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Synthesis of a Protein with the Properties of the Apolipoprotein C-I (ApoLP-Ser)

Sir:

We wish to report the synthesis of apolipoprotein C-I (apoLP-Ser), a constituent of very low density lipoproteins (VLDL) of human plasma.[1] Apolipoprotein C-I was shown to be an activator of lecithin:cholesterol acyltransferase[2] and thus may play an important role in cholesterol metabolism.[3] The amino acid sequence of C-I was recently determined[4] and was characterized by a high lysine content (16%) and the absence of cysteine, tyrosine, and histidine in the 57 residue sequence.

Heterogeneity of synthetic products is recognized as a serious problem in the solid phase synthesis[5] of peptides larger than ten residues.[6,7] The formation of incorrect sequences has often been attributed to incomplete coupling reactions[8-11] and premature removal of side chain protection.[12] Several variations were introduced into the automated synthetic procedure used to prepare apoC-I in an attempt to overcome some of these problems. A 1% cross-linked polystyrene resin support was used with a very low Boc(*O*-benzyl)serine substitution in an attempt to reduce deletion sequences.[8,13] The use of shrink and swell washes[8] and a mixed solvent system for coupling reactions[9] were used in an attempt to expose all of the reactive sites during the synthesis. The premature deprotection of the ϵ-amino group of lysine[14] was minimized by use of the *o*-chlorobenzyloxycarbonyl (*o*-Cl-Z)[15] derivative. The single Trp residue was protected by the N^{im}-formyl group.[16] Symmetrical anhydrides of Boc amino acids[17] were used in all coupling reactions, as it had been reported that this coupling method improves yields in longer syntheses.[18]

The synthesis was based on Boc(*O*-benzyl)serine esterified to the polystyrene resin[19] (0.086 mmol/g). The amino acid resin (4 g) was then treated to the automated stepwise addition[20] of a three- to fourfold excess of suitably protected amino acids. The Boc group was used for α-amino protection, and the side chains were protected as follows: Asp (β-OBzl), Glu (γ-OBzl), Ser (Bzl), Thr (Bzl), Lys (*o*-Cl-Z), Arg (NO_2), Trp (CHO). The coupling steps, including the addition of Asn and Gln, were carried out with a twofold excess of the symmetrical anhydride[17] (generated in situ by addition of half the required amount of DCC at the commencement of a coupling and the remainder after 1 h). With Asn and Gln the coupling reaction was carried out in the presence of an equimolar amount of 1-hydroxybenztriazole. An identical second coupling reaction was performed, except that a solvent system of dichloromethane and dimethylformamide (1:1, v/v) was used. Boc groups were removed by two treatments of the resin with trifluoroacetic acid in dichloromethane (50%, 25%, v/v) for 5 and 30 min, respectively. The peptide was cleaved from the resin (1 g) with liquid HF (20 ml) at 0° for 60 min in the presence of anisole (1 ml).[21] The formyl group was removed under mildly basic conditions (4°, pH 7.6, 3 days) and examination of the uv absorption at 280 and 300 nm[16] showed good removal of the protecting group. The crude protein was then purified by gel filtration (see Figure 1) and ion exchange chromatography (see Figure 2).

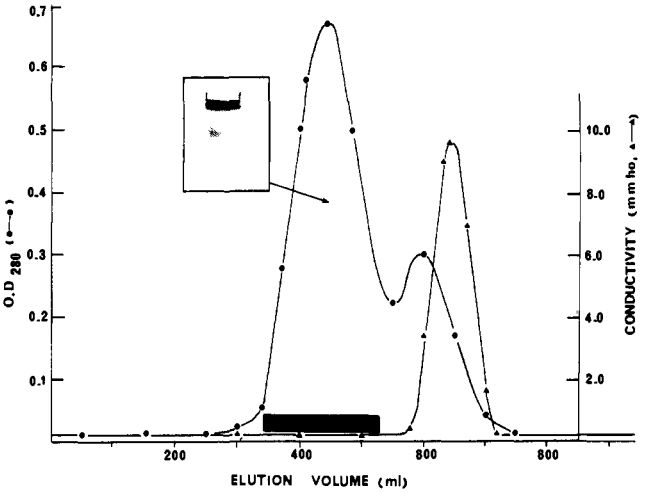

Figure 1. Gel filtration of the synthetic apoC-I after HF cleavage. The product was dissolved in 0.02 M potassium phosphate, pH 7.0 (50 ml), and applied to a column of Sephadex G50 (90 × 2.5 cm). The column was run with the same buffer as eluent, at a flow rate 100 ml/h and 10 ml/fraction. Each fraction was monitored by OD_{280} measurements (●—●) and conductivity (▲—▲). The solid bar shows the fractions which contained significant activity as an activator of LCAT, and the inset shows the result of polyacrylamide gel electrophoresis (7.5% cross-linked gel, pH 8.9, 8 M urea).

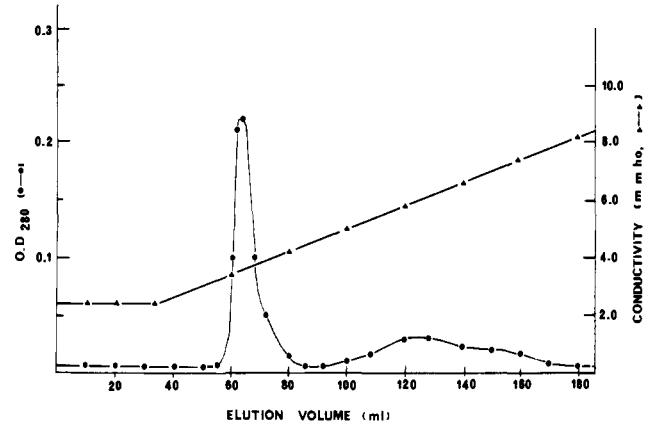

Figure 2. Purification of synthetic apoC-I by ion exchange chromatography on Sephadex SP-25. Synthetic apoC-I was applied to a column of SP-Sephadex (9 × 1.5 cm) equilibrated in potassium phosphate buffer (pH 4.0, 0.05 M) and the product was eluted with a linear gradient of potassium phosphate buffers (0.02 M, pH 4.0, 200 ml to 0.2 M, pH 7, 200 ml), at a flow rate of 200 ml/h. Fractions (7 ml) were monitored by OD_{280} (●—●) and conductivity (▲—▲). The recovery of purified synthetic apoC-I (first peak pH 4.2, conductivity 3.5 $m\Omega^{-1}$) was variable (20-50%), and was probably caused by the tendency of apoC-I to aggregate in the absence of denaturants.[28]

Examination of the crude product after HF cleavage indicated that the modifications to the synthetic procedure had indeed led to a more homogeneous product, as shown by a good yield of cleaved peptide,[22] by amino acid analyses at strategic points during the synthesis, and by electrophoretic and amino acid analysis of the crude product.[23]

The product after purification behaved as a homogeneous protein in that it eluted as a single narrow peak on gel filtration and ion exchange chromatography. Synthetic apoC-I migrated as a single band in a position identical with native apoC-I on

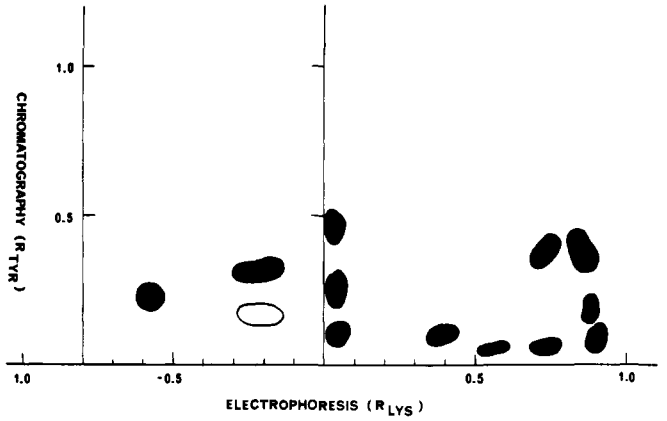

Figure 3. Two-dimensional peptide maps of a tryptic digest of synthetic apoC-I. The protein (1.5 mg) was dissolved in ammonium bicarbonate (0.5%), trypsin (15 μg) was added, and the digestion was performed at 37° for 4 h. The digest was then examined in two dimensions by electrophoresis at pH 6.5 in pyridine:acetic acid:water buffer (25:1:225) and by chromatography in butanol:acetic acid:water (4:1:5, upper phase). The chromatogram was visualized in ninhydrin and chlorine/starch reagent (both reagents gave similar results). The single open spot corresponds to the only Trp containing peptide (uv fluorescence).

polyacrylamide gel electrophoresis at pH 8 in the presence of 0.1% sodium dodecyl sulfate, and pH 8.2 in the presence of 8 M urea (the single band had the same appearance as shown in the Figure 1 inset but with the minor band absent). The synthetic product showed strong cross-reactivity with antiserum specific to native apoC-I.[25] The synthetic apoC-I was assayed by the procedure of Soutar et al.[2] and showed significant activity as an activator of LCAT.[24] Amino acid analysis of an acid hydrolysate was in good agreement with the expected values,[26] i.e., Lys 9.3 (9), Arg 3.1 (3), Trp 0.8 (1), Asp 5.0 (5), Thr 2.8 (3), Ser 7.1 (7), Glu 9.0 (9), Pro 0.9 (1), Gly 1.1 (1), Ala 3.1 (3), Val 2.2 (2), Met 1.0 (1), Ile 2.8 (3), Leu 5.8 (6), Phe 3.0 (3). Peptide maps of tryptic digests of synthetic apoC-I showed the expected number of fragments[4,27] and confirmed that the synthetic product was essentially pure.

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(23) Observations between this and another synthesis of apoC-I by standard procedures, see D. R. K. Harding, D. R. Husbands, and W. S. Hancock, Abstracts, New Zealand Institute of Chemistry Conference, Aug 1975, No. 24, 47. A similar improvement to the synthesis of apoC-I (G. F. Sigler, A. Soutar, A. M. Gotto, and J. T. Sparrow, Circulation Supplement II Abs 60 (1975)) was achieved by the use of a "spacer" arm (J. T. Sparrow, *J. Org. Chem.*, in press). It is probable that a significant improvement in both approaches is to limit the synthesis to the less sterically hindered regions of the matrix. As well, the mixed solvents, shrink and swell washes, and the use of highly reactive symmetrical anhydrides in this synthesis ensure that a greater percentage of more hindered regions continue to function throughout the synthesis.
(24) Synthetic apoC-I (15 μmol) activated LCAT to a specific activity of 200 pmol of cholesterol ester formed per hour, which compares favorably with the activity of the native protein.[2]
(25) The antibody assays were kindly performed by Dr. L. Simons, Department of Medicine, University of New South Wales.
(26) HCl (6N), 110 °C, and 24 h, the value for Trp was measured spectrophotometrically.[16]
(27) Based on the published sequence, apoC-I would be expected to give 12 peptide spots on peptide mapping. After three duplicate maps it was not possible to determine if the bottom right spots were one broad or two closely overlapping spots (the latter interpretation is shown and gives a total of 13 spots).
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Low Temperature Studies on Propionyl Benzoyl Peroxide and Propionyl Peroxide. The Ethyl Radical

Sir:

Recently we demonstrated that the infrared spectra of simple free radicals, in particular the infrared spectra of the phenyl and methyl radical, may be obtained by photochemical cleavage of asymmetric acyl peroxides.[1] Here we report that analogously ethyl and phenyl radicals may be obtained by photodestruction of propionyl benzoyl peroxide (I), and that high concentrations of alkyl radicals may be obtained by photolysis of symmetric diacyl peroxides as well. In fact, the concentrations of the radicals produced are so high that the complete vibrational spectrum is obtained.

Compound I was isolated in an argon matrix at 6 K and subsequently irradiated with a medium-pressure mercury lamp in conjunction with a water filter and a Corning No. 053 uv filter with no transmission below 2900 Å. During the irradiation bands due to CO_2 and ethyl benzoate[2] appeared together with an intense band at 710 cm^{-1} characteristic of the phenyl radical,[3] and another band at 541 cm^{-1}. Upon warming the matrix to 30 K the latter two bands disappeared[4] within several seconds, and simultaneously new bands due to ethylbenzene, ethylene, and benzene appeared. Since these new bands can conveniently be explained as being due to the combination and