig. 4A. □, control (15 μl of phosphate dilution buffer added); ○, 15 μl of 10 mM methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside inhibitor added at the beginning of the 37°C treatment; ▲, same amount of inhibitor added after 4 min of treatment.

during the time course of inactivation. The results (Fig. 5) indicate that the tosyl sugar can form a detergent-resistant complex with Enzyme $II^{lac}$ at any stage during the inactivation process.

*Effects of other compounds on inactivation of Enzyme $II^{lac}$ by Triton*

Although sugar substrates and analogs substantially slowed the rate of irreversible inactivation of Enzyme $II^{lac}$ in Triton, the reaction product galactose 6-phosphate slightly stimulated the inactivation rate. In 0.5% Triton at 37°C the inactivation rate constant, 0.14 $min^{-1}$, increased to 0.17 $min^{-1}$ in 33 mM galactose 6-phosphate and to 0.20 $min^{-1}$ at 66 mM. Sodium phosphate or glucose 6-phosphate added at similar concentrations had no effect.

As Eqn. 4 indicates, phospho-Factor $III^{lac}$ is a substrate for Enzyme $II^{lac}$, whereas the unphosphorylated protein is actually a product of the sugar phosphorylation reaction. Several preparations of Factor $III^{lac}$ were tested for their ability to protect Enzyme $II^{lac}$ against inactivation by Triton. Some of these preparations were purified to homogeneity as described under Materials and Methods; others (about 75% pure) were purified through the DEAE-cellulose step. In all cases the Factor $III^{lac}$ itself was shown not to be irreversibly inactivated by the Triton treatment (0.5% Triton, 37°C, 15 min). (Enzyme I and histidine protein were also stable under these conditions.) In several experiments, the presence of unphosphorylated Factor $III^{lac}$ (final concentration about 10 $\mu$M) significantly reduced the rate of Triton inactivation. This effect could not be reproduced using all of the Factor $III^{lac}$ preparations, however. Even the two homogeneous preparations used gave differing results. In all cases, though, phospho-Factor $III^{lac}$, the actual substrate for Enzyme $II^{lac}$, did not protect. Thus when a phosphogenerating system (phosphoenolpyruvate, $Mg^{2+}$, Enzyme I and histidine protein) was included in the detergent treatment mixture along with Factor $III^{lac}$, Enzyme $II^{lac}$ was inactivated at the same rate as in a control sample without added proteins. In a simultaneous experiment, using this particular Factor $III^{lac}$ preparation, in which the same proteins were included but phosphoenolpyruvate was omitted from the phosphogenerating system, the rate of inactivation was significantly less (about 20%) than that of the control sample.

It has been reported by Lanyi [11,12] that lipid-protein interactions in *Halobacterium cutirubrum* membranes could be stabilized against lysis by Triton X-100 by divalent cations. In the case of Enzyme $II^{lac}$, however, we found no change in the rate of inactivation by Triton in the presence of $MgCl_2$ (4—100 mM), or when EDTA (4.4 mM) was added and $Mg^{2+}$ was omitted. The effects of all agents on the Triton inactivation rate are summarized in Table II.

*Effects of other non-ionic detergents*

The effects of the non-ionic detergents Tween 40 and Brij 35 are qualitatively similar to those of Triton X-100, but are considerably milder. The maximal stimulation of the sugar phosphorylation assay occurs at much lower detergent concentrations (0.002% versus 0.1%) and the degree of stimulation is less. The extent of irreversible inactivation of Enzyme $II^{lac}$ by Tween and Brij is only 15% under conditions (0.5% detergent, 37°C, 15 min) where it is rapidly and completely inactivated by Triton.

TABLE II

SUMMARY OF EFFECTS OF SUBSTRATES, PRODUCTS, INHIBITORS AND $Mg^{2+}$ ON IRREVERSIBLE INACTIVATION OF ENZYME $II^{lac}$ BY TRITON X-100

| Compound | Role in reaction | Effect on inactivation rate |
|---|---|---|
| Methylthio β-galactoside | Substrate | Reduces inactivation |
| Methyl 6-*O*-(*p*-toluenesulfonyl) β-galactoside | Inhibitory substrate analog | Reduces inactivation |
| Methyl 6-*O*-(*p*-toluenesulfonyl) α-galactoside | Non-inhibitory anomer of substrate analog | No effect |
| Galactose 6-phosphate | Product | Slightly enhances inactivation |
| Factor $III^{lac}$ | Product | Reduces inactivation * |
| Phospho-Factor $III^{lac}$ | Substrate | No effect |
| $Mg^{2+}$ | — | No effect |

* Some Factor $III^{lac}$ preparations had no effect.

## Discussion

A dispersion in Triton X-100 of membrane fragments containing Enzyme $II^{lac}$ changes its properties as the detergent concentration or temperature (or both) are increased. The principle observations are: (1) maximum stimulation (2—3-fold) of Enzyme $II^{lac}$ at 25° C by 0.1% Triton; (2) solubilization (without inactivation) at 25° C by 0.3—0.5% Triton; (3) irreversible, rapid inactivation at detergent concentrations and temperatures above the narrow range for optimum solubilization; (4) protection of Enzyme $II^{lac}$ against irreversible inactivation by certain of its substrates and substrate analogs.

Triton X-100 is the detergent of choice for solubilization of many membrane-bound enzymes. The narrow range of temperatures and detergent concentrations for optimal solubilization observed here serves to reemphasize the importance of careful adjustment of these parameters. For instance, the half-life of Enzyme $II^{lac}$ in 0.2% Triton decreases 10-fold between 29 and 37° C. For this enzyme sugar substrate and substrate analogs provide strong protection against inactivation in the presence of detergent. This result suggests that some membrane-bound enzymes, refractory to solubilization under milder conditions, might be succesfully extracted by more drastic treatments in the presence of protective substrates. Our results further suggest that some substrate analogs may prove to be more protective than might be predicted on the basis of their apparent steady-state affinities under reaction conditions.

Protection by substrates against heat inactivation has been described for a variety of soluble enzymes [13—15]; a parameter analogous to the $K_{pr}$ here was determined by Sudi [15] for lactate dehydrogenase. Although protection of membrane-bound enzymes by substrates against loss of activity during extraction or storage has been described [16], neither the inactivation process

nor the protective effect was analyzed quantitatively. Lanyi [11,12] found that the plasma membrane of respiring cells of *H. cutirubrum* were much more resistant to lysis by Triton X-100 than those of cells poisoned with respiration inhibitors. It is not known whether or not this protection was a direct result of substrates interacting with respiratory enzymes in the membrane. In the present study, the inactivation is a well-defined first-order process, so that the effect of both reaction conditions and protective ligands can be determined quantitatively. This has two potentially useful consequences. First, it offers a novel probe for investigation of the nature of the interactions between this particular transport enzyme and other components of the membrane, and of the effect of substrates upon these interactions. Further studies should include isolation of both active and inactive membrane-detergent complexes, and their chemical and physical characterization, as in the studies of phospholipid-Triton complexes by Dennis and coworkers [17—20] and of detergent-protein complexes by Helenius and Simons [21]. Second, effects upon inactivation rate provide a convenient assay for the interaction of putative ligands with the enzyme, including interactions not demonstrable by other techniques. For instance, we demonstrate here a specific effect upon inactivation by the product analog, galactose 6-phosphate, which does not inhibit the action of Enzyme $II^{lac}$ in vitro.

The actual mechanism of the inactivation is not known. One possible trivial explanation, chemical attack by some contaminant in the Triton preparation, seems unlikely. Triton does not inactivate Factor $III^{lac}$, histidine protein, or Enzyme I (a very labile protein [3]) under the experimental conditions, and the dependence of the Enzyme $II^{lac}$ inactivation rate upon detergent concentration (Fig. 3) is not linear, but is instead more suggestive of a cooperative interaction over a critical concentration range. Another possible trivial explanation is detergent activation of a membrane-bound protease. However, the procedure used to prepare the membranes was quite effective in removing peripheral proteins, such as Factor $III^{lac}$. Furthermore, Factor $III^{lac}$ and the other soluble proteins were not inactivated by Triton in the presence of membranes. Thus the putative protease would have to be tightly membrane-bound, and, even when activated, ineffective against soluble proteins. The membrane preparation is too heterogeneous to permit identification of an Enzyme $II^{lac}$ band by gel electrophoresis in dodecyl sulfate, which would otherwise permit a specific assay for a putative protease. Thus we cannot rule out rate-limiting attack by a protease with the postulated properties, subsequent to a detergent-mediated step. Nevertheless, the most likely explanation would seem to be that the detergent perturbs the interactions among the lipid and protein components of the membrane.

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