Involvement of the Carboxy-Terminal Residue in the Active Site of the Histidine-Containing Protein, HPr, of the Phosphoenolpyruvate:Sugar Phosphotransferase System of *Escherichia coli*†

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Received November 20, 1990; Revised Manuscript Received July 8, 1991

ABSTRACT: Histidine-containing protein, HPr, of the *Escherichia coli* phosphoenolpyruvate:sugar phosphotransferase system has an active site that involves His-15, which is phosphorylated to form a $N^{\delta 1}$-P-histidine, Arg-17, and the carboxy-terminal residue Glu-85. Mutant HPrs with alterations to the three C-terminal residues, Glu-85, Leu-84, and Glu-83, were produced by site-directed mutagenesis. The properties of these mutants were assessed by kinetic analysis of enzyme I, enzyme $II^{mannose}$, enzyme $II^{N\text{-acetylglucosamine}}$, and enzyme $II^{mannitol}$, and the phosphohydrolysis properties of the HPr mutants. The results show that it is the C-terminal α-carboxyl of Glu-85 that is involved in the active site, and this involvement may be restricted to the phosphoryl donor action of HPr. The contribution of this α-carboxyl group is modest as the deletion of Glu-85 resulted in the reduction of the enzyme II activity (k_{cat}/K_m) to about 33%. Removal of both Glu-85 and Leu-84 yields an HPr that is an impaired substrate of both the enzyme I and enzyme II reactions. Glu-83 appears to have no role in the active site.

The histidine-containing protein, HPr,[1] is a phosphocarrier protein of the phosphoenolpyruvate:sugar phosphotransferase system (PTS). It is the acceptor of a phosphoryl group from enzyme I and the donor of the group to a sugar-specific factor III, which may be a separate phosphocarrier protein or a part of the membrane-bound enzyme II that carries out both sugar phosphorylation and translocation [see review by Meadow et al. (1990)]. HPr is a substrate of enzyme I, while phospho-HPr (P-HPr) is the substrate of enzyme II, and in both cases normal Michaelis–Menten kinetics can be demonstrated (Waygood, 1987). The early work of Anderson et al. (1971) showed that the phosphoryl group in P-HPr is carried in the form of a high-energy $N^{\delta 1}$-P-histidine, and the properties of the $N^{\delta 1}$-P-histidine were more fully described by Waygood et al. (1985). This later study led to the proposal that the active site of HPr had an arrangement that involved residues His-15, Arg-17, and Glu-66. Implicit in the term "active site" is the recognition that although HPr can be kinetically characterized as a substrate, it probably does participate in a catalytic manner to facilitate the phosphoryl transfer. Subsequently, the description of the tertiary structure of HPr by both two-dimensional nuclear magnetic resonance, 2D NMR (Klevit & Waygood, 1986), and X-ray diffraction (El Kabbani et al., 1987) revealed that the glutamate residue closest to the active site was the C-terminal residue Glu-85.

The evidence that led to the proposed active site is as follows. Dooijewaard et al. (1979) and Kalbitzer et al. (1982) had shown by NMR that the pK_a of His-15 was unusual, being 5.6 in HPr and 7.8 in P-HPr, which implies a deprotonated imidazole ring in HPr and a protonated imidazole ring in P-HPr at physiological pH. Hultquist (1968) had shown that the lability of the phosphoryl group in a $N^{\delta 1}$-P-histidine depended upon the ability of this phosphoryl group to interact with the amino group of the free histidine. As the $N^{\delta 1}$-P-histidine in HPr was at least as labile, the conserved Arg-17 residue was proposed to interact with the phosphoryl group leading to an increase in catalytic rate for phosphoryl transfer (Knowles, 1980; Vogel et al., 1982). The pH dependence of the phosphohydrolysis of P-HPr showed unusual features that were characterized by interactions with pK_as of about 7.5 and 4.0, presumably the imidazole of 1-P-histidine and a carboxyl group (Waygood et al., 1985). The carboxyl group is attributed to Glu-85 on the basis of the three-dimensional structure (Klevit & Waygood, 1986; El Kabbani et al., 1987).

As Glu-85 is the C-terminal residue of HPr, it provides two carboxyl groups. The X-ray diffraction structure determination showed ionic interactions between the α-carboxyl of Glu-85 and the imidazole ring and the γ-carboxyl of Glu-85 and the guanidino group of Arg-17. These interactions presumably occurred because the crystallization conditions were at pH 3.7, resulting in a protonated imidazole ring of His-15 in HPr (El Kabbani et al., 1987). The structural correlations are, however, complicated by the fact that the tertiary structures of HPr determined by the two independent methods of 2D NMR and X-ray diffraction are different, and it has been suggested that the structure determined by X-ray diffraction is the result of partial unfolding under the conditions of crystallization (El Kabbani et al., 1987). This suggests that the structure determined by 2D NMR is the physiologically correct structure, which has recently been confirmed with the

† This work was supported by an operating grant from the Medical Research Council of Canada to E.B.W. and by NIH Grant DK35187 to R.E.K. P.B. was supported by a Saskatchewan Health Research Board Fellowship.

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[1] Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase system; HPr, histidine-containing phosphocarrier protein of the PTS; 2D NMR, two-dimensional nuclear magnetic resonance; Mes, 2(*N*-morpholino)ethanesulfonic acid; man, mannose; mtl, mannitol; nag, *N*-acetylglucosamine.

0006-2960/91/0430-9601$02.50/0

use of epitope mapping (Sharma et al., 1991). The refinement of the 2D NMR structure is not at the stage that can distinguish which of the carboxyl groups is participating in the active site. We note, however, that P-HPr from *Streptococcus faecalis* showed similar phosphohydrolysis properties (Waygood et al,. 1988), although there is no conservation of the glutamate residue at the C-terminus in the HPrs from Gram-positive organisms (Reizer et al., 1988).

In order to investigate the role of Glu-85 in the active site of HPr, a number of site-directed mutants have been made, and their properties are reported here. A preliminary report of this work has been published (Waygood et al., 1989).

Materials and Methods

Materials. Restriction enzymes, Klenow fragment, T4 DNA ligase, and 2',3'-dideoxynucleotides were from Pharmacia and New England Biolabs. Radiolabeled compounds were purchased from NEN. Enzyme I was purified as previously described by Waygood and Steeves (1980) from *Escherichia coli* strain WA2127 containing plasmid pTSHIC9, which contained the genes *ptsHIcrr*, and which was generously provided by Dr. B. Erni. Homogeneous *E. coli* phosphoenolpyruvate carboxykinase was a gift from Dr. H. Goldie.

Enzyme Assays. The assays for enzyme $II^{mannose}$, enzyme $II^{N\text{-acetylglucosamine}}$, and enzyme $II^{mannitol}$ were carried out as described previously (Waygood et al., 1979; Waygood & Steeves, 1980). Enzyme II^{man} was the complex of the factor III and enzyme II that is isolated in crude membrane preparations of *Salmonella typhimurium* strain SB2950 grown on lactate minimal salts medium (Waygood et al., 1984). Enzyme II^{mtl} and enzyme II^{nag} were in membranes from *E. coli* strain CSH4 *trp rpsL* grown on minimal salts media with either 0.2% mannitol or 0.2% *N*-acetylglucosamine, respectively. In order to ensure the linearity of these discontinuous assays, the assays were sampled at both 10 and 20 min. Initial velocities were obtained for six to eight concentration values of HPr from 2 to 100 μM for the enzymes II. The lactate dehydrogenase coupled assay was used for enzyme I (Waygood et al., 1979), and nine HPr concentration values between 1.5 and 80 μM were used to obtain initial velocity data. To obtain reproducible enzyme I kinetics, care must be taken with the dilution and storage of enzyme I (Waygood et al., 1979; Waygood, 1986), and the following procedures were used. Enzyme I (about 1.3 mg/mL) was dialyzed against 0.01 M 2(*N*-morpholino)ethanesulfonic acid (Mes) buffer, pH 6.8, with 1 mM EDTA and 0.2 mM dithioerythritol. The preparation was then incubated at 37 °C for 30 min and diluted 1:200 into 0.01 M Mes buffer, pH 6.8, with 5 mM $MgCl_2$ and 4 mM phosphoenolpyruvate also at 37 °C. This dilution was left at room temperature (23 °C) for about 30 min before use and was stable at room temperature for about 60 h. (When enzyme I started to lose activity, both the K_m and V_{max} values would decrease together.) Assays (1 mL) were initiated with 0.005 mL of this enzyme I dilution. All solutions had to be filtered through either 0.22- or 0.45-μm filters to remove any particulate materials that interfered with the spectrophotometer response.

Phosphohydrolysis. The phosphohydrolysis measurements were carried out as previously described (Waygood et al., 1985). Phosphorylation of HPr and mutant HPrs was accomplished by incubation with [γ-^{32}P]ATP, oxaloacetic acid, and phosphoenolpyruvate carboxykinase to produce [^{32}P]-phosphoenolpyruvate. The standard incubation conditions were 50 mM *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid buffer, pH 7.5; 12.5 mM KF; 0.1 mg of phosphoenolpyruvate carboxykinase; 0.2 mM ATP; 0.2 mCi of [γ-^{32}P]ATP (specific activity 3000 Ci/mmol); 5 mM $MgCl_2$; 1 mM oxaloacetate. The incubations (1 mL) were started with the addition of oxaloacetate and were carried out at 37 °C for 5 min to label HPrs and for 10 min. The [^{32}P]P-HPr was isolated by G50-Sephadex chromatography as previously described (Waygood et al., 1985).

Antibody Binding. The competition antibody binding assays with purified mutant HPrs were carried out as previously described (Waygood et al., 1987).

HPr Cloning. The HPr gene was isolated from pAB65 (Lee et al., 1982), which was kindly provided by Dr. Hans Kornberg. The gene was transferred from this plasmid by use of digestion with the restriction endonucleases *Acc*I and *Sma*I into the *Cla*I and *Sma*I sites in both pUC13 and M13mp11 (Messing, 1983; Yanisch-Perron et al., 1985). This transfer left the HPr promoter region intact.

Oligonucleotide Site-Directed Mutagenesis. The site-directed mutagenesis was carried out by the general methods of Zoller and Smith (1984) with the modification described by Kunkel (1985). The primers were produced with use of an Applied Biosystems 380A DNA synthesizer and standard phosphoramidite chemistry (Mateucci & Caruthers, 1981). Sequencing was carried out according to the method of Sanger et al. (1977), and the complete gene for HPr was always sequenced. For protein production the HPr gene was transferred from M13mp11 to pUC13 with use of restriction enzyme digestions of *Hind*III and *Eco*RI. The primers used were as follows: E85Q, 5'-AAATTATTGGAGTTC-3'; E85D, 5'-AAATTAATCGAGTTC-3'; E85A, 5'-AAATTATGCGAGTTC-3'; E85K, 5'-AAATTATTTGAGTTC-3'; E85Term (▲85), 5'-AAATTACTAGAGTTC-3'; L84Term (▲84), 5'-GGAAATTACTATTATTCCGCC-3' (with ▲85 as template); E83A, 5'CTCGAGTGCCGCCATCAG-3'; E83Term (▲83), 5'-GGAAATTACTATTATTACGCC-3' (with ▲84 as template). Primers using ▲85 as the template: E83A,▲85, 5'-CTAGAGTGCCGCCATCAG-3'; E83D,▲85, 5'-CTAGAGATCCGCCATCAG-3'; E83Q,▲85, 5'-CTAGAGTTGCGCCATCAG-3'; E83K,▲85, 5'-CTAGAGTTTCGCCATCAG-3'.

Purification of HPr Proteins. Either *E. coli* strain ES7R *trp*, *rpsL*, *recA*, a *recA* derivative of *E. coli* strain ES7 (Waygood et al., 1987) or *E. coli* strain TP2811 F$^-$, *xyl*, *argH1*, *lacX74*, *aroB*, *ilvA*, (*ptsH*, *ptsI*, *crr*), Km^R (Levy et al., 1990), transformed by pUC(HPr) was grown in 6 L of LB-broth with 0.2% glucose and 100 μg/mL ampicillin. Only 1.5 L of broth per 6-L Erlenmeyer flask was used to ensure adequate aeration. Growth was usually for 18-20 h, and additional ampicillin (75 μg/mL) was added between 10 and 12 h after inoculation. The cells (15–25 g wet wt) were harvested, washed, broken, and centrifuged to remove membranes as previously described (Waygood & Steeves, 1980). The supernatant, about 100 mL, was loaded onto a 10 × 100 cm Ultrogel AcA54 molecular sieve column equilibrated with 0.01 M Tris-HCl, pH 7.5, with 1 mM EDTA. HPr eluted after the bulk of the protein had eluted. The fractions (500 mL) with HPr protein (fractions 7–9) were pooled and loaded onto a 150-mL DEAE-cellulose (Whatman DE32) or Q-Sepharose (Pharmacia) column equilibrated with the same buffer. HPr was eluted with use of a 500 mL + 500 mL gradient of 0–0.2 M KCl in buffer. The active fractions were pooled and dialyzed against 5 mM sodium acetate buffer, pH 5.5, using dialysis tubing with nominal pores <3500 MW, until both the pH and conductivity were equal to the dialysis buffer. The dialysate was chromatographed on a 150-mL CM-cellulose (Whatman CM23) or S-Sepharose (Pharmacia) column

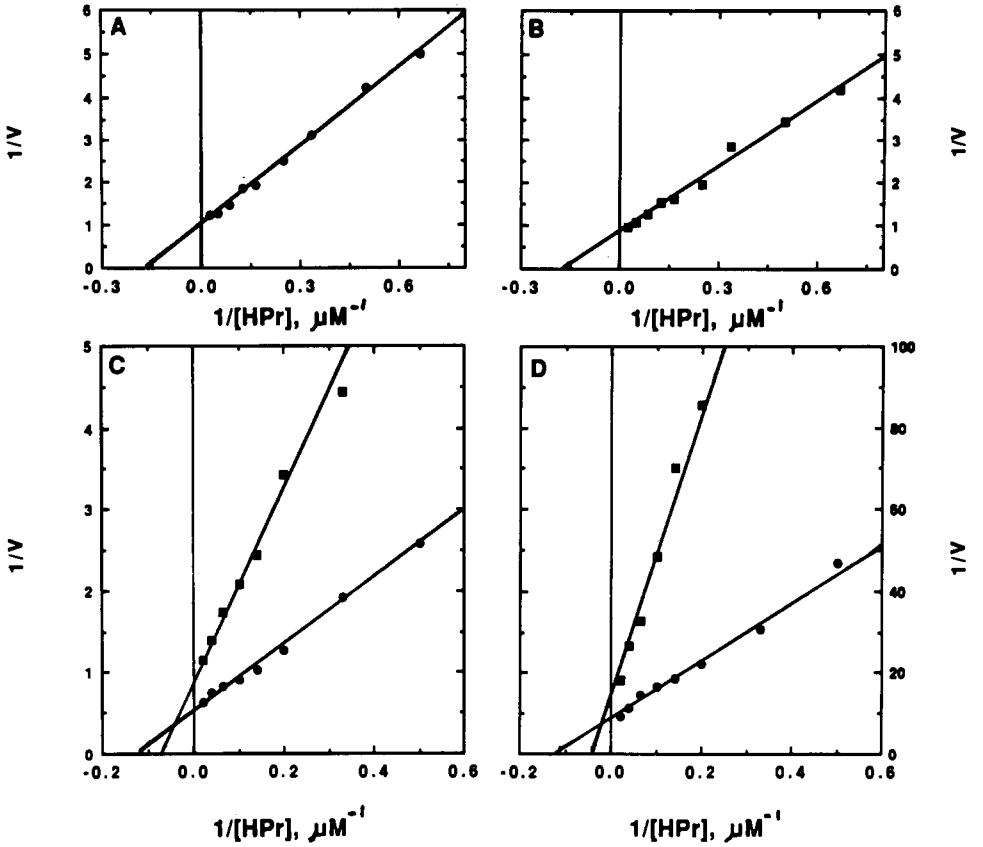

FIGURE 1: Kinetics of enzyme I and enzyme II. Examples of the kinetic measurements used to determine the results in Table I are shown: (A) Enzyme I with wild-type HPr; (B) enzyme I with ▲85 HPr; (C) enzyme II^{man} with wild-type and ▲85 HPr; (D) enzyme II^{mtl} with wild-type and ▲85 HPr. For enzyme I, v = nanomoles per minute. For enzyme II^{sugar}, v = micromoles per minute per milligram; the circles are wild-type HPr and the squares are ▲85 HPr.

equilibrated with the acetate buffer and eluted using a 500 mL + 500 mL gradient of 0–0.2 M NaCl in buffer. In both ion-exchange chromatography steps, the position of HPr elution varied as a result of the introduction or removal of ionizable residues. This procedure, which is a modification of that originally described by Beneski et al. (1982), produced 50–400 mg of homogeneously pure HPr as judged by isoelectric focusing gels.

Protein Determinations. HPr concentration was determined by the lactate dehydrogenase depletion assay (Waygood et al., 1979). The membrane protein concentration was determined by the method of Lowry et al. (1951).

Results

The Role of the C-Terminus in the Active Site. The results of structure determination and phosphohydrolysis have left a number of unresolved questions about the involvement of the C-terminus in the active site of HPr. These questions, some of which are addressed below, are as follows. Is Glu-85 involved in the phosphoryl-transfer process, and if so is it involved with the acceptor or donor role of HPr? Does the nearby Glu-83 residue have any role in the active site? Is there evidence for the involvement of some residue other than His-15, Arg-17, and Glu-85 in the active site? To help answer these questions, site-directed mutants have been constructed for the C-terminal residues Glu-85, Leu-84, and Glu-83.

Kinetic Measurements. The results and conclusions in this paper rely upon kinetic measurements of mutant HPrs, which are the substrates of two types of enzymes: the soluble, homogeneously pure enzyme I, which phosphorylates HPr, and the various enzymes II^{sugar} in crude membrane preparations, which phosphorylate sugars by use of phospho-HPr. The results in this paper do not show changes of orders of magnitude, and thus there is a necessity to understand the reliability of the kinetic measurements. In particular, our earlier report that the deletion of the C-terminal residue to give ▲85 HPr did not alter enzyme II^{mtl} kinetic parameters (Waygood et al., 1989), which is now clearly in error (Table I), serves to emphasize the care that must be taken with PTS assays (Waygood, 1987). The first source of variation is the determination of the concentration of HPr and the mutants HPrs. The spectrophotometric assay of Waddell (1956), which is often used for HPr protein determinations, although convenient, suffers from time to time from contaminants that affect the measurements at 215 and 225 nm. The lactate dehydrogenase depletion assay (Waygood et al., 1979), which is a specific assay for the amount of HPr that can be phosphorylated, was carried out on three concentrations of HPr (1×, 2×, and 4×), such that the concentration of the HPrs was known with a variation of less than 5%. Enzyme I kinetic measurements were carried out by use of a spectrophotometric method with pure enzyme preparations. Enzyme I must be handled with care as described under Materials and Methods, and duplicate assays gave K_m values within 10% variation. The enzyme I V_{max} for each mutant HPr was monitored with each series of assays by the use of assays with 50 μM wild-type HPr, and the values given (Table I) are rounded to the nearest 5%. Examples of these determinations are given in Figure 1A,B. The enzyme II^{sugar} kinetic measurements were carried out with dilute washed membranes (Waygood et al., 1979) whose activity in the absence of added HPr was not significantly different from the background values of the assay resulting from the radiolabeled sugars. With the appropriate dilution of membranes, each determination was carried out at least twice,

Table I: Kinetic Parameters[a] for Mutant HPrs

mutant	enzyme I		enzyme II^{man}		enzyme II^{mtl}		enzyme II^{nag}	
	K_m (μM)	V_{max} (%)	K_m (μM)	V_{max} (%)	K_m (μM)	V_{max} (%)	K_m (μM)	V_{max} (%)
wild type	6.3	100[b]	9	100[c]	7	100[d]	7	100[e]
▲85	5.9	110	**20**	**65**	**20**	**60**	**15**	**55**
E85Q	6.6	100	10	100	7	100	7	90
E85K	**4.0**	100	8	100	**12**	100	7	100
▲84	**15.0**	**85**	**30**	**40**	**35**	**45**	**40**	**50**
▲83	**6.2**	**60**	**50**	**25**	**65**	**40**	**80**	**60**
E83A	7.8	100	10	100	9	100	7	100
E83A,▲85	5.8	95	20	65	25	65	20	50
E83D,▲85	6.7	90	20	65	20	65	20	55
E83K,▲85	5.7	95	20	**100**	25	80	20	50
E83Q,▲85	5.6	100	20	65	20	65	20	50

[a] Parameters were derived from assays performed at pH 6.8 for enzyme I and pH 7.5 for enzymes II. Enzyme II^{man} was assayed by use of 1 mM [U-^{14}C]glucose (sp act. 4000 cpm/nmol) while enzyme II^{mtl} was assayed with 1 mM [U-^{14}C]mannitol (sp act. 2300 cpm/nmol). Enzyme II^{nag} was assayed with 1 mM [U-^{14}C]*N*-acetylglucosamine (sp act. 7200 cpm/nmol). [b] 100% activity was 57 μmol of P-HPr produced per min per mg of enzyme I protein. [c] 100% activity was 2.1 μmol of glucose-6-P produced per min per mg of membrane protein. [d] 100% activity was 0.15 μmol of mannitol-1-P produced per min per mg of membrane protein. [e] 100% activity was 0.14 μmol of *N*-acetylglucosamine-6-P produced per min per mg of membrane protein.

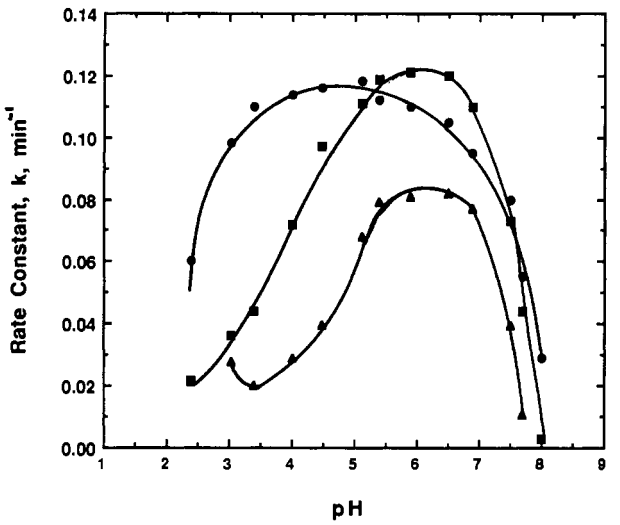

FIGURE 2: pH dependence of phosphohydrolysis of glutamate-85 mutants. The rates of phosphohydrolysis of the $N^{\delta 1}$-P-histidine in HPr (■), E85K (●) and ▲85 (▲) were determined at various pHs as described under Materials and Methods. This figure differs from and corrects a similar figure in Waygood et al. (1989).

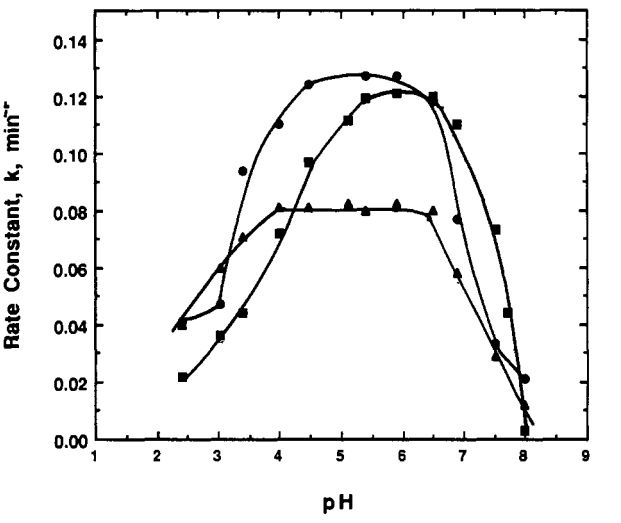

FIGURE 3: pH dependence of phosphohydrolysis of glutamate-83 and leucine 84 mutants. The rates of phosphohydrolysis of the $N^{\delta 1}$-P-histidine in HPr (■), ▲83 (●), and ▲84 (▲) were determined at various pHs as described under Materials and Methods.

and when other membrane preparations were used for the same enzyme II assay, the assays with wild-type HPr were repeated to normalize the results. Repeated assays with wild-type HPr and enzyme II^{man} gave a range in K_m values of 9 (±2) μM, and the values obtained are in good agreement with assays carried out under similar conditions (Waygood et al., 1985). The K_m values for all the enzymes II given in Table I have a variation of about ±20%. The V_{max} values given are rounded to the nearest 5%. The mutant HPrs that result in kinetic parameters that are judged to be significantly different from the parent HPr (either wild type or ▲85) are given in bold in Table I. Examples of enzyme II kinetics are shown in Figure 1C,D.

Properties of Mutants of Residue Glutamate-85. Glu-85 was changed to glutamine (E85Q), aspartate (E85D), alanine (E85A), and lysine (E85K) and was deleted (▲85). The pH dependence of the rate of phosphohydrolysis of the P-HPr for each of these showed that the mutations E85Q, E85D, and E85A gave essentially wild-type behavior; E85K resulted in a change in the apparent pK_a of 4.0 to 2.5, and ▲85 caused both a reduction in the phosphohydrolysis rate and a shift in the pK_a from about 4.0 to 4.7 (Figure 2). The apparent pK_as were determined from the midpoints between the extremes on the slopes of the curves such as seen in Figure 2. A screening assay in which enzyme II^{man} activity was measured with use of concentrations below the K_m value of HPr (i.e., where there is almost a first-order response between substrate concentration and enzyme initial velocity) showed that only ▲85 had impaired activity. More thorough kinetic measurements with E85Q, E85K, and ▲85 (Table I) show that alterations to the side chain of residue 85 have little or no effect upon the ability of HPr to act as a substrate for enzyme I or of P-HPr to act as a substrate for the enzymes II. ▲85 showed a normal response with enzyme I, but the ▲85 P-HPr had impaired activity with the enzymes II. These results would appear to eliminate a role for the side chain of Glu-85 in the active site but indicate that the C-terminal carboxyl has a role in the phosphoryl transfer from P-HPr to an enzyme II.

Properties of ▲84 and ▲83 Mutants. The mutant HPr ▲84 is terminated at position 84 to leave Glu-83 at the C-terminal. Compared to ▲85, ▲84 caused no change in the maximal rates of phosphohydrolysis properties, but it did change the apparent pK_as from about 4.7 to about 3.0 and from about 7.5 to 6.5 (Figures 2 and 3). The ▲84 mutation resulted in significant changes in the kinetic parameters with enzyme I and the enzyme IIs (Table I). The ▲83 mutant HPr is terminated at position 83 to leave Ala-82 at the C-terminus. There were further changes to the kinetic parameters with respect to ▲84. Unexpectedly, the maximal phosphohydrolysis rates for ▲83

Table II: Relative Binding Constants of Monoclonal Antibodies for Mutant HPrs

	monoclonal antibodies		
mutant	Jel42	Jel44	Jel323
wild type	1	1	1
▲85	1	1	1
E85Q	1	1	1
E85K	1	1	1
▲84	1	0.5	1
▲83	1	0.001	1
E83A	1	0.06	1
E83A,▲85	1	0.025	1
E83D,▲85	1	1	1
E83K,▲85	1	<0.01	1
E83Q,▲85	1	1	1

were restored to those of wild-type HPr (Figure 3), perhaps indicating either that another residue is allowed access to the active site or that the residue responsible for the remaining phosphohydrolysis rate in the ▲85 or ▲84 P-HPrs is now allowed better access. However, the pK_as do not return to wild-type values.

Properties of Mutants of Residue Glutamate-83. There was an inference from the results of a 2D NMR investigation of ▲85 HPr (see below) that the γ-carboxyl of Glu-83 was substituting for the α-carboxyl of Glu-85 (Waygood et al., 1989). This would have explained why the phosphohydrolysis rates had only reduced by about 50% (Figure 2). The following mutations were created in ▲85 HPr: E83D, E83A, E83Q, and E83K; and in wild-type HPr, E83A. None of these mutations caused a significant change in the phosphohydrolysis rates with respect to their background, ▲85, or wild type (results not shown), nor did they cause a significant change in the kinetic parameters except for the small changes that were found for E83K, ▲85 HPr (Table I). These results would appear to rule out a role for Glu-83 in the active site of HPr.

Histidine pK_as in ▲85 and E85K HPrs. The pK_a of His-15 in HPr has been reported as 5.6 (Dooijewaard et al., 1979; Kalbitzer et al., 1982). The pK_as in HPr, ▲85, and E85K were determined from NMR spectra by measuring the chemical shift of the histidine $C^{\delta}H$ and $C^{\epsilon}H$ peaks as a function of pH. The pK_a of His-15 in wild-type HPr is 5.7, while the pK_as in ▲85 and E85K are 5.9 and 5.7, respectively.

Assessment of Structural Changes by Antibody Binding. It is important when making site-directed mutations to attempt at least some evaluation of the potential structural changes in the resulting protein. Three monoclonal antibodies, Jel42, Jel44, and Jel323, bind to three different epitopes of HPr and, in effect, monitor over half of the surface of HPr (Sharma et al., 1991). If a mutation was to cause a general structural change, then a general disruption of antibody binding would occur. A mutation within an epitope would be expected to cause a binding change for only one antibody if the mutation had minor conformational consequences. The ability of the mutant HPrs to compete with wild-type HPr in an antibody binding assay helps to define the extent of structural change brought about by mutation, and the results of such assays are shown in Table II. The results suggest that mutation of residues 84 and 85 produces no significant changes to the overall structure of HPr and that mutation of residue 83 causes a change in binding to the Jel44. Residue 83 would appear to be part of the epitope for Jel44 (Sharma et al., 1991). The more drastic change in antibody binding seen for ▲83 may reflect a more significant change in structure. The changes in the ability of Jel44 to bind to the mutants with changes at residue 83 in the ▲85 background are consistent with the interpretation that Glu-83 is part of the epitope for Jel44 and that the conformational changes caused by mutations to this residue are local.

Discussion

The results in this paper support the contention that Glu-85 participates in the active site. However, it is the C-terminal α-carboxyl group of this residue that appears to be involved, rather than its side chain. The kinetic results suggest that the involvement occurs after phosphorylation of HPr, as the ▲85 HPr has normal activity with enzyme I but impaired activity with enzyme II. The relative enzyme II k_{cat}/K_m values for ▲85 and ▲84 are about 33% and 13%, respectively. Unfortunately, we had previously reported (Waygood et al., 1989) that ▲85 HPr kinetic parameters had no change with respect to enzyme II^{mtl}, but these results were incorrect. The results in this paper rule out any involvement of Glu-83 in either wild-type or ▲85 HPr active sites but do suggest a role for Leu-84. Leu-84 may be necessary to provide an appropriate hydrophobic environment for the phosphoryl-transfer reactions.

It would appear that HPrs from Gram-positive species have similar active site arrangements. 2D NMR studies of *Bacillus subtilis* HPr show that this HPr from a Gram-positive species has a tertiary structure that is similar to that of *E. coli* HPr and that *B. subtilis* HPr also has its C-terminus near the active site (Wittekind et al., 1990). An investigation of the phosphohydrolysis properties of *B. subtilis* P-HPr showed a behavior similar to that reported for *E. coli* HPr (J. W. Anderson and J. Reizer, unpublished results). In addition, the phosphohydrolysis properties of HPr from *Streptococcus faecalis* are similar to those for *E. coli* HPr (Waygood et al., 1988).

In a recent study, van Dijk et al. (1990) specifically incorporated [^{15}N]histidine into *E. coli* HPr and showed that both the $N^{\delta 1}$ and $N^{\epsilon 2}$ in the imidazole ring of His-15 are hydrogen-bonded. The $N^{\delta 1}$ hydrogen bond, which is weak, disappears upon phosphorylation of this nitrogen. It was also concluded on the basis of a theoretical calculation that there is no H bonding to the $N^{\epsilon 2}$ position in the phosphorylated state. It was therefore suggested that Glu-85 formed a hydrogen bond with the $N^{\epsilon 2}$ position in HPr but that in the P-HPr this involvement is broken. This is different from our suggestion that Glu-85 interacts with His-15 after phosphorylation (Waygood et al., 1986). The kinetic results presented suggest that Glu-85 is involved in the phosphoryl-transfer function rather than the phosphoacceptor function of HPr. As van Dijk et al. (1980) point out, such hydrogen bonding should increase the pK_a of the imidazole ring of His-15 while the experimental observations are the opposite (Dooijewaard et al., 1979; Kalbitzer et al., 1982). Elimination of Glu-85 does not alter the pK_a significantly; however the location of the α-carboxyl of Leu-84 is not known, and perhaps is compensating.

The pH dependence of the phosphohydrolysis of phosphohistidines (Hultquist et al., 1966, 1968) established that the dependence is not the same and that the $N^{\delta 1}$-P-histidine was about 10 times more labile than the $N^{\epsilon 2}$-P-histidine. This was attributed to an interaction between the amino group and the phosphoryl group at the $N^{\delta 1}$ position (Hultquist, 1968). The pH dependence of the $N^{\delta 1}$-P-histidine resembles that of phosphoimidazole (Jencks & Gilchrist, 1965). Denatured P-HPr has phosphohydrolysis properties remarkably similar to that of the free phosphoamino acid, and in native P-HPr the phosphohydrolysis properties change considerably (Waygood et al., 1985). We attribute the increased lability in native P-HPr to the active site arrangement. The increased lability in native P-HPr is characterized by two components: at physiological temperatures and below, the phosphohydrolysis shows a dependency on a moiety with an apparent pK_a of about

7.5 and another with an apparent pK_a of about 4.0. This behavior may be more than can be attributed to just the Arg-17 residue as suggested by van Dijk et al. (1990).

The pH dependence of phosphohydrolysis of native HPr does not resemble phosphoimidazole (Jencks & Gilchrist, 1965), either isomer of phosphohistidine (Hultquist et al, 1966, 1968), acyl phosphate (Koshland, 1952), and glucose-1-P (Bunton et al., 1958b). It does have some resemblance to methyl phosphate in which the dependence is attributed to the proportion of the monoanionic species (Bunton et al., 1958a). Similar suggestions about the monoanionic species and resemblances to methyl phosphate are found for phosphoarginine (Haake & Allen, 1971) and phosphocysteine (Herr & Koshland, 1957). For the pH dependence of phosphohydrolysis of native HPr, the most compelling resemblance is to the electrophilic catalysis of phosphoramidate (Jencks & Gilchrist, 1964) in which is described a requirement for first- and second-order reactions with formaldehyde and the involvement of RNH_3^+. Jencks and Gilchrist (1964) suggested that this type of catalysis, requiring three or four different molecules, would be greatly favored on the surface of an enzyme. Bell-shaped pH dependence of phosphohydrolysis has been attributed to the proportion of monoanionic phosphate present in methyl phosphate as determined by the pK_as of about 6.7 and 2.5 (Bunton et al., 1958a). Similarly, the increasing rates of phosphohydrolysis (moving from high to low pH) in phosphohistidines and phosphoimidazole have pK_as of 6.7–7.0 reflecting the dianionic/monoanionic equilibrium of the phosphate group (Jencks & Gilchrist, 1965; Hultquist et al., 1966; Hultquist, 1968). The pH dependence of the phosphohydrolysis curves for HPrs from *E. coli* (Figure 2), *S. faecalis* (Waygood et al., 1988), and *B. subtilis* (J. W. Anderson and J. Reizer, unpublished results) do not show a normal bell-shaped curve; the change of rates between pH 8.0 and 6.5 are too steep and suggest that more than one process is responsible. The mutants ▲84 and ▲83 do have bell-shaped curves with pK_as of about 2.5 and 6.7, which may simply reflect the occurrence of the monoanionic form of the phosphate group. This suggests that the C-terminal carboxyl group in wild-type HPr and the different C-terminal carboxyl group in ▲85 in effect cause a change in the proportion of the monoanionic form of the phosphoryl group, particularly in the physiological pH range of 6.5 and 8.0. The properties of the mutant E85K are consistent with this suggestion. E85K phosphohydrolysis shows an apparent pK_a of 2.5 instead of 4 (Figure 2), suggesting that the change to lysine in the side chain in effect ameliorates the action of the α-carboxyl. The modest changes in the kinetic properties (Table I) for E85K are consistent with this view.

The results in this paper suggest that, in conjunction with Arg-17, Glu-85 by itself may not be sufficient to cause the unusual phosphohydrolysis properties of HPr. Our results, and those of van Dijk et al. (1990), suggest the involvement of a yet unidentified residue in the active site. It is hoped that structural characterization of HPr along with the characterization of additional mutant HPrs will yield information regarding the nature of the unidentified moiety.

ACKNOWLEDGMENTS

We thank Alice Leung for her efficient and plentiful purification of HPr and S. Sharma for her enthusiastic interest. Dr. H. DeReuse and Dr. B. Erni are thanked for supplying *E. coli* strains. Drs. J. S. Lee, P. Hammen, and L. T. J. Delbaere are thanked for useful discussions.

Registry No. Leu, 61-90-5; Glu, 56-86-0; Gln, 56-85-9; Lys, 56-87-1; Ala, 56-41-7; Asp, 56-84-8; Enzyme I, 37278-09-4; Enzyme II, 37278-17-4.

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Ca^{2+}-Dependent Annexin Self-Association on Membrane Surfaces†

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Received June 14, 1991

ABSTRACT: Annexin self-association was studied with 90° light scattering and resonance energy transfer between fluorescein (donor) and eosin (acceptor) labeled proteins. Synexin (annexin VII), p32 (annexin IV), and p67 (annexin VI) self-associated in a Ca^{2+}-dependent manner in solution. However, this activity was quite labile and, especially for p32 and p67, was not consistently observed. When bound to chromaffin granule membranes, the three proteins consistently self-associated and did so at Ca^{2+} levels (pCa 5.0-4.5) approximately 10-fold lower than required when in solution. Phospholipid vesicles containing phosphatidylserine and phosphatidylethanolamine (1:1 or 1:3) were less effective at supporting annexin polymerization than were those containing phosphatidylserine and phosphatidylcholine (1:0, 1:1, or 1:3). The annexins bound chromaffin granule membranes in a positively cooperative manner under conditions where annexin self-association was observed, and both phenomena were inhibited by trifluoperazine. Ca^{2+}-dependent chromaffin granule membrane aggregation, induced by p32 or synexin, was associated with intermembrane annexin polymerization at Ca^{2+} levels less than pCa 4, but not at higher Ca^{2+} concentrations, suggesting that annexin self-association may be necessary for membrane contact at low Ca^{2+} levels but not at higher Ca^{2+} levels where the protein may bind two membranes as a monomer.

The annexins are a newly described group of homologous proteins that bind phospholipid membranes in a Ca^{2+}-dependent manner [for reviews see Klee (1988), and Burgoyne and Geisow (1989)]. Some members of this group are also commonly known as lipocortins (Huang et al., 1986), calpactins (Glenney, 1986), chromobindins (Creutz et al., 1983, 1987), calelectrins (Sudhof et al., 1984), or placental anticoagulant proteins (Tait et al., 1988). Currently, 10 distinct members of this gene family, termed annexins I-X, have been identified (Pepinsky et al., 1988; Burns et al., 1989; Hauptmann et al., 1989; Johnston et al., 1990). Comparison of their amino acid sequences reveals a common structural theme: Each protein has two regions, a variable-length amino-terminal region lacking homology with other members of the family and a core region of four or eight repeating 70 amino acid domains, which share 40-60% homology between family members.

The biological function of the annexin proteins is unknown, but they have been hypothesized to play a role in signal transduction (Hollenberg et al., 1988), in exocytosis (Creutz et al., 1978, 1983), in the organization of membrane phospholipid domains (Geisow et al., 1987), as structural/regulatory elements of the cytoskeleton (Glenney, 1986), and as regulators of phospholipase A_2 (Huang et al., 1986). These theories are based on the preferential cellular localization of some of these proteins to the plasma membrane/cortical cytoskeleton and their ability to bind in a Ca^{2+}-dependent manner to phospholipid membranes and in some cases to cytoskeletal elements (Geisow et al., 1987). However, the mode of interaction of the annexins with phospholipids and proteins at the membrane surface is unclear. In addition to interactions with other proteins, some annexins have been reported to self-associate. This phenemonon was first reported for isolated synexin in solution, which formed 50 × 150 Å rods, bundles of rods, and paracrystalline arrays in a Ca^{2+}-dependent manner (Creutz et al., 1979). A similar self-association event was seen with isolated Torpedo calelectrin, which formed morphologically different structures: circular forms composed of 50-Å globular subunits in the absence of calcium and amorphous aggregates of polygonal structures each composed of the 50-Å globular subunit in the presence of calcium (Walker et al., 1983). Other reports, however, have claimed that other members of the annexin family such as p32 (annexin IV) or calpactin (annexin II) do not self-associate (Shadle et al., 1985). Similarly, it has been debated whether annexins self-associate on membrane surfaces. Some investigators have

† This study was supported by a grant from the NIH (DK33151). C.E.C. was supported by an Established Investigator award from the American Heart Association with funds contributed in part by the Virginia Affiliate.
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0006-2960/91/0430-9607$02.50/0