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Synthesis by an Improved Solid-Phase Method of a Highly Acidic Peptide from Nucleolar Nonhistone Protein C23

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The total synthesis of a 42-amino acid residue peptide corresponding to the proposed sequence of a highly acidic fragment of nucleolar nonhistone protein C23 was accomplished by an improved, mild solid-phase method, employing a *p*-alkoxybenzyl alcohol polystyrene resin. The symmetrical anhydride and active ester coupling were used exclusively. Coupling monitoring and amino acid analyses were carried out during the stepwise synthesis. The synthetic product was purified by gel filtration and ion-exchange chromatography, and found to be homogeneous by seven additional criteria. Phosphorylation of serine residues was attempted enzymatically, and the phosphopeptides obtained had electrophoretic mobilities comparable to that of the natural product.

INTRODUCTION

The emerging biochemical studies on phosphorylation of nuclear and nucleolar acidic proteins are of increasing importance in the elucidation of cell biogenesis process. The nucleolus contains numerous phosphorylated proteins, some of which may be involved in the regulation of nucleolar physiology. The nucleolus also appears to possess the necessary kinases for phosphorylation of most of these phosphoproteins (*1*) and phosphatases for their dephosphorylation (*2*).

Recently Olson *et al.* (*3*) found that nonhistone protein C23 is the major protein phosphorylated by isolated nucleolar protein kinase. Previously, this protein and an additional nonhistone protein, namely B23, were isolated from nucleoli and found to contain phosphorylated, highly acidic tryptic peptides (*4*). More recently the complete amino acid sequence of one of these peptides, C23-Ca, has been determined, together with the probable positions of the phosphorylated residues by Mamrack *et al.* (*5*), who have proposed that peptide C23-Ca exists in three phosphorylated forms (C_0, C_1, and C_2) which differ only in phosphate content (3, 2, and 1 residues of phosphoserine, respectively). Only phosphoserine and not phosphothreonine was found in peptides C_0, C_1, and C_2.

Although at this time none of the functions of such strongly anionic structures has been defined, it may be possible that they are potential binding sites for cations, i.e., metal ions, amines, or clusters of basic amino acids in histones (*6*). Indeed it was recently demonstrated that silver selectively stains the nucleolus

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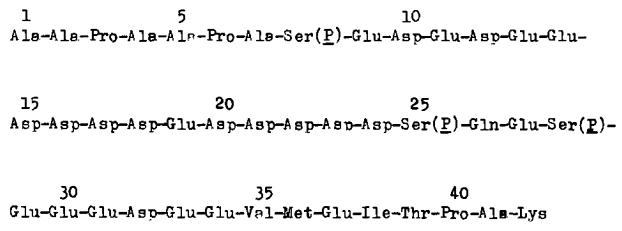

FIG. 1. Linear sequence of amino acids in the synthetic peptide C23-Ca. The letter P in parentheses indicates the probable positions of the phosphoryl groups in the three natural forms of C23-Ca (Mamrack *et al.* (*5*)).

organizer regions (NORs) on chromosomes (*7*), and isolated protein C23 also stains with silver on polyacrylamide gels (*8*). In order to provide material for further *in vitro* binding studies, I decided to undertake the chemical synthesis of the ditetracontapeptide corresponding to the amino acid sequence of C23-Ca, as determined by Mamrack *et al.* (Fig. 1).

Peptide synthesis may be carried out by conventional chemistry in solution or by the Merrifield solid-phase method (*9*). In either approach, the standard, routine methodology suffers from a series of shortcomings which usually do not allow one to obtain acceptable results for peptides with over 30–35 amino acid residues. However, a set of important improvements has been published recently, especially for the solid-phase method (*10*). For our purpose an improved solid-phase procedure is reliable since it is not exceedingly time consuming and has been proved to be particularly useful in studies on the structure–activity relationships of polypeptides of small to moderate weight.

A strategy of synthesis was selected that affords permanent protection of the threonine hydroxyl group as a benzyl ether and complete protection of all other functional groups in the amino acid side chains with mildly acid-labile groups (*tert*-butyl-type protecting groups) as well as the peptide to resin linkage (*p*-alkoxybenzyl ester), which is also mildly acid labile. A very acid-labile group (2-biphenylisopropyloxycarbonyl) was employed for temporary α-amino group protection. This scheme based on differential sensitivity to acid allows the synthesis to proceed under mild conditions even in the final cleavage and deprotecting step. The deprotection of *O*-benzyl threonine in position 39 was carried out by catalytic transfer hydrogenation after the enzymatic phosphorylation of serine residues. To secure high coupling yields, preformed symmetrical anhydrides were used together with coupling monitoring and repeated couplings when necessary. Extensive purification and careful characterization were accomplished at the end of the synthesis.

EXPERIMENTAL

General

All amino acids were of the L-configuration and were either derivatized by standard methods or purchased from Fluka A.G., Buchs, Switzerland. 2-Bipheny-

lisopropyloxycarbonyl (Bpoc) amino acids were obtained with satisfactory yields by the method of Sieber and Iselin (*11*). *tert*-Butyloxycarbonyl (Boc) alanine was prepared as described by Schnabel (*12*). Purity of solvents and reagents: CH_2Cl_2, $(CH_3)_3COH$, and ethanol (EtOH), water free, redistilled; dimethylformamide (DMF), distilled from CaH_2 at reduced pressure and stored at 4°C over molecular sieves; 1,4-dioxane (Diox), redistilled from Na and stored under nitrogen; trifluoroacetic acid (TFA), redistilled from calcium sulfate; acetic anhydride (Ac_2O), distilled (bp 140°C); pyridine (Py), water free, distilled (bp 115°C); diisopropylethylamine (DIEA), distilled from naphtylisocyanate; *N, N'*-dicyclohexylcarbodiimide (DCC), purchased from Fluka A.G. and used as received; 1,4-cyclohexadiene (Aldrich Chemical Co., Milwaukee, Wisc.) was kept refrigerated and used directly. All other chemicals were of reagent grade and used without further purification. Thin-layer chromatography was performed on silica gel G plates according to Stahl (E. Merck & Co., Darmstadt, Germany). Amino acid analyses were carried out on a Beckman 121-M amino acid analyzer. High-voltage paper electrophoresis was made with a Pherograph apparatus Mini 65. Catalytic transfer hydrogenation was carried out in a vibromixing apparatus similar to that described by Felix *et al.* (*13*).

Synthesis of the Protected Peptide Resin

The solid support was the alkoxybenzyl alcohol resin, prepared as described by Wang (*14*). The α-amino protecting group was Bpoc in all but the last step (Ala_1) where Boc was used. The following groups were employed for side-chain protection: N^{ϵ}-Boc for lysine, *O-tert*-butyl for serine, *O*-benzyl for threonine, and *tert*-butyl ester for aspartic and glutamic acids. N^{α}-Bpoc-N^{ϵ}-Boc-lysine (200 mg, 0.413 mmol) was allowed to react with *p*-alkoxybenzyl alcohol resin (2 g, 1.74 mmol) and DCC (85 mg, 0.413 mmol) in the presence of 33.3 ml of Py for 160 min. After washings, the Bpoc-Lys(Boc) resin was dried to constant weight (2.193 g) *in vacuo* at 40°C. A substitution of 0.192 mmol/g was estimated by weight gain and by the method of Gisin (*15*). The substituted resin (1.04 g, 0.20 mmol) was transferred to the glass reaction vessel of a manual apparatus and acetylated with a mixture of Py and Ac_2O (25 ml; 1 : 1, v/v, in the presence of a catalytic amount of 4-dimethylaminopyridine (*14*)) for 90 min to eliminate unreacted hydroxyl groups on the resin. After filtration and washing with DMF, CH_2Cl_2, $(CH_3)_3COH$, and CH_2Cl_2 the protected amino acid resin was submitted to the program of Table 1 for stepwise synthesis from the carboxyl terminus.

The purity of each amino acid derivative was checked by thin-layer chromatography and mp determination before use, and the product recrystallized if necessary. Bpoc-glutamine was coupled to the peptide resin as *p*-nitrophenyl ester (1.01 g, 2 mmol, 10 eq) in DMF in step 16, which was preceded and followed by DMF in steps 15 and 17. The "premix" reaction mixture (*16*) used in all other couplings was prepared as follows: protected amino acids (1.2 mmol, 6 eq) in 8 ml of CH_2Cl_2 were cooled to 0°C and mixed with DCC (0.6 mmol, 3 eq) in 2 ml of CH_2Cl_2. After this mixture was stirred for 20 min at 0°C, the white precipitate was filtered at room temperature and washed with 1 ml of CH_2Cl_2. The filtrate was immediately added to the resin with the aid of a pipet, followed by a rinse of 2 ml

TABLE 1

SYNTHETIC PROGRAM FOR C23-Ca

Operation	Reagent[a]	Mixing time (min)	Applications
1	CH_2Cl_2	1	3
2	0.5% TFA/CH_2Cl_2	1	1
3	0.5% TFA/CH_2Cl_2	10	1
4	CH_2Cl_2	1	3
5	$(CH_3)_3COH$[b]	1	3
6	CH_2Cl_2	1	3
7–9	Repeat operations 1–3		
10	CH_2Cl_2	1	3
11	Diox	1	3
12	CH_2Cl_2	1	3
13	5% DIEA/CH_2Cl_2	1	1
14	5% DIEA/CH_2Cl_2	5	1
15	CH_2Cl_2	1	6
16	Premix reaction mixture[c]	240	1
17	CH_2Cl_2	1	3
18	$(CH_3)_3COH$[b]	1	3
19–25	Repeat operation 12–18		
26	CH_2Cl_2	1	3
27	Coupling monitoring (if necessary, repeat operation 12–17)		
28	30% EtOH/CH_2Cl_2	1	3
29	CH_2Cl_2	1	3
30	5% Ac_2O/Py	1	1
31	5% Ac_2O/Py	20	1
32	CH_2Cl_2	1	3

[a] Percentages express v/v ratios; wash volumes were 18 ml.
[b] This operation was carried out at 30°C.
[c] For Gln_{26}, Bpoc-Gln *p*-nitrophenyl ester in DMF was employed.

of CH_2Cl_2. The exceptions to this procedure were for derivatives of isoleucine and methionine, which were first dissolved in DMF (2 ml) and then diluted to 8 ml with CH_2Cl_2 before the reaction with DCC (*17*). For these derivatives the premix reaction mixture was stirred for 30 min at 0°C.

Coupling Monitoring and Synthesis Checking

Completeness of coupling reactions was monitored both by the ninhydrin color test of Kaiser *et al.* (*18*) and the fluorometric method (*19*), which seem to be remarkably complementary (*20*). A small sample of peptide resin was removed from the vessel at step 27, divided into two parts, and free amino group presence tested. When positive or doubtful, the resin was thrice-coupled (repeat step 12–17). Thrice-coupling was necessary for Ile_{38}, Val_{35}, Glu_{30}, Ser_{28}, Gln_{26}, Asp_{21},

Glu_{19}, Asp_{16}, Asp_{15}, Glu_{11}, Asp_{10}, Ser_8, and for all the six N-terminal amino acids. After the second (or the third) coupling the peptide resin was acetylated.

The progress of the synthesis was checked seven times (after coupling of Glu_{37}, Glu_{31}, Ser_{35}, Glu_{19}, Glu_{13}, Ala_7, and Ala_2) by total acid hydrolysis with subsequent amino acid analysis. Small samples of peptide resin were treated with 12 *M* HCl per propionic acid (1 : 1, v/v, 2 hr, 135°C) (*21*) in sealed evacuated tubes. The filtered solutions were evaporated to dryness *in vacuo* and amino acid composition determined (Table 2).

Isolation and Purification of Thr_{39}(Bzl)-C23-Ca

At the end of the synthesis, after the last operation 32, the protected duotetracontapeptide resin was extensively washed with CH_2Cl_2 and dried to constant weight (2.23 g) *in vacuo* over P_2O_5 (50°C). To a suspension of the peptide resin in CH_2Cl_2 (10 ml), TFA (25 ml) in CH_2Cl_2 (15 ml) was added at 25°C with vigorous stirring. After 45 min at the same temperature, the resin particles were removed by filtration, and the solvent evaporated under reduced pressure (bath temperature: 30°C). The obtained residue was triturated with diethyl ether and ethyl acetate to give 283 mg of crude product as a white powder. The Thr_{39}-protected peptide was first desalted by gel filtration on a column of Bio-Gel P-2 in 1 *N* acetic acid; peptide-containing fractions were pooled and lyophilized (253 mg; 27% overall yield based on starting Bpoc-Lys(Boc) resin).

The desalted peptide was dissolved in 1 m*M* HCl and applied to a 2.5 × 100-cm column of Sephadex G-25. The peptide was eluted with 1 m*M* HCl and detected in the effluent by its absorbance at 210 nm (Fig. 2). Fractions corresponding to the main peak were pooled and lyophilized (198 mg; 21% overall yield). Further purification was accomplished by ion-exchange chromatography on a DEAE–Sephadex A-25 column (2.5 × 100 cm) with a running buffer of 6 *M* urea, 0.05 *M*

TABLE 2

CHECKUP OF SYNTHESIS PROGRESS BY AMINO ACID ANALYSIS

Amino acid	Sequences spanned by peptide resin[a]						
	37–42	31–42	25–42	19–42	13–42	7–42	2–42
Lys	1.07(1)	1.11(1)	1.05(1)	1.08(1)	1.16(1)	1.00(1)	1.09(1)
Asp	(0)	1.03(1)	0.98(1)	5.86(6)	10.04(10)	11.88(12)	11.91(12)
Thr	0.98(1)	0.95(1)	1.01(1)	0.95(1)	1.04(1)	0.93(1)	0.91(1)
Ser[b]	(0)	(0)	1.71(2)	1.60(2)	1.64(2)	2.54(3)	2.49(3)
Glx	1.11(1)	3.96(4)	8.12(8)	9.21(9)	11.03(11)	12.79(13)	13.11(13)
Pro	0.94(1)	0.92(1)	1.07(1)	1.10(1)	0.98(1)	1.07(1)	2.94(3)
Ala	1.03(1)	1.08(1)	0.97(1)	0.99(1)	1.04(1)	2.37(2)	5.09(5)
Val	(0)	0.89(1)	0.99(1)	1.08(1)	1.02(1)	0.97(1)	1.06(1)
Met	(0)	0.93(1)	0.91(1)	0.92(1)	0.97(1)	0.86(1)	0.97(1)
Ile	1.05(1)	1.09(1)	0.95(1)	1.11(1)	1.13(1)	0.98(1)	1.14(1)

[a] The theoretical number of residues is indicated in parentheses.

[b] Uncorrected for loss in hydrolysis.

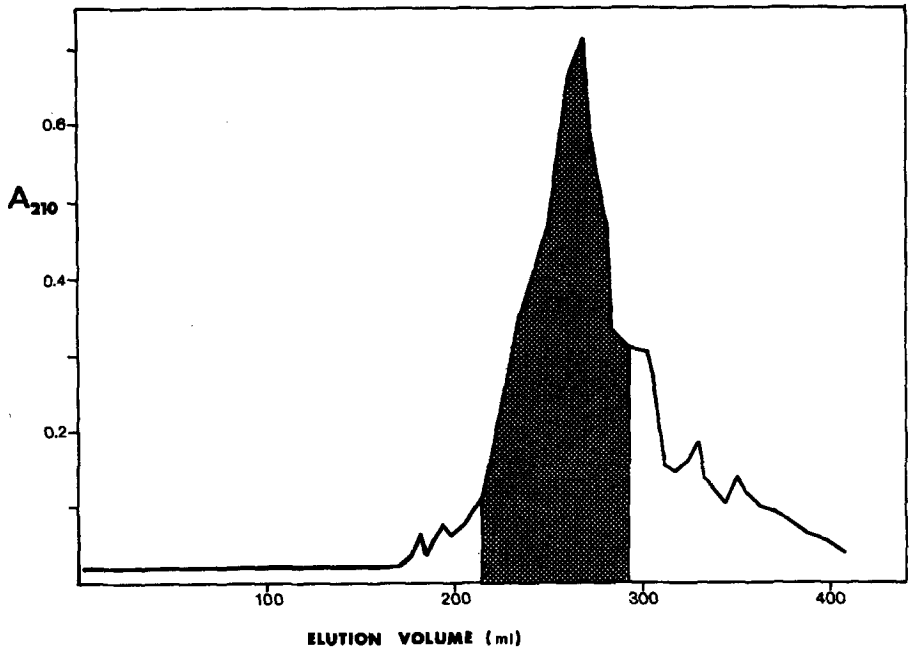

FIG. 2. Gel filtration chromatography of crude, desalted synthetic peptide Thr_{39}(Bzl)-C23-Ca on Sephadex G-25. The column (2.5 × 100 cm) was eluted with 1 m*M* HCl. Fractions from the main peak were pooled and lyophilized; 198 mg, 21% overall yield.

Tris pH 7.5 and a linear gradient of 0.1 to 0.6 *M* NaCl. Desalting on Bio-Gel P-2 of symmetrical main peak (Fig. 3) fractions in 1 *N* acetic acid followed by lyophilization gave a colorless residue of synthetic Thr_{39}-protected peptide, 161 mg (17% overall yield).

Samples of this final product were employed for analytical purposes. Paper chromatography on Whatman 3MM with the upper phase of *n*-butanol–acetic acid–water (4 : 1 : 4) gave a single ninhydrin-positive spot with R_f 0.31. Thin-layer chromatography in *n*-butanol–pyridine–acetic acid–water (5 : 5 : 1 : 3) gave a single chlorine-positive, ninhydrin-positive spot, R_f 0.49. High-voltage paper electrophoresis on Whatman 3MM at pH 1.8 (2% formic acid, 8% acetic acid) for 1 hr at 3000 V, gave one ninhydrin-positive spot, R_{Glu} 0.43. Alanine was shown to be the only amino-terminal amino acid by the dansyl technique (*22, 23*). Lysine was found to be the C-terminal amino acid by carboxypeptidase digestion; the digestion was carried out at 25°C in a total volume of 2 ml of 0.2 *M* sodium phosphate buffer, pH 7.6. The reaction mixture contained about 100 nmol (0.4–0.5 mg) of the purified peptide, 20 nmol of carboxypeptidase A, and 0.2 ml of an activated carboxypeptidase B solution (*24*). The carboxypeptidase B solution was added at zero time; the carboxypeptidase A was added after 30 min. Samples (0.5 ml) were removed after suitable intervals. The reaction was stopped with 0.2 ml of 1 *N* HCl, and each aliquot analyzed directly for free amino acid content determination (Table 3).

A sample, 10 mg, of the purified peptide was dissolved in acetic acid and

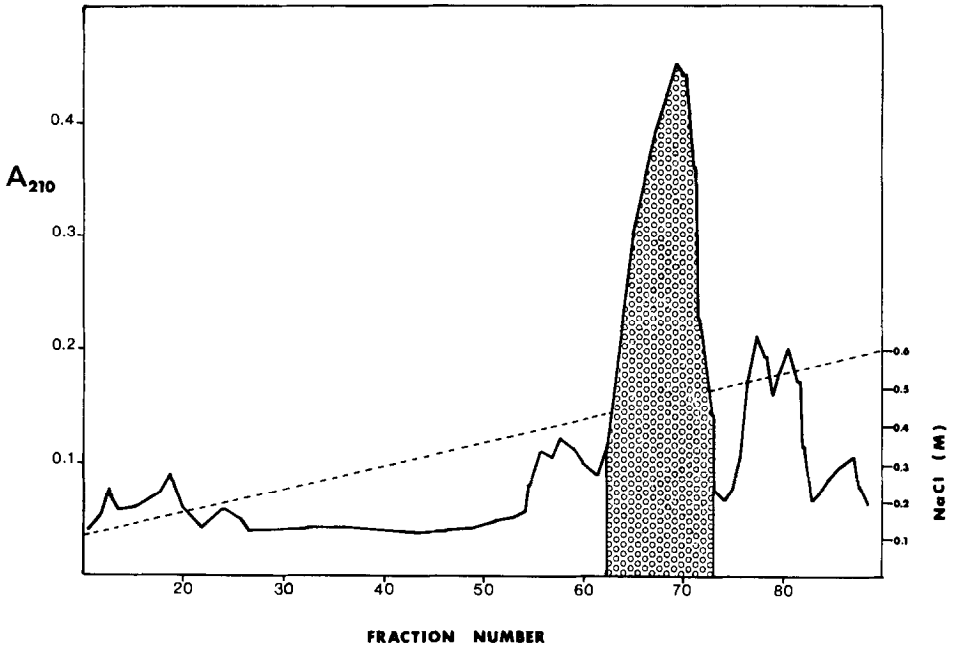

FIG. 3. Ion-exchange chromatography of pooled fractions from the main peak of gel filtration (Sephadex G-25, Fig. 2) on DEAE–Sephadex A-25. A running buffer of 6 *M* urea, 0.05 *M* Tris (pH 7.5) was used together with a linear gradient of 0.1 to 0.6 *M* NaCl. Fractions from the main peak were desalted (Bio-Gel P-2), pooled, and lyophilized; 161 mg, 17% overall yield.

submitted to catalytic transfer hydrogenation with 1,4-cyclohexadiene (*13*), in the presence of freshly generated palladium black as catalyst, to deprotect Thr_{39}-hydroxyl group. After 4 hr at 25°C the reaction mixture was filtered (celite), washed with acetic acid and evaporated under reduced pressure (bath temperature, 40°C). The free peptide, 8.7 mg, was submitted to amino acid analysis of acid and enzyme hydrolysates. The results are shown in Table 4.

TABLE 3

RESULTS OF CARBOXYL-TERMINAL DETERMINATION ON PURIFIED SYNTHETIC C23-Ca

Amino acid	Amino acid residues per molecule of peptide			
	Carboxypeptidase B		Carboxypeptidase A + carboxypeptidase B	
	10 min	30 min	60 min	180 min
Lys	0.91	0.98	1.02	1.00
Pro			0.41	0.89
Ala			0.59	1.04
Val			0.07	0.11
Met			0.09	0.17
Ile			0.21	0.63

TABLE 4

AMINO ACID ANALYSES OF SYNTHETIC C23-Ca

Amino acid	Theoretical	Acid hydrolysate[a,b]	Enzyme digest[b,c]
Lys	1	1.08	1.04
Asp	12	11.89	11.93
Thr	1	0.92	
Ser	3	2.61[d]	5.11
Gln	1	13.11	
Glu	12		12.09
Pro	3	2.96	2.18
Ala	6	6.05	6.13
Val	1	1.07	1.00
Met	1	0.89	0.98
Ile	1	1.19	1.15

[a] Hydrolysis was carried out with 6 *N* HCl for 24 hr in sealed evacuated tubes at 110°C.

[b] Average of three determinations.

[c] Digestion with acid protease for 48 hr at 37°C, followed by 48 hr digestion with leucine aminopeptidase.

[d] Uncorrected for loss in hydrolysis.

Enzymatic Phosphorylation of Synthetic C23-Ca

Normal liver nucleolar phosphoprotein kinase was extracted and purified as described by Olson *et al.* (*1*). The reaction mixture, with a final volume of 12 ml, contained 10 m*M* $MgCl_2$, 0.25 m*M* ATP, 24 m*M* 2,2-bis(hydroxymethyl)-2,2′,2″-nitriloethanol (pH 7), 6 m*M* dithiothreitol, 70 m*M* NaCl, 0.40 *M* enzyme extract, and peptide Thr_{39}(Bzl)-C23-Ca (4 mg/ml). Incubation was carried out at 37°C for 2 hr. The reaction was terminated with glacial acetic acid to give a final concentration of 30%. Phosphopeptides were desalted and separated from the enzyme by gel filtration on Sephadex G-25 and G-50 with 30% acetic acid. The peptide-containing fractions were pooled and lyophilized (3.8 mg).

Catalytic transfer hydrogenation was carried out as described above, and afforded 2.9 mg of phosphopeptides. Paper electrophoresis on Whatman 3MM at pH 1.8 for 1.5 hr at 3000 V was performed with a sample of Thr_{39}-deprotected phosphopeptides, and spots detected with ninhydrin (Fig. 4).

RESULTS AND DISCUSSION

Solid-Phase Synthesis of Peptide C23-Ca

The main features of this synthesis can be summarized as follows: (1) a strategy of synthesis was selected which is based on differential sensitivity to acid (TFA) of

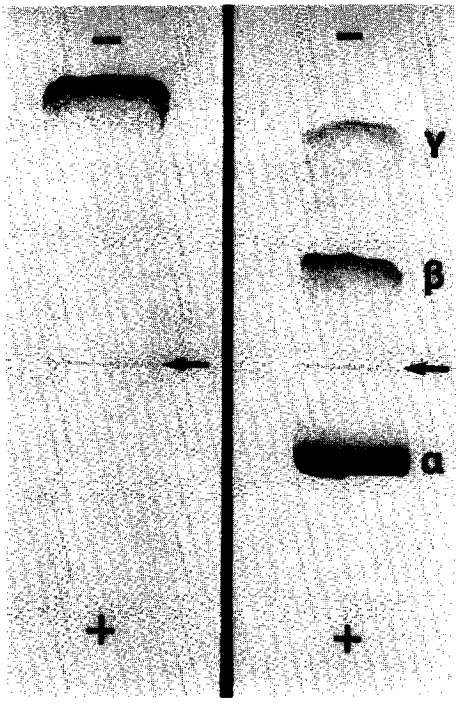

FIG. 4. High-voltage paper electrophoresis of unphosphorylated synthetic C23-Ca (left) and phosphorylated synthetic C23-Ca (right) peptides. The samples were desalted, placed on Whatman 3MM paper, and electrophoresed at pH 1.8 for 1.5 hr at 3000 V; spots were detected by ninhydrin spray. The arrow indicates the origin. The phosphopeptide components are indicated by greek letters; since phosphorylated peptide C23-Ca with one or two phosphoserine residues may exist in three isomeric forms, a subpopulation of positional isomers in the β- and γ-components may not be excluded.

Thr_{39}-hydroxyl protecting group (benzyl ether), all other side-chain protecting groups, peptide to resin linkage, and the α-amino protecting group (relative stability to TFA: 1,000,000:3000:3000:1, respectively (*25*)); (2) the final cleavage of the peptide from the *p*-alkoxybenzyl alcohol resin as well as all the other deprotecting steps was achieved avoiding the use of strong acidolysis, such as anhydrous HF or HBr-TFA, which causes serious product decomposition and often leads to the formation of undesirable and persistent side products; (3) the presence of an aspartyl-serine sequence (residues 24–25), which is known to give rise easily to aspartimide formation (*26*), suggested the use of the sterically hindered *tert*-butyl ester as protecting group for the β-carboxyl of aspartic acid (*27*) as well as prompted employment of mild acid conditions (*28*); (4) all the couplings, except for Gln_{26}, were performed with preformed symmetrical anhydrides of the protected amino acids, which allow more rapid and efficient reactions; (5) an effective monitoring system was obtained combining the ninhydrin color test (*18*) and the fluorometric method (*19*) in order to detect the presence of even small amounts of free amino groups due to incomplete couplings; (6) repeated couplings, first suggested by Weygand *et al.* (*29*), were employed to ensure more

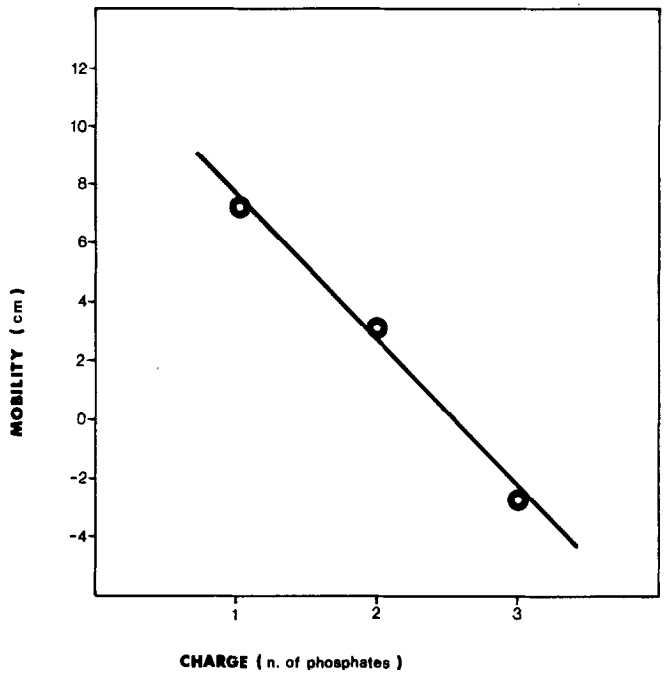

FIG. 5. Plot of relative mobility vs presumed number of phosphate residues per peptide C23-Ca synthetic molecule. The data from paper electrophoresis (Fig. 4) were plotted (Offord, 1977 (*36*)) to test the linearity of the relationship between electrophoretic mobility and integral multiples of the negative charge carried by each of the three forms of phosphorylated synthetic C23-Ca. The phosphopeptides were assumed to have almost identical molecular weights as deduced from amino acid composition.

complete reactions; (7) acetylation of the peptide resin was used to block unreacted amino groups (*9*); (8) a synthetic program was used which included swelling and shrinking of the resin (*30*) between the two deprotection cycles and also between the double couplings (*31*); (9) synthesis progress was checked by amino acid analysis of hydrolized samples of peptide resin to give the up-to-date status of the synthetic growing peptide; and (10) the non acidolytic removal of Thr_{39}-hydroxyl benzyl protecting group, carried out by catalytic transfer hydrogenation with 1,4-cyclohexadiene (*13*), was possible even in the presence of a sulfur-containing amino acid (Met_{36}). Although literature reports (*13*, *32*, *33*) on this matter are conflicting, we have observed that the use of a large excess of freshly generated catalyst allows us to overcome this traditional obstacle.

It must be emphasized that the use of the above-mentioned improved procedures for solid-phase peptide synthesis (*34*) became necessary in order to obtain acceptable results, since intermediate peptides were not isolated and purified during the synthesis.

Purification and Yield in the Synthesis

Careful purification of crude products and scrupulous characterization of the

TABLE 5

CARBOXYPEPTIDASE Y DIGESTION OF COMPONENTS β AND γ FROM PAPER ELECTROPHORESIS OF PHOSPHORYLATED SYNTHETIC C23-Ca PEPTIDE

Amino acid[a]	C23-Ca β			C23-Ca γ (30 min)
	15 min	30 min	120 min	
Lys	1.0 (47%)	1.0 (89%)	1.0 (63%)	1.0 (81%)
Asp			0.09	
Thr		0.78	0.94	0.81
Glu		1.03	1.11	1.17
Pro		0.81	0.98	0.91
Ala		0.96	1.00	0.94
Val			0.69	
Met			0.65	
Ile		0.48	0.93	0.53

Note. Peptides C23-Ca β and γ (0.4 nmol) were digested for various times at 37°C as described by Mamrack *et al.* (*5*). Results are expressed as ratios of released amino acids to lysine (percentage yield given in parentheses).

[a] Free amino acids were separated on Sephadex G-25, and a sample applied to the amino acid analyzer.

final peptides obtained by solid-phase peptide synthesis is indispensable and still remain a vital part of the chemical work. The use of gel filtration together with ion-exchange chromatography is a well-established procedure, although excellent purifications may be achieved by other separation techniques.

Elution profile of the crude desalted Thr_{39}(Bzl)-peptide on Sephadex G-25 (Fig. 2) clearly indicates that a major product has been obtained in a large quantity and only small amounts of minor peptides were present. Amino acid analysis on samples taken from different parts of the elution profile indicated that the desired product was present only in the main peak. Fractions from this peak were rechromatographed on ion-exchange resin for further purification. This afforded a major symmetrical peak surrounded by some minor peaks (Fig. 3), which were discarded owing to their lower contents of Asp, Glu, and Ala on amino acid analysis.

The purified peptide had the expected overall amino acid composition (Table 4), and was homogeneous in paper electrophoresis as well as on thin-layer and paper chromatography. Alanine was found to be the only amino-terminal amino acid, and carboxypeptidase A digestion indicated that lysine was the C-terminal residue (Table 3).

The overall yield of the crude product obtained was 27%. This yield, which could be considered low in comparison with ordinary organic syntheses, is not unusual for solid-phase peptide synthesis and can be explained as follows: (1) 41 coupling reactions were necessary for assembling the entire amino acid sequence; (2) repeated removal of resin samples for coupling monitoring and amino acid analysis; (3) manipulations in the final cleavage step and during the desalting procedure resulted in a loss of peptide; and (4) acidolytic cleavage of the *p*-

alkoxybenzyl ester linkage between the peptide chain and the polymeric support during repeated treatments with 0.5% TFA in deprotection steps.

Characterization of Phosphorylated Peptides C23-Ca

O-Phosphorylation of Thr_{39}(Bzl)-C23-Ca peptide with di-*p*-nitrobenzylphosphochloridate (*35*) and with other chemicals was unsuccessful. Enzymatic phosphorylation was then attempted with liver-extracted nucleolar phosphoprotein kinase (*1*). Paper electrophoresis at pH 1.8 of the phosphorylated, threonine-deprotected peptide showed that three components were present in the product. One peptide moved slightly off the origin toward the positive pole, while the other two peptides moved toward the cathode (Fig. 4). Since no detectable change in amino acid composition was observed, and since both electrophoretic pattern and the plot (Fig. 5) of relative mobility vs presumed number of phosphate residues per peptide molecule (*36*) are very similar to those reported for the natural phosphopeptide fraction C23-Ca by Mamrack *et al.* (*5*), it may be argued that the three forms α, β, and γ differ only in phosphate content, having three, two, and one phosphoryl groups, respectively. In addition to amino acid analysis, further evidence for the similarity of phosphopeptides β and γ was obtained by carboxypeptidase Y digestion (*5*) at 37°C for various times (Table 5). The α-form should be the phosphopeptide C23-C_0 isolated by Mamrack *et al.* (*5*) whereas it remains to be ascertained whether the other two components, β and γ, correspond to the phosphopeptides C_1 and C_2 reported by the same authors, or are two of their positional isomers (peptide C23-Ca with one or two phosphoserine residues may exist in three isomeric forms). Furthermore, a subpopulation of positional isomers in the β and γ components may not be excluded.

From a quantitative point of view, the negative-charged phosphopeptide α formed in a higher quantity than the one which is present in the total mass of the natural phosphopeptide fraction C23-Ca (peptide C_0 accounts for only 5% (*5*)). This may be explained if we assume that, besides primary sequence of amino acids around the phosphorylation sites, the tertiary structure of a protein is important, at least in a negative sense (*37*), in determining the extent of phosphorylation by protein kinase. The intact native structure of nonhistone protein C23 is probably required for its correct degree of phosphorylation, which is perhaps the key to the mystery that surrounds the structure–activity relationships of such highly negatively charged proteins.

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