Phosphoryl Transfer between Phosphorylated Histidine-Containing Protein and Histidine-Containing Protein Is Not Autocatalytic†

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ABSTRACT: Histidine-containing protein, HPr, is a phosphocarrier protein that is part of the bacterial phosphoenolpyruvate:sugar phosphotransferase system. HPr is phosphorylated by enzyme I, and P-HPr transfers the phosphoryl group to the IIA domain of a number of sugar-specific enzyme II complexes. Autocatalytic phosphoryl transfer between P-HPr and HPr has recently been reported [van Dijk, A. A., Eisermann, R., Hengstenberg, W., & Robillard, G. T. (1991) *Biochemistry 30*, 2876–2882]. Our results show that this phosphoryl transfer is due to an unidentified contaminant of HPr preparations. The phosphoryl transfer activity is not present in all HPr preparations. When present, the phosphoryl transfer activity can be removed by further purification or destroyed over time by resuspension of HPr preparations in water. There is no autocatalytic phosphoryl transfer between P-HPr and HPr.

Many bacterial species transport sugars into their cells via the phosphoenolpyruvate:sugar phosphotransferase system, PTS,[1] which also phosphorylates the sugar [see review by Meadow et al. (1990)]. There are several steps in this process that involve phosphoryl transfer reactions between protein components, and most often these involve phosphohistidines at the active sites. One of these components, histidine-containing protein (HPr), has a $N^{\delta 1}$-P-histidine, which is derived from a $N^{\epsilon 2}$-P-histidine in the active site of enzyme I and yields a $N^{\epsilon 2}$-P-histidine in the IIA domain of a sugar-specific enzyme II.

van Dijk et al. (1991) have reported that there can be an autocatalytic phosphoryl exchange between P-HPr_a + HPr_b ↔ HPr_a + P-HPr_b without the involvement of other protein components. This is a $N^{\delta 1}$-P-histidine giving rise to an $N^{\delta 1}$-P-histidine at the active sites of these HPrs. This activity as pointed out by van Dijk et al. (1991) provides a facile method by which mutants of HPr could be evaluated in respect to their phosphoryl transfer equilibrium.

Hultquist (1968) showed that phosphoryl transfer between phosphohistidines and histidines occurred, and phosphoryl migration has often been a concern in the correct identification of phosphoamino acids, especially phosphohistidines [see Meadow et al. (1990)]. These facts presumably helped to give acceptance to the demonstration of autocatalytic transfer of phosphoryl groups between HPrs. However, in the case of inter-HPr phosphoryl exchange, this could be catalyzed by any sugar-specific IIA domain or enzyme I. van Dijk et al. (1991) went to considerable lengths to eliminate the possibility that the IIA^{glc} protein (the most likely candidate) was catalyzing the phosphoryl exchange but were not as rigorous with potential contamination by enzyme I or other IIA proteins.

In this paper, we will show that the inter-HPr phosphoryl transfer activity can be removed from HPr preparations.

MATERIALS AND METHODS

Protein Preparations. Enzyme I was a homogeneous preparation purified as described by Anderson et al. (1991). HPr and mutant HPrs were plasmid-derived and were purified to homogeneity as has previously been described (Sharma et al., 1991; Anderson et al., 1991, 1993; Sharma, 1992). The pUC13 plasmids containing HPr genes were in *Escherichia coli* strain TP2811 [F^- *xyl argH1 lacX74 aroB ilvA* (*ptsH ptsI crr*) Km^R] (Levy et al., 1990) which was grown in Luria broth (Maniatis et al., 1982) with 100 μg/mL ampicillin. All HPr preparations were judged to be homogeneous by isoelectric focusing and were stored lyophilized at −70 °C. The HPr proteins were resuspended in water at about 10 mg/mL and filtered (0.22 μm) into sterile tubes.

Mono-Q FPLC Chromatography. The Mono-Q chromatography for the separation of HPr, P-HPr, mutant HPr, and P-(mutant)HPr was carried out using the conditions described by van Dijk et al. (1991). Elution of HPr was detected using a 214-nm UV monitor, and peak heights provided adequate quanititation.

Rechromatography. Rechromatography of HPr or P-HPr required dilution with 0.05 M Tris-HCl buffer, pH 9.0, to decrease salt concentrations.

Isolation of P-HPrs. HPr (≃0.8 mg) was incubated for 5 min at room temperature with 5 μg of enzyme I, 10 mM phosphoenolpyruvate, 5 mM $MgCl_2$, 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer, pH 7.0, 1 mM EDTA, and 0.2 mM dithioerythreitol in 0.2 mL. This incubation gave >90% P-HPr. The reaction mixture was loaded onto the Mono-Q column, and P-HPr was collected manually as it eluted. Separation of P-HPr from enzyme I, phosphoenolpyruvate, and HPr was as described by van Dijk et al. (1991).

Phosphoryl Transfer. Phosphoryl transfer was carried out between HPrs with different p*I*s so that they could be distinguished by their elution positions from Mono-Q chromatography. The phosphoryl transfer incubation was usually 5 min at room temperature (23 °C) in 0.05 M Tris-HCl buffer, pH 9.0, in 0.05–0.2 mL.

HPr Quantitation. HPr activity was assayed in an enzyme II^{man}-dependent reaction as previously described (Waygood et al., 1979). HPr protein concentrations were determined by

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[1] Abbreviations: PTS, phosphoenolpyruvate:sugar phosphotransferase system; HPr, histidine-containing phosphocarrier protein of the phosphotransferase system; P-HPr, phosphorylated histidine-containing protein; FPLC, fast protein liquid chromatography; glc, glucose; man, mannose.

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the lactate dehydrogenase depletion assay (Waygood et al., 1979) or by the spectrophotometric method of Waddell (1956). For FPLC, samples of HPr with different quantities were chromatographed separately. Peak heights vs HPr quantity gave a linear plot. In mixed samples, the quantities in each peak were determined on the basis of the relative peak heights.

Phosphohydrolysis. Phosphohydrolysis of E70A was carried out as previously described (Anderson et al., 1991).

RESULTS AND DISCUSSION

There are a number of known PTS components that could, if present, catalyze inter-HPr phosphoryl transfer: enzyme I, IIA^{glc}, other IIA proteins such as those for mannose, glucitol, and fructose, and even IIA domains released by proteolysis from enzymes II specific for mannitol and *N*-acetylglucosamine (Meadow et al., 1990). The HPr preparations were derived from genes in pUC13 plasmids grown in *E. coli* strain TP2811, which contains a deletion through the genes for HPr and enzymes I and IIA^{glc}. Thus these components of the PTS could not contaminate the HPr or mutant HPr preparations used in the experiments reported here. Except for IIA^{man}, the other PTS components are inducible by growth on their specific sugars, and growth on Luria broth would not be expected to produce these inducible proteins in high amounts [for example, see Waygood et al. (1984)]. IIA^{man} is membrane-associated, and although constitutively produced it is not normally considered to be in the soluble fraction. However, it would be difficult to account for all the known components of the PTS in the HPr preparations, and this was not attempted in either this work or the previous report by van Dijk et al. (1991). The two PTS components other than HPr that are found constitutively at high levels are IIA^{glc} and enzyme I, which are eliminated from our HPr preparations by the choice of *E. coli* strain.

Our purification procedure for HPr contains extra chromatography steps as compared to that used by van Dijk et al. (1991). Briefly, the purification is removal of membranes by ultracentrifugation, Ultragel AcA54 molecular sieve chromatography, Q-Sepharose chromatography, S-Sepharose chromatography, and usually another Q-Sepharose chromatography. When the amino acid replacements introduced into HPr cause a change in charge for the residue, the purification steps involving ion-exchange chromatography yield HPr eluting at different positions in a gradient, thus giving preparations of HPr mutants different potential contaminants. However, all preparations were judged to be homogeneously pure by isoelectric focusing gels.

Initially, we tried to use the interphosphoryl exchange assay to assess several substitutions of Arg17, which is an invariant active-site residue and which we have investigated in some detail (Anderson et al., 1993). No phosphoryl transfer was obtained between HPr and the mutants R17G and R17S, even at high ratios between P-HPr and mutant or P-mutant and HPr and extended incubation times (Table I). This could be explained by proposing that the Arg17 residue was essential for the phosphoryl exchange process. However, two positive controls presented two different causes for concern. In the E70A/P-HPr exchange, there was always more dephosphorylation than phosphoryl transfer (Table I). Phosphorylated E70A has phosphohydrolysis properties (results not shown) similar to those of wild type (Anderson et al., 1993). Of more concern, however, was the inability to demonstrate phosphoryl transfer from P-HPr to E83A, while P-E83A would phosphorylate either HPr (Figure 1A, Table II) or Q3E (result not shown). For some of the phosphoryl exchange reactions,

Table I: Phosphoryl Exchange between HPr and Mutant HPrs

		starting concn (μM)		equilibrium concn (μM)			
HPr_a	HPr_b	HPr_a	P-HPr_b	HPr_a	P-HPr_a	HPr_b	P-HPr_b
E70A	HPr	40	40	35	8	15	19
E70A	HPr	45	35	37	8	16	20
E70A	HPr	27	36	14	5	17	24
R17S	HPr	300[a]	50	300	0	0	50
R17G	HPr	300[a]	50	300	0	0	50
HPr	R17S	18	20	18	0	0	20
HPr	R17S	600[a]	20	600	0	0	20
HPr	R17G	30	35	30	0	0	35

[a] Attempted phosphoryl transfer was carried out for 5- and 40-min incubations with the same result. The imbalance in the sample concentration led to the peak of the larger component obscuring some of the other peak positions. However, there was no reduction in the P-R17S or P-HPr peak height as compared to a control.

Table II: Removal of Phosphoryl Transfer Activity[a]

			starting concn (μM)		equilibrium concn (μM)			
HPr_a	HPr_b	sample	HPr_a	P-HPr_b	HPr_a	P-HPr_a	HPr_b	P-HPr_b
E83A	HPr	1	50	50	47	2	2	45
		2	100	50	97	2	2	47
HPr	E83A	3	100	50	52	48	24	26
		4	100	50	70	30	14	36
		5	100	50	95	3	2	48
		6	60	100	60	<1	<1	100
Q3E	HPr	7	40	50	18	22	28	23
		8	40	100	32	8	10	90
		9	60	100	60	<1	<1	100

[a] E83A was used from a purified sample without further treatment. Samples 1 and 2 were incubated for 5 and 30 min. (2) E83A was rechromatographed on Mono-Q. (3) P-83A was used after isolation by chromatography. (4) P-E83A and HPr were rechromatographed on Mono-Q once. (5, 6) HPr was chromatographed two more times. (7) Q3E was used from a purified sample without further treatment. (8) Q3E was produced from dephosphorylation of isolated P-Q3E, and P-HPr was produced from rechromatographed HPr. (9) Q3E from (8) was rechromatographed on Mono-Q two more times, as was the HPr that was used to produce HPr.

e.g., P-E83A/HPr exchange (Figure 1B), stoichiometric transfer and a $K_{eq} = 1$ were observed, which is consistent with the results reported by van Dijk et al. (1991). Both Q3E with HPr and Q3E with E83A phosphoryl exchanges were found to occur in both directions (results not shown).

The lack of reversible transfer for E83A and HPr was fundamentally anomalous. The homogeneously pure (as judged by isoelectric focusing) E83A was chromatographed on the FPLC Mono-Q column to attempt to remove anything that may inhibit the phosphoryl transfer reaction. This did not result in any phosphoryl transfer. The P-E83A, which like other P-HPrs was isolated by similar chromatography following an incubation with enzyme I and phosphoenolpyruvate, was rechromatographed, as was HPr. The subsequent phosphoryl exchange was reduced (Figure 1C). HPr was rechromatographed two more times, and the phosphoryl exchange activity was eliminated (Figure 1D). At each round, the P-E83A/HPr phosphoryl transfer was measured using a standard 5-min incubation. The results suggest that the HPr rather than E83A contained a contaminant that promoted phosphoryl transfer. Because Q3E with P-E83A completed phosphoryl exchange, Q3E must have contained the contaminating activity. If HPr contained the contaminating activity, then the phosphoryl transfer would seem to require Arg17 in both HPr and P-HPr (Table I).

A rechromatography of P-Q3E did not reduce the phosphoryl transfer capacity. However, because of the nature of the mutations, P-Q3E elutes at a different position during

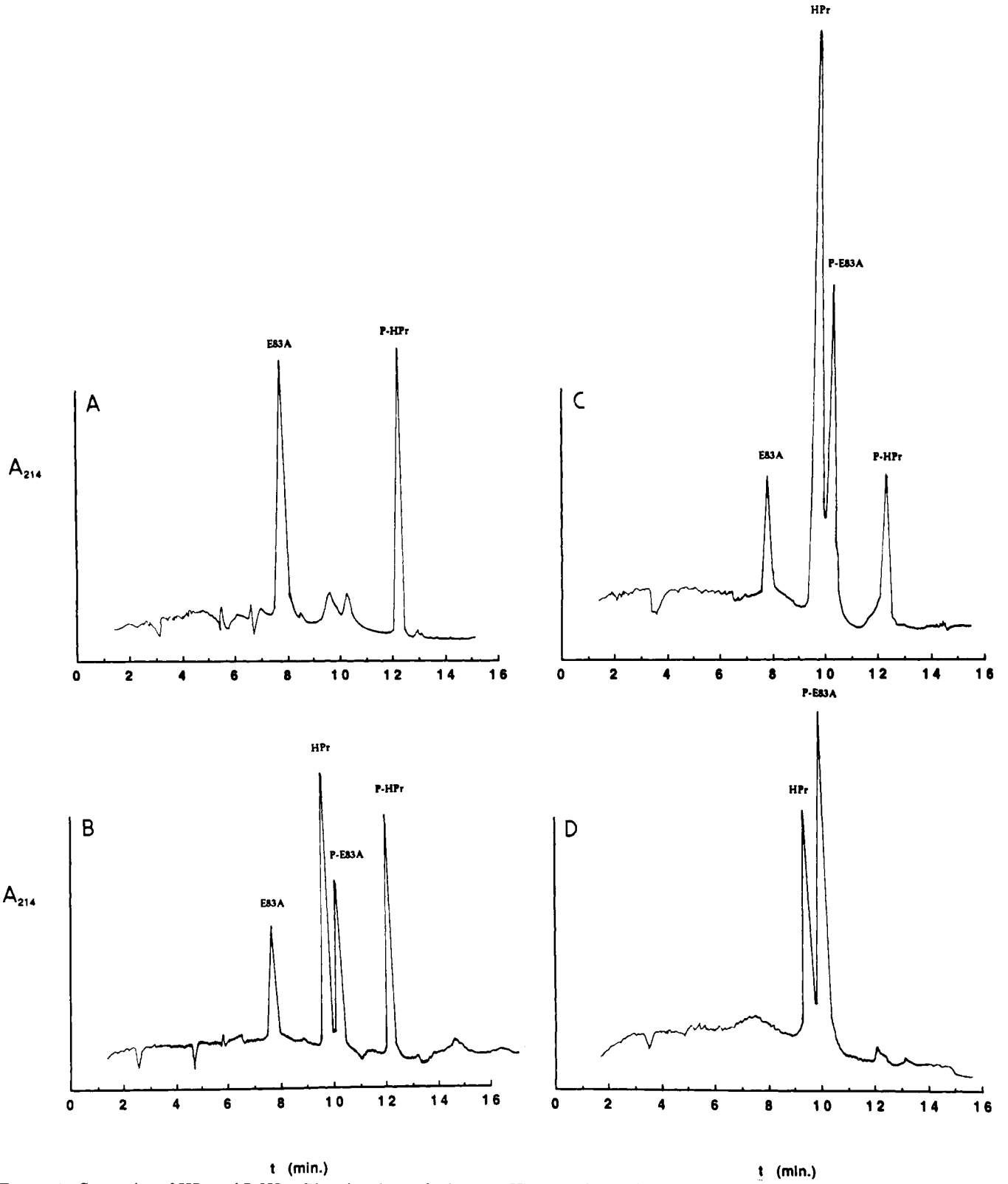

FIGURE 1: Separation of HPr and P-HPr. Phosphoryl transfer between HPrs was detected using HPrs with different p*I*s. Incubations were for 5 min, and samples were placed in a sample loop and loaded onto the FPLC Mono-Q column after 2 min. Elution positions of either phosphorylated or nonphosphorylated HPrs were determined by chromatography of individual protein preparations, and the phosphoryl exchange conditions are described in Table II. (A) E83A and P-HPr; Table II, sample 1. (B) P-E83A and HPr; Table II, sample 3. (C) P-E83A and HPr after rechromatography of P-E83A and HPr; Table II, sample 4. (D) P-E83A and HPr after two more rounds of rechromatography of HPr; Table II, sample 5. The peak heights vary because of different loading conditions.

chromatography from the other HPrs used in this study. Therefore P-Q3E was treated as follows: rechromatography; dephosphorylation by incubation at pH 6, where P-HPr has a half-life of about 5 min at 37 °C; and rechromatography of the dephosphorylated Q3E three times. Also, at each step HPr was rechromatographed. This regime led to a removal of phosphoryl transfer ability (Table II), which was apparently contaminating both HPr and Q3E protein. Attempts at rechromatography of either Q3E or HPr on their own did not remove the phosphoryl transfer activity.

In all the above, it appears that some activity can be purified away from the HPrs in experiments that lasted about 3 days in each case. However, it was found that when HPr, E83A, Q3A, and HPr were filtered into sterile tubes and left suspended in water for about 4 weeks on ice, P-E83A, when isolated, would not carry out phosphoryl transfer with either HPr or Q3E. This suggests that the preparations contained a labile contaminant. The HPrs all chromatographed as they did when they were freshly prepared solutions, which suggests no chemical alteration to HPr causing a change in charge; they could all be fully phosphorylated by enzyme I and retained 100% phosphoryl donor activity with respect to enzyme II^{man}.

Rechromatography of enzyme I prior to use in incubations to yield P-HPr or P-HPr(mutant) did not alter the phosphoryl transfer properties that were detected. The experimental protocol used to remove the phosphoryl transfer activity was tested each time with freshly prepared P-HPr or P-mutant HPr; thus the contamination does not come from the enzyme I preparation. Phosphoryl transfer between P-HPr and E83A was not detected in the presence of the following compounds at 0.1 mM: histidine, lysine, arginine, pyridine, and imidazole.

The series of experiments described above show that the inter-HPr phosphoryl exchange is not autocatalytic as reported by van Dijk et al. (1991) but rather catalyzed by some component that contaminates some HPr preparations. Even the purest of HPr preparations, which may be as high as 99% HPr, are contaminated with other proteins at very low concentrations. For most applications this presents no problem, but clearly events that may appear autocatalytic could be due to catalytic contaminants present in very small amounts. How to routinely remove this activity has not been determined, and the procedures to be used may vary with different purification procedures applied to HPr. The lability of the phosphoryl transfer activity in water may be generally applicable.

It had been our intention to use this phosphoryl exchange to help in the evaluation of mutants: we were simply lucky that one of our mutant preparations, E83A, gave such anomalous results. We must conclude that HPr/P-HPr autocatalysis of phosphoryl exchange does not occur.

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REFERENCES

Anderson, J. W., Bhanot, P., Georges, F., Klevit, R. E., & Waygood, E. B. (1991) *Biochemistry 30*, 9601–9607.

Anderson, J. W., Pullen, K., Georges, F., Klevit, R. E., & Waygood, E. B. (1993) *J. Biol. Chem.* (in press).

Hultquist, D. E. (1968) *Biochim. Biophys. Acta 153*, 329–340.

Levy, S., Zeng, G.-Q., & Danchin, A. (1990) *Gene 86*, 27–33.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Meadow, N. D., Fox, D. K., & Roseman, S. (1990) *Annu. Rev. Biochem. 59*, 497–542.

Sharma, S. (1992) Ph.D. Thesis, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Sharma, S., Georges, F., Delbaere, L. T. J., Lee, J. S., Klevit, R. E., & Waygood, E. B. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4877–4881.

van Dijk, A. A., Eisermann, R., Hengstenberg, W., & Robillard, G. T. (1991) *Biochemistry 30*, 2876–2882.

Waddell, W. J. (1956) *J. Lab. Clin. Med. 48*, 311–314.

Waygood, E. B., Meadow, N. D., & Roseman, S. (1979) *Anal. Biochem. 95*, 293–304.

Waygood, E. B., Mattoo, R. L., & Peri, K. G. (1984) *J. Cell. Biochem. 25*, 139–159.