# Characterization of an Associated Equilibrium Folding Intermediate of Bovine Growth Hormone

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ABSTRACT: In the preceding paper [Havel, H. A., Kauffman, E. W., Plaisted, S. M., & Brems, D. N. (1986) *Biochemistry* (preceding paper in this issue)], an associated intermediate was shown to be highly populated during the equilibrium denaturation of bovine growth hormone. In this paper, we describe its partial characterization and propose a mechanism for association. The associated equilibrium intermediate is populated under conditions that induce partial denaturation and at protein concentrations greater than 0.2 mg/mL. The remaining nativelike helical structure present in the partially denatured species is implicated in the mechanism of association as demonstrated by similar concentration dependencies and thermal stabilities of the helix and the associated equilibrium intermediate. Furthermore, it is suggested that a putative amphiphilic helix from residues 110–127 plays a critical role in the association as demonstrated by a diminution of the associated equilibrium intermediate when mixed with the peptide fragment 96–133. A model is proposed to account for these results in which partial denaturation exposes the segment of the protein corresponding to the hydrophobic face of the putative amphiphilic helix 110–127. This metastable form is the species from which association occurs. Association is stabilized by the hydrophobic interactions resulting from intermolecular packing of the lipophilic faces of the helices. The implications of these results to protein folding studies are described.

The guanidine hydrochloride (Gdn-HCl), urea, and acid induced equilibrium denaturation of bovine growth hormone (bGH) has been studied previously (Burger et al., 1966; Edelhoch & Burger, 1966; Holladay et al., 1974; Brems et al., 1985; Havel et al., 1986). Denaturation of bGH cannot be explained by a two-state transition: equilibrium intermediates have been invoked in order to explain the noncoincidence of transitions as monitored by multiple probes. The unfolding process can best be described by preferential loss of tertiary structure followed by loss of the more stable secondary structure. To date, both monomeric and associated equilibrium intermediates have been reported.

In the preceding paper (Havel et al., 1986), we identified an associated equilibrium intermediate that is populated during the equilibrium denaturation of bGH. This intermediate was identified by classical light scattering (LS), photon correlation spectroscopy (PCS), size-exclusion high-performance liquid chromatography (SE-HPLC), second-derivative absorption spectra, fluorescence, and circular dichroism (CD) at 300 nm. LS, PCS, and SE-HPLC are intrinsic measures of molecular size and would be expected to be measures of protein association. The CD at 300 nm, second-derivative absorption spectra, and fluorescence are measures of the intermediate due to spectral changes in the tryptophan moiety as a result of this association.

The associated equilibrium intermediate was shown to be of defined size, completely reversible, and populated in a variety of solution conditions providing they induce partial denaturation. Because of the requirement for partially denaturing solvents, the possibility exists that the affinity for association may be due to interactions between regions of remaining nativelike structure, random coil, or both. In order to address these possibilities, we have further characterized the nature of the intermolecular interactions of the associated equilibrium unfolding intermediate.

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## Materials and Methods

*Materials.* Gdn-HCl was ultrapure grade from Schwarz/Mann. Other reagents and solvents were of analytical grade. The fragment 96–133 was obtained by partial trypsin digestion and isolated as described by Graf and Li (1974). The identity of fragment 96–133 was established by amino acid analysis and complete sequence analysis by gas phase sequencing (Applied Biosystems Inc. Model 470-A protein sequencer). The purity of fragment 96–133 was determined by the criteria of a single component by HPLC reverse-phase chromatography (Brems et al., 1985) and polyacrylamide gel electrophoresis (Swank & Munkres, 1971). All protein solutions contained 50 mM $NH_4HCO_3$, pH 8.5.

*Methods.* Protein concentration was determined by the absorbance at 278 nm utilizing an extinction coefficient of 15 270 $M^{-1}$ $cm^{-1}$ (Burger et al., 1966). The concentration of peptide 96–133 was measured by ninhydrin determination following alkaline hydrolysis (Rosen, 1957). All thermal- and Gdn-HCl-induced transitions were completely reversible. CD data were obtained on a Jasco J-500C spectropolarimeter, and the UV absorbance data were obtained on an IBM 9420 spectrophotometer. Both instruments were thermostated and maintained at the desired temperature.

## Results

*Denaturation As Monitored by CD at 222 nm Is Dependent on Protein Concentration.* The effect of protein concentration on the denaturation as measured by CD at 222 nm was investigated and is illustrated in Figure 1A. At low protein concentration (less than 0.1 mg/mL) the denaturation is independent of concentration and can be characterized as a monophasic transition. At high protein concentration (more than 1.0 mg/mL) the denaturation is dependent on concentration and demonstrates a biphasic transition. At high protein concentrations, the biphasic transition is a result of a decrease in stability to Gdn-HCl in the pre-midpoint transition region and an increase in stability to Gdn-HCl in the post-midpoint

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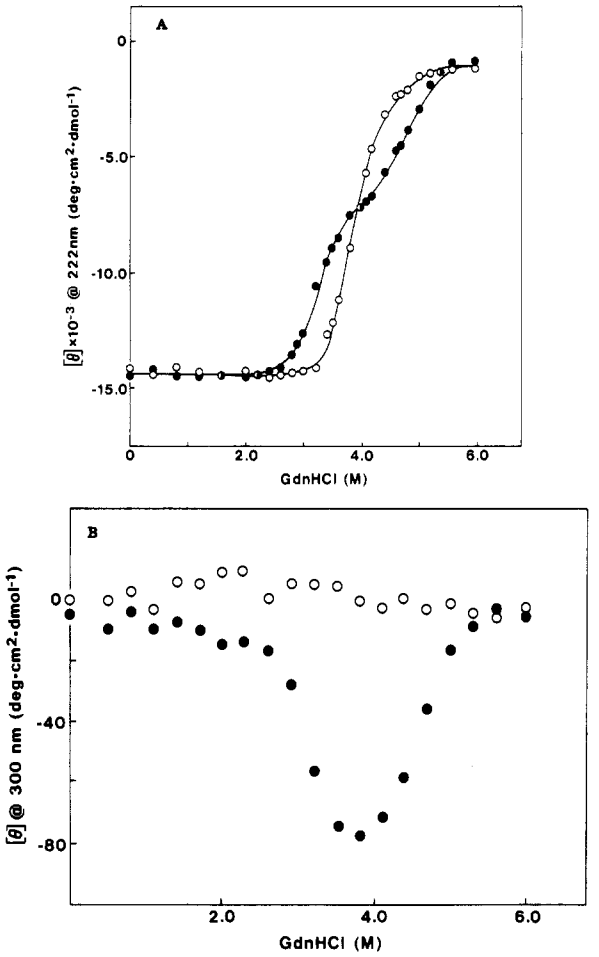


FIGURE 1: Effect of protein concentration. Equilibrium denaturation of bGH at 1.6 (●) and 0.01 mg/mL (○) at 25 °C as monitored by CD at 222 (A) and 300 nm (B).

transition region. We note that Gdn-HCl-induced denaturation as monitored by the absorbance at 290 nm is not dependent on protein concentration within the range of 0.1–2 mg/mL. As described previously (Havel et al., 1986), the CD at 300 nm is a convenient probe of the associated equilibrium intermediate. This effect is further illustrated in Figure 1B at the two extreme protein concentrations which were used in Figure 1A. At high protein concentration (1.6 mg/mL), the transition measured by CD at 222 nm is biphasic, and the CD at 300 nm reflects the presence of the associated equilibrium intermediate whereas at low protein concentrations (0.01 mg/mL) the associated equilibrium intermediate cannot be detected and the corresponding transition measured by CD at 222 nm is monophasic. Since the maximum concentration of the associated equilibrium intermediate occurs at the same Gdn-HCl concentration (3.7 M) as the inflection point for the denaturation transition at high protein concentrations (Figure 1A), the potential relationship between the two was explored.

*Thermal Stability of the Associated Equilibrium Intermediate Is Different Than Thermal Stability of Nativelike Tertiary Structure.* The UV absorbance at 290 nm reflects the solvent environment of the aromatic amino acid chromophores. Increasing concentrations of denaturant cause the aromatic UV spectra to blue shift, indicating an increased exposure to bulk solvent or unfolding of the tertiary structure. The thermal stability of the associated equilibrium intermediate as detected by CD at 300 nm and the nativelike tertiary structure as detected by UV absorbance at 290 nm are shown in Figure 2A. The thermal stability of the associated equilibrium intermediate shows maximum stability at 15 °C higher

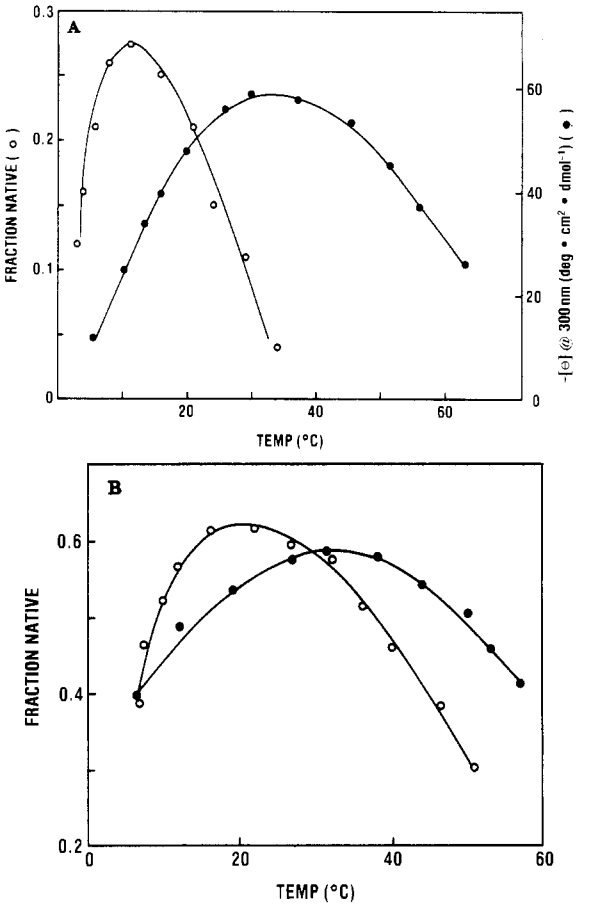


FIGURE 2: Thermal stability of bGH in 3.7 M Gdn-HCl. (A) Thermal stability of nativelike tertiary structure as measured by absorbance at 290 nm (○) and the associated equilibrium intermediate (●) as measured by CD at 300 nm. Measurements were obtained at a protein concentration of 1.6 mg/mL. (B) Thermal stability of secondary structure or helix as measured by CD at 222 nm under conditions where the associated equilibrium intermediate is not populated, 0.016 mg/mL (○), and under conditions where it is populated, 1.6 mg/mL (●). The fraction native as measured by UV absorbance at 290 nm was determined by $(A_{sample} - A_{denatured})/(A_{native} - A_{denatured})$ and for CD at 222 nm by $([\theta]_{sample} - [\theta]_{denatured})/([\theta]_{native} - [\theta]_{denatured})$, where $[\theta]$ is the mean residue ellipticity.

temperature than the nativelike tertiary structure.

*Thermal Stability of the Secondary Structure Is Dependent on the Presence of the Associated Equilibrium Intermediate.* The thermal stability of the secondary structure was measured in 3.7 M Gdn-HCl at high (1.6 mg/mL) and low (0.016 mg/mL) protein concentrations (Figure 2B). At high protein concentrations where the equilibrium intermediate is populated, the secondary structure resembles the temperature dependence of the associated equilibrium intermediate. At low protein concentrations where the associated equilibrium intermediate is not populated, the thermal stability is decreased by at least 10 °C. The different temperature stabilities observed by CD at 222 nm at two different protein concentrations are further indication that the denaturation of bGH, as detected by a single probe, cannot be explained by a simple two-state process.

The concentration effect on the helix transition and the similar thermal stabilities of the helix and the associated equilibrium intermediate imply a relationship between the two. To understand the relationship between the helix and the associated equilibrium intermediate, we have explored the properties of the active core fragment 96–133 that has been reported to possess helical structure in aqueous solutions (Chen & Sonenberg, 1977).

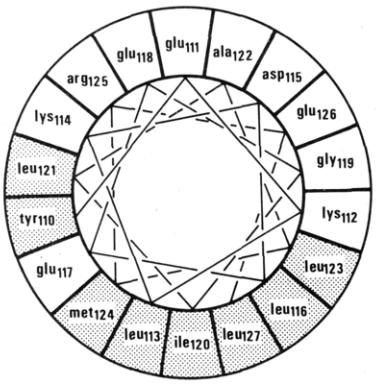


FIGURE 3: Axial projection of a potential $\alpha$-helix structure of residues 110–127. The segregation of the hydrophobic and hydrophilic residues is shown.

*Residues from 110–127 Are Predicted To Form an Amphiphilic Helix.* Secondary structure prediction algorithms (Robson & Pain, 1971; Chou & Fasman, 1974) indicate that residues 110–127 are involved in an $\alpha$-helix structure. We have analyzed this proposed helix for amphiphilicity. As illustrated in Figure 3, residues 110–127 are an ideal example of an amphiphilic helix. The isolated peptide fragment 96–133 obtained by limited proteolysis has been shown to contain approximately 50% helical structure in aqueous solutions (Chen & Sonenberg, 1977). Generally, fragments (which are derived from portions that are helical in the intact protein) have been found to be devoid of helical structure in the isolated state in aqueous solution. The prime exception is the S-peptide helix from ribonuclease S (Brown & Klee, 1971; Shoemaker et al., 1985). We have further studied the helix formation of fragment 96–133 and have found that the helical content is strongly dependent on fragment concentration (results to be published). It appears that the unusual helical stability of fragment 96–133 is a consequence of intermolecular interactions.

*Formation of the Associated Equilibrium Intermediate Is Decreased by Addition of Fragment 96–133.* As observed in Figure 4B, under conditions for maximal population of the associated equilibrium intermediate, addition of a 10-fold molar excess of fragment 96–133 to bGH results in a substantial decrease in the 300-nm band. Since the 300-nm band has been shown to be diagnostic of the associated equilibrium intermediate, we conclude that fragment 96–133 diminishes the concentration of this intermediate. Figure 4A demonstrates that at identical conditions as in Figure 4B, addition of a 10-fold molar excess of fragment 96–133 to bGH results in a slight increase in the far-UV CD spectrum. For conditions in which the associated equilibrium intermediate is not populated (0–3.0 M Gdn-HCl), no increase in the far-UV CD signal is observed by addition of fragment 96–133 to bGH. At the conditions for maximal population of the associated intermediate, both the intermediate and the isolated fragment are partially denatured. The increased ellipticity observed upon addition of the fragment to the associated equilibrium intermediate can be accounted for by induction of helicity in the fragment alone. However, it is not certain whether this is a result of increased helicity in the fragment or the intermediate or both.

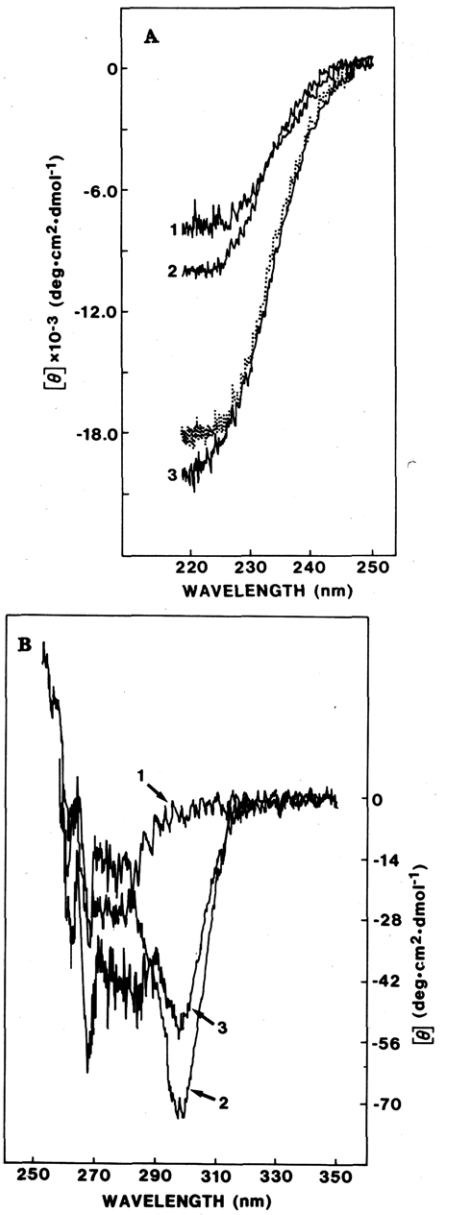


FIGURE 4: Effect of fragment 96–133 on the near- and far-UV CD spectra at 3.7 M Gdn-HCl. (A) Spectrum 1 is that of fragment 96–133 alone at 2.0 mg/mL, spectrum 2 is that of bGH alone at 1.0 mg/mL, and spectrum 3 is the spectrum of bGH at 1.0 mg/mL mixed with fragment 96–133 at 2.0 mg/mL together. The dotted spectrum is the mathematical sum of spectra 1 and 2. (B) Spectrum 1 is that of fragment 96–133 alone, spectrum 2 is of bGH alone, and spectrum 3 is of bGH mixed with fragment. The protein and peptide concentrations were the same as in (A).

## Discussion

Equilibrium denaturation of bGH has been previously studied (Holladay et al., 1974; Brems et al., 1985). The results are atypical with respect to the two-state transitions reported for most small globular proteins (Kim & Baldwin, 1982). For bGH, different probes of conformation demonstrate noncoincident denaturation transitions. The noncoincident transitions can be explained by preferential loss of the tertiary structure as monitored by UV absorbance and tryptophan fluorescence followed by loss of the more stable secondary or helical structure as monitored by CD at 222 nm. The observation that secondary structure is retained after loss of tertiary interactions has been interpreted as evidence for a framework model of protein folding (Kim & Baldwin, 1982; Brems et al., 1985). The presence of such equilibrium intermediates in the denaturation pathway makes bGH an attractive model for protein folding studies providing that the equilibrium intermediates are kinetic intermediates on the direct pathway of protein folding. The role of these equilibrium intermediates in the kinetic pathway of folding is yet to be established.

In addition to the noncoincident transitions, a noncontinuous transition was observed for a single probe, that of HPLC size exclusion (Brems et al., 1985). The nature of the noncontinuous transition was described previously (Havel et al., 1986) and was found to be due to the formation of an associated equilibrium intermediate. It is not certain whether it represents a single unique form or a population of multiple species. Until further evidence is available for multiple forms, we will assume the simplest singular case. The associated equilibrium intermediate contains approximately 50% of the original helical structure. This retained secondary structure (helix) and the stability of the associated intermediate are interrelated as demonstrated by similar dependencies to concentration and temperature. Figure 1A,B demonstrates that under conditions in which the associated equilibrium intermediate is not populated (low protein concentration), the Gdn-HCl-induced unfolding of the helix is a monophasic transition. Under conditions that the associated equilibrium intermediate is populated (high protein concentration), the transition is biphasic, with approximately 50% of the helix demonstrating increased stability to Gdn-HCl and 50% showing a decreased stability. The biphasic transition obtained at high protein concentration demonstrates the presence of two populations of helical structure. Our interpretation of these results is that in order to form the associated equilibrium intermediate, a conformational change occurs which strains or decreases the stability of one helix population, yet increases the stability of the other population.

Figure 2A compares the thermal stability of nativelike tertiary structure as monitored by absorbance at 290 nm and of the associated equilibrium intermediate as measured by CD at 300 nm under identical solution conditions of 3.7 M Gdn-HCl. The 300-nm CD signal has a broad temperature dependence with a maximum stability of approximately 33 °C, and the UV absorbance at 290 nm shows a more narrow temperature dependence with a maximum near 11 °C. Figure 2B depicts the effect of temperature on the helical content in 3.7 M Gdn-HCl. Under selected conditions where the associated equilibrium intermediate is not populated (0.016 mg/mL), the CD at 222 nm has a temperature dependence at least 10 °C lower than under conditions where the associated intermediate is populated (1.6 mg/mL). Because the helical content present in 3.7 M Gdn-HCl at high protein concentrations and the associated intermediate have very similar thermal dependencies, it is probable that they are mutually dependent. Perhaps the increased helix stability observed at high protein concentration results from the intermolecular packing of helices from adjacent protein molecules, e.g., intermolecular packing of amphiphilic helices. Figure 3 shows that a predicted helix from residues 110–127 would have a strong preponderance for amphiphilicity. Previous workers have demonstrated that the fragment 96–133, commonly referred to as the "active core" of bGH, contains as much as 50% helix structure in aqueous solutions (Chen & Sonenberg 1977). Our solution studies of the fragment (results to be published) show that the helix content is strongly dependent on peptide concentration, indicating that intermolecular interactions are responsible for its unusual stability. Figure 4B demonstrates that this fragment, when added in a 10-fold molar excess to bGH, significantly reduces the concentration of the associated equilibrium intermediate. Concomitant to the decrease in the associated equilibrium intermediate is an increase in overall helical content. We suggest that the fragment specifically interacts with partially denatured bGH and is manifested by an induced helix of the fragment. The observation that bGH helical structure is dependent on intermolecular association and that a specific helix-containing fragment diminishes the association strongly implies that the secondary structure plays a critical role in the formation of the associated equilibrium intermediate. We propose the following model to explain our results. As bGH unfolds, the first detectable event is a change in the UV absorbance at 290 nm. We believe this to reflect the opening up of the tertiary structure; at this stage, the secondary structure predominantly remains stable. However, the opening of the tertiary structure exposes the hydrophobic face of the amphiphilic helix 110–127. If the concentration of bGH is sufficiently high, the hydrophobic face of one amphiphilic helix will interact with similar surfaces of adjacent molecules, resulting in the formation of the associated intermediate. Upon association of two or more amphiphilic helices, a net stabilization of the involved helices is observed.

The results reported here have significant relevance to both the solubility and folding of bGH. For example, we previously reported the observation (Brems et al., 1985) that the denaturation transition of bGH is completely reversible within the transition zone (2.0–6.0 M Gdn-HCl). However, when bGH samples containing denaturing amounts of Gdn-HCl are diluted to less than 2.0 M Gdn-HCl, irreversible turbidity developed. Yet, if the denatured samples are diluted to 2.0 M Gdn-HCl, allowed to fold for several minutes, and then diluted to lower Gdn-HCl concentrations, turbidity did not appear. Such an observation can be explained by the associated equilibrium intermediate if one assumes that it is insoluble in Gdn-HCl solutions of less than 2.0 M.

The effect of protein concentration has been reported to play a role in the renaturation of other proteins. For *E. coli* tryptophanase, the presence of folding intermediates in the renaturation process at high protein concentrations caused aggregation that resulted in the formation of an inactive form and was characterized by incorrect quaternary interactions (London et al., 1974). Incorrect interchain interactions were also reported for two monomeric enzymes: the $\alpha$ chain of tryptophan synthetase (Jackson & Yanofsky, 1969) and ribonuclease A (Crestfield et al., 1963). In both cases, a denaturation–renaturation process at high protein concentration was required for the preparation of artificial dimers.

Our results have interesting relevance to the framework model of protein folding. In the framework model, folding is a sequential process with the secondary structure appearing early due to short-range stabilizing interactions. The stability of this archetype intermediate is increased by subsequent long-range intramolecular interactions. At low protein concentrations, the bGH associated intermediate is not detectable, yet the denaturation transitions as measured by the absorbance at 290 nm and CD at 222 nm are still distinct, with the CD showing a greater stability. Therefore, a bGH monomeric intermediate exists with the aromatic groups considerably exposed to solvent but with retained helix. Because the putative helix 110–127 is highly stable, as evidenced by its presence in the isolated fragment 96–133, we speculate that formation of this helix in an early folding intermediate could provide considerable stability by making available a hydrophobic face for long-range intramolecular interactions. At high protein concentrations, the same hydrophobic surface provides the stabilizing force through intermolecular interactions for the formation of the associated equilibrium intermediate.

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# Streptococcal Phosphoenolpyruvate–Sugar Phosphotransferase System: Amino Acid Sequence and Site of ATP-Dependent Phosphorylation of HPr†

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ABSTRACT: The amino acid sequence of histidine-containing protein (HPr) from *Streptococcus faecalis* has been determined by direct Edman degradation of intact HPr and by amino acid sequence analysis of tryptic peptides, V8 proteolytic peptides, thermolytic peptides, and cyanogen bromide cleavage products. HPr from *S. faecalis* was found to contain 89 amino acid residues, corresponding to a molecular weight of 9438. The amino acid sequence of HPr from *S. faecalis* shows extended homology to the primary structure of HPr proteins from other bacteria. Besides the phosphoenolpyruvate-dependent phosphorylation of a histidyl residue in HPr, catalyzed by enzyme I of the bacterial phosphotransferase system, HPr was also found to be phosphorylated at a seryl residue in an ATP-dependent protein kinase catalyzed reaction [Deutscher, J., & Saier, M. H., Jr. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6790–6794]. The site of ATP-dependent phosphorylation in HPr of *S. faecalis* has now been determined. [$^{32}$P]P-Ser-HPr was digested with three different proteases, and in each case, a single labeled peptide was isolated. Following digestion with subtilisin, we obtained a peptide with the sequence -(P)Ser-Ile-Met-. Using chymotrypsin, we isolated a peptide with the sequence -Ser-Val-Asn-Leu-Lys-(P)Ser-Ile-Met-Gly-Val-Met-. The longest labeled peptide was obtained with V8 staphylococcal protease. According to amino acid analysis, this peptide contained 36 out of the 89 amino acid residues of HPr. The following sequence of 12 amino acid residues of the V8 peptide was determined: -Tyr-Lys-Gly-Lys-Ser-Val-Asn-Leu-Lys-(P)Ser-Ile-Met-. Thus, the site of ATP-dependent phosphorylation was determined to be Ser-46 within the primary structure of HPr.

Histidine-containing protein (HPr)[1] functions as a phosphate carrier protein in the phosphoenolpyruvate (PEP)–sugar phosphotransferase system (PTS) mediated uptake reaction of carbohydrates in most anaerobic and facultatively anaerobic bacteria (Hengstenberg, 1977; Robillard, 1982; Meadow et al., 1984). HPr becomes phosphorylated at the N-1 position of His-15 in a PEP-dependent reaction catalyzed by enzyme I of the PTS (Simoni et al., 1973; Beyreuther et al., 1977;

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[1] Abbreviations: HPr, histidine-containing protein; P-His-HPr, HPr phosphorylated at histidyl residue 15; P-Ser-HPr, HPr phosphorylated at seryl residue 46; PTS, phosphoenolpyruvate–sugar phosphotransferase system; PEP, phosphoenolpyruvate; III, factor III or enzyme III; P-III, phosphorylated III protein; TFA, trifluoroacetic acid; Pth, phenylthiohydantoin derivatives of amino acids; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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