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†

G. T. Robillard,* G. Dooijewaard, and J. Lolkema

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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and sugar transport, chemical and kinetic studies should be able to provide substantial insight into these processes and their regulation. Such studies, however, are best executed with pure enzyme. Unfortunately, no purification procedure has ever been published which resulted in a homogeneous preparation of E_I, despite a number of attempts at the purification (Hengstenberg, 1977; Roseman, 1972; Kundig & Roseman, 1971a).

In our early attempts to purify E_I, we observed that E_I activity could be recovered in more than one molecular weight range during elution from Sephadex G-200. Such an observation could be explained by self-aggregation or complex formation with other macromolecules, either of which could result from hydrophobic interactions. Subsequent studies showed that E_I was retarded on a number of hydrophobic resins. The procedure presented below uses this one feature of E_I for its complete purification.

Materials and Methods

Octyl-Sepharose was purchased from Pharmacia Fine Chemicals. Throughout this study we have used only one lot, no. 9080, which contains 40 μmol of ligand/mL of gel bed.

Phenyl-Sepharose, lot no. 9705, was also purchased from Pharmacia Fine Chemicals.

Methyl α-D-[U-^{14}C]glucopyranoside (180 mCi/mmol) was purchased from the Radiochemical Centre, Amersham.

Phosphoenolpyruvate (monocyclohexylammonium salt) and *dithiothreitol* were purchased from Sigma.

Absolute ethanol, 1-butanol, ethylene glycol (analytical grade), sodium cholate, and deoxycholate were obtained from Merck.

Sodium dodecyl sulfate was purchased from BDH Chemicals Ltd., England, as sodium lauryl sulfate, specially pure.

Bacteria. E. coli K235 were grown and harvested as described by Dooijewaard et al. (1979). The *Salmonella typhimurium* ptsI mutant SB1690 was the source of mutant crude cell extract containing HPr and E_{II}. The conditions for growth of the cells and preparation of the mutant crude cell extract are identical with those used for the ptsH mutant SB2226 (Dooijewaard et al., 1979).

Measurement of Phosphotransferase Activity. E_I activity was measured following the method of Kundig & Roseman (1971b) by using methyl α-D-[U-^{14}C]glucopyranoside as the phosphate acceptor and a crude cell extract (48000*g* supernatant) of the ptsI mutant SB1690.

Protein concentrations were determined by the procedure of Lowry et al. (1951).

Polyacrylamide disc gel electrophoresis was performed on 7.5% or 10% polyacrylamide gels in a pH 9.5 buffer system. DTT was incorporated in the gels and buffers to a final concentration of 1 mM. Electrophoresis in the presence of 1% sodium dodecyl sulfate was also done on 10% polyacrylamide gels containing 1 mM DTT. Protein samples were first denatured by heating at 37 °C for 2 h in the presence of 3% sodium dodecyl sulfate, 5% 2-mercaptoethanol. The gels were stained with Coomassie Brilliant Blue.

Partial Purification of E_I by DEAE Chromatography. E. coli K235 cells suspended in 20 mM K_2HPO_4, 1 mM DTT, 1 mM EDTA (1-g cells, wet weight/5 mL) were ruptured by passage through a French press at 10000 psi at 5 °C. After centrifuging the suspension for 30 min at 48000*g*, 5 °C, the supernatant was brought to 0.33% protamine sulfate by dropwise addition of a 2% protamine sulfate solution. The suspension was stirred 30 min at 5 °C and centrifuged at 48000*g* for 30 min. The supernatant was made 0.25 M in KCl by addition of solid KCl and loaded on a 5 × 25 cm DEAE-23 cellulose column preequilibrated in 10 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, 0.25 M KCl, pH 7.6. After loading, the column was washed until the absorbance reached zero, and then a batchwise elution of the E_I activity was performed by using the same buffer containing 0.45 M KCl. The E_I containing fractions were concentrated on an Amicon ultrafiltration apparatus equipped with a UM 20 membrane. The concentrate was frozen at −20 °C until use.

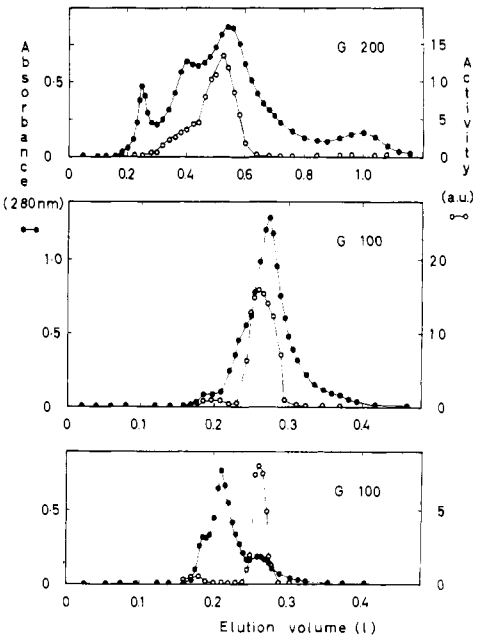

FIGURE 1: Sephadex chromatographies of a partially purified E_I fraction from a DEAE chromatography as described under Materials and Methods. The columns were eluted in 10 mM Tris-HCl, 1 mM EDTA, and 1 mM DTT, pH 7.6. The upper trace is the profile from a Sephadex G-200 column (5 × 50 cm). The middle and lower traces are the profiles from a Sephadex G-100 column (2.5 × 100 cm). The open circles represent the E_I activity; the filled circles represent the absorbance at 280 nm.

Chromatography in Ethylene Glycol. The ethylene glycol concentration gradient was measured after each chromatography by measuring the index of refraction at room temperature. The stated pH of ethylene glycol containing buffers was the pH adjusted before addition of ethylene glycol. No correction was made for the effect of dilution or the presence of ethylene glycol.

Determination of Yields in the Isolation Procedure. After each step in the isolation, several samples were taken and stored at −20 °C. When the entire isolation was completed (within 1 week), the activity of all samples was measured at the same time. The same procedure was used for protein determinations.

Results

The upper trace in Figure 1 shows a Sephadex G-200 elution profile of a concentrated E_I fraction obtained from a DEAE-cellulose chromatography as described under Materials and Methods. The main peak of the E_I activity profile eluted in a molecular weight range of 80000, but a shoulder in the E_I activity profile also occurred in the molecular weight range of 160000. These two portions were separately pooled and chromatographed over Sephadex G-100 columns. The middle trace in Figure 1 shows that the 80000 mol wt fraction from the G-200 column eluted from Sephadex G-100 in the same molecular weight range. Furthermore, the activity and absorbance profiles coincided. Upon chromatography of the

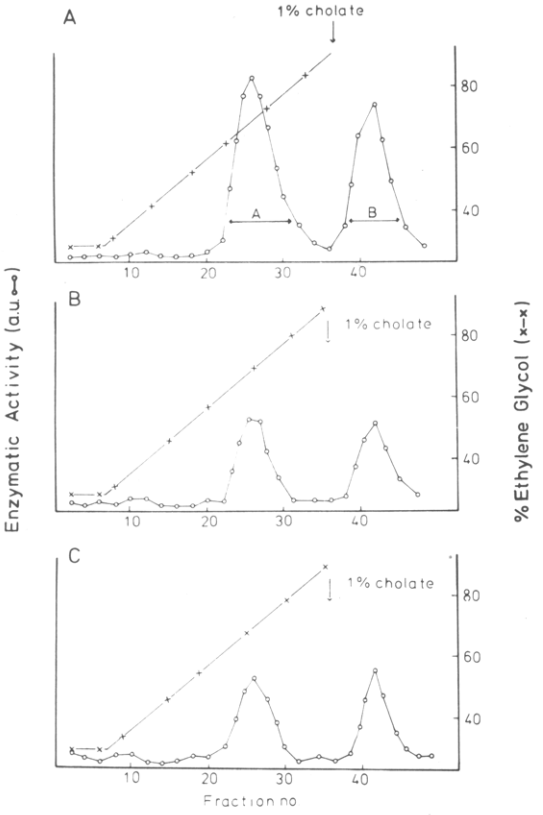

FIGURE 2: Octyl-Sepharose elution profiles from a 5 × 25 cm column eluted with a 3-L gradient of 30–90% ethylene glycol in 5 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, pH 7.6. The sodium cholate batchwise elution was performed with the same buffer containing 1% sodium cholate (w/v) instead of ethylene glycol. Each of the three elutions performed in this figure was done on a new column containing resin not previously used. (A) The E_I used on this column was a partially purified preparation obtained from DEAE-cellulose chromatography (see Materials and Methods). (B) Peak A from panel A, rechromatographed. Before loading, the sample was diluted with 2 volumes of buffer lacking ethylene glycol. (C) Peak B from panel A, rechromatographed. Before loading, the sample was extensively dialyzed to remove sodium cholate (see text).

160 000 mol wt fraction over Sephadex G-100 (Figure 1, lower trace), virtually all the E_I activity eluted in a molecular weight range of 80 000 separated from the bulk of the protein which eluted in the breakthrough as shown by the absorbance profile. The most straightforward interpretation of these results is that E_I monomers with a molecular weight of 80 000 are in equilibrium with a dimeric E_I species. It is possible, however, that E_I also forms aggregates with other macromolecules. Either process could occur via hydrophobic interactions.

Binding of E_I to Octyl-Sepharose. Figure 2A presents an elution profile of E_I activity from an octyl-Sepharose column in which newly purchased resin was used. The source of E_I was the concentrated DEAE-cellulose chromatography fraction described under Materials and Methods. Approximately 50–60% of the total E_I activity eluted in an ethylene glycol gradient at approximately 70% ethylene glycol. The remainder of the E_I activity could only be removed by washing the column with buffers containing 1% sodium cholate (w/v), as shown in Figure 2A, 1% deoxycholate (w/v), or 1% Triton X-100 (v/v). The total recovery of E_I activity was approximately 90%. To determine whether the two E_I fractions represented two types of E_I, the peaks of activity in Figure 2A were separately pooled and separately rechromatographed on new octyl-Sepharose. The elution profile in Figure 2B shows that the ethylene glycol E_I fraction from the first octyl-Sepharose column (peak A) redistributed itself again equally into an

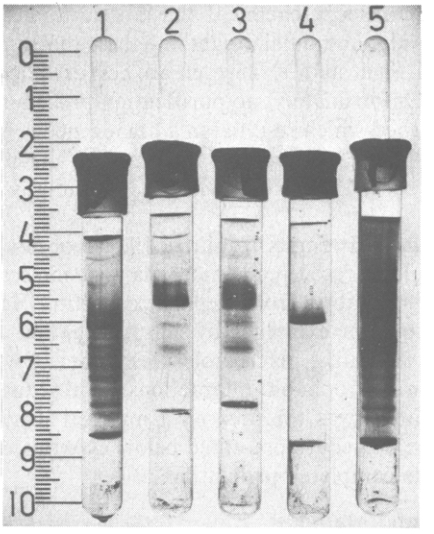

FIGURE 3: Ten percent polyacrylamide gels run in a pH 9.5 buffer system. Prior to electrophoresis, the enzyme samples were concentrated on an Amicon ultrafiltration apparatus equipped with a UM 20 membrane and then washed with 20 volumes of buffer (5 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, 1 mM NaN_3, pH 7.6) by using the same apparatus. (Gel 1) Peak B, Figure 2A. (Gel 2) Ethylene glycol eluting peak, Figure 2B. (Gel 3) Sodium cholate eluting peak, Figure 2B. (Gel 4) Ethylene glycol eluting peak, Figure 2C. (Gel 5) Sodium cholate eluting peak, Figure 2C.

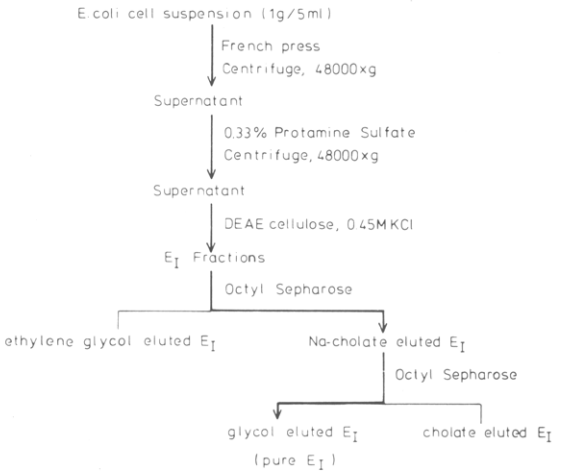

FIGURE 4: Flow diagram of the E_I purification procedure using untreated octyl-Sepharose.

ethylene glycol fraction and a cholate fraction. Identical results are presented in Figure 2C for the rechromatographed cholate eluting E_I fraction (peak B), after extensive dialysis to remove the cholate. The disc electrophoresis patterns in Figure 3, for the various fractions obtained by this procedure, show that pure E_I can be obtained by using two octyl-Sepharose columns, starting from a partially purified E_I fraction. The cholate eluting E_I fraction (peak B) from the first octyl-Sepharose column was impure (gel 1, Figure 3). When this peak was rechromatographed on octyl-Sepharose, however, only E_I appeared to redistribute itself in two fractions, while the rest of the protein impurities remained in the cholate fraction (compare gels 4 and 5, Figure 3). The resulting ethylene glycol fraction, Figure 2C, was pure as shown by gel 4, Figure 3. Rechromatography of peak A, Figure 2A, also resulted in a further, but not complete, purification as shown by the electrophoresis patterns, gels 2 and 3, Figure 3. The additional bands have intensities 10–15% that of the E_I band. The streaking of the E_I band arises from overloading the gels with E_I. A flow chart of the procedure just described, is presented

Table I: Binding Capacity of Octyl-Sepharose for E_I[a]

resin bed vol (mL)	vol of extract (mL)	E_I (%) breakthrough	ethylene glycol	cholate
58	5		30 (1.5)	70 (3.5)
58	18	10 (1.8)	50 (9)	40 (7.2)
58	80	90 (72)	10 (8)	(0)

[a] The numbers in parentheses represent the amount of E_I in each fraction expressed in arbitrary units.

in Figure 4. It is obvious that redistribution of E_I into two fractions during each octyl-Sepharose chromatography substantially diminished the yields of pure E_I.

The redistribution phenomenon itself indicates that the two E_I fractions found on octyl-Sepharose chromatography do not arise from two distinct, noninterconverting populations of E_I. Having once demonstrated that fact, the following step was to find a method to force E_I to behave as a single, homogeneous population during the chromatography. Table I lists the amounts and percentages of E_I elutable with ethylene glycol and sodium cholate as a function of the volume of crude cell extract containing E_I which was loaded, holding the resin bed volume constant. When a small volume was loaded, twice as much E_I was found in the cholate fraction as in the ethylene glycol fraction. Upon increasing the volume loaded, the absolute amount of E_I in the cholate fraction first increased and, with even larger volumes of crude cell extract, eventually decreased to zero. This result suggests that either the properties of the resin itself or the E_I binding affinity to the resin was altered by a component in crude cell extract which binds strongly to octyl-Sepharose. As the amount of E_I in the cholate fraction decreased to zero, the amount in the ethylene glycol fraction increased and reached a plateau. At this point, the resin appeared to be saturated and the extra E_I did not bind.

Pretreatment of Octyl-Sepharose. The distribution of E_I into two fractions can be prevented by pretreating the resin in the following manner. A column of octyl-Sepharose is equilibrated in 30% ethylene glycol containing 5 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, 1 mM NaN_3, pH 7.6. One-half a bed volume of crude cell extract (48000*g* supernatant) is brought to 30% in ethylene glycol by using pure ethylene glycol and loaded on octyl-Sepharose at 2 mL/min. After being washed with 1 volume of equilibration buffer, the column is eluted, batchwise, with the same buffer containing 90% ethylene glycol. The column is then unpacked and the resin washed thoroughly with water, followed by 2 bed volumes of absolute ethanol, 2 volumes of 1-butanol, again 2 volumes of absolute ethanol, and, finally, water. After degassing the resin, a new column is poured and the entire procedure is repeated two more times. By using resin pretreated in such a manner, all the E_I activity can be recovered in the ethylene glycol elution. This procedure modifies octyl-Sepharose in a more or less irreversible manner in as much as the standard cleaning procedures, recommended by Pharmacia (ethanol, butanol, ethanol washes) (Pharmacia Fine Chemicals, 1977), are not capable of regenerating an octyl-Sepharose resin on which E_I distributes itself in two fractions.

Complete Purification of E_I via Octyl- and Phenyl-Sepharose Chromatography. All procedures were done at 5 °C. Sixty grams of *E. coli* cells (wet weight) was suspended in 300 mL of 20 mM K_2HPO_4, 1 mM DTT, 1 mM EDTA, 1 mM NaN_3, pH 7.6. Immediately before the suspension was put through the French press, the suspension was brought to a final concentration of 1 mM DFP by using a stock solution

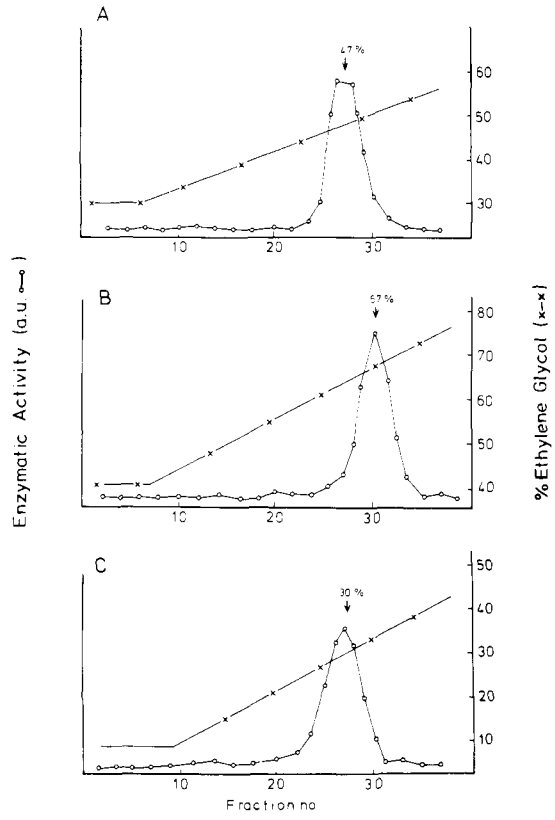

FIGURE 5: Elution patterns of E_I activity from the isolation procedure employing only hydrophobic interaction chromatography. (A) First octyl-Sepharose column eluted with 5 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, 1 mM NaN_3, pH 7.6, by using a gradient of 30–60% ethylene glycol. (B) Second octyl-Sepharose column eluted with 35 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, 1 mM NaN_3, pH 7.6, by using a gradient of 40–80% ethylene glycol. (C) Phenyl-Sepharose column eluted with the same buffer as in (A) by using a gradient from 10% to 50% ethylene glycol. See text for details.

of 0.1 M DFP in isopropyl alcohol. The cells were ruptured by passage through the French press at 10000 psi and centrifuged for 30 min at 48000*g*. The pellet was discarded.

First Octyl-Sepharose Chromatography. The supernatant (225 mL) was brought to 30% in ethylene glycol by addition of pure ethylene glycol. The solution was loaded, at 2 mL/min, on a 5 × 25 cm column of pretreated octyl-Sepharose equilibrated in 30% ethylene glycol containing 5 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, 1 mM NaN_3, pH 7.6. After loading, the column was rinsed with 1 column volume of equilibration buffer, at 3 mL/min, and eluted, at the same rate, with a linear gradient from 30% to 60% ethylene glycol containing the same buffer components. The total volume of the gradient was 3 L. E_I eluted at approximately 47% ethylene glycol, Figure 5A. After this, and the following chromatographic steps, the columns were unpacked and the resin was regenerated with the standard ethanol, butanol, ethanol wash procedure recommended by Pharmacia Fine Chemicals (1977).

Second Octyl-Sepharose Chromatography. The peak of E_I activity from the first column was pooled and diluted to a final concentration of 40% ethylene glycol, and the salt concentration was raised to 35 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, 1 mM NaN_3, pH 7.6, and immediately loaded on a 5 × 15 cm column of pretreated octyl-Sepharose, at 2 mL/min, equilibrated in the same solution. After washing the column with 1 bed volume of equilibration buffer, the E_I was eluted with a linear gradient of 40–80% ethylene glycol (1 L each) containing the same buffer components. The elution rate was

Table II

purifcn step	total protein (mg)	sp act.[a]	purifcn	recovery (%)	purifcn time (days)
crude cell extract	2340	0.27	1	100	
first octyl-Sepharose chromatogr	34	16	58	85	1
second octyl-Sepharose chromatogr	9	42	151	60	2
phenyl-Sepharose chromatogr	1.5	235	870	55	4–5

[a] μmol of sugar-P min^{-1} (mg of protein)$^{-1}$.

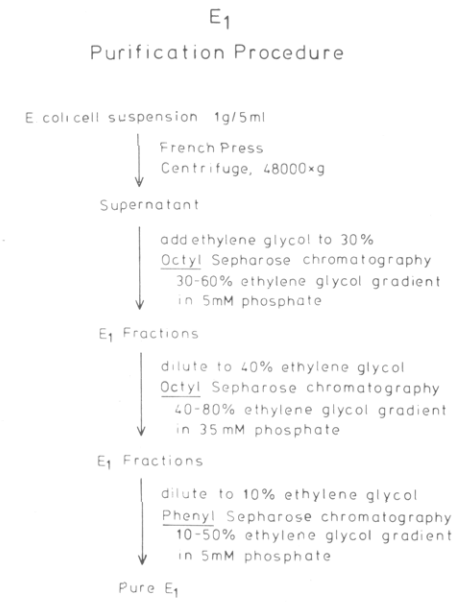

FIGURE 6: Scheme for the complete purification of E_I by employing only hydrophobic interaction chromatography.

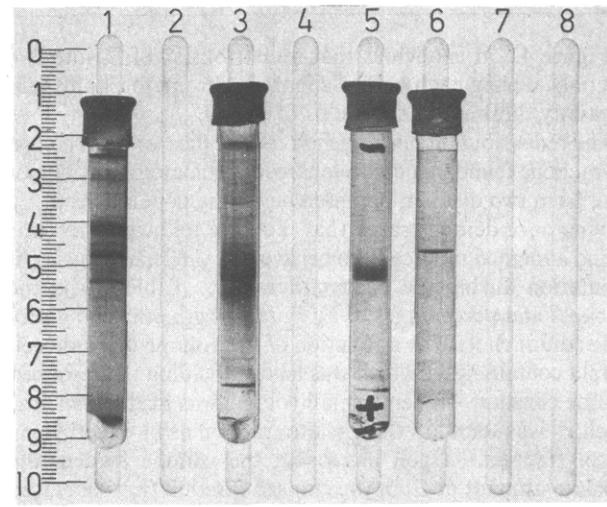

FIGURE 7: Polyacrylamide disc gel electrophoresis of E_I fractions taken at each stage in the isolation procedure. (Gel 1) E_I pool from the first octyl-Sepharose column. Ten percent polyacrylamide gel in a pH 9.5 buffer system. (Gel 3) E_I pool from the second octyl-Sepharose column. Polyacylamide gel (7.5%) in a pH 9.5 buffer system. (Gel 5) E_I pool from the phenyl-Sepharose column. Polyacrylamide gel (7.5%) in a pH 9.5 buffer system. (Gel 6) $NaDodSO_4$ containing 10% polyacrylamide gel on which E_I from the phenyl-Sepharose column was electrophoresed. The polarity of the electrophoresis, shown on gel 5, was the same for all gels in the figure. Prior to electrophoresis, the enzyme samples were concentrated and washed as described in the legend to Figure 3.

3 mL/min. E_I activity eluted at approximately 67% ethylene glycol (see Figure 5B).

Phenyl-Sepharose Chromatography. The peak of E_I activity was pooled, diluted with 5 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, 1 mM NaN_3, pH 7.6, to a final concentration of 10% ethylene glycol. It was immediately loaded, at 2 mL/min, on a 24 × 5 cm column of phenyl-Sepharose equilibrated with 10% ethylene glycol containing the same buffer. After washing with 1 bed volume of equilibration buffer, the E_I was eluted with a linear gradient of 10–50% ethylene glycol (1.5 L each) in the same buffer, at 3 mL/min. E_I activity eluted at 30% ethylene glycol (see Figure 5C). At this point, E_I is pure. The E_I fractions can be pooled and stored at −20 °C. The ethylene glycol can be most easily removed by gel filtration over a Sephadex G-25 column in buffer containing 1 mM DTT, after which E_I may be lyophilized and stored at −20 °C. In the lyophilized form, at −20 °C, E_I activity is stable indefinitely.

A flow chart of the purification procedure is presented in Figure 6.

Summary of the Purification. Figure 7 presents the disc gel electrophoresis patterns from the E_I pool after each chromatography. The most striking result is the purification achieved by the first octyl-Sepharose column. With a single step, E_I can be purified from crude cell extract 58-fold, to a point where only two or three major protein components are present (Figure 7, gel 1). Electrophoresis in the presence of $NaDodSO_4$ demonstrates that this preparation contains chains of three distinct molecular weights 115000, 84000, and 60000. After the second octyl-Sepharose chromatography, the E_I preparation is approximately 95% pure relative to the remaining bands as analyzed by spectrophotometric traces of the gels, Figure 7, gel 3. At this stage, there is only one dominant molecular weight species on $NaDodSO_4$ containing gels. There is a faint trace of the 115000-dalton species, the 60000-dalton species has been completely eliminated, and the 84000-dalton species remains as the principle component. The final chromatography over phenyl-Sepharose produces completely pure E_I as judged by both regular gel electrophoresis and gels containing $NaDodSO_4$ (see Figure 7, gels 5 and 6). From the $NaDodSO_4$ containing gels, we estimate that the pure preparation consists of single polypeptide chains with a molecular weight in the range of 84000. E_I does not electrophorese on gels in a pH 4.5 or pH 2.3 buffer system. Since E_I activity is unstable at these pH values, denaturation or aggregation may cause this difficulty.

Table II lists the specific activity, purification, and recovery data for each step in the purification procedure. It should be stated that the flow rates, wash volumes, and elution volumes are critical to the reproducibility of the procedure. Alteration of these does change the point of elution of the E_I peak from the various columns.

Discussion

The first procedure, presented above, summarized in Figure 4, had several disadvantages in the time required for the purification and the yield of pure E_I. (i) The yields from the DEAE column were low and variable, approximately 50%. (ii) Before the cholate eluting fraction from the first octyl-Sepharose column could be rechromatographed, the cholate had to be removed. Dialysis for 24 h, with a calculated dilution of 400 in the cholate concentration, was insufficient to allow

E_I to bind again to octyl-Sepharose. Calculated dilutions of 10^4 over 60–72 h of dialysis were required. This could arise from a binding of cholate to E_I, or simply a low efficiency of cholate removal by dialysis. (iii) The yield of pure E_I could not be higher than 25% of what was loaded in the first octyl-Sepharose step since only one-quarter of the initial E_I loaded on the first column would find its way into the ethylene glycol fraction of the second column. Usually the yield of the whole procedure was 10% or less. (iv) In principle, the remaining impure fractions could be repeatedly rechromatographed until all the E_I found its way first into a cholate fraction and, subsequently, into an ethylene glycol fraction, at which point it would be pure. However, the number of individual fractions and chromatographic steps could rapidly multiply to a sizable number as shown in Figure 4. In the interest of rapidity, simplicity, and higher yields, the second procedure was developed.

The success of the second procedure rests largely in our ability to force E_I to elute from octyl-Sepharose nearly quantitatively, as a single population in an ethylene glycol gradient. The behavior of E_I on octyl-Sepharose, distributing itself in two fractions, and our ability to alter this behavior by pretreating the resin, could be interpreted in one of two ways: (i) octyl-Sepharose could possess two classes of sites, one of which could be blocked by pretreatment of the resin, or (ii) E_I could exist in different states having widely differing affinities for octyl-Sepharose; the distribution between these states could then be influenced by a component which is fixed to the resin in the pretreatment step.

The first possibility cannot be entirely ruled out. However, at least one result has been presented above which is in conflict with such an interpretation. If there were more than one class of binding sites on the resin, we would expect to find other proteins also distributing themselves in two populations, in addition to E_I. The results in Figures 2 and 3 show, however, that, upon rechromatography of the cholate eluting fraction, only E_I redistributes itself in two fractions. The large number of contaminating proteins are all found back in the cholate eluting fraction (compare gels 4 and 5, Figure 3). Furthermore, there have been no reports from other investigators, suggesting that a heterogeneity of sites exists on octyl-Sepharose.

The second interpretation suggests that the existence of different affinities for octyl-Sepharose is an inherent property of E_I. We have evidence, in Figure 1, that E_I appears to be able to exist in more than one molecular weight form suggestive, for instance, of a monomer–dimer equilibrium. In addition, it appears that the fractional distribution between these forms can be altered by removal of some component during chromatography. A similar process might also account for the behavior of E_I on octyl-Sepharose. It is not unreasonable, for instance, to expect one form of the enzyme to bind more strongly (the cholate eluting fraction) than another form (the ethylene glycol eluting fraction). Something which is surprising and which we do not claim to fully understand is that, by pretreating the resin with crude cell extract, a factor apparently binds to the resin irreversibly, but in a functional form, which is capable of altering the equilibrium between these E_I populations, so that the tighter binding form no longer occurs.

The procedure presented in this paper demonstrates, conclusively, that E_I possesses surface regions which are very hydrophobic in character. Furthermore, these hydrophobic interaction sites appear to be rather aspecific inasmuch as E_I binds such diverse resins as octyl- and phenyl-Sepharose as well as valine-Sepharose (unpublished data). In the past, the hydrophobic nature of E_I had not been recognized and, moreover, was not expected since E_I has always been found in the soluble fraction of extracts from mechanically ruptured cells. The fact that repeated chromatography over the same resin results in increasing purification of the enzyme suggests that the enzyme may be bound, via hydrophobic interactions, to other cellular components and that, during chromatography, there may be a competition between the octyl groups of the resin and these other components for the hydrophobic sites on E_I. As these interactions are broken, E_I can be separated and isolated in a homogeneous form. This is consistent with the results shown in Figure 3, gels 2 and 3. When peak A from the first untreated octyl-Sepharose column (Figure 2A) is rechromatographed, resulting again in two E_I fractions (Figure 2B), both fractions are identical as judged by electrophoresis. Since the elution conditions are very different for the two peaks, one would expect different proteins in one fractions as opposed to the other, just as seen in gels 4 and 5. Since this is clearly not the case, it must indicate that the protein impurities are binding to and eluting with E_I. This interpretation is also supported by the following observation. If the pool from the first octyl-Sepharose column, Figure 5A, which eluted at 47% ethylene glycol, is reloaded on a second octyl-Sepharose column, preequilibrated with buffers containing the same salt concentrations but *50%* in ethylene glycol, all of the E_I binds. A concentration of 60–63% ethylene glycol is then required to remove it from the column.

Whether these proteins are functionally related to E_I has not yet been determined. However, activity measurements for E_I on slices of electrophoresed polyacrylamide disc gels showed that only one component possessed E_I activity. Furthermore, removal of these other proteins during the purification did not cause a noticeable change in the turnover number as judged by the high recovery of E_I activity in the purified preparation.

Studies on the physical and chemical characterization of E_I are currently in progress.

Acknowledgments

We gratefully acknowledge the excellent technical assistance of R. Ten Hoeve-Duurkens.

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