Vol. 213, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 4, 1995 Pages 239-248

# Purification and Characterization of Human Pancreatic Polypeptide Expressed in *E. coli*

**Yuri V. Griko*+ and Marina D. Kapanadze****

**Department of Biology, The Johns Hopkins University, Baltimore, MD 21218*

***Institute of Protein Research of the Russian Academy Sciences, Pushchino, Russia*

Received June 22, 1995

The region of cDNA encoding human pancreatic polypeptide (hPP) was obtained by polymerase chain reaction (PCR) and subcloned into an expression vector. The pancreatic polypeptide gene was expressed in *Escherichia coli* in two versions: as a cleavable fusion protein with IgG-binding synthetic ZZ domains of protein A from *Staphylococcus aureus* or with the 1-48 fragment of λ Cro repressor. Site-specific hydrolysis by hydroxylamine was used to cleave the fusion protein, releasing the human polypeptide. The structure of the obtained hPP has been studied by scanning microcalorimetry and circular dichroism spectrometry. It has been shown that hPP in solutions close to neutral has a compact and unique spatial structure with an extended hydrophobic core. This structure is stable at 20 °C and co-operatively breaks down upon heating from this temperature. 

Human pancreatic polypeptide is a small hormone, consisting of 36 amino acid residues, that plays a key role in the regulation of the secretion of many physiologically active peptides produced by the pancreas [1]. Its three dimensional structure, similar to that of avian pancreatic polypeptide, was found to be rather simple [2,3]. The structure consists of an N-terminal polyproline helix, a central turn and an α-helical region. Since it does not have co-factors or prosthetic groups, it is a most attractive object for studying the mechanism of protein folding and stabilization. The cloned DNA containing the gene encoding human pancreatic polypeptide has previously been described [4,5]. Like other biologically active polypeptides, hPP is a hormone generated through post-translational modification of a larger precursor molecule [6,7]. The 465 base pair long fragment of pancreatic polypeptide cDNA encodes a peptide of 95 amino

+Address correspondence to this author at the Department of Biology at The Johns Hopkins University, 144 Mudd Hall, 3400 Charles Strs., Baltimore, MD 21218. FAX: (410) 516 5213.

0006-291X/95 $12.00

acids in the coding region. In this peptide, 29 amino acids are assigned to the leader sequence, 36 amino acids to a pancreatic polypeptide, and 27 amino acids to a peptide at the carboxyl terminus of which the first 20 amino acids correspond to the icosapeptide described earlier Fig.1 [7].
Chemical synthesis or purification of pancreatic polypeptides from the native source in the amounts required for physical studies is, however, too expensive [6]. To overcome these difficulties we have developed a system to produce hPP of high purity and in considerable amounts in *E.coli* .
In this report, we describe two different constructions of chimerical genes in which the gene encoding human pancreatic polypeptide was fused to the gene encoding the Cro repressor of phage λ [8] or the ZZ domains of Staphylococcal protein A (an IgG-binding fragment). These constructions facilitate the expression and secretion of the recombinant protein. The fusion gene product was found to be stable in *E.coli.*

## Materials and Methods

**Reagents and enzymes:** Chemicals, restriction endonuclease, T4-DNA ligase, DNA Polymerase I Large Fragment (Klenow fragment), and T4 polynucleotide kinase, obtained from United States Biochemicals were used according to the supplier's recommendations.
**Plasmids, Bacterial strains:** *E. coli* HB101 [9], TG-2 and W3110 [10] were used for plasmid propagation and for the expression of the genes for Cro-hPP and ZZ-hPP fusion proteins. The expression vector pEZZ318 for producing fusion protein with IgG-binding domains of Staphylococcal protein A was kindly provided by Dr. M. Uhlén (Royal Institute of Technology, Sweden). The plasmid contains the staphylococcal protein A promoter and signal sequence followed by a gene encoding IgG-binding synthetic ZZ domains from protein A [11].
**DNA construction:** The starting plasmid pBR322 containing cDNA encoding the native pancreatic polypeptide was kindly donated by Dr. Yamada (University of Michigan). PCR was used to subclone the gene encoding hPP into the vector pEZZ318 and pTZ18U(Cro) [12]. In order to create a BamHI site at the 5' end and a PstI restriction site at the 3' end of the hPP gene fragment, as well as the codons encoding a Asn-Gly bond enabling hydroxylamine cleavage [13,14], PCR primer construction was performed. The primers used were:
$P_1$ 5' GGA TCC AAT GGT CCA CTG GAG CCA GTG T
$P_2$ 5' AAC CTG CAG TTA ATA CCT AGG CCT GGT CAG C
In the primer $P_1$, which is complementary to the 3' region of the antisense strand of hPP, bases 1-12 were changed to create a Bam HI and a site for hydrolysis by hydroxilamine. In primer $P_2$, bases 1-9 encode the sequences creating a PstI site and inserting termination a codon TAA. A 136 base pair long fragment of the DNA was generated by PCR using Taq DNA polymerase (Amersham). Amplified double-stranded fragments were separated on a low-melting point agarose gel, cut by BamHI and PstI restriction enzymes, and subcloned into the BamHI/PstI site of the replicative forms of M13mp18 and M13mp19. The nucleotide sequence of the construct was verified by DNA sequencing using a Sequenase kit (USB) and α $^{35}$S-dATP (Amersham).

**Construction of various gene fusion vectors:**
Two different recombinant plasmids were designed to express pancreatic polypeptide as a fusion product. One of them contains an additional amino-

terminal polypeptide chain of IgG-binding synthetic ZZ domains of protein A from *Staphylococcus aureus.* The other contains a fragment of Cro repressor of phage λ. Both of them have a unique hydroxylamine hydrolysis site downstream from the hPP coding region.

**I. Fusion with protein A:** The fusion gene encoding pancreatic polypeptide under the control of the staphylococcal protein A promoter was constructed as summarized in Fig.1. Before subcloning the 0.2 kDa fragment into pEZZ318, its BamHI end was "blunted" with a Klenow reaction. The vector pEZZ318 was opened by BamHI digestion, "blunted", and subsequent digestion was done by PstI. The 0.2 kD BamHI-PstI PCR fragment was subcloned into pEZZ318.

**II. Fusion with the fragment of Cro repressor:** Plasmid pTZ18(Cro) containing a Cro repressor gene [12] was cleaved by BamHI and PstI endonucleases. The fragment containing pancreatic polypeptide was inserted into the unique BamHI/PstI site. The plasmid pTZ18(CrohPP) has the truncated λCro repressor gene fused to the pancreatic polypeptide as shown in Fig.1.

We compared these two different plasmid constructions for their level of product expression, proteolytic degradation, and other qualities of the hybrid protein in order to choose the more efficient one.

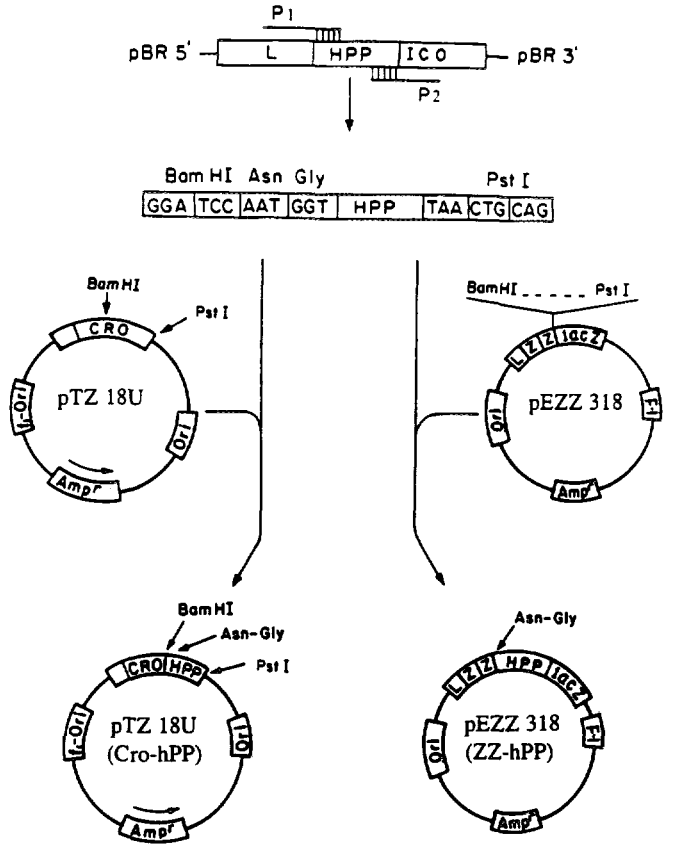



**Figure 1.** Schematic representation of the construction of the plasmids pEZZ 318 and pTZ18U containing human pancreatic polypeptide as fusion with ZZ domains of Staphylococcal protein A and Cro repressor of phage λ, respectively.

Transformation of competent cells was carried out according to [15]. Proteins were analyzed by electrophoresis in an acidic urea system [16]. SDS/PAGE was performed according to Laemmli [17].

**Expression and Purification:** The pTZ18(Cro-hPP) was expressed in E.coli W3110. Expression of fusion protein was induced at mid-logarithmic phase ( OD = 0.9 ) by the addition of 0.5 mM IPTG. After 2-3 hours the cells were harvested, collected and resuspended in 20 ml. of lysis buffer, containing 50 mM Tris (pH=7.5), 150 mM NaCl, and 10 mM DDT. The suspension was sonicated, and urea was added to a final concentration of 2 M. The supernatant after the first extraction consisted of about 70% fusion protein. To obtain all of the protein, the pellet fraction was washed twice with lysis buffer containing 2 M urea. The solubilized protein was purified by FPLC Mono-S chromatography (Fig.2a) and gel filtration on the TSKG 3000W column (Fig.2b). The purity of the fusion protein after chromatography was 98% when assayed by SDS-PAGE (Fig.3a, lane 4). According to the electrophoretic mobility, apparent molecular weight of the polypeptide is 10 kDa, what is close to that expected for the fusion protein containing 48 amino acid residues of λCro repressor and the 36 amino acids of human pancreatic polypeptide.
*E.coli* TG-2 cells transformed with the plasmid pEZZ 318 were grown at 37° C in LB medium in the presence of ampicillin (100μg/ml). The cells containing the hPP gene fused with IgG-binding ZZ domains grew more slowly than those containing the fusion with the native bacterial gene of the λCro repressor. The periplasmic fraction was collected by osmotic shock and passed through an affinity column of IgG-Sepharose Fast Flow (Pharmacia) as described in [18]. The protein fraction was eluted with 0.5 M AcOH (pH=2.8), collected, and lyophilized. As shown by electrophoretic analysis (Fig.3b), the full-length fusion protein, migrating at 19 kDa, is not the major component of the affinity bound proteins. The presence of additional bands on the gel with MW 14 kDa and 10 kDa suggest that there could be some partial degradation of the synthetic ZZ domains after IgG chromatography. Because a 19 kDa protein of a size close to that expected of the fusion ZZ-hPP was produced, and because all of these proteins react with IgG-Sepharose, we collected and purified them.

**Site Specific Cleavage of the Fusion Protein:**
The IgG-Sepharose purified fusion protein, as well as the Mono S purified pool, were dialyzed in water. hPP was cleaved from the fusion protein by treatment with 2M hydroxylamine at pH 9.0 for 4 hours at 45° C to cleave the Asn-Gly residues flanking the hPP. The reaction was performed in the presence of 6 M GuHCl as described previously [14]. The efficiency of the hydrolysis reaction was about 60%. The protein mixture was desalted using Sephadex-25, and after extensive dialysis was again applied to the IgG-Sepharose column. As shown by SDS/PAGE, the hydroxylamine cleavage resulted in a decrease in the intensity of the 19 kDa band in Fig. 3b (lane 2) and yielded new major bands of 14 kDa and 4.5 kDa. The band corresponding to a protein with MW 10 kDa also disappeared after treatment with hydroxylamine. Only the fraction with a molecular weight about 14 kDa corresponded to the size of the synthetic ZZ domains of protein A, bound to IgG-Sepharose. The low molecular weight fraction did not interact with IgG-Sepharose and passed through the column. Its molecular weight was around

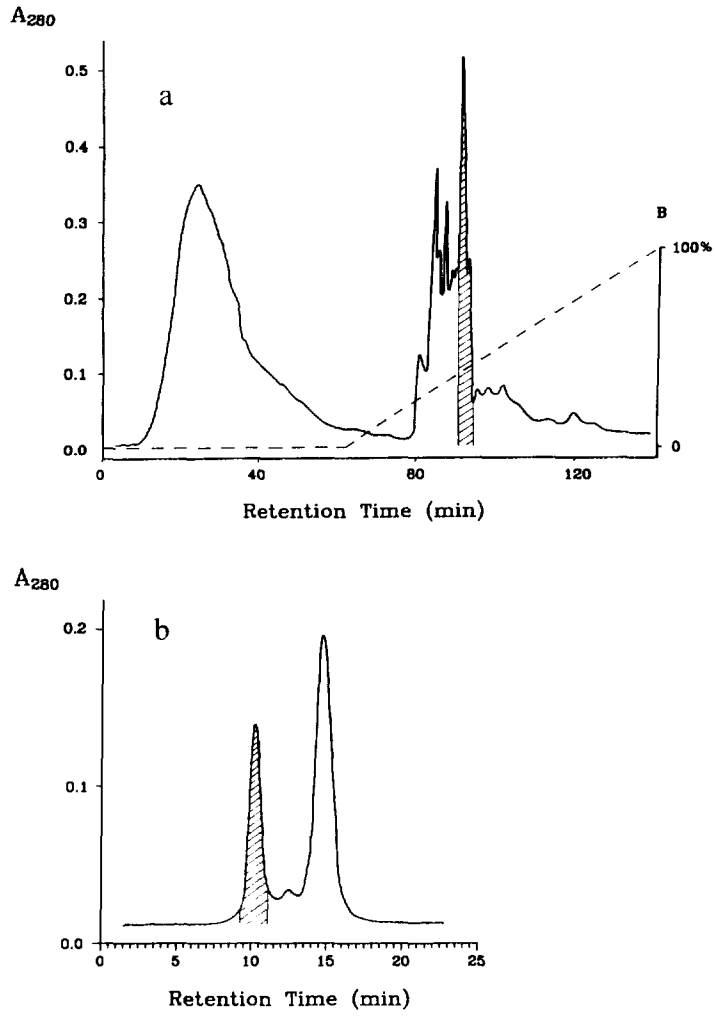



**Figure 2.** Purification of the Cro-hPP fusion protein from *E.coli* cells crude extract.
**(a)** Initial fractionation by FPLC (Mono S column). About 250 µl of the crude extract was dissolved in 250 µl of 10 mM Na-oAc buffer containing 4 M urea, pH 4.0,and applied on the Mono S column. The column was eluted at 1.0 ml/min with linear gradient of NaCl (from 0 to 1 M) in same buffer.
**(b)** Gel filtration profile of the Cro-hPP containing fraction after FPLC on the TSKG 3000 W column.

4.5 kDa, which is close to the 4.2 kDa expected for the full length hPP polypeptide chain.
The same procedure for the cleavage of the Asn-Gly bond was done for the Cro-hPP fusion protein. In this case, the digestion mixture was loaded on a Mono S FPLC column equilibrated with 10 mM Na OAc buffer, pH 4.0. The material was eluted with a linear gradient of NaCl (see Fig.2a). Fig.3a (line 5) shows the appearance of a new protein band with a mobility corresponding to 4.5 kDa after hydrolysis by hydroxylamine.

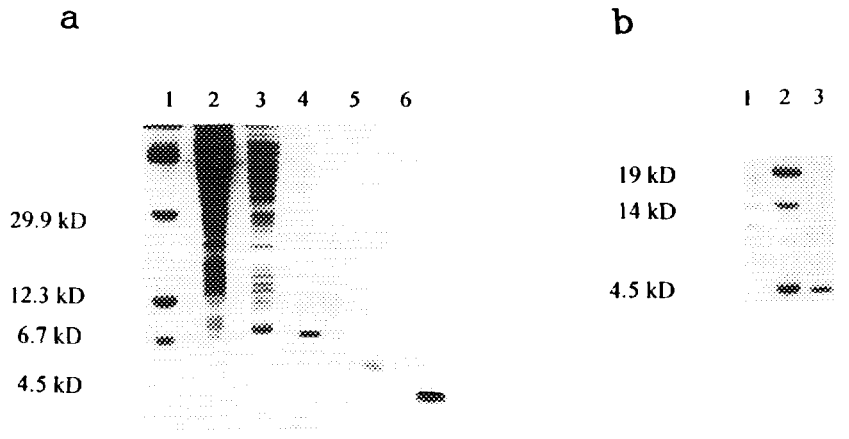



**Figure 3.** SDS/PAGE analysis of the different steps in hPP purification from *E.coli* extract containing pTZ18U(Cro-hPP) (a) and pEZZ(ZZ-hPP)318 (b). Lanes of the 18% Tris/glycine gel are:

**(a).** Containing pTZ18U(Cro-hPP)

1. Markers: carbonic anhydrase B, MW 29.9 kD; Cytochrome C, MW 12.3 kD; Aprotinin, MW 6.7 kD.
2. *E.coli* lysate before induction by IPTG (4 μl of the lysate was loaded).
3. *E.coli* lysate after induction by IPTG (3μl of the lysate was loaded).
4. The chromatographically purified Cro-hPP fusion protein before cleavage by hydroxylamine (10μg of the protein was loaded).
5. Mobility of the digested mixture after 4 hours of the reaction with hydroxylamine (8μg of the mixture was loaded).
6. Pancreatic polypeptide moiety after FPL chromatography. (20μg of the protein was loaded).

**(b).** Containing pEZZ(ZZ-hPP)

1. Proteins eluted after affinity binding with IgG-Sepharose (5 μg of the protein was loaded).
2. Mobility of the digested mixture after 4 hours of the hydroxylamine reaction (25 μg).
3. Mobility of the pancreatic polypeptide moiety after the chromatography (10 μg).

**N-Terminal Amino Acid Sequence Determination:**

In addition to the nucleotide sequences of the both constructs, the sequence of 4 amino acid residues from the N-terminal end of the 4.5 kDa fragment has been determined by Edman degradation [19]. The amino acid composition obtained from this analysis is consistent with the N-terminal sequence Gly-Pro-Leu-Glu- expected for the hPP [4,5]. Analysis of the light absorption spectrum of hPP shown that its polypeptide chain contain 4 tyrosines. Because hPP has C-terminal Tyr 36, no any heterogeneity at the carboxyl terminus could be expected.

**Calorimetric and circular dichroism measurements:**

Scanning calorimetric measurements of the protein solution was performed using DASM-4 scanning microcalorimeter at the heating rate 1 K/min. The protein

concentration in these measurements was determined spectrophotometrically using 280 nm absorption coefficient of $E^{1\%}_{1cm}$= 12.1. The concentration used in the calorimetric experiments varied between 1-3 mg/ml in the different experiments. The partial specific heat capacity of hPP was determined as described [20]. The CD spectra were obtained in the range 180-320 nm with Jasco-41A spectropolarimeter equipped with thermostatic cell holders and a data processor. The temperature was controlled by water-bath thermostat RTE-210 (Neslab).

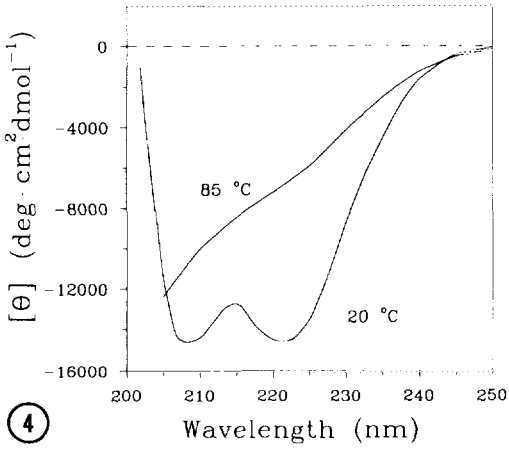
## Results and Discussion

The results obtained show that both the expression systems - the fusion protein with λCro repressor and the fusion with ZZ domains of protein A - can be used to produce the full length human pancreatic polypeptide in *E. coli*. Concerning the production of fusion ZZ-hPP, it is necessary to note that in spite of the fact that the efficient protein secretion in this system allowed us to easily obtain full size hPP, we could not completely exclude some degradation of the fusion protein during purification under acidic conditions. In contrast to the ZZ-hPP system, the fusion protein of the pancreatic polypeptide with a fragment of λCro repressor gave higher yield. This construct yields as much as 20-25 mg/L on induction by IPTG. Separation of *E.coli* lysate into soluble and insoluble fractions indicated that the fusion protein was found predominantly in the insoluble fraction. Lower solubility is typical for proteins which are interacting with cellular components and/or precipitating in the cells, forming inclusion bodies [21]. The protein could, however, be solubilized by suspension of the pellet in 2-4 M urea. Since the denaturation of the pancreatic polypeptide is completely reversible [22], this approach seems effective to increase overall yield.

The expressed hPP consisting 36 amino acids was identical to the natural hPP with exception that the Ala → Gly in first position introduced during construction of the fusion proteins. However, this substitution is not essential for the protein structure and activity because many pancreatic polypeptide hormones from different sources have Gly in the first position [21, 23]. In addition, the C-terminal Tyr in the produced pancreatic polypeptide is not converted to a carboxyl-terminal α-amid group as observed for the natural protein after post-translational processing and subsequent modification by specific enzyme. While this modification may have physiological importance, it is not critical for the protein structure [23, 24].

In order to obtain definitive proof that the obtained pancreatic polypeptide is in native conformation in solution, it has been characterized by CD and scanning microcalorimetry. Figure 4 shows the circular dichroism spectra of hPP at 20 and 85 °C in 10 mM sodium acetate buffer, pH 5.2. The CD spectrum at 20 °C had two negative maxima at 208 and 222 nm, which is characteristic of the α-helix. Judging by the ellipticity at 222 nm, the helicity of the obtained hPP is similar to the intact PP [22,23,25]. The CD spectrum of hPP changed greatly on heating to 85 °C. Figure 6 shows that the heat denaturation lead to an almost complete loss of helicity in the protein.

Scanning microcalorimetric recording of the heat effect observed upon heating of the hPP solution is shown in Figure 5. The heating is accompanied by an extensive heat absorption peak occurring in the temperature range in which the breakdown of the secondary structure of hPP is observed by CD. Disruption of the ordered structure of hPP caused by heating leads to a significant increase of



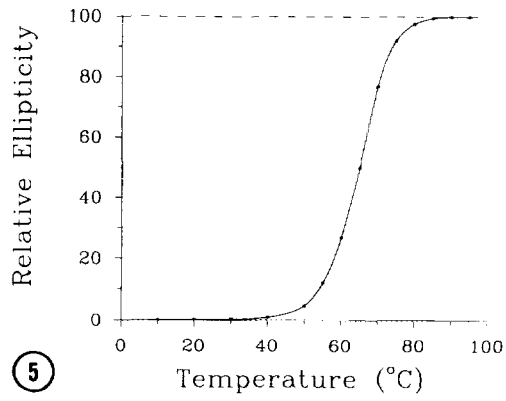




**Figure 4.** Circular dichroism spectra of hPP in 10 mM Na-acetate buffer, pH 5.2, at various temperatures.

**Figure 5.** Temperature dependence of the normalized ellipticity of hPP at 222 nm in 10 mM Na-acetate buffer, pH 5.2.

heat capacity. From the observed increase of heat capacity of hPP upon disruption of its compact ordered structure, one can conclude that this molecule has a well-developed hydrophobic core arranged by non-polar groups of amino acid residues [26] and in this respect the obtained hPP does not differ from the native PP [24] and other globular proteins. The structure of hPP is maximally stable at room temperature and breaks down reversibly upon heating from this temperature. The process of breakdown of the structure is highly co-operative with the denaturational enthalpy $\Delta H$ = 191 kJ/mol at temperature 61 °C. The value of the

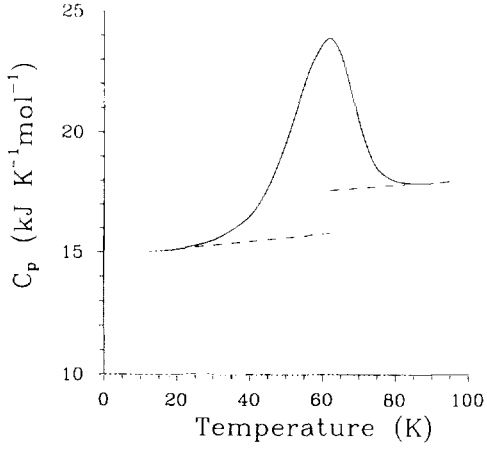



**Figure 6.** Temperature dependence of the partial molar heat capacity of hPP in 10 mM Na-acetate buffer, pH 5.2. The dashed lines show the functions of the partial heat capacity native and denatured state of hPP extrapolated to temperature of the denaturational transition. Scanning rate is 1 K/min. Protein concentration is 2.5 mg/ml.

calorimetric enthalpy is close to van't Hoff enthalpy (196 kJ/mol) determined from the sharpness of the peak. This suggests that the heat denaturation of hPP represents a two-state transition between the native and denatured states. The extreme co-operativity of the hPP structure can be regarded as an indication of the uniqueness of its structure which is characterized by specific packing of residues [27]. From the results obtained it follows that the polypeptide chain of hPP folds into unique conformation similar to the native PP and can serve as a good model system for studies of protein folding and stability.

**Acknowledgments**

Some of the experimental work was carried out in the laboratory of Dr. Alan Fersht (Cambridge University, Chemical Laboratory). We are deeply indebted to him for his support. We also thank Peter Privalov for reading of the manuscript and discussion. We thank Dr. J. Carra for helpful comments and correction of the manuscript in its preparation. We are indebted to Dr. Oleg Yarchuk for his help with PCR.

## References

1. Whitcomb, D.C., Taylor, I.L., & Vigna, S.R. (1990) Am. J. Physiol. 259, 687-691.
2. Blundell, T.L., Pitts, J.E.,Tickle, I.J., Wood, S.P., & Wu, C.-W. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 4175-4179.
3. Glover, I.D., Hancef, I., Pitts, J.E., Wood, S.P., Moss, D.,Tickle, I.J., & Blundell, T.G. (1983) *Biopolymers* 22, 293-304.
4. Takeuchi, T. & Yamada, T. (1985) *Proc. Natl. Acad. Sci. USA* 82, 1536-1539.
5. Leiter, A. B., Keutmann, H. T. & Goodman, R. H. (1984) J. Biol. Chem. 259, 14702-14705.
6. Kimmel, J.R., Hayden, L.G., & Pollock, H.G. (1975) J. Biol. Chem. 250, 9369-9376.
7. Schwartz, T. W., Hansen, H. F., Hakanson, R., Sundler, F. & Tager, H. S. (1984) Proc. Natl. Acad. Sci. USA 81, 708-712.
8. Sutton, H.W. (1985) Thesis University of Colorado, Boulder.
9. Boyer, H.W. & Roulland-Dussoix, D. (1969) J. Mol. Biol. 41, 459-472.
10. Brent, R. & Ptashne, M. (1981) Proc. Natl. Sci. USA 78, 4204-4208.
11. Nilsson, B., Moks, T., Jansson, B., Abrahmsén, L., Elmblad, A., Holmgren, E., Henrichson, Ch., Jones, T.A., & Uhlén, M. (1987) Protein Engineering 1, 107-113.
12. Rogov, V.V. & Griko, Yu.V. (1993) Mol. Biol.(Russia) 27, 488-491.
13. Frohman, M., Dush, M. K. & Martin, G. R. (1988) Proc. Natl. Acad. Sci. USA 85, 8998-9002.
14. Bornstein, P. and Balian, G. (1977) Methods in Enz. 47, 132-145.
15. Maniatis, T., Fritsch, E. F. & Sambrook, J., (1989) In Molecular Cloning: A Laboratory Manual, Vol. I, pp. 249-255. Cold Spring Harbor, New York Harbor Laboratory. 249-255.
16. Spiker, S. (1980) Anal. Biochem. 108, 263-265.

17. Laemmli, U. K. (1970) Nature (London), 227, 680-685.
18. Shein, C.H. (1989) Bio/Technology 7, 1141-1149.
19. Edman, P. (1950) Acta Chem. Scand. 4, 277-288.
20. Privalov, P.L. & Potekhin, S.A. (1986) Methods in Enzymology 131, 5-51.
21. Litthauer, D. & Oelofsen, W. (1987) Int. J. Peptide Protein Res. 29, 739-745.
22. Kanazawa, I. & Hamaguchi, K. (1986) J Biochem. 100, 207-212.
23. Glover, I.D., Barlow, D.J., Pitts, J.E., Wood, S.P., Tickle, I.J., Blundell, T.L., Tatemoto, K., Kimmel, J.R., Wollmer, A., Strassburger, W., Zhang, Y.S. (1984) Eur. J. Biochem. 142, 379-385.
24. Schoemaker, J.M., Brasnett,A.H. and Marston, F.A.O. (1985) EMBO J. 4, 775-780.
25. Tonan, K., Kawata, Y. & Hamaguchi, K. (1990) Biochemistry 29, 4424-4429.
26. Kauzmann, W. (1959) Adv. Protein Chem. 14, 1-63.
27. Privalov, P.L. (1979) Adv. Protein Chem. 33, 167-241.