Deamidation of HPr, a Phosphocarrier Protein of the Phosphoenolpyruvate:Sugar Phosphotransferase System, Involves Asparagine 38 (HPr-1) and Asparagine 12 (HPr-2) in Isoaspartyl Acid Formation*

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Histidine-containing protein, HPr, of the phosphoenolpyruvate:sugar phosphotransferase system in *Escherichia coli*, when incubated at elevated temperatures forms many species of protein. The two major species are HPr-1 and HPr-2, which have been shown to lack one or two amides, respectively (Anderson, B., Weigel, N., Kundig, W., and Roseman, S. (1971) *J. Biol. Chem.* 246, 7023-7033). The formation of HPr-1 and HPr-2 is shown to be pH-dependent and does not occur readily below pH 6. Investigation of the identities and properties of the two residues that deamidate involved creation of site-directed mutants at the 6 glutamine and 2 asparagine residues of HPr; description of their deamidation species by isoelectric focusing; determination of their relative antibody binding properties; assay of their phosphoacceptor and phosphodonor activities; characterization of tryptic and V8-protease peptides; obtaining two-dimensional nuclear magnetic resonance spectra of HPr, HPr-1, and several mutants. It was determined that the sequential deamidation of Asn-38 and Asn-12 yields HPr-1 and HPr-2. Both residues exist as Asn-Gly pairs, and both deamidations probably form isoaspartyl acid. HPr from *Bacillus subtilis* and *Staphylococcus carnosus* which also have Asn-Gly at residues 38 and 39 form HPr-1 species presumably by deamidation. HPr from *Streptococcus faecalis* which does not have Asn-38 does not form a HPr-1 species. The *E. coli* mutant HPrs, N12D and Q51E, residues that may be involved in the active site, had impaired phosphohydrolysis properties and decreased phosphoenolpyruvate:sugar phosphotransferase system activity.

The histidine-containing phosphocarrier protein, HPr, of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) is well described for a number of bacterial species. Tertiary structures of HPr from *Escherichia coli* (Klevit and Waygood, 1986; El-Kabbani et al., 1987; Hammen et al., 1991; van Nuland et al., 1992), *Bacillus subtilis* (Wittekind et al., 1990, 1992; Herzberg et al., 1992), *Staphylococcus aureus* (Kalbitzer et al., 1991), and *Streptococcus faecalis* (Jia et al., 1993) have been reported. HPr has been considered a stable protein (Kundig et al., 1964), yet upon heating or storage, HPr from *E. coli* is susceptible to two major deamidation events to yield HPr-1 and HPr-2 (Anderson et al., 1971; Waygood et al., 1985). It was reported that both HPr-1 and HPr-2 had impaired activity in a sugar phosphorylation assay, and careful amino acid analysis indicated deamidation caused these two species (Anderson et al., 1971). However, Waygood et al. (1985) showed that HPr-1 retained full activity while HPr-2 had impaired activity with both enzyme I and enzyme II* supplement.* In addition, HPr-2 has impaired phosphohydrolysis properties in that the phosphohistidine, Hist(P)-15, is more stable. Neither HPr-1 nor HPr-2 has altered binding to several HPr-specific monoclonal antibodies (Waygood et al., 1987), which suggests that conformational changes caused by deamidation are local (Sharma et al., 1991).

Deamidation in proteins is a widespread occurrence; generally a non-enzymatic event, but one for which there may be a repair system, protein carbamylmethyl transferase. Deamidation is suggested as a potential biological clock in aging; a signal that initiates protein degradation; an important contributing factor in protein denaturation; and both enzymatic deamidation and carbamylmethylation are important features in signal transduction in chemotaxis in *E. coli* (see reviews, Clarke (1985) and Wright (1991a)). The most rapidly occurring deamidation events occur where asparaginyl-glycine pairs are found (Wright, 1991b), and this deamidation involves a β-aspartyl shift mechanism with a number of potential products including D- and L-isomer forms of aspartic and isoaspartic acid. The major species produced are the L-isomers of

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1 The abbreviations used are: PTS, phosphoenolpyruvate:sugar phosphotransferase system; HPr, histidine-containing phosphocarrier protein of the PTS; MES, 2-(N-morpholino)ethanesulfonic acid; DQF-COSY, two-dimensional double-quantum filtered coherence spectroscopy; TOCSY, two-dimensional total coherence spectroscopy; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; Mtl, mannitol; HPLC, high performance liquid chromatography.
aspartic and isoaspartic acid in an approximate 1:3 ratio (Bornstein and Baiian, 1976; Geiger and Clark, 1987). This mechanism is not the only mechanism that leads to deamidation of asparagines (Kossiakoff, 1988; Wright, 1991a). In general, deamidation of asparaginyl residues is increased by alkaline pH, phosphate and arsenate anions, and inhibited by secondary and tertiary structure. Glutamine deamidations are not as well characterized (Robinson and Rudder, 1974; Wright, 1991a).

In HPr, there are two asparaginyl-glycine pairs, Asn-12-Gly-13 and Asn-38-Gly-39; the former is located in a loop, which contains the active site; the latter is part of a β-turn structure (Klevit and Waygood, 1986; El-Kabbani et al., 1987). There are also 6 glutamine residues: Gln-3, Gln-4, Gln-21, Gln-51, Gln-57, and Gln-71. When HPr is boiled in phosphate buffer at pH 7.5, conditions which lead to rapid deamidation, there are many species of HPr formed with pI values that are more acidic. The major species formed are HPr-1 and HPr-2, and at least for HPr-1 the species appears identical whether formed under the above conditions or during long term storage (Waygood et al., 1985). HPr-1 was studied by two-dimensional NMR, and it was concluded that there were two glutamine deamidations, Q57E and Q71E, in this deamidated species (Klevit et al., 1988). However, the spectral evidence was not conclusive, and other approaches appeared necessary to confirm this conclusion, and to identify the events leading to deamidation and kinetic functions of HPr (Waygood et al., 1988). Glutamine deamidations are to be involved in chemotaxis that is dependent upon the PTS (Lengeler and Vogler, 1989; Giubil et al., 1991). Deamidated HPr at Ser-17696 was studied by three-dimensional NMR, and it was concluded that there were two glutamine residues (Waygood and Steeves, 1980), and subsequently HPr-1 and HPr-2 were derived as wild type with the two mutagenic primers. The double mutant N12D and N38D was made using N12D DNA as the template.

Deamidation of HPr mutants was carried out using synthetic oligonucleotides as described by Sharma et al. (1991) and Anderson et al. (1991). The primers used were as follows: Q3E, 5'-TCTCTGTCGAAATC-3'; Q5K, 5'-AACCTTCTGTTGGA-3'; Q5S, 5'-AACTCTCTTGCTTGAACTG-3'; Q4S, 5'-GGTACA-CTTCTCTTGCGAAATC-3'; N12D, 5'-CACAGCTCCGCGGAG-3'; Q21E, 5'-TACAACTCGGCAGC-3'; QZL, 5'-TTTTACAAA- GGTGCCAGCAGG-3'; N38D, 5'-TTTGCCTGCGAGGT-3'; Q51E, 5'-CCAGGCTCCGTTGAGT-3'; Q57E, 5'-GGTACCTTGCTGAGT-3'; Q71E, 5'-CCGTTTCTCCTGCT-3'. The double mutant Q57E/Q71E was made using the wild type template with the two mutagenic primers. The double mutant N12D/N38D was made using N12D DNA as the template.

Demonstration of Deamidation Using Isotope-Focusing Gels—HPr and mutant HPrs were incubated in different buffers: pH 3 and 5, citrate-phosphate buffer; pH 6.0, MES; pH 7.5, potassium phosphate buffer. All buffers were adjusted at room temperature. The procedure used and the isoelectric focusing was as described by Waygood et al. (1985). Peptide Analysis—Samples of various HPrs (1 mg) were incubated with 0.1 mg of the proteolytic enzymes in 0.2 ml of 50 mm ammonium bicarbonate, pH 8.0. For the V8 protease incubations, 1 mm EDTA was included. Incubations were for 1.5, 5, 6, and 20 h. After incubation, the samples were diluted to about pH 9.0 in 100:1 mixture of spectroscopy grade H2O and stored frozen (−20 °C). Separation of the peptides was performed on a reverse-phase column (Sera RP8, 10 mm, 4.6 mm) using a linear gradient at 1 ml/min. The gradient was solvent A (0.1H trifluoroacetic acid in water) 100 to 60% in 60 min; solvent B (0.1H trifluoroacetic acid in 100% acetonitrile) 100 to 60% in 60 min; the solvent B was 65% acetonitrile in water. The effluent was monitored at 230 nm, and samples were collected manually. Samples applied were from 1-2 nmol of protein.

Amino Acid Analysis—Amino acid analysis was performed on 200-400 pmol of peptide using the methodology Heinidrkon and Meredith (1984) by Dr. Meyer at the Amino Acid Analysis Facility, University of Washington, Seattle.

Two-dimensional NMR Spectroscopy—Purified proteins were dialyzed against 5 mM potassium phosphate buffer, pH 6.5, containing 0.01 mm EDTA. The solutions were lyophilized and dissolved in 90% H2O and 10% D2O (or in 99.96% atom D2O) to give samples that were ~4 mm in protein, 50 mM in buffer, and 0 mM in EDTA. Two-dimensional NMR spectra were acquired on a Bruker AM-500 spectrometer at 30 °C. They were obtained in the pure-phase absorption mode using time proportional phase incrementation (Marion and Davis, 1985) was used for D2O TOCSY spectra with mixing of 70 ms. Clean TOCSY spectra (Greisinger et al., 1988) were acquired for HPr and HPr-1 with mixing times of 46 ms. The NOESY spectrum of HPr-1 was acquired using a 52-step phase cycle and a mixing time of 100 ms. The two-dimensional NMR spectra were acquired on a Bruker AM-500 spectrometer at 30 °C. They were obtained in the pure-phase absorption mode using time proportional phase incrementation (Marion and Wiirth, 1983). Data were processed using the FELIX 1.0 (Hare Research, Woodinville, WA) on a Silicon Graphics 4D-35 workstations.

Materials—Enzymes used in the manipulation of plasmid DNA were obtained from either Pharmacia LKB Biotechnology Inc. or New England Biolabs. Ampholytes were obtained from Pharmacia. Radioactive chemicals were obtained from Du Pont-New England Nuclear. Q-Sepharose was obtained from Pharmacia. Acetoniitric acid was HPLC grade from Merck. Guanidine HCl was enzyme grade from Schwarz-Mann Biochemical. Trypsin (1:1-trypanolamido-2-phenyl) was treated with Panserose (Daehn, 1983, and an improved separation was achieved using Q-Sepharose in place of DEAE-cellulose. HPr, Q4K, was obtained from Salmonella typhimurium strain SB3093 (Benesi et al., 1982) as described by Waygood et al. (1987). B. subtihile HPr was obtained as described by Reizer et al. (1989); S. faecalis HPr was obtained as described by Kalibitzer et al. (1982) and Staphylococcal HPr was obtained as described by Endo and Hara (1987). Enzyme I was obtained as described by Anderson et al. (1991). HPr-specific monoclonal antibodies were obtained as described by Waygood et al. (1987). Phosphoenolpyruvate carboxykinase was a gift from Dr. Hughes Goldie (University of Saskatchewan).
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Fig. 1. pH and temperature dependence of HPr deamidation. HPr (0.1 mg/ml) was heated at various temperatures and pH values in a thin-walled glass test tube. Samples (0.05 ml) were taken at the times (minutes) indicated, cooled, and applied to an isoelectric focusing gel. The gels contained pH 3-10, 1:2:2 ampholytes (1:2:2) and were run for 2 h at 8 watts (voltage limiting to 1000 V). The 0.01 M buffers used were: pH 6, MES; pH 7.5, potassium phosphate; pH 10, borate. The gels shown had the cathode on top. H indicates a sample of HPr, which in some cases contains HPr-1 as indicated.

**RESULTS**

*Deamidation of HPr*—Deamidation reactions in proteins occur more readily at alkali pH and are accelerated by the presence of anions such as phosphate (Robinson and Rudd, 1974; Wright, 1991a). Deamidation of HPr was assessed by heating HPr in buffers of different pH values and resolving the samples on isoelectric focusing gels (Fig. 1). HPr at pH 3.0 and pH 5.0 did not deamidate to any significant extent for the time period studied, 2 h at 80°C (results not shown). Even at pH 6.0 deamidation was not observed, but for increasing pH values, the rate of deamidation increased (Fig. 1, B, D, and E). The rate of deamidation in phosphate buffer was somewhat faster than in HEPES and Tris-HCl buffers at the same concentration and pH (results not shown). At all pH values, except pH 10 (Fig. 1D), the total amount of HPr observed on the isoelectric focusing gels (Figs. 1–3) remained approximately constant with time. At pH 10, the amount of HPr found on the gel declined with time indicating that alkali-dependent chain cleavage may occur at the two Asn-Gly pairs found in HPr (see Wright (1991a)). The rate of deamidation at 60°C (Fig. 1A) was clearly slower than would have been anticipated by comparison to the 80 and 100°C rates (Fig. 1, B and C). Also at 60°C, the formation of HPr-2 was not observed. This suggests that some conformational change or partial unfolding enhances the rate of deamidation. When deamidation occurred, the major species formed were HPr-1 and HPr-2, however, as has previously been noted (Waygood et al., 1985) there were other species formed which are particularly evident following boiling (Fig. 1A). Lower temperatures appeared to produce fewer observable species (Fig. 1, B and C). However, deamidation during long term storage of frozen solutions of HPr in 10 mM Tris-HCl buffer, pH 7.5, resulted in many species of HPr (Waygood et al., 1985).

*Deamidation Properties of HPr Mutants*—A series of mutants were created for all 6 glutamine and 2 asparagine residues. The initial mutagenesis was carried out at residues Gln-57 and Gln-71 because of the identification of these residues as the sites of deamidation (Klevit et al., 1988). Both these residues were proposed to deamidate in HPr-1, and because mutation of these residues did not completely reproduce the HPr-1 properties, other residues were mutated. The rationale for mutant construction was as follows. If the amide residue is not involved in the formation of either HPr-1 or HPr-2, then a neutral change in the residue (e.g. to serine or threonine) should not alter the formation of HPr-1 and HPr-2, nor change the pI values of HPr and its deamidated species. A change to an acidic residue would allow the formation of HPr-1 or HPr-2, but would lead to major deamidation species with pI values lower than those observed for HPr-1 and HPr-2. If the residue was involved in the formation of HPr-1, then the neutral change would limit the subsequent formation of the major deamidated species to only one. A change to an acidic residue would mimic either HPr-1 and allow HPr-2 formation, or be equivalent to the deamidation that leads to HPr-2, and allow the formation of a species with a pI equivalent to HPr-2 following the deamidation at the HPr-1 residue(s). In addition, the appearance of doublet bands on the isoelectric focusing gels, which are characteristic of HPr-2 preparations was closely followed. These doublets (examples seen in Figs. 1 and 2) appear to be characteristic of isoaspartyl and aspartyl
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residue formation. There are several well characterized deamidations in calbindin and cholera toxin, where isoelectric focusing gels show doublet bands with approximately the 1:3 or 1:4 ratio (Chazin et al., 1989; Spangler and Westbrook, 1989).

The results of heating experiments to induce deamidation in preparations of Q57E, Q71E, and the double mutant Q57E+Q71E are shown in Fig. 2. Both Q57E and Q71E had PI values that were intermediate between HPr and HPr-1, and the double mutant had a PI that was very close to HPr-1 (Fig. 2A). These results appeared to confirm the identification of HPr-1 (Klevit et al., 1988). However, neither Q57E nor Q71E when heated yielded major species with PI values equivalent to HPr-1 or HPr-2 (Fig. 2, B and C). The double mutation, Q57E+Q71E, which has a PI close to HPr-1, when heated gave a doublet band with a PI similar to that of HPr-2, but also a doublet band with a PI lower than that of HPr-2 (Fig. 2B). All three mutant HPrs gave deamidation species that were consistent with the proposal that none of these species are equivalent to HPr-1 or HPr-2 (Fig. 2, B and C). The double mutant has a PI that is very close to HPr-1 (Klevit et al., 1988). However, neither Q57E nor Q71E when heated yielded major species with PI values equivalent to HPr-1 or HPr-2 (Fig. 2A). These results appeared to confirm the identification of HPr-1 (Klevit et al., 1988). However, neither Q57E nor Q71E when heated yielded major species with PI values equivalent to HPr-1 or HPr-2 (Fig. 2A). These results appeared to confirm the identification of HPr-1 (Klevit et al., 1988). However, neither Q57E nor Q71E when heated yielded major species with PI values equivalent to HPr-1 or HPr-2 (Fig. 2A). These results appeared to confirm the identification of HPr-1 (Klevit et al., 1988). However, neither Q57E nor Q71E when heated yielded major species with PI values equivalent to HPr-1 or HPr-2 (Fig. 2A). These results appeared to confirm the identification of HPr-1 (Klevit et al., 1988). However, neither Q57E nor Q71E when heated yielded major species with PI values equivalent to HPr-1 or HPr-2 (Fig. 2A). These results appeared to confirm the identification of HPr-1 (Klevit et al., 1988). However, neither Q57E nor Q71E when heated yielded major species with PI values equivalent to HPr-1 or HPr-2 (Fig. 2A). These results appeared to confirm the identification of HPr-1 (Klevit et al., 1988). However, neither Q57E nor Q71E when heated yielded major species with PI values equivalent to HPr-1 or HPr-2 (Fig. 2A). These results appeared to confirm the identification of HPr-1 (Klevit et al., 1988). However, neither Q57E nor Q71E when heated yielded major species with PI values equivalent to HPr-1 or HPr-2 (Fig. 2A). These results appeared to confirm the identification of HPr-1 (Klevit et al., 1988). However, neither Q57E nor Q71E when heated yielded major species with PI values equivalent to HPr-1 or HPr-2 (Fig. 2A). These results appeared to confirm the identification of HPr-1 (Klevit et al., 1988). However, neither Q57E nor Q71E when heated yielded major species with PI values equivalent to HPr-1 or HPr-2 (Fig. 2A).

Other mutant HPrs were examined with respect to their PI values and their deamidation properties. Some of the results are presented in Fig. 3 and other results have been presented by Sharma (1992). The following neutral mutations, Q3S, Q4S, and Q61T, gave HPrs with PI values and deamidation patterns identical to HPr. In addition, Q3K and Q4K, which have higher PI values than that of HPr, gave deamidation patterns which resembled HPr, but with species for which the PI values were shifted to higher values. This confirms earlier reports that HPr-1 and HPr-2 generation in Q4K was similar to wild type (Beneski et al., 1985). The principal effect of Q4E was a change in the apparent pK, from about 7.8 for wild type to about 7.4. However, the effect of the aspartic acid at residue 12 was phosphiroylhydrolysis properties that had the most similarity to HPr-2. As the other results above exclude Gln-51 from being involved in the deamidation, the phosphohydrolysis results suggested that deamidation of Asn-12 is involved in the conversion of HPr-1 to HPr-2.

**Antibody Binding**—Three monoclonal antibodies for HPr, Je142, Je144, and Je1323, have been extensively investigated with respect to the effects of residue replacement in HPr upon antibody binding (Waygood et al., 1987; Sharma et al., 1991; Sharma, 1992). The deamidations that yield HPr-1 and HPr-2 do not affect the binding of these three antibodies (Waygood et al., 1987). Competition binding experiments revealed that, with the exception of Q71E, all the mutants of HPr with acidic residues, Q3E, Q21E, Q51E, Q57E, N12D, and N38D, did not alter the binding of all three antibodies. Q71E gave a 100-fold decrease in the binding to Je142 (Sharma et al., 1991) as did the double mutant Q57E+Q71E. Similarly, the neutral mutants, Q3S, Q4S, Q21T, and Q51T, had no effects upon binding. However, Q3K and Q4K have already been shown to alter Je142 and Je144 binding, respectively (Sharma et al., 1991). These results provided further evidence against the involvement of Gln-71 in the deamidation events.

**Phosphohydrolysis**—HPr-1 has phosphohydrolysis properties that are essentially identical to HPr, while HPr-2 has impaired phosphohydrolysis properties (Waygood et al., 1985). The mutant HPrs, Q21E, Q71E, and N38D, had phosphohydrolysis properties identical to wild-type HPr and HPr-1 (results not shown). Q57E and the double mutant Q57E+Q71E had lower maximal phosphohydrolysis rates (k = 0.09 versus 0.12 min⁻¹ for wild-type HPr). Q51E and N12D had phosphohydrolysis rates that were different from either HPr and HPr-1 or HPr-2 (Fig. 4). The principal effect of Q51E was a change in the apparent pK, from about 7.8 for wild type to about 7.4. However, the effect of the aspartic acid at residue 12 was phosphohydrolysis properties that had the most similarity to HPr-2. As the other results above exclude Gln-51 from being involved in the deamidation, the phosphohydrolysis results suggested that deamidation of Asn-12 is involved in the conversion of HPr-1 to HPr-2.

**Peptide Analysis**—Peptides were generated from HPr, HPr-1, HPr-2, and mutant HPrs, Q3S, Q21T, Q21E, Q51E, Q51T, Q57E, Q71E, the double mutant Q57E+Q71E, N12D, and N38D by digestions with trypsin and V8-protease. The peptides were separated by HPLC chromatography as described under "Experimental Procedures." The elution patterns of the peptides generated from HPr and HPr-1 were identical. The peptides were identified by amino acid analysis and they are given in Tables I and II. For the tryptic digests, the Arg-Pro linkage, residues 17–18, hydrolyzed slowly (Weigel et al., 1982) such that after a 1.5-h digestion, peptides containing residues 1–24, 18–24, and 1–17 were usually detected (Fig. 5A). After 3 h, there was complete digestion at the Arg-Pro linkage. However, with time of incubation at pH 8.0, peptide T6 (residues 1–17) was gradually converted into peptides T5 and T7 (Fig. 5, A and C). All three peptides had identical amino acid composition (Table I), and contained Asn-12-Gly-13. The half-life of peptide T6 was less than 24 h. No such
Preparations were heated at 80 °C in potassium phosphate buffer, pH 7.5, and samples taken at the times shown (minutes). H are HPr standards containing different amounts of HPr and HPr-1. H2 a sample of HPr boiled to yield a HPr-2 standard.

FIG. 3. Deamidation of selected mutants. Conditions for the isoelectric focusing gels were as described in Fig. 1. The mutant HPr preparations were heated at 80 °C in potassium phosphate buffer, pH 7.5, and samples taken at the times shown (minutes). H are HPr standards containing different amounts of HPr and HPr-1. H2 a sample of HPr boiled to yield a HPr-2 standard.

Fig. 4. Phosphohydrolysis of N12D and Q51E. The pH-dependent phosphohydrolysis rates of N12D (□-□), Q51E (■-■), wild-type HPr which is identical to HPr-1 (○-○), and HPr-2 (■-■).

Changes were observed for peptide T4, which eluted as a broad peak (Fig. 5), and contained Asn-38-Gly-39. Except for HPr-2, the double mutant Q57E+Q71E, and N12D, similar properties were observed for the elution of peptides from all the HPr proteins. The double mutant Q57E+Q71E resulted in peptide T6 that eluted as a broad peak (Fig. 5B). When HPr and HPr-2 tryptic digests were mixed, the HPr peptide T6 was found between the HPr-2 peptides T5 and T7 (Fig. 5D). HPr-2 peptides T5 and T7 amino acid analysis gave the same composition as shown in Table I. The mutant N12D gave only peptide T7, and neither peptide T5 nor T6 were detected in any of the peptide preparations. The N12D peptides when mixed with HPr-2 peptides resulted in the superimposing of the peptides T7 from both sources (results not shown). The relative abundance of peptide T5 and T7 in HPr-2 was 1:3 which is typical of the isoaspartyl shift reaction. Presumably, peptide T5 contains an isoaspartyl residue. These results identify the site of deamidation responsible for the conversion of HPr-1 to HPr-2 as asparagine 12.

The conversion of Asn-12 into the two deamidated isomers was much more rapid in the peptides than in HPr. In order to confirm that the conditions (other than the protease) used for proteolysis did not lead to Asn-12 deamidation, HPr was preincubated in 50 mM ammonium bicarbonate, pH 8.0, at 37 °C for 24 h (the tryptic digest conditions), prior to the addition of trypsin. The time-dependent appearance of peptides T5, T6, and T7 was indistinguishable from that observed for HPr peptides derived from freshly prepared lyophilized HPr. The 1.5-h digest yielded an elution profile identical to that seen in Fig. 5A.

The digestion of HPrs with V8 protease should generate different peptides as the result of the introduction of acidic residues in place of the amide residues. The peptides that were separated are shown in Table II. Again HPr and HPr-1 had identical peptides, and peptide V7 which contained Asn-12 converted to peptides V6 and V8 in a manner essentially identical to that described for the tryptic peptides T5, T6, and T7. Similarly, HPr-2 gave peptides V6 and V8, and the mutant N12D gave only peptide V8. Peptide V9 ends in the sequence -EGEDE, residues 66-70, and there was no indication that this acidic sequence was further digested by the V8-protease. Mutant HPrs Q51E and Q57E gave the appropriate peptides, while Q71E resulted in peptide V9 with an amino acid composition that suggested the sequence -EGEDE at the end of the peptide, residues 66-70. Because this peptide has seven rather than 6 Glx residues, it was not possible to be completely certain about the quantitation. Moreover, the peptides 71-75 or 72-75 eluted in conjunction with a variable amount of the dipeptide, Leu-84-Glu-85 (Table II), which made quantitation difficult. Mutant HPrs, Q3E and Q21E, did not produce additional sites for V8 protease digestion under the conditions used. These results provided further evidence against the involvement of Gln-51, Gln-57, and Gln-71 in the process of deamidation, but still left the roles of Gln-3, Gln-4, and in particular Gln-21 in question. The role

![Graph](image-url)

**TABLE I**

| Peptide | Elution | Amino acid sequence (residue number) |
|---------|---------|-------------------------------------|
| T1      | 9.9     | SLFK (46-49)                       |
|         |         | AVEHLVK (73-79)                    |
| T2      | 13.7    | PAAQFVK (18-24)                    |
| T3      | 18.5    | LMAELE (80-85)                     |
| T4      | 22.5    | GTSEITVTSGK (28-40)                |
| T5      | 31.2    | MFQQEVTITAPNLHTR (1-17)            |
| T6      | 33.0    | MFQQEVTITAPNLHTR (1-17)            |
| T7      | 34.5    | MFQQEVTITAPNLHTR (1-17)            |
| T8      | 37.4    | LQTLGLTGQTVTISAEDEEQK (50-72)      |
| T9      | 39.0    | MFQQEVTITAPNLHTRPAAFQVK (1-24)     |

*Peptides were analysed for all three incubation times used in the tryptic hydrolysis, for HPr, HPr-1, HPr-2 and for selected peptides of all mutant HPrs.

*Peptides not found: residues 24-28 and 41-45.

The sequences have been determined by both protein sequencing (Weigel et al., 1982; Powers and Roseman 1984) and DNA sequencing (DeReuse et al., 1985). The amino acid determinations did not distinguish amide residues from acidic residues.
TABLE II

V8-protease peptides of HPr

| Peptide | Elution Amino acid sequence (residue number)* |
|---------|------------------------------------------------|
| V1      | 1.0   LE (84-85) |
| V2      | 1.5   QKAVE (71-75) |
| V3      | 4.0   QKAVE (71-75) |
| V4      | 7.7   AKGFTSE (26-32) |
| V5      | 18.0  MFQQE (1-5) |
| V6      | 30.2  HLVKMAE (78-83) |
| V7      | 31.5  HLVKMAELE (78-85) |
| V8      | 32.5  VTTTPNLHTRPAQFVE (6-25) |
| V9      | 45.0  VTTTPNLHTRPAQFVE (6-25) |

* See Table I, footnote a.

** See Table I, footnote c.

Fig. 5. Tryptic peptides containing residues 1–17. Part of the elution (right to left) profile (A90, nm) of peptides generated by tryptic digests and separated as described under “Experimental Procedures” is shown. The composition and times of elution are given in Table I. The samples illustrated are: A, wild-type HPr, 1.5-h digestion (1-nmol sample); B, HPr-2, 1.5-h digestion (1-nmol sample); C, wild-type HPr, 17-h digestion (1.5-nmol sample); D, wild-type HPr (2-nmol sample) and HPr-2 (1-nmol sample), both 1.5-h digests.

Deamidation of HPr

Two-dimensional NMR Spectroscopy—The assignment of chemical shifts for HPr-1 was carried out using DQF-COSY, TOCSY, and NOESY spectra. A pair of methylene protons, with chemical shifts at 2.47 and 3.01 ppm, were observed that had not been found in the HPr spectra. Analysis of the DQF-COSY and TOCSY spectra placed these residues an AMXY spin system. Sequential assignment, using the NOESY spectrum, revealed cross-peaks connecting the amide proton (8.70 ppm) of this spin system with the $\beta$ protons of Ser-37 and the $\alpha$-methylene protons of Gly-39 (Fig. 6). The absence of NOEs correlating the $\alpha$ proton (4.77 ppm) and the amide proton with the amide proton of Gly-39 are consistent with an isoaspartyl residue (Chazin et al., 1989), which includes the methylene group as part of the backbone. Both of the latter interactions are observed in the NOESY spectrum of HPr (Hammen et al., 1991).

The initial identification of the deamidated residues in HPr-1 (Klevit et al., 1988) was based on chemical shift assignments that have since been revised (Hammen et al., 1991; van Nuland et al., 1992). DQF-COSY spectra were obtained for HPr-1, Q57E, Q71E, Q57E+Q71E, NUD, and N38D. The spectra for NUD, Q57E, Q71E, and the double mutant Q57E+Q71E gave changes in chemical shift for protons in residues near the site of each mutation, but not at all similar to the HPr-1 spectra (results not shown).

Comparison of the DQF-COSY spectra of HPr-1 and wild-type HPr clearly showed that the chemical shift changes are for residues 38, residues near 38 in sequence, and residues near 38 in the tertiary structure (Fig. 7). The DQF-COSY
FIG. 7. Chemical shift differences between HPr and HPr-1. Backbone 1H chemical shift differences between HPr and HPr-1 greater than the 0.04 ppm. Amide protons (○) and 3H protons (△). spectra of HPr-1 and N38D were not identical (Fig. 8). Aside from obvious differences in the position of cross-peaks assigned to residue 38, there are significant differences in the position of cross-peaks associated with residues 39-41. Additional differences in the spectra were observed, mainly involving residues 51-54 which are close to residue 38 in the structure. In general, the spectrum of N38D compares more favorably with that of wild-type HPr-1 than with HPr-1.

Low intensity cross-peaks were found in the spectra of HPr-1, corresponding to the positions of the cross-peaks for the aspartate residue created in N38D. To obtain values for the relative amounts of aspartic acid and isoaspartic acid present in HPr-1, the DQF-COSY, and TOCSY cross-peaks connecting the methylene protons of these residues were integrated. The intensities of these cross-peaks should be insensitive to local conformation, and therefore provide an unbiased measure of the mixture proportion. The integration showed that the HPr-1 samples contained about 6:1 isoaspartic:aspartic acid.

PTS Activity—Previously it has been found that HPr-1 has no impairment to its ability to act as a substrate for enzyme I or enzyme II^max, and had a small change in K_m for enzyme II^mut. HPr-2 however was impaired for enzyme I and enzyme II^mut, but not enzyme II^max (Waygood et al., 1985). All the mutants that were produced were screened for activity using an enzyme II^max assay (Sharma et al., 1991), and the results indicated that only N12D and Q51E had impaired activity. Q51E, Q57E, Q71E, N12D, N38D, and the double mutant N12D+N38D have been more thoroughly investigated. The kinetic parameters for enzyme I and various enzymes II are given in Table III. The N38D mutant resulted in normal kinetic responses, as would be expected for a deamidation leading to isoaspartyl at residue 12. However, the double mutant N12D+N38D was significantly impaired in the enzyme II^mut which is similar to HPr-2. The impairment of HPr-2 in its interaction with enzyme I appears to be much more dependent upon the formation of a isoaspartyl at residue 12. Previously it had been reported that N12D was impaired in an enzyme II^max screening assay (Sharma et al., 1991), which was not found in the more careful kinetic analysis (Table III). The discrepancy probably resulted from insufficient enzyme I being provided in the screening assay.

While all the above results indicate that Gln-51 is not involved in the formation of either HPr-1 nor HPr-2, its kinetic parameters were described as it is located near the active site. The deamidation of Gln-51 has the most effect of any of the deamidation mutants on PTS activity. All four measured activities were impaired to some degree (Table III).

**Deamidation in HPrs from Other Species—** HPr from either _B. subtilis_ or _Staphylococcus_ spp. have Asn-38-Gly-39 sequences (Gonzalez-Treboul et al., 1989; Eisermann et al., 1991), and both of these forms a major species, similar to HPr-1, upon heating (Fig. 9). HPr from _S. faecalis_ which does not have this sequence (Deutscher et al., 1986) does not form any other species under these conditions (results not shown). It should be noted that the HPr-1 species generated in _B. subtilis_ HPr and _S. carnosus_ gave a doublet band on the isoelectric focusing gel which indicates isoaspartyl formation.

**Discussion**

The results in this paper identify the deamidation of residues Asn-38 and Asn-12 as being responsible for the formation of HPr-1 and HPr-2, respectively. The evidence is consistent with the proposal that HPr-2 derives from HPr-1, although no direct evidence for the identification of Asn-38 deamidation in HPr-2 has been obtained. Isolated HPr-1 yields HPr-2 upon heating, and HPr-1 is always accumulated before HPr-2 appearance (Figs. 1-3). The identification of these two asparagine residues involved the elimination of all other amide residues. The identification of the involvement of Asn-12 in the formation of HPr-2 was obtained from several experiments because HPr-2 has significant differences in properties from HPr. The identification of Asn-38 involvement in the formation of HPr-1 came from the elimination of other residues, and the altered NMR spectra.

Deamidation of proteins is wide-spread, and for the most part appears to affect asparagine residues (Wright, 1991a). The phenomenon is associated with protein aging and denaturation, and possibly is one of the more common spontaneous changes that occurs in protein preparations. There are various mechanism by which it can occur, but the fastest deamidations occur at Asn-Gly sequences by the mechanism shown in Fig. 10 (Bornstein and Balian, 1970; Clarke, 1985; Wright, 1991a, 1991b). This deamidation is well studied in peptides, and the principle products are both D- and L-aspartic acid and D- and L-isoaspartyl acid in a 1:3 proportion (Geiger and Clarke, 1987). Several reports indicate that this proportionality is also found in other small proteins that deamidate at Asn-Gly sequences (Chazin et al., 1989; Spangler and Westbrook, 1989). Deamidation of glutamine residues as a spontaneous event is not as well characterized, but enzymatic deamidation of glutamines and methylation of glutamates are processes in chemotaxis in _E. coli_ (Clarke, 1985; Stock et al., 1989). The PTS is involved in chemotaxis, and it has been proposed that HPr may be involved in the interaction between the PTS and chemotaxis (Lengeler and Vogel, 1998; Grubel et al., 1990).

The rates of asparagine deamidation in HPr observed in _vitro_ suggest that HPr-1 could be present in _vivo_ at about 1% concentration, *i.e.* about 1 μM (Mattoo and Waygood, 1983). Assessment of the possible role of deamidated HPr in chemotaxis, although it is an asparagine deamidation, will be conducted using a gene replacement method.

The deamidation of _E. coli_ HPr has been described since the first characterization (Anderson et al., 1971), but has not been described in HPrs from other species. It is now clear that HPrs from _B. subtilis_ and _S. carnosus_ with the conserved
Asn-38-Gly-39 sequence (Gonzyl-Treboul et al., 1989; Eisermann et al., 1991) also form an HPr-1 species, presumably by deamidation (Fig. 9). S. faecalis HPr, which lacks this sequence (Deutscher et al., 1986) does not form an HPr-1 species, and none of these HPrs from Gram-positive bacteria form the HPr-2 species because Asn-12 is not conserved.

In the purification of E. coli HPr and derived mutants, variable amounts of the HPr-1 species are always generated. The principal cause appears to be the exposure during the purification of HPr to buffers with pH values >6.0. Clearly maintenance of reduced temperatures is an important factor in reducing the amount and nature of deamidation (Fig. 1). In general, the formation of HPr-2 is not observed unless HPr is treated harshly as was the case for early purification methods (Anderson et al., 1971). HPr-1 species can be relatively easily separated from HPr by the chromatography steps used in HPr purification: Q-Sepharose at alkali pH, or S-Sepharose at acidic pH. The results in this paper suggest that the latter is the safer method. The stability of HPr at acid pH, in respect to deamidation, has been observed in E. coli HPr crystals. The protein in crystals that take months to grow at pH 3.7 and 15 °C (El-Kabbani et al., 1987) have been examined by isoelectric focusing, and the amount of HPr-1 detected was typical of that found in preparations at the beginning of crystallization.3

We had previously reported that the deamidation of both Gln-57 and Gln-71 was responsible for the formation of HPr-1. These identifications were made by comparing NMR COSY spectra (Klevit et al., 1988) using residue assignments that

Fig. 8. DQF-COSY spectra. Comparison of the DQF-COSY spectra of A, HPr; B, HPr-1; and C, N38D. The main part of each panel correlates the amide proton with the Hα protons of each residue, while the inserts show the methyl proton correlations of residue 38 in each spectrum.

3 M. Vandonselaar, E. B. Waygood, and L. T. J. Delbaere, unpublished results.
have since been corrected (Hammen et al., 1991; van Nuland et al., 1992). The NMR spectral data presented in Figs. 6–8 leave no doubt that it is deamidation of Asn-38 that is responsible for HPr-1 formation. By chance, the double mutation Q57E+Q71E had several properties that were very similar to those of HPr-1, but it was the differences that led to the investigation of other amide residues. This double mutation had an almost identical PI to that of HPr-1, but other single glutamine to glutamate or asparagine to aspartate mutations had an almost identical PI to that of HPr-1, but other single mutations gave wild type kinetic parameters: Q57E, Q71E, Q57E + Q71E.

* Parameter values are rounded as described previously (Anderson et al., 1991).

Table III: Kinetic parameters for enzyme I and enzyme IIs

| HPi | Enzyme I | Enzyme II* | Enzyme II* | Enzyme II* |
|-----|----------|------------|------------|------------|
|     | \(\mu M\) | \(V_{\text{max}}\) | \(K_{n}\) | \(K_{s}\) | \(V_{\text{max}}\) | \(K_{s}\) | \(V_{\text{max}}\) | \(K_{s}\) |
| HPr | 6 100* | 15 7 100* | 7 100* | 7 100* | 8 100* | 8 100* | 8 100* | 8 100* |
| N12D | 15 60 | 15 7 100 | 8 100 | 8 100 | 8 100 | 8 100 | 8 100 | 8 100 |
| N38D | 8 90 | 15 8 100 | 8 100 | 8 100 | 8 100 | 8 100 | 8 100 | 8 100 |
| N12D + N38D | 15 60 | 45 6 100 | 6 100 | 6 100 | 6 100 | 6 100 | 6 100 | 6 100 |
| HPr-1 | 7 100 | 20 8 100 | 8 100 | 8 100 | 8 100 | 8 100 | 8 100 | 8 100 |
| HPr-2 | 15 30 | 50 8 100 | 8 100 | 8 100 | 8 100 | 8 100 | 8 100 | 8 100 |
| Q51E | 20 50 | 40 15 30 | 30 100 | 30 100 | 30 100 | 30 100 | 30 100 | 30 100 |

* All gave 100% \(V_{\text{max}}\).

**Fig. 9.** Deamidation of *B. subtilis* and *S. carnosus* HPiRs. The HPiRs were treated as described in Fig. 3. The gel used for *S. carnosus* HPiPr had pH 3–10 and pH 3.5–5 ampholytes (1:4 ratio) because of its lower pl. The control samples H1 were *E. coli* HPiPr containing amounts of HPi and HPi-1. H1 was isolated HPi-1.

**Fig. 10.** Pathways of spontaneous deamidation, isomerization, and racemization for aspartyl and asparaginyl residues. These pathways are proposed by Geiger and Clarke (1987) based on studies with hexapeptides.

date readily in HPi under normal conditions, and it is more susceptible under conditions in which the tertiary structure is destroyed as in the peptide, or under conditions which are leading to denaturation as in the case of elevated temperatures (Fig. 1). Wearne and Creighton (1989) showed a similar behavior for Asn-67 in RNase that exists in a type III β-turn with Asn-Gly at positions 3 and 4. The mobility of the glycine residue at position 4 is constrained by the hydrogen bond of the turn, and presumably inhibits the cyclization reaction (Fig. 10). That the deamidation of Asn-12-Gly-13 occurs more rapidly in a less structured polypeptide is consistent with the proposed mechanism of isoaspartyl residue formation (Bornstein and Balian, 1970; Clarke, 1985; Geiger and Clarke, 1987; Wright, 1991a). Asn-38-Gly-39, which more easily yields an isoaspartyl residue, is in a type III' β-turn structure in *E. coli* HPiPr (Jia, 1992). The two-dimensional NMR results suggest a type I' β-turn for *E. coli* HPiPr and B. subtilis HPiPr (Hammen et al., 1991; Wittekind et al., 1992), but the distinction is probably not real due to overlapping bounds of the \(\phi\) and \(\varphi\) angles used to classify these turns. The Asn-Gly occupy positions 2 and 3 of the turn, and are unencumbered by hydrogen bonds. This Asn-Gly pair in HPiRs from various species deamidates under mild conditions in the native protein to yield HPi-1 and appears to more readily deamidate at elevated temperatures (Fig. 1). No indication was found that the peptides containing this pair were more susceptible. The β-turn with Asn-38-Gly-39 is in a conformation that causes it to be very solvent exposed, and away from the body of the structure in both *E. coli* and *B. subtilis* HPiRs (Herzberg et al., 1992; Wittekind et al., 1992; Jia, 1992). However, the amide exchange rates with solvent suggest that the turn is more exposed in *E. coli* than *B. subtilis* HPiPr (Wittekind et al., 1992).

Two of the deamidation mutants, N12D and Q51E, and HPi-2 have alterations in their ability to carry out phosphoryltransfer. The structural information that is available for HPiRs from different species shows that the overall folding patterns are similar, but the active site descriptions have significant differences. A number of reports indicate the involvement of residues 12 and 51, but with differences in the details. The *E. coli* HPiPr structures were the first reported (Klevit and Waygood, 1986; El-Kabbani et al., 1987), and both have now been modified (Hammen et al., 1991; van Nuland et al., 1992; Jia, 1992). A major difference between the two...
early E. coli HPr tertiary structures has been resolved with a new 2Å resolution structure (Jia, 1992). It is, however, not clear what roles residues 12 and 51 have in the active site of E. coli HPr. In the HPrs from S. faecalis and B. subtilis, residue 12 is threonine and serine, respectively, and residue 51 is a methionine, and both residues are implicated in interactions with the active site His-15 (Herzberg et al., 1991; Jia, 1992; Jia et al., 1993). Unfortunately, the structural information on the B. subtilis HPr has inadvertently been carried out on a M5IV mutant (Wittekind et al., 1992).

In E. coli HPr, we have now investigated by site-directed mutagenesis, all the residues that so far have been implicated in hydrogen bonding to His-15 in any HPr structure. The acidic replacements of Asn-12 and Gln-51 and the deletion of G1u-85 (Anderson et al., 1991) have all resulted in changed phosphorylation properties, but only modest changes to the kinetic parameters investigated. The lack of extent of these kinetic changes is particularly evident when these results are compared to the effects of alterations to Arg-17 which produce 100-1000-fold reductions in PTS activity (Anderson et al., 1993). Some potential insensitivity in the kinetic measurements have been discussed in respect to the Arg-17 mutants (Anderson et al., 1993), and those comments apply to the assessment of these deamination mutants in respect to active site mechanism roles.

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