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Nucleophile in the Active Site of *Escherichia coli* Galactose-1-phosphate Uridylyltransferase: Degradation of the Uridylyl-enzyme Intermediate to N^3-Phosphohistidine†

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ABSTRACT: The [^{32}P]uridylyl-enzyme intermediate form of *Escherichia coli* galactose-1-P uridylyltransferase can be converted to a [^{32}P]phosphoryl-enzyme by first cleaving the ribosyl ring with $NaIO_4$ and then heating at pH 10.5 and 50 °C for 1 h. After alkaline hydrolysis of the [^{32}P]phosphoryl-enzyme the major radioactive product is N^3-[^{32}P]-phosphohistidine. A lesser amount of $^{32}P_i$ is also produced as a side product of the hydrolysis of N^3-[^{32}P]phosphohistidine. No N^1-phosphohistidine, N^ϵ-phospholysine, or phosphoarginine can be detected in these hydrolysates. It is concluded that the nucleophile in galactose-1-P uridylyltransferase to which the uridylyl group is bonded in the uridylyl-enzyme intermediate is imidazole N^3 of a histidine residue. This degradation procedure should have general applicability in the degradation and characterization of nucleotidyl-proteins.

Structural characterization of covalently bonded enzyme–substrate intermediates and covalently modified regulated enzymes remains a challenging task in biochemistry. Among the known covalently modified enzymes, only the phosphoryl-enzymes and the aldimine(or ketimine)-enzymes are routinely subject to general degradative procedures for identifying the nature of covalent bonding and the amino acid residue involved.

The nucleotidyl-enzymes which have been discovered and studied over the past 10 years present special degradation problems, some of which are well exemplified by the uridylylgalactose-1-P uridylyltransferase discovered and studied in this laboratory (Wong & Frey, 1974a; Wong et al., 1977). This uridylyl-enzyme is the intermediate in the interconversion of galactose-1-P and UDP-glucose with glucose-1-P and UDP-galactose (Wong & Frey, 1974b; Wong et al., 1977). The uridylyl group is known from the hydrolytic properties of the uridylyl-enzyme to be bonded to a nitrogen atom in the enzyme. On the basis of data from chemical modification with diethyl pyrocarbonate, it appears that this may be one of the nitrogen atoms in the imidazole ring of a histidine residue (Wong et al., 1977). Direct degradation of the intermediate to uridylylhistidine for structural characterization is not presently feasible because the protein is refractory to enzymatic degradation. Moreover, the acid lability of the phosphoramidate linkage and the instability of the uracil ring in strongly alkaline solutions rule out the possibility that the uridylyl-enzyme might be hydrolyzed directly to a uridylyl amino acid.

In this paper we report on the degradation of this uridylyl-enzyme to a phosphoryl-protein and then to N^3-phosphohistidine by a procedure which should have general applicability for characterizing nucleotidyl-proteins when the nucleotidyl group is bonded to serine, threonine, lysine, arginine, or histidine.

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Materials and Methods

Enzymes, Coenzymes, and Substrates. Galactose-1-P was purified from a regulatory mutant of *Escherichia coli* (ATCC-27797) by the procedure of Saito et al. (1967) as modified by Wong et al. (1977). Phosphoglucomutase and glucose-6-P dehydrogenase used in the assay of uridylyltransferase were purchased from Sigma Chemical Co. UDP-glucose, UDP-galactose, glucose-1-P, galactose-1-P, Bicine buffer, NADP,[1] and phosphoarginine were purchased from Sigma Chemical Co. [α-^{32}P]UTP was purchased from New England Nuclear, and [*uracil*-4-^{14}C]UTP was purchased from Amersham/Searle. Uridine [α-^{32}P]diphosphate glucose and [*uracil*-4-^{14}C]UDP-glucose were prepared from correspondingly labeled UTP as described by Wong et al. (1977). N^3-Phosphohistidine and N^1-phosphohistidine were synthesized as described by Hultquist et al. (1966). N^ϵ-Phospholysine was synthesized as described by Zetterqvist & Engstrom (1967).

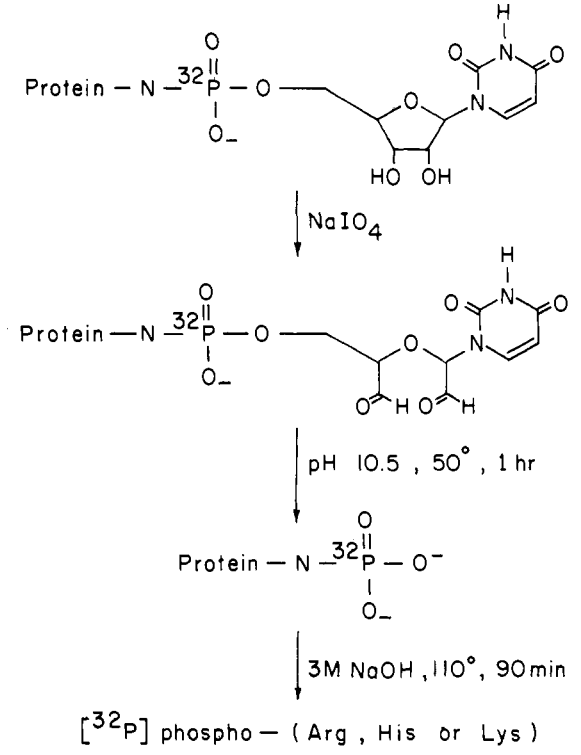
Assays. Galactose-1-P uridylyltransferase was assayed by the standard method described by Wong & Frey (1974a,b). α-Amino acids in column effluents were assayed by the ninhydrin method described by Moore (1968). The Pauly test for histidine in column effluents was carried out by the procedure described by DeLuca et al. (1963). Phosphate in column effluents was measured as described by Lowry & Lopez (1946). Radiochemical assays were carried out on aliquots of aqueous samples by liquid scintillation counting in a Packard Model 3310 Tri-Carb liquid scintillation spectrometer.

Preparation of [^{32}P]Phosphoryl-enzyme. [^{32}P]Uridylylgalactose-1-P uridylyltransferase and [*uracil*-4-^{14}C]uridylylgalactose-1-P uridylyltransferase were prepared by reacting the enzyme with a mixture of correspondingly labeled UDP-glucose samples and precipitating the intermediates with perchloric acid as described by Wong et al. (1977). The washed precipitate was dissolved in a minimal volume of 0.1 M NaOH and dialyzed against 4 L of 50 mM sodium carbonate buffer at pH 10.5 for 4 h. All operations were carried out at 4 °C, and the labeled protein was stored at −10 °C. The doubly labeled uridylyl-enzyme was prepared to contain 6×10^6 cpm/μmol of ^{32}P and 4.5×10^6 cpm/μmol of ^{14}C. Samples of [^{32}P]phosphoryl-enzyme were prepared as needed from aliquots of labeled uridylyl-enzyme. To the labeled uridylyl-enzyme was added $NaIO_4$ in 10-fold excess of bound uridylyl groups. After 30 min at room temperature, a fivefold excess of ethylene glycol relative to periodate was added to destroy periodate. The solution was heated for 60 min at 50 °C to effect the elimination of the cleaved nucleoside, and the [^{32}P]phosphoryl-enzyme was isolated by gel exclusion chromatography through a column of Bio-Gel P-2. The periodate degradation and alkaline elimination were carried out in 50 mM Na_2CO_3 buffer at pH 10.5, and chromatography was carried out in 5 mM $NaHCO_3$ at pH 10.5.

Chromatographic Identification of N^3-Phosphohistidine. Samples of [^{32}P]phosphoryl-enzyme (0.5 mg) were subjected to hydrolysis in 3 M NaOH at 110 °C for 90 min in very dilute solution (24 mL, 0.02 mg of protein/mL, 3 M NaOH) to minimize randomization of the label. Wålinder (1969) did not observe serious randomization of ^{32}P in phosphoryl-proteins hydrolyzed under otherwise similar conditions at 1 mg of protein/mL. The hydrolysates were diluted 15-fold with water and adsorbed on 1.4×33 cm columns of Dowex 1-X8 resin which had been equilibrated with 5 mM $KHCO_3$. Samples of carrier sodium phospholysine, sodium phosphoarginine, and lithium N^3-phosphohistidine were then adsorbed on the resin, and the columns were eluted with a 600-mL linear gradient of 0.25–0.60 M $KHCO_3$ essentially as described by Wålinder et al. (1968). Column fractions were analyzed for ^{32}P, α-amino acids (ninhydrin), histidine (Pauly test), and P_i.

[1] Abbreviations used: NADP, nicotinamide adenine dinucleotide phosphate; UTP, uridine 5′-triphosphate.

Scheme I



Results

Our strategy for structurally characterizing uridylylgalactose-1-P uridylyltransferase is outlined in Scheme I. The [^{32}P]uridylyl-enzyme is prepared and degraded to the corresponding [^{32}P]phosphoryl-protein by specific C–O bond cleavage at ribosyl C-5 of the uridyl group. The [^{32}P]-phosphoryl-protein is then subjected to the general degradation procedures that have been used for other phosphoryl-proteins. In the present case, since it is known that the uridylyl group is bonded to a nitrogen atom in the enzyme (Wong et al., 1977), the [^{32}P]phosphoryl-protein is subjected to alkaline hydrolysis and the hydrolysates are analyzed for ^{32}P-labeled phosphoarginine, phospholysine, and phosphohistidine.

Appropriate conditions for effecting the degradation of the uridylyl-enzyme to a phosphoryl-enzyme were devised in ^{14}C/^{32}P double labeling experiments. The results given in Figure 1 were obtained. The [*uracil*-4-^{14}C,^{32}P]uridylyl-enzyme was prepared to contain comparable amounts of ^{14}C and ^{32}P. This was reacted with a 10-fold excess of sodium periodate at pH 10.5 for 30 min. Ethylene glycol was then added in fivefold excess over periodate, and the solution was heated at 50 °C for 1 h. Passage of this solution over a Bio-Gel P-2 column effected a clean separation of ^{32}P, which eluted with the protein, from ^{14}C, which eluted in back fractions with small molecular weight materials (Figure 1A). The ^{14}C and ^{32}P were eluted together with the protein when periodate was omitted (Figure 1B). These conditions were adopted for preparing the [^{32}P]phosphoryl-protein.

The [^{32}P]phosphoryl-protein was subjected to alkaline hydrolysis in 3 M NaOH at 110 °C for 90 min, 10 °C higher temperature and half the time described by Wålinder et al.

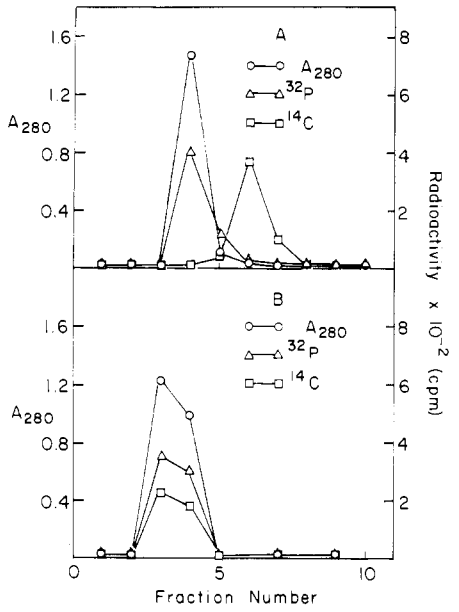


FIGURE 1: Degradation of [^{32}P,^{14}C]uridylyl-enzyme to [^{32}P]-phosphoryl-enzyme. (A) A sample of [^{32}P,^{14}C]uridylyl-enzyme consisting of 0.57 mg of protein, 8.9×10^4 cpm of ^{14}C, and 7×10^4 cpm of ^{32}P in 1.0 mL of 50 mM sodium carbonate buffer at pH 10.5 was treated with 0.5 mM $NaIO_4$ at room temperature for 30 min. After the addition of ethylene glycol to 2.5 mM, the solution was heated at 50 °C for 1 h. The cooled solution was applied to the top of a 0.7 × 18 cm column of Bio-Gel P-2 (100–200 mesh) that had been equilibrated with 5 mM sodium carbonate buffer at pH 10.5 and 4 °C. The column was eluted with the same buffer at 0.7 mL/min, and 1.2–1.5-mL fractions were collected. The fractions were analyzed for A_{260}, ^{32}P, and ^{14}C. (B) An identical sample of [^{32}P,^{14}C]uridylyl-enzyme was subjected to the identical procedures with the exception that $NaIO_4$ was omitted.

(1968). These conditions were adequate to hydrolyze the protein without fully hydrolyzing phosphorylated basic amino acids, and the hydrolysis was carried out in dilute solution to minimize randomization of the [^{32}P]phosphoryl groups among the endogenous basic amino acids in the hydrolysate. The hydrolysate was subjected to anion-exchange chromatography together with carrier phosphoryl amino acids on a column of Dowex 1-X8 resin under conditions known to separate the phosphorylated basic amino acids from phosphate and from one another. In numerous experiments the ^{32}P was invariably found to cochromatograph with orthophosphate and N^3-phosphohistidine, which were well separated from each other and from phosphoarginine and phospholysine. The latter two were never found to be labeled with ^{32}P. The overall recovery of ^{32}P was 95%, of which 50–60% was associated with N^3-phosphohistidine.

As shown in the elution profile given in Figure 2, the first radioactive band eluted from the column was well separated from phosphorylated amino acid standards. This was shown in other experiments to cochromatograph with Na_3PO_4, and it no doubt originated with partial hydrolysis of the major product, N^3-phosphohistidine. The second radioactive band, the major product, corresponded exactly with the elution position of N^3-phosphohistidine.[2] This latter material was rechromatographed with carrier N^1-phosphohistidine in Figure 3, and the radioactivity failed to cochromatograph with

[2] The minor products eluted after N^3-phosphohistidine in Figure 2 have not been identified. They are present in variable amounts and are thought to be [^{32}P]oligopeptides resulting from incomplete hydrolysis in the minimal alkaline hydrolysis conditions. Minimal hydrolytic conditions are used to minimize secondary hydrolysis of phosphoryl amino acids.

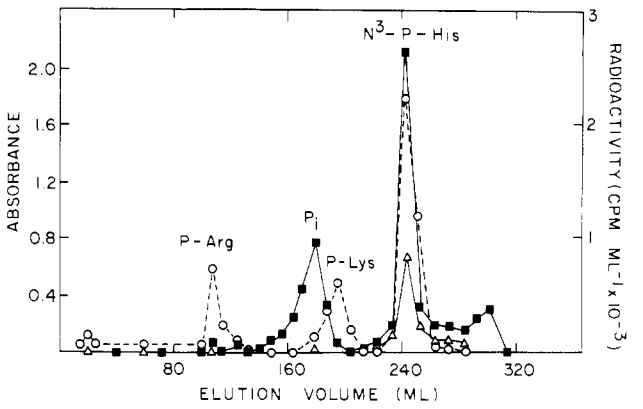


FIGURE 2: Ion-exchange chromatography of the alkaline hydrolysate of [^{32}P]phosphoryl-enzyme. The hydrolysis of [^{32}P]phosphoryl-enzyme and chromatography on Dowex 1-X8 ion-exchange resin are described in detail under Materials and Methods, as are the analytical methods used for surveying the column effluent: (○) amino acids detected by the ninhydrin method; (■) ^{32}P; (△) Pauly test for the imidazole ring of histidine after acid hydrolysis of phosphohistidine.

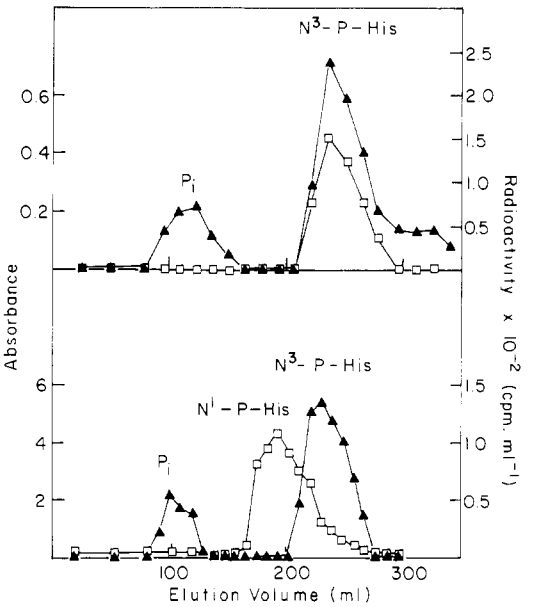


FIGURE 3: Identification of [^{32}P]phosphohistidine as N^3-[^{32}P]-phosphohistidine. In the upper panel the fractions containing [^{32}P]phosphohistidine and carrier N^3-phosphohistidine in Figure 2 were pooled, diluted sixfold with H_2O, readsorbed on the Dowex 1-X8 ion-exchange column, and again chromatographed by the same elution schedule used in Figure 2. In the lower panel the fractions containing carrier N^3-phosphohistidine and [^{32}P]phosphohistidine were pooled, diluted sixfold with H_2O, combined with 45 mg of carrier N^1-phosphohistidine, readsorbed on the ion-exchange column, and again rechromatographed. (▲) ^{32}P; (□) absorbance at 480 nm from the Pauly test for histidine.

N^1-phosphohistidine. In Figure 3 the amount of N^1-phosphohistidine carrier added to the sample was 10 times the amount of N^3-phosphohistidine carrier present in the sample from Figure 2, so that the N^3-phosphohistidine carrier appears only as a shoulder of Pauly-positive material corresponding to the position of the ^{32}P in Figure 3. The leading ^{32}P-containing band eluted in Figure 3 was inorganic phosphate, which originated with a small degree of hydrolysis of N^3-[^{32}P]-phosphohistidine obtained from the column elution in Figure 2. This appeared upon rechromatography of N^3-[^{32}P]-phosphohistidine independently of whether other carrier phosphoryl amino acids were present, as shown in Figure 3. The separation of N^1- and N^3-phosphohistidine in Figure 3 was very comparable with that reported by Wålinder (1969).

We conclude that the radioactive phosphoryl amino acid resulting from alkaline hydrolysis of the [^{32}P]phosphoryl-enzyme is N^3-[^{32}P]phosphohistidine and that the active site nucleophile is N^3 of the imidazole ring of a histidine residue. The possibility that the actual site of phosphorylation may be the N^1 nitrogen is difficult to exclude rigorously because N^1-[^{32}P]phosphohistidine might have rearranged to N^3-[^{32}P]phosphohistidine during alkaline hydrolysis. This happens when N^1-phosphohistidine is subjected to the hydrolytic conditions in the presence of a pool of histidine. However, this is very unlikely to have occurred under the conditions used in our experiments because they were carried out in dilute solution without carrier amino acids or phosphoryl amino acids, and no other randomization of the ^{32}P, i.e., [^{32}P]phosphoarginine or [^{32}P]phospholysine formation, was detected. Moreover, Wålinder (1969) reported that these hydrolytic conditions do not lead to randomization of ^{32}P between N^1- and N^3-phosphohistidine even at 1 mg of protein/mL.

Discussion

The degradation of [^{32}P]uridylyl-enzyme to a [^{32}P]-phosphoryl-protein described here is similar to the methods which have been developed for removing the 3′-terminal ribonucleoside from RNA (Brown et al., 1953; Whitfield & Markham, 1953; Neu & Heppel, 1964). In RNA degradation the elimination step is promoted by a primary amine which may function both as a general base to abstract the C-4′ proton and as a covalent catalyst to labilize the C-4′ proton by iminium ion formation with the C-3′ aldehyde group resulting from periodate cleavage. In the degradation of uridylyl-enzyme we do not include the amine because of the possibility that the uridylyl or phosphoryl groups might undergo transfer to the primary amine. Phosphoramidates are known to be susceptible to transphosphorylation. We instead carry out the elimination reaction at a higher pH and rely upon hydroxide ion to promote the elimination. This procedure appears to give satisfactory results while avoiding the potential complications associated with the presence of amines. Brown et al. (1953) and Whitfield & Markham (1953) originally described the facile elimination at pHs 10.5 and 10.

The general approach adopted here could probably also be applied to nucleotidyl-proteins in which the nucleotide is bonded to the hydroxyl group of serine or threonine. The [^{32}P]phosphoryl-protein produced from periodate cleavage and elimination of the nucleoside would be subjected to minimal acid hydrolysis to produce *O*-[^{32}P]phosphoserine or *O*-[^{32}P]phosphothreonine. The elimination conditions are not sufficiently alkaline to lead to β elimination of $^{32}P_i$ from [^{32}P]phosphoserine, which requires 0.1 M NaOH (Perlmann, 1955), so conversion of such a nucleotidyl-protein to a phosphoryl-protein in good yield can be expected. To date, however, none of the known nucleotidyl-proteins appear to involve serine or threonine as recipients of nucleotidyl groups.[3]

Other nucleotidyl-proteins that have been studied are the adenylyl-DNA ligases from *E. coli* and T4 (Lehman, 1974), the adenylylglutamine synthetase from *E. coli*, and the uridylyl uridylyltransferase which regulates adenylylation and deadenylylation of *E. coli* glutamine synthetase (Stadtman & Ginsburg, 1974). The latter two have been shown by spectral and amino acid analyses of adenylyl- and uridylyl-peptides obtained from enzymatic degradations to involve the phenolic hydroxyl group of tyrosine as the site of nucleotide binding (Shapiro & Stadtman, 1968; Adler et al., 1975). The adenylyl-DNA ligases have been degraded to inosylyl-N^{ϵ}-lysine in modest yield by alkaline hydrolysis (Gumport & Lehman, 1971).

The degradation of [^{32}P]uridylylgalactose-1-P uridylyltransferase to N^3-[^{32}P]phosphohistidine described here directly implicates a histidyl residue at the active site of this enzyme, and the uridylyl group in the uridylyl-enzyme we have isolated is bonded to N^3 of this residue.

Our earlier postulate that the active site nucleophile may be a histidyl residue was based upon the hydrolytic properties of the uridylyl-enzyme and upon the interactions of the enzyme with diethyl pyrocarbonate. This reagent was shown to inactivate the enzyme at pH 6 very efficiently, whereas in the presence of UDP-glucose the inactivation was blocked. That this inactivation probably resulted from the reaction of a histidine residue with diethyl pyrocarbonate was indicated by the fact that NH_2OH reversed the inactivation. That the histidyl residue was probably the nucleophile bonded to the uridylyl group in the active site was indicated by the fact that galactose-1-P failed to protect the enzyme from inactivation by diethyl pyrocarbonate and, more directly, by the fact that the isolated uridylyl-enzyme was found to be highly resistant to inactivation by this reagent. All of these provisional conclusions, which were based on indirect evidence, have now been verified by the direct degradation of the uridylyl-enzyme to N^3-phosphohistidine.

The only residual doubt that the active site nucleophile is N^3 of the imidazole ring of a histidine at the active site is based on the highly remote possibility that the uridylyl-enzyme we have isolated is not the true intermediate but is merely in equilibrium with the true uridylyl-enzyme intermediate. We consider this very unlikely to be the case for the following reasons.

First, as discussed in detail in our earlier paper, we are unable to find evidence of the involvement of any other nucleophilic group in the active site (Wong et al., 1977). If there is another nucleophile involved in catalysis, it must be shielded from reaction with reagents other than substrates, it must be in close proximity to the histidine we have identified in the active site, and the uridylyl group in the intermediate must be partitioned between N^3 of this histidine and the hypothetical nucleophile.

Second, if the uridylyl group in the intermediate is partitioned in this way, we must conclude on the basis of our data that the uridylyl-N^3-histidyl species dominates the equilibrium. Otherwise we could not expect the uridylyl-enzyme to be so unreactive with diethyl pyrocarbonate, and N^3-phosphohistidine would not have been isolated in such good yield. If such an equilibrium involved an N^{ϵ}-uridylyllysyl species as a significant component, we should have detected part of the ^{32}P in N^{ϵ}-phospholysine in this work. If the hypothetical nucleophile were to have the serine, threonine, or tyrosine hydroxyl groups, the carboxyl group of a glutamate or aspartate residue, or the sulfhydryl group of a cysteine as significant components, the alkaline hydrolysis conditions would have converted all of these to [^{32}P]phosphate or [^{32}P]thiophosphate, with the result that the elution profile in Figure 2 would have exhibited an atypically large percentage of ^{32}P associated with P_i rather than with N^3-phosphohistidine. The ratio of ^{32}P in P_i to that in N^3-phosphohistidine in several experiments similar to that of Figure 2 was between 1.0 and 0.6, which is typical of that expected for phosphoryl-proteins that have been subject to minimal alkaline hydrolysis when the phosphoryl group is bonded to basic amino acids (Wålinder

[3] The hydrolytic properties of adenylyl-5′-nucleotide phosphodiesterase suggest that the adenylyl group may be bonded to a seryl or threonyl residue (Landt & Butler, 1978).

et al., 1968). Kreil & Boyer (1964) found this ratio to be as small as 0.15 when the hydrolysis was carried out under milder conditions (3 M NaOH, 80 min, 100–105 °C), but about one-fourth of the phosphoryl groups remained as phosphoryl-peptides under these conditions.

Third, if the uridylyl group were partitioned between the histidyl residue and another nucleophile in close proximity, the required dominance of the N^3-uridylylhistidyl form in the equilibrium limits the possibilities for the hypothetical nucleophile. Phosphoramidates are very high-energy species relative to esters, so it is highly improbable that a minor equilibrium component could involve serine, threonine, tyrosine, or cysteine. This leaves the ϵ-amino group of lysine or the β- or γ-carboxyl groups of aspartate or glutamate as plausible alternative nucleophiles in such an equilibrium. Yet our group-selective chemical modification studies give no evidence of the involvement of such functional groups (Wong et al., 1977).

We conclude that N^3 of the imidazole moiety of a histidyl residue at the active site of *E. coli* galactose-1-P uridylyltransferase is bonded to the uridylyl group in the uridylyl-enzyme intermediate and that the possibility of any other nucleophilic group being involved in bonding the uridylyl group is remote.

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Escherichia coli Phosphoenolpyruvate Dependent Phosphotransferase System. Complete Purification of Enzyme I by Hydrophobic Interaction Chromatography†

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ABSTRACT: We have observed that E_I possesses extremely hydrophobic surface regions. In light of this property, a high yield, rapid procedure has been developed for the complete purification of E_I using solely hydrophobic interaction chromatography on commercially available resins. The entire procedure can be completed in 4–5 days with a 55–65% recovery of E_I activity and an 870-fold purification.

E_I catalyzes the first reaction in the phosphoenolpyruvate dependent transport of sugar via the phosphotransferase system as described by Roseman and co-workers (1969).

$$PEP^1 + HPr \underset{Mg^{2+}}{\overset{E_I}{\leftrightarrow}} P\text{–}HPr + \text{pyruvate}$$

It is one of the common components of all phosphoenolpyruvate dependent phosphotransferase systems (Hengstenberg, 1977; Saier, 1977; Cirillo & Razin, 1973). Along with HPr, it is found in the soluble portion of *Escherichia coli* crude cell extract and has been classified as cytoplasmic in origin.

Since E_I is the initial catalyst in the process coupling phosphoenolpyruvate hydrolysis, phosphoryl group transfer,

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[1] Abbreviations used: PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate dependent phosphotransferase system; $NaDodSO_4$, sodium dodecyl sulfate; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol; Sepharose and Sephadex are registered trademarks of Pharmacia Fine Chemicals; ptsI is a mutant deficient in E_I.

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