Ross, P. D., & Shapiro, J. T. (1974) *Biopolymers 13*, 415–416.
Scruggs, R. L., Achter, E. K., & Ross, P. D. (1972) *Biopolymers 11*, 1961–1972.
Shapiro, J. T., Leng, M., & Felsenfeld, G. (1969) *Biochemistry 8*, 3219–3232.
Shiffman, M. L., Mciewicz, R. A., Hu, A. W., Howard, J. C., & Li, H. J. (1978) *Nucleic Acids Res. 5*, 3409–3426.
Sipski, M. L., & Wagner, T. E. (1977) *Biopolymers 16*, 573–590.
Stryer, L. (1968) *Science 162*, 526–533.
Thomas, G., Lange, H., & Hempel, K. (1975) *Eur. J. Biochem. 51*, 609–615.
Tidwell, T., Allfrey, V. G., & Mirsky, A. E. (1968) *J. Biol. Chem. 243*, 707–715.
Wehling, K., Krauss, S., & Wagner, K. G. (1976) *Nucleic Acids Res. 3*, 149–164.
Wilson, W. W., & Bloomfield, V. A. (1979) *Biochemistry 18*, 2192–2196.

Solid-Phase Synthesis of Thymosin α_1 Using *tert*-Butyloxycarbonylaminoacyl-4-(oxymethyl)phenylacetamidomethyl-resin†

Tai Wai Wong and R. B. Merrifield*

ABSTRACT: Thymosin α_1 and its desacetyl analogue were synthesized by the solid-phase method. Use of aminoacyl-4-(oxymethyl)phenylacetamidomethyl-resin resulted in an improved yield and allowed the synthetic products to be purified by simple ion-exchange and gel filtration chromatography. Success of the synthesis was largely due to enhanced stability of the peptide-resin linkage to trifluoroacetic acid and to the elimination of hydroxy functions on the resin. This improved quality of the solid support helps eliminate chain loss and chain termination during the synthesis. The purified synthetic peptides were found to be homogeneous by paper electrophoresis, isoelectric focusing in polyacrylamide gel, and thin-layer chromatography. They also had biological activity in the azathioprine-sensitive rosette assay. Use of the new 9-(2-sulfo)fluorenylmethyloxycarbonyl chloride reagent for purification of protected peptides was also demonstrated and discussed.

In the past few years, there have been various reports of the isolation of a family of polypeptides that are believed to play an important role in the function of the thymus gland in the immune system (Friedman, 1975; Bach, 1977). Many of these thymic factors have been suggested to be involved in a hormonal mechanism in which the thymus affects T cell development. A few of these polypeptides have been chemically characterized, sequenced, and synthesized (Bach, 1977). Thymosin α_1, which was isolated and sequenced by Goldstein et al. (1977), was shown to be an acidic peptide whose sequence is as shown in Figure 1. The octaeicosapeptide was shown to be 10–1000 times as active as thymosin fraction 5, from which it was isolated, in vivo and in vitro (Low et al., 1979).

The chemical synthesis of thymosin α_1 has been achieved by both solution- and solid-phase methods (Wang et al., 1979, 1980; Birr & Stollenwerk, 1979). We now report a greatly improved solid-phase synthesis of thymosin α_1. The key to the new synthetic strategy was the use of aminoacyl-4-(oxymethyl)phenylacetamidomethyl-resin as the solid support (Scheme I). The ester linkage between the peptide and this oxymethyl-Pam-resin[1] was previously shown to have enhanced acid stability compared to the usual benzyl ester resin and to prevent loss of peptide chains during the synthesis (Mitchell et al., 1976a). Use of this polymeric support was also shown recently to help avoid trifluoroacetylation (Kent et al., 1979), which has been a major terminating side reaction in solid-phase peptide synthesis. The new synthesis of thymosin α_1 on Pam-resin resulted in an improved yield, and the purified synthetic product has been shown to be homogeneous in a number of chromatographic and electrophoretic systems. The desacetyl analogue of thymosin α_1 was also synthesized and purified (Scheme I), and both peptides were tested for biological activity in the rosette inhibition assay.

Experimental Procedures

Materials

All chemicals were reagent grade. Dichloromethane was distilled from sodium carbonate and DIEA was distilled from sodium hydride. Protected amino acids were purchased from Chemical Dynamics and Vega Biochemicals and were checked for purity by TLC. Unsubstituted resin was copoly(styrene–1% divinylbenzene) beads, 200–400 mesh, from Bio-Rad. Fluorescamine (Fluram) was obtained from Hoffmann-La Roche, [^{3}H]acetic anhydride (50 mCi/mmol) was from New England Nuclear, and PTH-amino acid standards were purchased from Pierce Chemical Co.

Methods

Peptide Synthesis. N^{α}-Boc-amino acids were used in the synthesis. Trifunctional amino acids were protected as N^{α}-Boc-Lys(Tfa), N^{α}-Boc-Thr(Bzl), N^{α}-Boc-Ser(Bzl), N^{α}-Boc-Asp(OBzl), and N^{α}-Boc-Glu(OBzl).

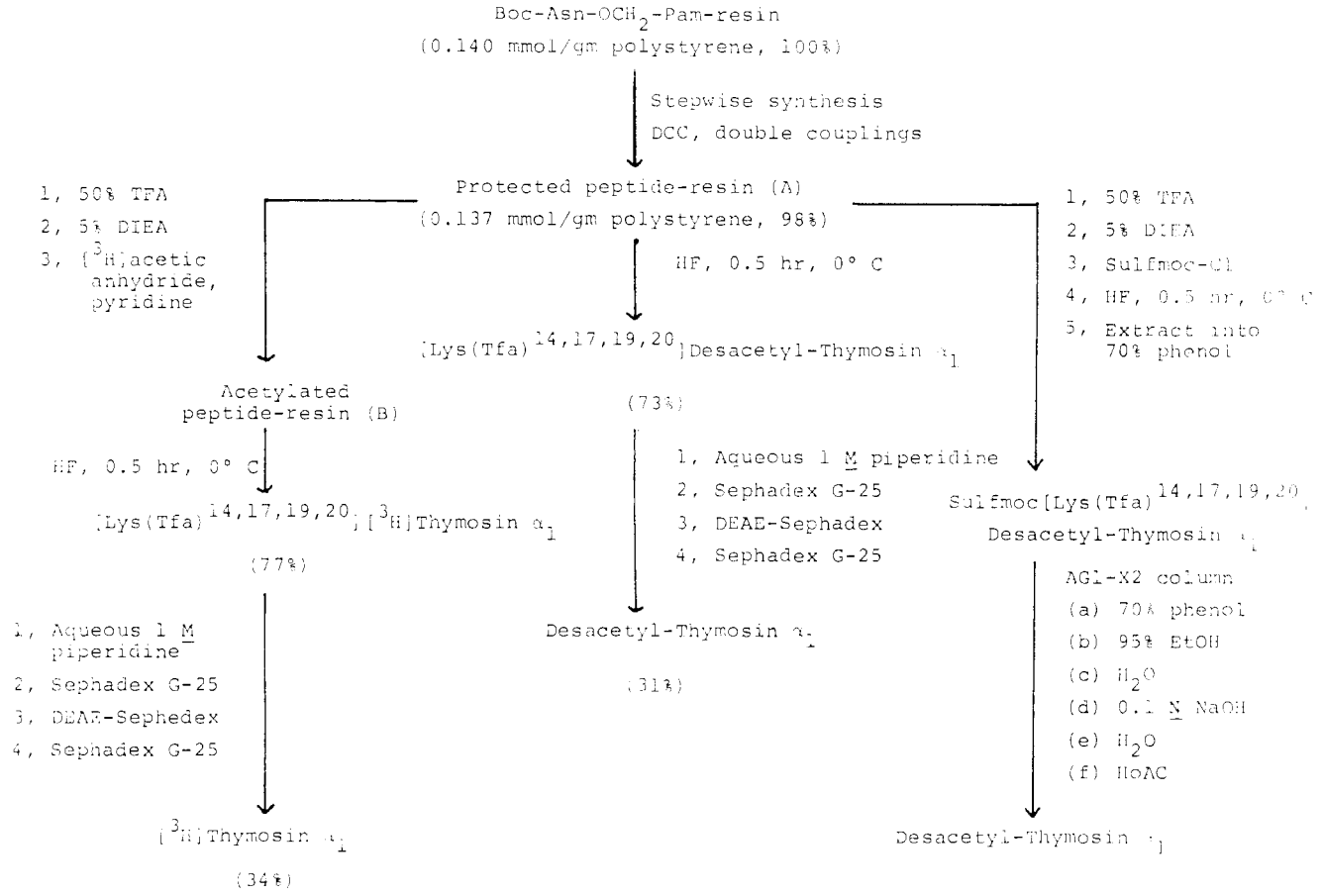
The syntheses of Boc-Asn-4-(oxymethyl)phenylacetic acid and of aminomethyl-resin have been described elsewhere (Tam

† From The Rockefeller University, New York, New York 10021. *Received February 6, 1980.* This work was supported by National Institutes of Health Grant AM01260 and by a grant from the Hoffmann-La Roche Foundation.

[1] Abbreviations used: Pam, phenylacetamidomethyl; TLC, thin-layer chromatography; PTH, phenylthiohydantoin; Boc, *tert*-butyloxycarbonyl; Tfa, trifluoroacetyl; Bzl, benzyl; DCC, dicyclohexylcarbodiimide; Sulfmoc, 9-(2-sulfo)fluorenylmethyloxycarbonyl; CF_3COOH, trifluoroacetic acid; DIEA, *N,N*-diisopropylethylamine; high-pressure LC, high-pressure liquid chromatography.

0006-2960/80/0419-3233$01.00/0

Scheme I



Ac-Ser-Asp-Ala-Ala-Val-Asp-Thr-
Ser-Ser-Glu-Ile-Thr-Thr-Lys-
Asp-Leu-Lys-Glu-Lys-Lys-Glu-
Val-Val-Glu-Glu-Ala-Glu-Asn-OH

FIGURE 1: Amino acid sequence of bovine thymosin α_1.

et al., 1979; Mitchell et al., 1976b, 1978). Boc-Asn-4-(oxymethyl)-Pam resin was prepared by coupling Boc-Asn-4-(oxymethyl)phenylacetic acid (0.492 g, 1.30 mmol) to aminomethyl-resin (3.2 g, 0.15 mmol/g) in the presence of DCC (0.269 g, 1.30 mmol) in a 60-mL mixture of DMF–CH_2Cl_2 (1:2 v/v) for 21 h. The resin was washed with CH_2Cl_2 and treated with acetic anhydride–pyridine (1:1 v/v) for 30 min to acetylate any remaining amino groups. HF cleavage of the dried resin and amino acid analysis indicated a substitution of 0.140 mmol/g polystyrene. Amino acid analysis of resin hydrolysate (12 N HCl–phenol–acetic acid, 2:1:1 v/v; 24 h; 110 °C) also gave the same result for resin substitution.

Peptide synthesis was done with 3 g of substituted resin in a stepwise fashion with double couplings of each amino acid. All solvents were used in a ratio of 10–20 mL/g. The protocol for a typical cycle included (1) 50% CF_3COOH–CH_2Cl_2 for 1 min, (2) 50% CF_3COOH–CH_2Cl_2 for 30 min, (3) CH_2Cl_2 4 times for 1 min, (4) 5% DIEA–CH_2Cl_2 for 2 min, (5) CH_2Cl_2 for 1 min, (6) repeat steps 4 and 5, (7) 2-propanol twice for 1 min, (8) CH_2Cl_2 6 times for 1 min, (9) 3 equiv of Boc-amino acids–CH_2Cl_2 for 5 min, followed by 3 equiv of DCC for 60 min, (10) CH_2Cl_2 once for 2 min, (11) repeat step 9, and (12) CH_2Cl_2 6 times for 2 min.

Coupling efficiency was monitored by the fluorescamine method (Felix & Jimenez, 1973) and was judged to be >99% after two couplings for all residues monitored except for residues 14 and 22, which were negative only after three couplings, and for residue 17, which remained slightly positive even after a third coupling.

A sample of the peptide-resin (A) was cleaved with HF in the absence of anisole to yield lysine-protected desacetyl-thymosin α_1, and another sample was acetylated as follows. After the last Ser residue had been incorporated, the Boc group was removed with 50% CF_3COOH–CH_2Cl_2 and the peptide-resin was neutralized with DIEA. The amino terminus was then acetylated by treating the peptide-resin with an excess of a 1:1 mixture of pyridine and [^{3}H]acetic anhydride (3.12 mCi/mmol) in CH_2Cl_2 for 1 h. The acetylated peptide-resin (B) was then washed with CH_2Cl_2 and dried in vacuo.

HF Cleavage and Deprotection of Peptides. Acetylated peptide-resin (B) (508 mg) was treated with 10 mL of anhydrous hydrogen fluoride without anisole at 0 °C. At the end of 30 min, the hydrogen fluoride was evaporated at 0 °C, and the resin was extracted 3 times with CF_3COOH. The trifluoroacetic acid was immediately removed by evaporation with N_2. The residue was suspended in water and lyophilized to give 129 mg of [Lys(Tfa)^{14,17,19,20}][^{3}H]thymosin α_1 (77% yield by amino acid analysis). Peptide-resin (A) was also cleaved with HF to give [Lys(Tfa)^{14,17,19,20}]desacetyl-thymosin α_1 (73% yield).

The trifluoroacetyl groups on the side chains of the four lysines were removed by treatment of either protected peptide with aqueous 1 M piperidine (10 mg of protected peptide per mL). At the end of 1 h, the reaction mixture was neutralized with acetic acid, diluted with water, and lyophilized.

Purification of Synthetic Peptides. Part of the crude thymosin α_1 (57.5 mg) was passed through a Sephadex G-25 column (2.6 × 70 cm) equilibrated in 5% acetic acid. The peptide was eluted with 5% acetic acid and lyophilized. This was followed by chromatography on a DEAE-Sephadex G-25

column (1 × 15 cm) equilibrated in pH 7.4 phosphate buffer (9.6 mM KH_2PO_4 plus 29.7 mM K_2HPO_4), and the peptide was eluted with a 0.4 M NaCl gradient in the phosphate buffer. Eluted fractions were monitored by radioactivity measurement and/or measurement of absorbance at 570 nm after ninhydrin reaction of aliquots of samples (Stewart & Young, 1969). The peptide from the main peak was lyophilized and desalted on a Sephadex G-25 column in 5% acetic acid. The eluted fractions were pooled and lyophilized to give 22.4 mg of thymosin α_1 (34% overall isolated yield based on C-terminal Asn). Desacetyl-thymosin α_1 was purified by the same procedure in 31% overall isolated yield.

Affinity Purification of Desacetyl-peptide. The N terminus of peptide-resin (A) was deprotected with 50% CF_3COOH–CH_2Cl_2, neutralized with 5% DIEA–CH_2Cl_2, and then derivatized with 9-(2-sulfo)fluorenylmethyloxycarbonyl chloride as previously described (Merrifield & Bach, 1978) to yield Sulfmoc-peptide-resin. After HF cleavage from the resin, the crude Sulfmoc-[Lys(Tfa)^{14,17,19,20}]desacetyl-thymosin α_1 (30 mg) was extracted into 70% phenol and applied to an AG1-X2 column (1 × 8.5 cm) (Bio-Rad) which had been equilibrated in 70% phenol. The column was then washed with the following solvents: (a) 45 mL of 70% phenol; (b) 100 mL of 95% ethanol; (c) 150 mL of H_2O; (d) 40 mL of 0.1 N NaOH; (e) 75 mL of H_2O. Finally, the deprotected desacetyl-peptide was eluted as one major peak with a linear gradient of 0–50% acetic acid. Fractions collected were monitored for peptides by the ninhydrin method as described under Purification of Synthetic Peptides.

Tryptic Digestion. Synthetic thymosin α_1 (1 mg) purified as described above was digested with 60 μg of TPCK–trypsin (Worthington) in 0.5 mL of 0.5 M pH 8.0 ammonium bicarbonate for 20 h at 25 °C. The reaction was stopped by addition of a few drops of glacial acetic acid and the mixture was lyophilized. The residue was dissolved in water and subjected to electrophoresis on cellulose TLC plates (MN 300; Analtech) in acetic acid–formic acid–water buffer (10:15:75) at pH 1.2. This was followed by TLC in the second dimension in butanol–pyridine–acetic acid–water (65:50:10:40). The plate was air-dried and sprayed with chlorine–tolidine. A similar tryptic map was also obtained with a sample of thymosin α_1 synthesized and characterized as described previously (Wang et al., 1980).

Electrophoresis and Isoelectric Focusing. Synthetic thymosin α_1 was examined by paper electrophoresis on Whatman No. 1 paper in 0.2 M pyridine acetate buffer at pH 5.0, and the peptide was visualized with ninhydrin. The synthetic peptide was also subjected to isoelectric focusing in polyacrylamide gel according to Scheele (1975). The focused gel was fixed in a solution containing 10% trichloroacetic acid, 3.5% sulfosalicylic acid, and 30% methanol for 2 h. It was then treated with a solution of 25% ethanol and 8% acetic acid for 10 min. Staining was done overnight in the same solution containing 0.1% Coomassie Blue R-250 (Bio-Rad).

Solid-Phase Sequencing. Sequence and preview analyses of the synthetic desacetyl-peptide were performed on a Sequemat Mini-15 Solid Phase sequencer coupled to a Sequemat P-6 Auto converter (Sequemat Inc., Watertown, MA). This was done while the peptide was still fully protected and attached to the resin. The reaction column was packed with silanized glass beads and 25 mg of protected peptide-resin. A single column program was started by advancing to CF_3CO-OH treatment in order to remove the Boc group. A total of 29 cycles of degradation were carried out and the PTH-amino acids were collected in fractions. The PTH-amino acids were identified by TLC on silica GF plates (Analtech) by using the solvent system chloroform–methanol (95:5 v/v) and were visualized under short-wavelength UV. Those of the aliphatic amino acids were also quantitated by high-pressure liquid chromatography according to Bhown et al. (1978).

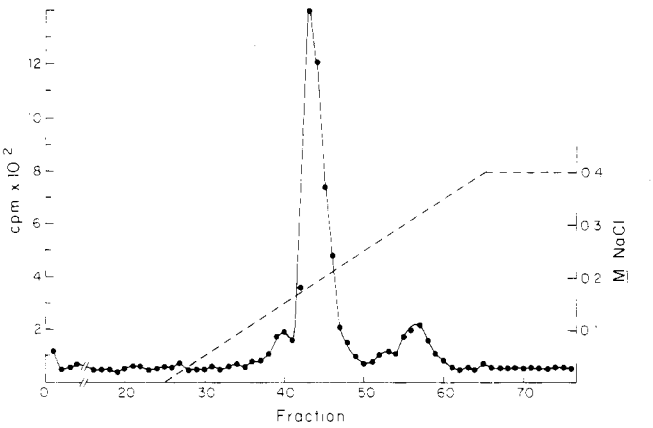


FIGURE 2: Ion-exchange chromatography of synthetic thymosin α_1. Crude peptide after desalting on Sephadex G-25 was applied to a DEAE-Sephadex G-25 column (1 × 15 cm) equilibrated in pH 7.4 phosphate buffer. The flow rate was 10 mL/h and 3-mL fractions were collected. Peptide was eluted with a 0.4 M NaCl linear gradient in phosphate buffer. Aliquots (10 μL) were removed for scintillation counting, and fractions 41–48 were pooled and lyophilized.

Bioassay. Spleen cells from normal mice form rosettes when incubated with sheep erythrocytes, and the rosette formation can be suppressed with the immunosuppressive drug, azathioprine. Rosette-forming cells from adult thymectomized animals lose their sensitivity to azathioprine which can be restored by incubation with certain thymic extracts (Dardenne & Bach, 1975). Synthetic thymosin α_1 and desacetyl-thymosin α_1 were tested for ability to restore azathioprine sensitivity in rosette formation by spleen cells of adult thymectomized mice.

All assays were performed by Drs. T. Iwata and G. Incefy of the Memorial Sloan-Kettering Cancer Center as described by Dardenne & Bach (1975). Spleen cells (2 × 10^6) from adult C67BL/6 mice (Cumberland View Farm, Clinton, TN) which had been thymectomized 2 weeks prior to the experiment were incubated at 37 °C with azathioprine (final concentration 7 μg/mL) in a total volume of 110 μL of Hanks' balanced salt medium. After 10 min of preincubation, 60 μL of synthetic peptide solutions at various concentrations was added, and the mixtures were incubated for 75 min. This was followed by the addition of 60 μL of 1% sheep erythrocytes in Hanks' medium, and the cell suspension was centrifuged at 150*g* at 4 °C for 5 min. The cells were then resuspended and the E-rosette formation was evaluated on a hemocytometer. The active dilution of peptide was defined as the minimum concentration which gave greater than 50% inhibition of rosette formation.

Results

Purification and Characterization of Synthetic Peptides. The experimental design and procedures for obtaining purified thymosin α_1 and desacetyl-thymosin α_1 are outlined in Scheme I. The crude thymosin α_1, after HF cleavage and removal of Tfa groups, consisted of only one peak in gel filtration, showing the absence of detectable levels of short chains. Paper electrophoresis, however, detected two or three other minor components (data not shown). In addition, Figure 2 shows that, in ion-exchange chromatography, the peptide consisted of one major peak which eluted at 0.19 M NaCl and could be separated from two other minor peaks. The final product obtained

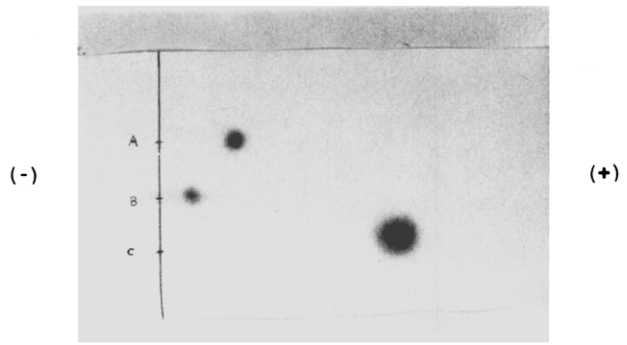


FIGURE 3: Paper electrophoresis of purified peptides. Purified thymosin α_1 (A) and desacetyl-thymosin α_1 (B), 120 μg each, were electrophoresed on Whatman No. 1 paper in 0.2 M pyridine acetate buffer at pH 5.0. Electrophoresis was for 2 h at 1000 V and glutamic acid (C) was used as a standard. The paper was oven-dried and sprayed with ninhydrin.

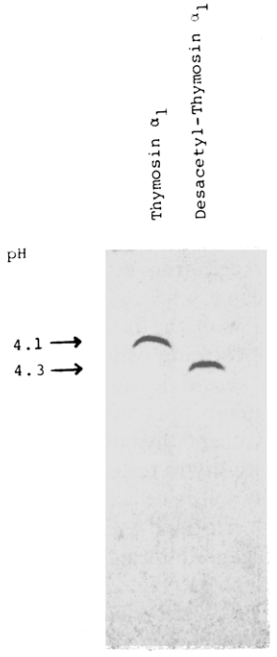


FIGURE 4: Isoelectric focusing of purified peptides. Purified thymosin α_1 and desacetyl-thymosin α_1 (200 μg each) were focused in 5% polyacrylamide gel at 4 °C by using pH 3.5–10 Ampholines (LKB). The gel was focused at 300 V for 1 h and then at 400 V for 3 h. One lane of the gel was cut into 5-mm slices and eluted overnight with deionized water at 4 °C, and the pH of the Ampholines was measured with a microelectrode. The gel was stained overnight with 0.1% Coomassie Blue R-250.

after these chromatographic procedures was shown to be homogeneous by the criteria of paper electrophoresis (R_{Glu} = 0.31 at pH 5.0; Figure 3), isoelectric focusing in polyacrylamide gel (pI = 4.1; Figure 4), and TLC in the following systems: (a) butanol–pyridine–acetic acid–water (30:50:10:40) and (b) butanol–ethyl acetate–acetic acid–water (1:1:2:2); R^a = 0.68 and R^b = 0.45.

Purified thymosin α_1 was hydrolyzed in 6 N HCl at 110 °C for 24, 48, and 72 h, and the results were extrapolated to 0 h for Ser and Thr to give the expected amino acid analysis (numbers in parentheses are theoretical values): Lys = 3.87 (4); Asp = 3.92 (4); Glu = 6.20 (6); Ala = 2.93 (3); Val = 2.77 (3); Leu = 1.00 (1); Ile = 0.93 (1); Ser = 2.90 (3); Thr = 2.70 (3). Tryptic maps of the two samples of synthetic thymosin α_1 are shown in Figure 5. The number and positions of products are consistent with the structure of the peptide, and the two maps are essentially identical.

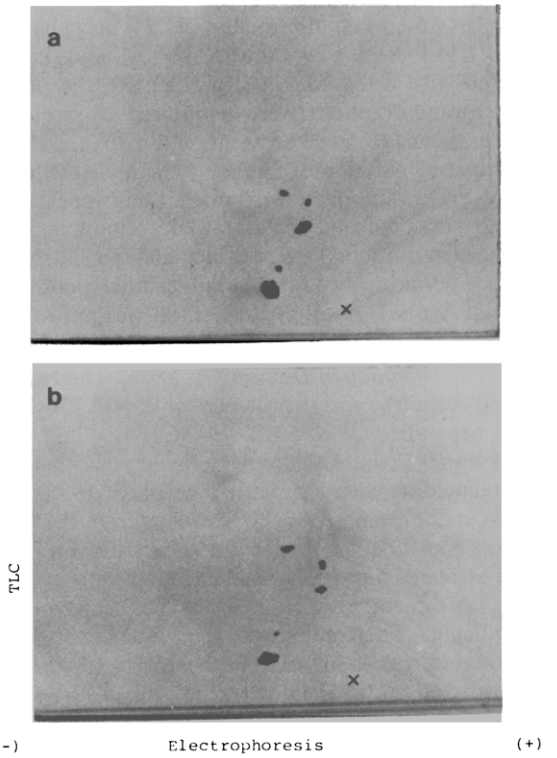


FIGURE 5: Tryptic maps of synthetic thymosin α_1. Tryptic digests were spotted 7 cm from one end (×) of the TLC plate (20 × 20 cm), and electrophoresis was carried out in the first dimension in formic acid–acetic acid–water (10:15:75) buffer at pH 1.2. Electrophoresis was for 25 min at 450 V. The TLC plate was air-dried, and thin-layer chromatography was carried out in the second dimension in butanol–pyridine–acetic acid–water (65:50:10:40). The plate was then dried and sprayed with chlorine–tolidine. Sample a was thymosin α_1 synthesized by using Pam-resin, and sample b was thymosin α_1 synthesized by using benzhydrylamine-resin (Wang et al., 1980).

Desacetyl-thymosin α_1 was purified by similar procedures and eluted as a major peak at 0.15 M NaCl from a DEAE-Sephadex column. The purified preparation was also homogeneous by paper electrophoresis (R_{Glu} = 0.14 at pH 5.0, Figure 3), isoelectric focusing (pI = 4.3, Figure 4), and TLC (R^a = 0.68 and R^b = 0.34). Hydrolysis gave the following amino acid analysis: Lys = 4.21 (4); Asp = 3.75 (4); Glu = 6.29 (6); Ala = 2.72 (3); Val = 2.73 (3); Leu = 1.00 (1); Ile = 0.92 (1); Ser = 2.75 (3); Thr = 2.55 (3).

Desacetyl-peptide obtained after chromatography and deprotection of crude Sulfmoc[Lys(Tfa)[14,17,19,20]]desacetyl-thymosin α_1 was examined by paper electrophoresis. Results indicated that, in addition to a major spot corresponding to desacetyl-thymosin α_1, there were also detected three minor side products with mobilities different from that of thymosin α_1 or desacetyl-thymosin α_1. The electrophoretic pattern corresponded closely to that of crude material before Sulfmoc purification.

Solid-Phase Sequencing of Synthetic Peptide. Phenylthiohydantions of aliphatic amino acids from the unpurified synthetic peptide-resin were compared with standard PTH's by TLC, and the results indicated that those residues were correctly incorporated. By use of quantitative high-pressure LC, it was found that total previews at residues 15, 21, and 25 were 4.5, 14.8, and 15.4%, respectively. This indicates an average of only 0.3% deletion per step for the first 15 synthetic cycles, followed by an abrupt increase at one or more steps between residues 15 and 21. Fluorescamine monitoring data suggested that the large deletion may have occurred at Lys-(Tfa)[17].

Bioassay. Thymosin α_1 and desacetyl-thymosin α_1 synthesized on Pam-resin were compared with thymosin α_1 synthesized on benzhydrylamine resin in the rosette inhibition assay, and all three peptides were found to be active. The last peptide had been shown earlier to have activity equal to that of natural thymosin α_1 (Wang et al., 1980). The minimum concentration required for restoration of azathioprine sensitivity was found to be between 10^{-7} and 10^{-8} M for all three compounds. Within the precision of this assay, the three peptides were essentially equally active.

Discussion

Thymosin α_1 was previously synthesized by the solid-phase method using a benzhydrylamine resin for attachment through the side chain of Asn (Wang et al., 1980). The product was obtained in 6.5% yield and was shown to be chemically and biologically indistinguishable from natural thymosin α_1 and is thus a reasonable standard for comparison in these studies. Acid hydrolysis and amino acid analysis indicated that 98% of the peptide chains remained on the resin at the end of the synthesis and that over 30% of the peptide was recovered in purified form after ion-exchange and gel filtration chromatography. These high recovery yields can be accounted for by the use of Pam-resin and thereby minimizing chain loss in the course of the synthesis.

The trifluoroacetyl group was used to protect lysine side chains with the intention of making use of the new 9-(2-sulfo)fluorenylmethyloxycarbonyl chloride purification procedure (Merrifield & Bach, 1978). The Sulfmoc reagent serves to remove any terminated chains which might have been present in the crude product at the end of the synthesis. In the previous synthesis, a dramatic improvement in the homogeneity of the peptide was achieved by this simple technique (Merrifield et al., 1980). In that synthesis, the lysines were not selectively protected, with the result that the N^{α}-acetyl group could not be specifically introduced and only desacetyl-thymosin α_1 was obtained after the Sulfmoc purification procedure. With the present synthetic design, the use of N^{ϵ}-Tfa protection of Lys would allow the selective acetylation of the amino terminus after HF cleavage. After purification, the Sulfmoc group could be removed with anhydrous morpholine and the N^{α}-acetyl group could be introduced before removal of Tfa groups with aqueous piperidine. Although the Tfa-protected peptide was very insoluble, and introduction of the Sulfmoc group did not render it any less insoluble, the difficulty was overcome by performing the chromatographic purification in 70% phenol (Scheme I) (Merrifield et al., 1980). Both the N^{α}-Sulfmoc and N^{ϵ}-trifluoroacetyl groups were removed by washing the AG1-X2 column with 0.1 N NaOH while the peptide was still on the column. Electrophoretic analysis of the desacetyl-peptide obtained after Sulfmoc purification indicated that the low-level impurities present before purification were not removed by the procedure (data not shown). Since the Sulfmoc method was specifically designed to remove terminated peptides, the observed impurities were interpreted to be deletion or modified peptides. This speculation is also consistent with chemical properties of Pam-resin. Therefore, the main portion of the synthetic desacetyl-peptide was purified directly (without Sulfmoc derivatization) by ion-exchange and gel filtration chromatography. Both the synthetic thymosin α_1 and desacetyl-thymosin α_1 were shown to be homogeneous by the criteria of paper electrophoresis, isoelectric focusing, and thin-layer chromatography.

The synthesis presented in this report serves to illustrate the improvement of the solid-phase synthesis of thymosin α_1 using Pam-resin. The improved yield is attributed to the enhanced stability of the anchoring bond of this support to trifluoroacetic acid and to the elimination of chain terminations. The sequencing data indicated that more then 80% of the chains synthesized were of the desired sequence, and the chromatographic results supported this finding. Results from the Sulfmoc purification serves to demonstrate that this strategy can be used to purify relatively insoluble peptides and that an extension of the scheme would also allow the purification of peptides containing a blocked amino terminus. Since the sequence of thymosin α_1 contains no amino acid which may be susceptible to alkylation, HF cleavage of peptide-resin was done without including anisole as a trap for carbonium ions. This precaution avoided the potentially serious irreversible side reaction involving acylation of anisole by the six glutamic acid residues (Feinberg & Merrifield, 1975).

The synthetic thymosin α_1 and desacetyl-thymosin α_1 were found to be equally active in the azathioprine-sensitive rosette assay, and both peptides had activity equal to that of natural thymosin α_1. While it is necessary to show that the products had biological activity, it must be emphasized that the assay described is very imprecise. For the purpose of assessing the purity of the synthetic products, the data on composition, sequence, and homogeneity are therefore particularly important.

Acknowledgments

The authors thank Drs. R. Good and J. Hadden for arranging the bioassay and Drs. T. Iwata and G. Incefy for performing the assays. We also thank Dr. G. Scheele for his help with the isoelectric focusing.

References

Bach, J.-F. (1977) *Annu. Rev. Pharmacol. Toxicol. 17*, 281.

Bhown, A. S., Mole, J. E., Weissinger, A., & Bennett, J. C. (1978) *J. Chromatogr. 148*, 532.

Birr, C., & Stollenwerk, U. (1979) *Angew. Chem. 18*, 394.

Dardenne, M., & Bach, J.-F. (1975) in *The Biological Action of Thymic Hormones* (van Bekkum, D. K., Ed.) p 235, Kooyker Scientific Publications, Rotterdam.

Feinberg, R. S., & Merrifield, R. B. (1975) *J. Am. Chem. Soc. 97*, 3485.

Felix, A. M., & Jimenez, M. H. (1973) *Anal. Biochem. 52*, 377.

Friedman, H. (1975) *Thymus Factors in Immunity*, New York Academy of Sciences, New York.

Goldstein, A. L., et al. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 725.

Kent, S. B. H., Mitchell, A. R., Englehard, M., & Merrifield, R. B. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2180.

Low, T. L. K., Thurman, G. B., McAdoo, M., McClure, J., Rossio, J. L., Naylor, P. H., & Goldstein, A. L. (1979) *J. Biol. Chem. 254*, 981.

Merrifield, R. B., & Bach, A. E. (1978) *J. Org. Chem. 43*, 4808.

Merrifield, R. B., Kent, S. B. H., Tam, J. P., Tjoeng, F. S., Sarin, V., Mojsov, S., Riemen, M., Wong, T. W., & Voss, C. (1979) in *Peptides* (Gross, E., & Meienhofer, J., Eds.) pp 29–47, Pierce Chemical Co. Publ., Rockford, IL.

Mitchell, A. R., Erickson, B. W., Ryabtsev, M. N., Hodges, R. S., & Merrifield, R. B. (1976a) *J. Am. Chem. Soc. 98*, 7357.

Mitchell, A. R., Kent, S. B. H., Erickson, B. W., & Merrifield, R. B. (1976b) *Tetrahedron Lett. 42*, 3795.

Mitchell, A. R., Kent, S. B. H., Englehard, M., & Merrifield, R. B. (1978) *J. Org. Chem. 43*, 2845.

Scheele, G. A. (1975) *J. Biol. Chem. 250*, 5375.

Stewart, J. M., & Young, J. D. (1969) *Solid Phase Peptide Synthesis*, p 57, W. H. Freeman, San Francisco.
Tam, J. P., Kent, S. B. H., Wong, T. W., & Merrifield, R. B. (1979) *Synthesis*, 955.
Wang, S.-S., Kulesha, I. D., & Winter, D. P. (1979) *J. Am. Chem. Soc. 101*, 253.
Wang, S.-S., Makofske, R., Bach, A. E., & Merrifield, R. B. (1980) *Int. J. Pept. Protein Res. 15*, 1.

Histone 2A, a Heteromorphous Family of Eight Protein Species†

Michael H. P. West and William M. Bonner*

ABSTRACT: The histone 2A family of proteins is shown to consist of eight protein species. In addition to the previously described mammalian 2A variants H2A.1 and H2A.2, we describe two variants which are separable from each other and from variants 1 and 2 on both sodium dodecyl sulfate and acetic acid–urea gels. These two proteins H2A.X and H2A.Z are termed heteromorphous variants to distinguish them from the predominating form and its homeomorphous variants which require nonionic detergents for their resolution. The two heteromorphous variants are present in nucleosomal core particles isolated from mouse L1210 cells. In addition, these variants are found in normal mouse tissues, human HeLa cells, and chicken erythrocytes. On sodium dodecyl sulfate gels, one variant, H2A.X, has an apparent molecular weight ~1000 larger than H2A.1 and comprises ~11% of the total 2A in mouse L1210 cells. The second variant, H2A.Z, has an apparent molecular weight ~600 smaller than H2A.1 and comprises ~4% of the total 2A in mouse L1210 cells. The two heteromorphous variants have the same arginine/lysine ratio as H2A.1. In addition, a fraction of each of the four variants (~11% in L1210 cells) is combined with ubiquitin. The molar sum of these eight H2A species approximately equals the number of moles of H4, H2B, or H3 in chromatin.

The remarkable progress in elucidating chromatin structure and function over the past few years has implicated histones in the primary condensation of deoxyribonucleic acid (DNA) into a nucleosomal structure (Felsenfeld, 1978). This more recent appraisal of the structural role of histones contrasts with earlier notions of histones in gene regulatory roles. However, histones are known to undergo many modifications including acetylation (Dixon et al., 1975), phosphorylation (Dixon et al., 1975), methylation (DeLange et al., 1969, 1973), condensation with ubiquitin (Goldknopf et al., 1978), and poly(adenosine 5'diphosphate) ribosylation (Wong et al., 1977; Giri et al., 1978). These many modifications presumably reflect alterations in chromatin which relate to one or more of its various functions.

Another level of complexity involves the sequences of histones. In vertebrates, histones 3, 2B, and 2A can have nonallelic variants which are related by simple amino acid substitutions (Franklin & Zweidler, 1977). Newrock et al. (1977) have shown that patterns of nonallelic variants may change during embryogenesis. A second type of variation, which includes changes in length due to insertions and deletions, has been reported (von Holt et al., 1979) in plants and invertebrates (Rodrigues et al., 1979). These latter heteromorphous variations are separable on acetic acid–urea or sodium dodecyl sulfate ($NaDodSO_4$) gels.

In some cases heteromorphous variants have been reported in the same organism. Wheat embryo contains heteromorphous H2A variants (Rodrigues et al., 1979), and sea urchin sperm contains heteromorphous H2B variants (Strickland, M., et al., 1977, 1979; Strickland, W. N., et al., 1977). In this paper we report the existence of two heteromorphous variants of H2A in mouse L1210 cells. These two variants, H2A.X and H2A.Z, contain peptides which comigrate with peptides from H2A.1 and H2A.2. In addition, H2A.X and H2A.Z have the same arginine/lysine ratio as variants 1 and 2 and have the same proportion bound to ubiquitin[1] as do variants 1 and 2. H2A.X and Z are also found in HeLa cells and chicken erythrocytes, findings which suggest that the heteromorphous nature of the H2A family may be a general phenomenon.

Materials and Methods

N^{α}-Tosylmethylalanine chloromethyl ketone (TPCK)–trypsin and micrococcal nuclease were obtained from Worthington Biochemicals. Sodium dodecyl sulfate was from BDH chemicals. Agarose (Type 1:low EEO), cysteamine hydrochloride (2-mercaptoethanolamine), Triton N-101, Triton X-100, and hexadecyltrimethylammonium bromide (CTAB) were from Sigma. ^3H- or ^{14}C-labeled amino acids were from New England Nuclear or ICN Pharmaceuticals; $^{32}PO_4$ was from New England Nuclear.

Cell Culture. Mouse L1210 leukemia cells were maintained in Roswell Park Memorial Institute (RPMI) Medium 1630 supplemented with 20% fetal calf serum without antibiotics as a static culture. When required, cells were diluted to 2×10^5 cells/mL in the presence of penicillin and streptomycin and were harvested at approximately 1×10^6 cells/mL after 18–24 h.

Labeling. For fingerprinting, cells were labeled with [^{14}C]arginine at 5 μCi/mL in RPMI 1630 prepared with arginine at 14% of the normal level and supplemented with 20% fetal calf serum. Cells were labeled with [^{35}S]methionine,

† From the Laboratory of Molecular Pharmacology, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205. *Received November 30, 1979.* M.H.P.W. is a Fogarty Fellow.

[1] The ubiquitin–H2A complex was discovered and named A_{24} by Goldknopf et al. (1975) and Olson et al. (1976). In this paper we use the prefix u to denote the ubiquitin (Cook et al., 1979) adduct; hence u2A.1, u2A.2, u2A.X, and u2A.Z are the ubiquitin adducts of 2A.1, 2A.2, 2A.X, and 2A.Z, respectively. Albright et al. (1979) have also reported that the A_{24} of Goldknopf & Busch (1977) is a mixture of u2A.1 and u2A.2.