Int. J. Peptide Protein Res. **34**, 1989, 118–123

Influence of ions on cyclization of the amino terminal glutamine residues of tryptic peptides of streptococcal PepM49 protein

Resolution of cyclized peptides by HPLC and characterization by mass spectrometry

KIRAN M. KHANDKE, THOMAS FAIRWELL[1], BRIAN T. CHAIT, and BELUR N. MANJULA

The Rockefeller University, New York, NY, and [1]Molecular Diseases Branch, National Heart, Lung and Blood Institute, Bethesda, MD, USA

Received 20 July, accepted for publication 27 December 1988

RPHPLC of the tryptic digest of lysine blocked group A streptococcal PepM49 protein (DHP-PepM49) consistently yielded, among others, two pairs of peptides which were well resolved, eluted in tandem, and had identical amino acid compositions. In each pair, the earlier eluting peptide was readily amenable to sequencing and yielded an amino-terminal glutamine whereas the later eluting peptide could not be sequenced. Mass spectral analysis revealed that each of these pairs of peptides differed in mass corresponding to the loss of ammonia. These data suggested that the later eluting peptide in each pair is a result of cyclization of the amino-terminal glutamine residue to pyroglutamic acid, which apparently leads to an increase in the hydrophobicity of the peptide. A kinetic analysis of the tryptic digestion of the DHP-PepM49 protein revealed that the cyclized form of the peptides were essentially absent during the initial time and increased with time of incubation, with a concomitant decrease in the uncyclized form. In 0.2 M ammonium bicarbonate at 37°, nearly 44% conversion of the glutaminyl peptides to the pyroglutamyl peptides was observed in 24 h. This conversion was accelerated in sodium phosphate buffer relative to that in ammonium bicarbonate whereas it had a significantly lower rate in ammonium acetate buffer. The conversion was also temperature dependent, with essentially no cyclization at 0°, in all the three buffers. Thus, an extended digestion at 0° or a brief digestion at 37° in ammonium acetate was found to be a suitable condition for limiting the cyclization of amino-terminal glutamine residues of PepM49 peptides. The results of the present study suggest that use of ammonium acetate should be preferred over the ammonium bicarbonate and phosphate buffers in the handling of peptides and proteins known to contain amino-terminal glutamine in order to minimize the possibility of cyclization of these residues.

Key words: HPLC; mass spectrometry; pyroglutamic acid; streptococcal PepM49

During the course of our studies on the isolation of arginyl peptides of lysine-modified group A streptococcal PepM49* protein by reverse phase HPLC (RPHPLC), we obtained multiple peaks with identical amino acid compositions. In view of the highly repetitious nature of the sequence of the other streptococcal M protein serotypes (1–4), it appeared likely that the multiplicity of the peptides was only a reflection of the repetitious nature of the PepM49 sequence. However, the total number of peptides isolated by HPLC was more than could be accounted for by the number of arginines in the PepM49 protein. It was, therefore, essential to establish if the multiplicity of the peptides was due to post-translational modification of the protein *in vivo*, which could result in isoforms of the protein. This in turn could generate multiple peptide species from the same region of the molecule, but the difference between them could go undetected by conventional amino acid analysis. Alternatively, the multiplicity of the peptides could also result from chemical modification of the protein or peptides during the course of the structural studies. In the present study we demonstrate that two of the peptides

*The abbreviations used are: PepM49, the biologically active peptic fragment of streptococcal type 49 M protein (5, 29); DHP, dihydroxypropyl; RPHPLC, reverse phase high performance liquid chromatography; TFA, trifluoroacetic acid.

are the result of cyclization of the amino terminal glutaminyl peptides, generated by tryptic cleavage, to pyroglutamyl peptides. The formation of amino terminal pyroglutamic acid is a major impediment in structural studies, since it precludes sequencing of peptides by Edman degradation. We have, therefore, investigated conditions under which such cyclization reactions can be virtually prevented.

MATERIALS AND METHODS

Dihydroxypropylation of PepM49 protein

PepM49, a peptic fragment of group A streptococcal type 49 M protein, was purified as previously described (5). In order to limit the tryptic cleavage to the arginyl peptide bonds, the ε-amino groups of lysine residues of PepM49 were blocked by reductive alkylation with glyceraldehyde (1, 6, 7). Briefly, PepM49 (2 mg/mL) in 0.01 M sodium phosphate-0.15 M chloride, pH 7.4, was reacted with 0.1 M glyceraldehyde in the presence of 1 M sodium cyanoborohydride at 37° for 30 min. The excess reagents were removed by dialysis against 0.05 M ammonium bicarbonate, and the sample lyophilized.

Tryptic digestion of dihydroxypropylated PepM49

Dihydroxypropylated-PepM49 (DHP-PepM49) (1–3 mg/mL) in the buffer of choice was digested with TPCK-trypsin (E/S 1:100) at 37° or at 0° for the appropriate times. The reaction was terminated by acidification with either acetic acid or 0.1% TFA.

HPLC

Reverse phase HPLC was carried out with a Shimadzu HPLC system on a Waters μ-Bondapak C18 column using an acetonitrile-0.1% TFA gradient for elution. The elution of peptides was monitored at 210 nm. Appropriate peaks were pooled and taken to dryness in a Savant Speedvac concentrator centrifuge.

Amino acid and sequence analyses

Amino acid analysis was carried out on 6 N HCl hydrolyzates of the peptide samples as described earlier (8). Amino-terminal sequence analysis of peptides (10–25 nmol) was carried out by automated Edman degradation in a Beckman 890 M sequencer, as described (1).

Mass spectrometry

Mass spectrometric measurements were performed on the ^{252}CF fission fragment ionization time-of-flight mass spectrometer described previously (9, 10). Samples were prepared for measurement by adsorption of ~1 nmol of peptide from solution (0.2 nmol/μL in 0.1% TFA) on a thin nitrocellulose film, as described (11). The accuracy of the mass measurements was generally greater than 200 ppm.

RESULTS

Tryptic digestion of DHP-PepM49 in ammonium bicarbonate

DHP-PepM49 in 0.2 M ammonium bicarbonate, pH 7.9, was digested with trypsin at 37° for 24 h to cleave at its arginyl peptide bonds. RPHPLC of the tryptic digest of DHP-PepM49 revealed more peptides than could be accounted for by the 11 arginine residues in the molecule (two of these are in Arg-Arg linkage (5)) (Fig. 1). Among these, we noted two pairs of peptides, namely A, B and C, D, that were of interest. The two peptides in each of these pairs eluted adjacent to each other, were very well resolved and were present in approximately equal amounts. Amino acid analysis revealed that the peptides in each pair had identical compositions (Table 1). Sequence analyses revealed that peptides A and C could be readily sequenced and both peptides contained glutamine at the amino-terminus (Fig. 2). In contrast, peptides B and D were refractive to Edman degradation. These results suggested that peptides B and D may be formed from peptides A and C, respectively, due to the formation of pyroglutamic acid by cyclization of their amino-terminal glutamine (12). Apparently, this cyclization increases the hydrophobicity of the peptides, resulting in an increase in their retention times on the reverse phase column.

Formation of peptides B and D from purified peptides A and C

In order to demonstrate that peptides B and D are indeed formed from peptides A and C, respectively, purified peptides A and C themselves were incubated in 0.2 M ammonium bicarbonate, pH 7.9, in the absence of trypsin, and their conversion to the cyclic forms was monitored by HPLC (Fig. 3). As can be

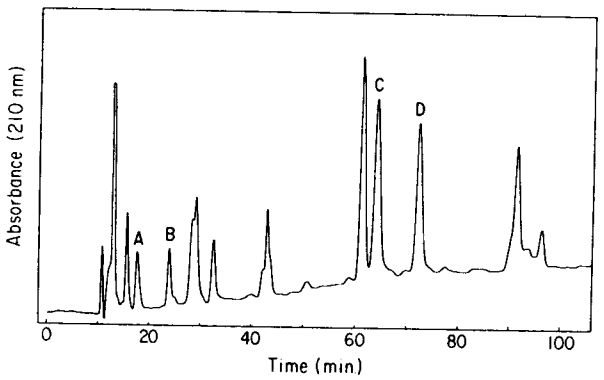

FIGURE 1

Tryptic digestion of DHP-PepM49 in 0.2 M ammonium bicarbonate, pH 7.9. Fractionation of a 24 h tryptic digest of DHP-PepM49 by HPLC on a Waters μ-Bondapak C18 reverse phase column. A gradient of 5%–25% acetonitrile-0.1% TFA over a period of 100 min was employed. The flow rate was 1 mL/min.

TABLE I

Amino acid composition of peptides A, B, C, and D

Amino acid	Peptide A	Peptide B	Peptide C	Peptide D
Asp	1.0(1)	1.4(1)	2.0(2)	2.1(2)
Ser	–	–	0.9(1)	1.1(1)
Glu	5.0(5)	4.8(5)	14.7(15)	14.8(15)
Ala	–	–	3.2(3)	3.0(3)
Val	–	–	1.1(1)	0.9(1)
Leu*	1.1(1)	1.3(1)	2.0(2)	2.0(2)
Tyr	–	–	1.8(2)	1.9(2)
DHP-Lys + His**	1.0(1)	0.9(1)	8.7(9)	9.0(9)
Arg*	1.0(1)	1.0(1)	1.0(1)	0.9(1)

*Normalized for Arg=1 for peptides A and B, and Leu=2 for peptides C and D.

**Both mono- and di-DHP-Lys were detected in all the samples. Mono-DHP-Lys eluted just prior to histidine, but was not completely resolved from it. DHP-Lys was quantitated as described earlier (1, 7). Therefore, the values shown represent the total of DHP-Lys and His. Subsequent sequencing of peptides A and C (see Fig. 2) revealed that peptide A contained one Lys and peptide C contained six Lys and three His.

seen, there was a progressive conversion of peptide A to peptide B (Fig. 3a) and peptide C to peptide D (Fig. 3b) with time. Prolonged incubation of peptide A resulted in the formation of other minor peaks, suggesting conversion to other forms. It may also be seen from Fig. 3b that the conversion of peptide C to D proceeded through an intermediate, C′.

It was also observed that trace amounts (1–3%) of peptides B and D were formed from peptides A and C, respectively, during the processing steps involving drying of the peptide samples in a Speedvac to remove the acetonitrile and TFA. Furthermore, it was also found that solutions of both peptides A and C in either 50 mM ammonium bicarbonate or 0.1 M acetic acid very gradually converted to peptides B and D, respectively, during storage even at −20°. About 15% conversion was observed in 7 months in samples stored in 0.1 M acetic acid, and a slightly lower conversion in samples stored in ammonium bicarbonate. The presence of an intermediate in the stored samples of peptide C was also observed.

```
Peptide A   Gln Lys Asn Gln Glu Gln Leu Glu Arg
Peptide C   Gln Lys Asn Leu Glu Lys Leu Glu His Gln Ser Gln
            Val Ala Ala Asp Lys His Tyr Gln Glu Gln Ala Lys
            Lys His Gln Glu Tyr Lys Gln Glu Gln Glu Glu Arg
```

FIGURE 2

Amino acid sequence of peptides A and C. Identities between the two sequences are underlined.

Mass spectral analysis

Mass spectrometry is a high resolution technique which allows for the precise determination of molecular masses. Recently, this technique has been found to be highly useful in characterizing the modifications such as *N*-acetyl and pyroglutamic acid at the amino terminus of the *N*-blocked peptides and proteins (10, 13–16). Therefore, to determine the nature of the blocking groups in peptides B and D, these peptides as well as the corresponding parent peptides A and C were subjected to mass spectral analysis. As can be seen from Table 2, the measured mass of peptide A was in good agreement with the calculated mass. The mass of peptide B was less than that of peptide A by 17 mass units, as expected for the loss of ammonia due to cyclization of the amino-terminal glutamine. Similarly, as can be seen from Table 3, the observed masses for peptides C and D corresponded well with the calculated values and the difference in mass between the two peptides corresponded well for the loss of ammonia. These results, therefore, indicated

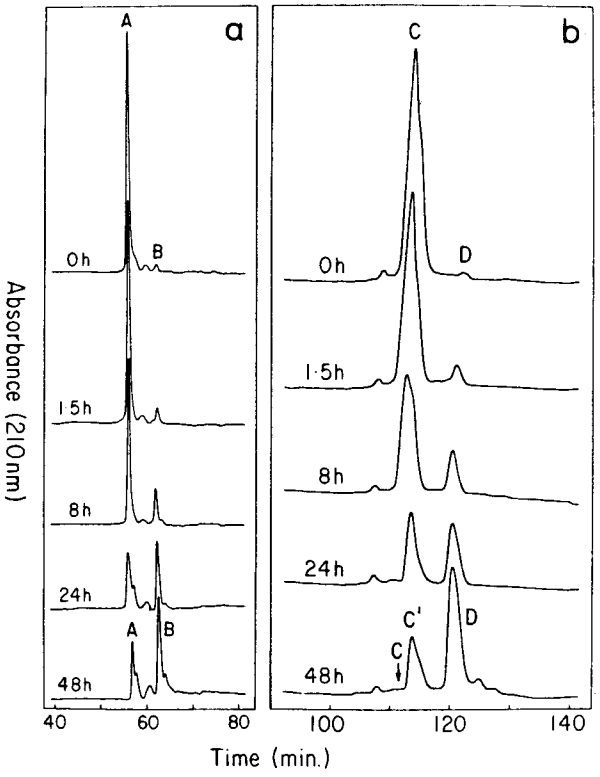

FIGURE 3

a, Formation of peptide B from purified peptide A. **b**, Formation of peptide D from peptide C. Peptides A and C were incubated in 0.2 M ammonium bicarbonate, pH 7.9, for 1 h, 8 h, 24 h, 48 h and their conversion to B and D, respectively was monitored by HPLC. The column was eluted with 0.1% aqueous TFA for the first 28 min followed by a gradient of 0%–30% acetonitrile-0.1% TFA in 180 min. The flow rate was 1.2 mL/min.

TABLE 2

Mass of peptides A and B

Peptide A*			Peptide B*		
Peak	Mass		Peak	Mass	
	Obs.**	Calc.		Obs.**	Calc.
M1 (mono-DHP)	1246.6	1246.4	M1 (mono-DHP)	1229.5	1229.4
M2 (di-DHP)	1320.5	1320.4	M2 (di-DHP)	1303.5	1303.4

*Two most abundant species M1 and M2 were observed for both peptides A and B. Peptide A contains one DHP-lysine; nearly 75% of this is DHP-lysine and the remainder is di-DHP-lysine. The relative intensities of the mass ions observed were in approximate agreement with this proportion of substitution.

**From protonated peaks. Similar values were obtained from the sodium cationized peaks.

TABLE 3

Mass of peptides C and D

Peptide C			Peptide D		
Peak*	Mass		Peak*	Mass	
	Obs.**	Calc.		Obs.**	Calc.
1	5057.8	5057.4	1	5042.2	5040.4
2	5131.2	5131.4	2	5114.8	5114.4
3	5205.1	5205.5	3	5188.7	5188.4

*Peptide C contains 6 lysines, which are dihydroxypropylated to varying extents. Therefore, the masses of peptides C and D were calculated assuming different degrees of dihydroxypropylation. Peaks 1, 2, and 3 represent the peptide ion species containing 8, 9, and 10 dihydroxypropyl substituents, respectively.

**The values shown are averages determined from the masses observed for the singly protonated and doubly protonated species.

that the formation of B and D from A and C, respectively, was due to cyclization of their amino-terminal glutamine residues.

Influence of different ions on the cyclization reaction

In order to study the influence of different ions on the cyclization of the amino-terminal glutamine in peptides A and C, in addition to the 0.2 M ammonium bicarbonate buffer, tryptic digestion of DHP-PepM49 was carried out in two other buffers, 0.01 M sodium phosphate, pH 8.0 and 0.2 M ammonium acetate pH 7.9. The results of a time course tryptic digestion of DHP-PepM49 in the three buffers at 37° revealed that only small amounts of peptides B and D were present during the initial time and their amounts increased progressively with time of enzymic digestion with a concomitant decrease in the amounts of peptides A and C, respectively (Table 4). Furthermore, as can be seen from Table 4, the rate of cyclization of peptides A and C varied widely depending upon the ion present. The conversion of both peptides A and C was significantly accelerated in the 0.01 M phosphate buffer relative to that seen in 0.2 M ammonium bicarbonate buffer. In contrast, acetate ions lowered the rate of conversion markedly for both peptides A and C.

In contrast to peptides A and C, the amounts of

TABLE 4

Influence of various ions on the cyclization of peptides A and C

Time (h)	% Peptide B* (B/A + B × 100)			% Peptide D* (D/C + D × 100)		
	Ammonium acetate (0.2 M) pH 7.9	Ammonium bicarbonate (0.2 M) pH 7.9	Sodium phosphate (0.01 M) pH 8.0	Ammonium acetate (0.2 M) pH 7.9	Ammonium bicarbonate (0.2 M) pH 7.9	Sodium phosphate (0.01 M) pH 8.0
1½	ND	ND	9.8	ND	ND	9.2
4	5.9	11.6	21.5	5.7	11.3	21.1
8	9.7	23.1	ND	9.7	22.8	ND
24	21.0	43.9	61.1	22.2	41.3	61.9

*Time course tryptic digestions of DHP-PepM49 were carried out in the buffers indicated, and the digests subjected to RPHPLC on Waters μ-Bondapak C18 column. The amounts of peptides B and D present at different periods of enzymic digestion, relative to peptides A and C, respectively, are indicated. ND: Not determined.

other peaks were virtually the same in the initial and the later digests, indicating that the enzymic digestion was essentially complete within the first few hours of incubation. Thus, the cyclization reaction could be minimized without affecting the enzymic digestion. These results suggested that peptides B and D are the consequence of the secondary modification of peptides A and C that are generated during the enzymic digestion and are not due to the presence of post-translational modifications of the protein.

Influence of temperature on the cyclization reaction

In order to determine if the cyclization reaction could be minimized at lower temperatures without affecting the completeness of the enzymic digestion, tryptic digestion of DHP-PepM49 in the above three buffers was also carried out at 0°. The results of these experiments (data not shown) revealed that in all the three buffers, by 24 h, the enzymic digestion was complete whereas peptides B and D were virtually absent in these digests. Thus, cyclization reaction could indeed be prevented at 0°, while at the same time achieving complete tryptic digestion.

DISCUSSION

Pyroglutamic acid has been found at the amino-terminus of several proteins and natural peptides (16, 17), and is believed to be a result of post-translational modification of glutamine. Analysis of the structural genes for several of these proteins (18–21) has suggested that glutamine and not glutamate is the amino acid antecedent of the *N*-terminal pyroglutamic acid. In view of the amino acid sequences of the larger precursors of these proteins/peptides, the post-translational pyroglutamyl formation has been suggested to involve proteolytically generated glutaminyl peptides as conversion intermediates (22). Both nonenzymic (23) and enzymic (15, 22) conversions have been reported. Studies with model peptides have demonstrated that internal glutamic acid residues can convert into internal pyroglutamic acid residues (24). It was suggested that some of the observed pyroglutamic acid residues in the *N*-blocked proteins/peptides might also arise by regioselective hydrolysis with peptide chain fragmentation of a precursor polypeptide containing an internal pyroglutamic acid residue.

The results of the present study demonstrate that two of the arginyl peptides of PepM49 having amino-terminal glutamine readily undergo cyclization at their amino termini, during the course of tryptic digestion, to yield pyroglutamyl peptides. Since the cyclized peptides were absent during the early stages of the digestion, internal pyroglutamyl residues do not appear to be present in the PepM49 protein. The formation of the cyclized peptides during the course of enzymic digestion could be readily monitored by RPHPLC because of their significantly higher retention time relative to their uncyclized counterparts.

Formation of ninhydrin negative, amino-terminal blocked peptides has been observed previously during enzymic digestion of ribonuclease A and insulin A chain (25–28). This was attributed to the formation of pyroglutamic acid by cyclization of amino-terminal glutamine (12, 17), either during enzymic digestion or during the storage of the digests at low pH prior to their fractionation by ion-exchange chromatography. However, the conditions that would limit this conversion were not investigated.

In the present study, the formation of the pyroglutamyl peptides of PepM49 was found to be highly dependent on the reaction conditions such as the buffer, temperature and the time of incubation. In both 0.2 M ammonium bicarbonate and 0.2 M ammonium acetate buffers at 37°, the cyclized peptides were present only in trace amounts during the initial time when the enzymic digestion was essentially complete, and accumulated only during subsequent incubation. Cyclization was also virtually absent at 0° even after 24 h of enzymic reaction, by which time the digestion was essentially complete. Sodium phosphate buffer accelerated the conversion relative to the ammonium bicarbonate and ammonium acetate buffers. The relative rates of conversion in the three buffers tested was, 0.2 M ammonium acetate $<$ 0.2 M ammonium bicarbonate $<$ 0.01 M sodium phosphate.

Together, these results suggest ammonium acetate to be a suitable buffer for limiting the cyclization of amino-terminal glutamine residues in enzymic digests of proteins. The cyclization reaction can be minimized either by a brief digestion at 37° or an extended digestion at 0°. These observations could also have implications for the isolation and processing of other proteins and peptides that are known to contain amino terminal glutaminyl residues.

ACKNOWLEDGMENTS

We wish to thank Drs. Emil C. Gotschlich and Vincent A. Fischetti for their comments on the manuscript and Drs. A. Seetharama Acharya and Sabir Khan for valuable discussions. This work was supported by the USPHS grant HL36025 to B.N.M. The mass spectrometric measurements were made by the Rockefeller University Mass Spectrometric Biotechnology Research Resource supported by the Division of Research Resources, National Institutes of Health (Grant RR00862-15). We thank Ms. Tanuja Chaudhary for her skillful technical assistance in the mass spectrometric measurements.

REFERENCES

1. Manjula, B.N., Acharya, A.S., Mische, S.M., Fairwell, T. & Fischetti, V.A. (1984) *J. Biol. Chem.* **259**, 3686–3693
2. Hollingshead, S.K., Fischetti, V.A. & Scott, J.R. (1986) *J. Biol. Chem.* **261**, 1677–1686

3. Mouw, A.R., Beachey, E.H. & Burdett, V. (1988) *J. Bacteriol.* **170**, 676–684
4. Miller, L., Gray, L., Beachey, E. & Kehoe, M. (1988) *J. Biol. Chem.* **263**, 5668–5673
5. Khandke, K.M., Fairwell, T. & Manjula, B.N. (1987) *J. Exp. Med.* **166**, 151–162
6. Manjula, B.N., Acharya, A.S., Fairwell, T. & Fischetti, V.A. (1986) *J. Exp. Med.* **163**, 129–138
7. Acharya, A.S., Sussman, L.G. & Manjula, B.N. (1984) *J. Chromatography* **297**, 37–48
8. Manjula, B.N. & Fischetti, V.A. (1980) *J. Immunol.* **124**, 261–267
9. Chait, B.T., Agosta, W.C. & Field, F.H. (1981) *Int. J. Mass Spectrom. Ion Phys.* **39**, 339–366
10. Chait, B.T., Gisin, B.F. & Field, F.H. (1982) *J. Am. Chem. Soc.* **104**, 5157–5162
11. Chait, B.T. & Field, F.H. (1986) *Biochem. Biophys. Res. Commun.* **134**, 420–426
12. Greenstein, J.P. & Winitz, M. (1961) *Chemistry of the Amino Acids*, Wiley & Sons, New York
13. Gibson, B.W., Daley, D.J. & Williams, D.H. (1988) *Anal. Biochem.* **169**, 217–226
14. Gibson, B.W., Yu, Z., Aberth, W., Burlingame, A.L. & Bass, N.M. (1988) *J. Biol. Chem.* **263**, 4182–4185
15. Bubsy, W.J. Jr., Quackenbush, G.E., Humm, J., Youngblood, W.W. & Kizer, J.S. (1987) *J. Biol. Chem.* **262**, 8532–8536
16. Gerber, G.E., Anderegg, R., Herlihy, W.C., Gray, C.P., Biemann, K. & Khorana, H.G. (1979) *Proc. Natl. Acad. Sci. US* **76**, 227–231
17. Blomback, B. (1967) *Methods Enzymol.* **11**, 398–411
18. Richter, K., Kawashima, E., Egger, R. & Kreil, G. (1984) *EMBO J.* **3**, 617–621
19. Lechan, R.M., Wu, P., Jackson, I.M.D., Wolf, H., Cooperman, S., Mandel, G. & Goodman, R.H. (1986) *Science* **231**, 159–161
20. Seeburg, P.H. & Adelman, J.P. (1984) *Nature* **311**, 666–668
21. Schechter, I., Wolf, O., Kantor, F. & Schechter, B. (1980) *Ann. N.Y. Acad. Sci.* **343**, 218–227
22. Fischer, W.H. & Spiess, J. (1987) *Proc. Natl. Acad. Sci. US* **84**, 3628–3632
23. Jones, K.F., Manjula, B.N., Johnston, K.H., Hollingshead, S.K., Scott, J.R. & Fischetti, V.A. (1985) *J. Exp. Med.* **161**, 623–628
24. Khan, S.A. & Erickson, B.W. (1984) *J. Am. Chem. Soc.* **106**, 798–799
25. Smyth, D.G., Stein, W.H. & Moore, S. (1962) *J. Biol. Chem.* **237**, 1845–1850
26. Hirs, C.H.W., Stein, W.H. & Moore, S. (1956) *J. Biol. Chem.* **221**, 151–169
27. McWherter, C.A., Thannhauser, T.W., Fredrickson, R.A., Zagotta, M.T. & Scheraga, H.A. (1984) *Anal. Biochem.* **141**, 523–537
28. Sanger, P., Thompson, E.O.P. & Kitai, R. (1955) *Biochem. J.* **59**, 509–518
29. Khandke, K.M., Fairwell, T., Acharya, A.S., Trus, B.L. & Manjula, B.N. (1988) *J. Biol. Chem.* **263**, 5075–5082

Address:

Belur N. Manjula
The Rockefeller University
1230 York Avenue
New York, NY 10021
USA