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Chemical and Physical Characterization of a Phosphoprotein, Protein C, from Human Saliva and Comparison with a Related Protein A

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The isolation of a highly purified phosphoprotein, previously named protein C, from human parotid saliva is described. A chemical and physical characterization of protein C was undertaken and the properties of protein C were compared with those of a related protein A. The content of glycine, proline and dicarboxylic amino acids accounts for 83% of the total residues of protein C and it contains 2.0 mol of P/mol of protein, most likely as phosphoserine. The protein also contains 1.2% glucose, but no hexosamine. The *N*-terminus is blocked and the proposed *C*-terminal sequence is -Ser(Gly,Pro)Gln. The molecular weight determined from ultracentrifugation is 16300. Circular dichroism and nuclear magnetic resonance fail to demonstrate the presence of polyproline structure, and there are no conformational changes under a variety of conditions. With specific antisera to protein C the protein can be detected in submandibular as well as in parotid saliva, but there is only reaction of partial identity of proteins A and C. It is proposed that at least part of the difference between proteins A and C is due to the presence of an additional length of peptide at the *C*-terminus of protein C.

The demonstration in human parotid saliva of two proteins named proteins A and C with similar and unusual composition has previously been reported (Bennick & Connell, 1971). Subsequently a physical and chemical characterization was undertaken of protein A, which was also found to be a Ca^{2+}-binding phosphoprotein (Bennick, 1975, 1976).

It was decided to investigate the chemical and physical properties of protein C more closely, since this would allow a more detailed comparison of proteins A and C, which are known to have common antigenic determinants, and to compare these proteins with structural proteins such as collagen and enamel proteins, with which they share compositional characteristics.

Experimental

Materials

The sources of the materials were the same as used in previous studies (Bennick, 1975, 1976).

Collection and concentration of saliva and chromatographic procedures

Human parotid and submandibular saliva was collected and stored as described previously from one donor in 100ml volumes (Bennick & Connell, 1971; Bennick, 1976).

The chromatographic resins were treated according to the manufacturer's instructions. Saliva was desalted in 200ml volumes, concentrated and stored as previously described (Bennick, 1975).

Gel filtration of 1 litre volumes of concentrated parotid saliva was performed as described by Bennick & Connell (1971) and cation-exchange chromatography by a previously developed method (Bennick, 1975).

Anion-exchange chromatography was carried out on different columns of DEAE-Sephadex A-25 as follows. A 2.6cm×19cm column was used and the resin was equilibrated before it was applied to the column. A linear gradient of 250ml of 0.05M-NaCl and 250ml of 0.3M-NaCl both in 0.05M-Tris/HCl buffer, pH8.0, was used to develop the column. A 1.6cm×40cm column was also used, the resin was equilibrated in imidazole/HCl buffer, *I* 0.1, pH6.2, and the sample was dialysed against the same buffer before it was applied to the column. A linear gradient of 200ml of imidazole/HCl, *I* 0.1, pH6.2, and 200ml of imidazole/HCl, *I* 0.1, pH6.2, containing 0.15M-NaCl, was used to develop the column. In a subsequent purification step the same column was used, but the gradient that was used to develop the column consisted of 200ml of 0.05M-NaCl and 200ml of 0.1M-NaCl, both in imidazole/HCl, *I* 0.1, pH6.2.

Purified samples of protein C were desalted by two successive gel filtrations on columns (0.9cm×30cm) of Sephadex G-25. The columns were equilibrated and developed in 0.03M-NH_4HCO_3. All chromatographic procedures were performed at 3–5°C, and chloroform was used as an antibacterial agent.

Throughout the purification the protein fractions were concentrated by ultrafiltration through UM2 membranes or by freeze-drying solutions of protein in water.

Concentrated protein fractions were quick-frozen in liquid N_2 and stored at −10°C. The protein concentrations of pure samples of protein C were determined from the amino acid composition. Protein A was purified as previously described (Bennick, 1975).

Analytical polyacrylamide-gel electrophoresis

In order to evaluate the composition of the fractions containing protein C obtained during the purification procedure, electrophoresis of the fractions was performed in 0.1M-Tris adjusted to pH9.2 with glycine as described previously (Bennick, 1975). The purity of the final preparations of protein C was evaluated by electrophoresis in 4.5 and 15% polyacrylamide gels in either 0.1M-Tris/glycine buffer, pH9.2, or 0.05M-sodium formate buffer, pH2.8. In the latter system, components migrating towards the cathode were separated. The gels were stained with Coomassie Blue as described by Chrambach *et al.* (1967).

Amino acid analysis

The amino acid composition was determined as described previously (Bennick, 1975) on three different preparations of protein C. Tryptophan was determined as described by Matsubara & Sasaki (1969). The protein samples were hydrolysed for 24h and the mean content ± S.D. of each amino acid was calculated. No corrections were made for loss of threonine and serine during hydrolysis, and the values for valine and isoleucine were not corrected for incomplete recoveries.

Analysis for γ-carboxyglutamic acid

Analysis for γ-carboxyglutamic acid was performed by Dr. S. Magnusson, University of Aarhus, Aarhus, Denmark.

Samples of 500μg each of proteins A and C were hydrolysed in 2M-KOH for 10h. $HClO_4$ was added to pH7, the samples were centrifuged and the supernatants subjected to paper electrophoresis at pH6.5 and then at pH2.1 at right angles (Magnusson *et al.*, 1975).

Carbohydrate analysis

The total carbohydrate content was determined by the anthrone method (Seifter *et al.*, 1950) with a glucose standard. In order to determine the nature of carbohydrate in proteins C and A, 1mg samples of the proteins were hydrolysed in 4M-HCl at 100°C for 4h. The sample was dried, redissolved in water and passed through a column of Amberlite MPO-3 Mixed-Bed Resin. The sample was dried and chromatographed on paper in pyridine/ethyl acetate/water (12:5:4, by vol.), together with standards of glucose, mannose, galactose, fucose, glucosamine and galactosamine. The chromatograph was stained with $AgNO_3$ (Smith, 1960).

The presence of hexosamine was also evaluated from the chromatograms obtained from amino acid analysis.

Phosphorus analysis

The method described by Bartlett (1959) was used to determine total as well as inorganic phosphorus. To determine whether the phosphorus was bound in an ester linkage to the polypeptide, a sample of protein was hydrolysed in 1M-NaOH at 37°C for 4h before analysis for inorganic and total phosphorus. Further, samples of protein C as well as protein A were digested with alkaline phosphatase in molar protein/enzyme ratios of 25:1 and 100:1 respectively for 48h in 1M-Tris/HCl buffer, pH8.0, as described by Garen & Levinthal (1960), before analysis for P_i. The presence of proteinase activity in the phosphatase preparation was tested by incubating 1% casein with phosphatase in 1M-Tris/HCl, pH8.0, for 48h and determining the degree of proteolysis by the method of Kunitz (1947).

N-Terminal determination

Samples (25μg) of protein were dansylated, hydrolysed and subjected to t.l.c. on polyamide plates by the method of Percy & Buchwald (1972) in three different solvent systems consecutively. Standards of dansyl-amino acids were run along with the hydrolysate.

C-Terminal amino acid sequence

Samples (768μg) of protein C were incubated with penicillo-carboxypeptidase-S1 (Jones & Hofmann, 1972) in 0.05M-pyridine/formic acid, pH4.2. The molar ratio of protein/enzyme was 50:1 and hydrolysis proceeded at 37°C for periods varying from 30min to 8h. Additional samples of the same amounts of protein C or enzyme as used for digestion were incubated separately for 8h. At the end of the incubation period, the digests were heated on a boiling-water bath for 10min and subjected to amino acid analysis on a Beckman Spinco model 120C amino acid analyser by using a lithium salt system designed for amino acid analysis of physiological fluids (Benson *et al.*, 1967).

Determination of molecular weight

Samples of protein C were subjected to analytical ultracentrifugation by the low-speed method of

Richards & Schachman (1959) as described previously (Bennick, 1975). Two different preparations of protein C were used. A total of three mol.wt. determinations were made on a solution containing 1.25 or 2.00 mg of protein/ml at speeds of 17000 and 21000 rev./min. The partial specific volume was calculated from the amino acid composition.

Circular dichroism

C.d. (circular-dichroism) spectra were recorded at room temperature (23°C) on a Jasco ORD/CD-15 spectropolarimeter equipped with an SS-20 modification. Spectra were recorded between 320 and 250 nm, in a cell with a 1 cm optical pathway, of an aqueous solution of protein C containing 4.9 mg of protein/ml. A cell with a 1 mm optical pathway was used to record spectra between 250 and 190 nm. In the far-u.v. region spectra were recorded of solutions of protein C containing approx. 0.1 mg/ml. Spectra were recorded in the following solutions: water; 5 mM-Tris/HCl, pH 7.5; 5 mM-Tris/HCl, pH 7.5, containing 1 mM-$CaCl_2$; 5 mM-Tris/HCl, pH 7.5, containing 50 mM-NaCl; and 5 mM-acetic acid/sodium acetate, pH 3.5. Spectra were also recorded in the far-u.v. region of solutions of protein A containing 0.1 mg of protein/ml in the following solvents: 5 mM-Tris/HCl, pH 7.5; 5 mM-Tris/HCl, pH 7.5, containing 1 mM-$CaCl_2$; 5 mM-Tris/HCl, pH 7.5, containing 29 mM-NaCl and 10 mM-KCl; and 5 mM-acetic acid/sodium acetate, pH 3.5.

The results are given as mean amino acid residue ellipticity $[\theta]$ measured in degrees·cm^2·$dmol^{-1}$. A mean residue weight for protein C of 101 was calculated from the amino acid composition. The mean residue weight of protein A had previously been found to be 104 (Bennick, 1975).

Proton n.m.r. spectroscopy

N.m.r. spectra were recorded by continuous-wave spectroscopy on a Varian 220 MHz spectrometer at the Canadian 220 MHz n.m.r. centre as previously described (Bennick, 1975).

Protein A or C was freeze-dried and redissolved in 99.8% ^2H_2O, and this procedure was repeated before the proteins were dissolved at a concentration of 6–9 mg/ml.

Spectra were recorded of protein A or C dissolved in the following solutions: 5 mM-$C^2H_3CO_2{}^2H$ was adjusted to nominal pH 6.0 with 5 mM-NaO^2H (5 mM-deuterated sodium acetate, pH 6.0); 5 mM-deuterated sodium acetate, pH 6.0, containing 1 M-NaCl; 5 mM-deuterated sodium acetate containing 1 mM-$CaCl_2$; 10 mM-$C^2H_3CO_2{}^2H$.

A spectrum of denatured protein C was computed from the amino acid composition by the method of McDonald & Phillips (1969). Measurements of the spectra were in Hz relative to the position of a 2,2-dimethyl-2-silapentane-5-sulphonate reference signal as described previously (Bennick, 1975).

Preparation of antiserum to protein C, immunodiffusion and immunoelectrophoresis

An adult goat was immunized with an aqueous solution containing 4 mg of protein C mixed with 2 vol. of complete Freund's adjuvant as previously described (Bennick, 1976). The antiserum was quick-frozen in 1 ml batches and stored at −10°C.

Double radial immunodiffusion was performed as described previously (Bennick, 1976). Samples of stimulated and unstimulated parotid and submandibular saliva were subjected to electrophoresis on polyacrylamide gels polymerized in the space between two concentrically placed cylindrical glass tubes. The conditions for electrophoresis were otherwise as described previously (Bennick, 1976). After electrophoresis, the central glass tube was removed and replaced with an agar-gel solution containing antiserum to protein C. After setting of the gel solutions, the tubes were incubated at 24°C. Electrophoresis was also performed on duplicate gels which were stained with Coomassie Blue (Bennick, 1976).

Results

Purification

After desalting, the concentrated saliva was fractionated by gel filtration on Sephadex G-50. This gave rise to four fractions, I, II, III and IV, as previously described (Bennick & Connell, 1971). Protein C, which was located in fraction I, was further purified by cation-exchange chromatography on CM-cellulose (Bennick, 1975). By means of polyacrylamide-gel electrophoresis at pH 9.2 protein C was located in the fraction indicated in Fig. 1. Protein C was further purified by anion-exchange chromatography by using a Tris/HCl buffer, pH 8.0, containing a NaCl gradient as described in the Experimental section. The elution diagram is shown in Fig. 2. The fractions containing protein C were pooled and concentrated and resubmitted to cation-exchange chromatography with the use of an imidazole/NaCl gradient to develop the column. The elution diagram is shown in Fig. 3. This purification procedure was repeated with a shallower NaCl gradient as described in the Experimental section. After desalting of the purified protein C by gel filtration, it was concentrated by ultrafiltration, filtered through Millipore filters, pore size 0.22 μm, and stored at −10°C. From 1 litre of unconcentrated parotid saliva about 50 mg of protein C was recovered, as determined from amino acid analysis.

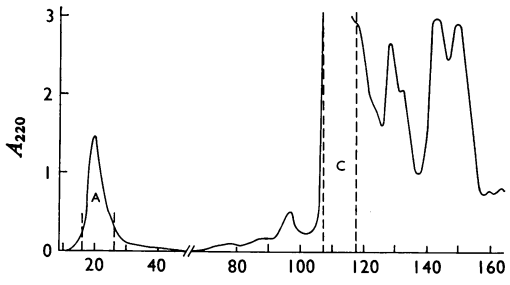

Fig. 1. *CM-cellulose chromatography of fraction I*
Fraction I obtained from gel filtration of 1.0 litre of concentrated parotid saliva on Sephadex G-50 was fractionated on a CM-cellulose column (2.6cm× 75.5cm) equilibrated in sodium acetate buffer, pH4.4, *I* 0.01. After sample application, a volume of 520ml of acetate buffer, pH4.4, *I* 0.01, was passed through the column before the gradient was started. The column was developed with a linear gradient composed of 1000ml of sodium acetate buffer, pH4.4, *I* 0.01, and an equal volume of sodium acetate buffer, pH4.4, *I* 0.2. The flow rate was 40ml/h. The volume of the fractions was 13ml, and A_{220} was read in a cell with a 1mm optical path-length. A and C indicate the location of proteins A and C in the diagram.

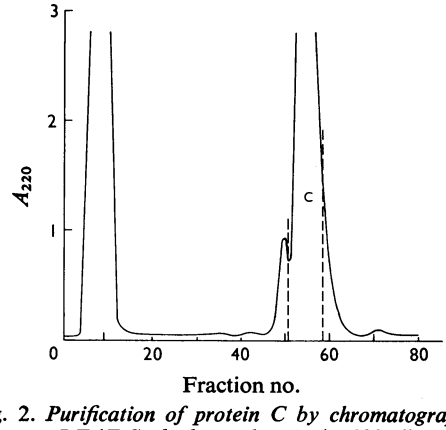

Fig. 2. *Purification of protein C by chromatography on DEAE-Sephadex with a Tris/HCl buffer*
The fraction containing protein C obtained from CM-cellulose chromatography was concentrated and dialysed against the starting buffer of the DEAE-Sephadex column. The protein sample was applied to a column of DEAE-Sephadex (2.6cm×19cm) equilibrated in 0.05M-Tris/HCl buffer, pH8.0, containing 0.05M-NaCl. The column was developed with a volume of 98ml of the equilibrating buffer followed by a linear gradient of 250ml of the equilibrating buffer and a equal volume of 0.05M-Tris/HCl buffer, pH8.0, containing 0.3M-NaCl. The flow rate was 15ml/h and the volume of the fractions was 7.5 ml. The A_{220} of the fractions was read in a cell with a 1mm optical pathway. The location of the fraction containing protein C is indicated in the diagram.

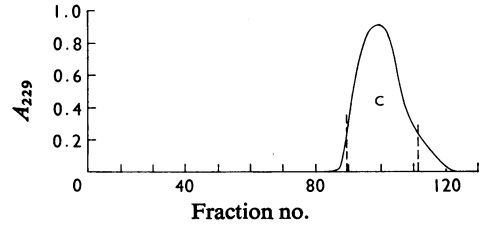

Fig. 3. *Purification of protein C by chromatography on DEAE-Sephadex with an imidazole/HCl buffer*
The fraction containing protein C obtained from chromatography on DEAE-Sephadex by means of a Tris/HCl buffer was concentrated and dialysed against an imidazole/HCl buffer, *I* 0.1, pH6.2. The protein sample was applied to a column (1.6cm×40 cm) of DEAE-Sephadex equilibrated in imidazole/HCl buffer, *I* 0.1, pH6.2. The column was developed with a volume of 60ml of the same buffer followed by a linear gradient of 200ml of imidazole/HCl buffer, *I* 0.1, pH6.2, and 200ml of the same buffer containing 0.15M-NaCl. The flow rate was 10ml/h and the volume of the fractions was 3ml. A cell with an optical pathway of 1mm was used to read A_{229} of the fractions. The location of protein C is indicated on the diagram.

Criteria of purity

Samples (80μg) of pure protein C were subjected to analytical gel electrophoresis as described in the Experimental section. In all gels only a single heavily stained band could be seen.

Amino acid composition

The result of the amino acid analysis is given in Table 1. For comparison the amino acid composition of protein A (Bennick, 1975) is also given.

Protein C lacks sulphur-containing amino acids as well as tryptophan, tyrosine and threonine. The four most prominent amino acids are glutamic acid, proline, glycine and aspartic acid. Together these amino acids account for 83% of all the residues in the protein. From the amino acid analysis a minimum mol.wt. of 15300 was calculated.

Analysis for γ-carboxyglutamic acid

No γ-carboxyglutamic acid could be detected in proteins A and C. The limit of the technique is such that protein A contains less than 0.13mol of γ-carboxyglutamic acid per mol of protein and the corresponding value for protein C is 0.22mol/mol.

Carbohydrate analysis

The carbohydrate content of protein C as determined with a glucose standard was 1.2μg/100μg of protein C. Paper chromatography revealed that only

Table 1. *Amino acid composition*

The results are mean values ± S.D. of 24h hydrolysates of three different preparations of protein C, except for tryptophan (determined from a sample hydrolysed in the presence of thioglycollic acid). The value for serine has not been corrected for loss during hydrolysis and the recoveries of valine and isoleucine have also not been corrected.

	Protein C		Protein A (Bennick, 1975)
Amino acid	% of total residues	Nearest integral value (mol.wt. 15300)	Nearest integral value (mol.wt. 10800)
Lysine	1.42 ± 0.16	2	1
Histidine	1.94 ± 0.12	3	2
Arginine	3.57 ± 0.44	5–6	4
Aspartic acid	7.57 ± 0.24	11–12	11
Threonine	0	0	0
Serine	3.78 ± 0.35	6	4–5
Glutamic acid	27.4 ± 0.15	41	29
Proline	26.9 ± 1.13	40	25
Glycine	21.1 ± 0.69	31–32	20
Alanine	0.82 ± 0.12	1	1
Half-cystine	0	0	0
Valine	1.74 ± 0.21	3	3
Methionine	0	0	0
Isoleucine	1.03 ± 0.08	2	2
Leucine	2.01 ± 0.06	3	3
Tyrosine	0	0	0
Phenylalanine	0.67 ± 0.11	1	1
Tryptophan	0	0	0

Table 2. *Phosphorus analysis*

Inorganic and total phosphorus were determined by the method of Bartlett (1959). For details of analysis, see the text.

		Phosphorus μg/100 μg of protein	
Pretreatment	Nature of phosphorus	Protein A	Protein C
None	Inorganic	0.08	0.04
None	Total	0.57	0.41
NaOH hydrolysis	Inorganic	0.48	0.36
	Total	0.55	0.39
Alkaline phosphatase digestion	Inorganic	0.46	0.37
Chloroform/methanol extraction			
Supernatant	Total	0	—
Residue	Total	0.55	—

glucose was present in proteins A and C. No hexosamine was found in protein C.

Phosphorus analysis

The results of the phosphorus assays are given in Table 2 together with results previously obtained for protein A (Bennick, 1975). The total phosphorus concentration was 0.41 μg/100 μg of protein or 2.0 μg-atoms of P/μmol of protein determined as the minimum molecular weight from the amino acid analysis. The small amount of inorganic phosphorus in the sample is probably due to release of organic phosphate during the assay, which includes brief heat-treatment in the presence of acid. After hydrolysis either in NaOH or with alkaline phosphatase, 91% of the phosphorus in protein C and 80% of that in protein A could be recovered as P_i. No proteolytic activity was detected in the alkaline phosphatase under the conditions of incubation.

N-Terminal determination

Because of the difficulty of distinguishing dansyl-alanine from dansylamine in chromatography on thin-layer polyamide plates, a standard of dansyl-alanine was co-chromatographed in the second and third dimension. No dansyl-amino acids, including dansyl-alanine, could be detected in the digest. These results suggest that the *N*-terminus is blocked.

C-Terminal amino acid sequence

The results of digestions of protein C with penicillocarboxypeptidase-S1 are summarized in Table 3. The amounts of amino acids listed in Table 3 have been corrected for small amounts of amino acids found when the enzyme or protein was incubated alone. From these experiments it can be concluded

that the *C*-terminal amino acid is glutamine. The small amounts of glutamic acid present in the hydrolysates are probably due to deamidation of the *C*-terminal glutamine during heat-inactivation of the enzyme. If the small amount of glycine that was detected in samples of enzyme or protein C incubated alone is added to the values given for glycine in Table 3, the amount of glycine would be slightly larger than the amount of proline in all three hydrolysates. The presence of small amounts of other amino acids in the enzyme and protein-C preparations had negligible influence on the amounts of the corresponding amino acids in the enzymic hydrolysates.

On the basis of these results it is proposed that the *C*-terminal sequence is -Ser(Gly,Pro)Gln. Although it is difficult to be certain of the sequence of glycine and proline, it is most likely that the sequence is -Ser-Gly-Pro-Gln.

Table 3. *Release of amino acids from protein C by penicillo-carboxypeptidase-S1*

Samples (50nmol) of protein C were incubated with penicillo-carboxypeptidase-S1 in an enzyme/substrate ratio of 1:50 (w/w). The amount of protein C digested is based on a minimum mol.wt. of 15300 as determined from amino acid analysis, The presence of glutamic acid is probably due to deamidation of glutamine due to heat-inactivation of the enzyme. The values given in the Table are the amounts of amino acids found in the hydrolysates after subtracting small amounts of amino acids found in the protein and enzyme preparations after incubation for 8h under the experimental conditions.

Time of incubation (h)	Amino acid released (nmol)				
	Ser	Gly	Pro	Glu	Gln
½	—	—	—	—	3.4
1	0.6	1.3	3.9	0.7	7.4
3	10.8	12.8	15.6	3.1	32.6
8	22.3	30.5	32.7	5.0	45.7

Determination of molecular weight

Corresponding values of r^2 (distance from rotor)^2 and log C (concentration of protein expressed as fringe displacements) were plotted. All the data points in a given plot could be fitted to a straight line, indicating homogeneity of the protein sample and absence of non-ideality of the solution. The partial specific volume was 0.695ml/g and the average mol.wt. of three determinations was 16340 ± 560 (S.D.). There were no indications that the molecular weight was dependent on the speed of the ultracentrifuge or the concentration of the sample.

Circular dichroism of protein A

In the near-u.v. region a minimum can be seen at 267nm (Fig. 4). This may be assigned to the phenylalanine residue in the protein. The bands that are observed at 257, 260 and 264nm in *N*-acetylphenylalanine (Horwitz *et al.*, 1969) cannot be seen in protein C, most likely because the protein only contains one phenylalanine residue out of approx. 152 residues.

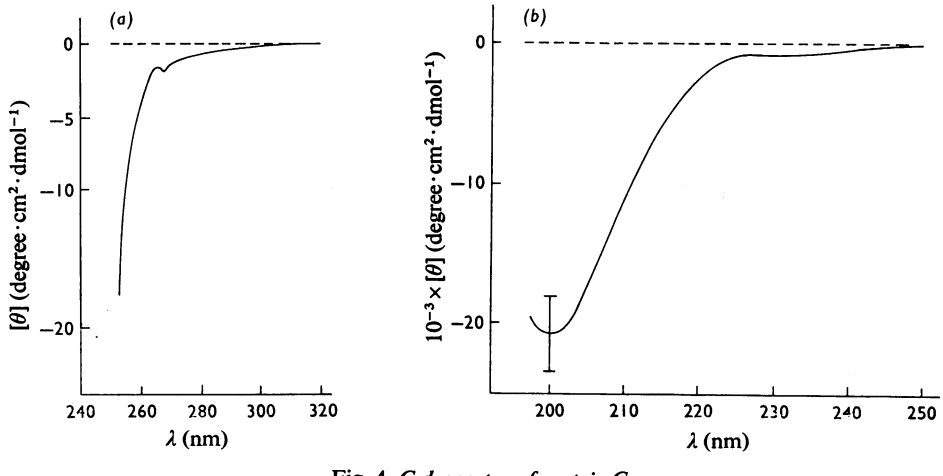

Fig. 4. *C.d. spectra of protein C*

In the near-u.v. region (250–320nm; *a*) the spectrum was recorded in a cell with an optical path of 1cm of a solution containing 4.9mg of protein/ml of water. In the far-u.v. region (190–250nm; *b*) a cell with an optical path of 1mm was used and the concentration of the solution was 0.098mg/ml. Duplicate scans were made of each solution. The magnitude of the error at 200nm is indicated by a vertical bar. The error in the near-u.v. region was estimated to be 0.2 degrees·cm²·dmol⁻¹. ——, Spectrum of the protein solution; ----, blank recording.

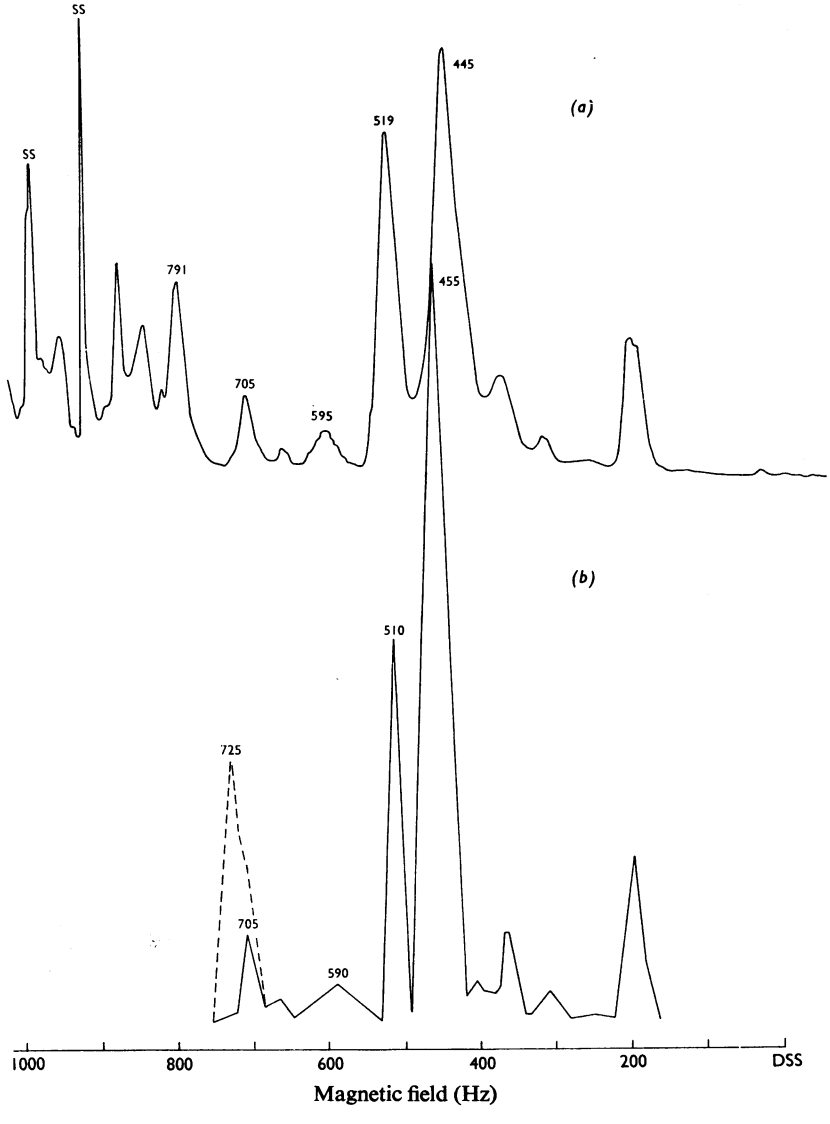

Fig. 5. *N.m.r. spectrum of protein C*

Only the part upfield to the H_2O resonance is illustrated. All measurements are in Hz downfield to the 2,2-dimethylsilyltetradeuteriopropionic acid (DSS) signal. (*a*) N.m.r. spectrum of a solution containing 9mg of protein/ml of ^2H_2O. (*b*) ——, Spectrum computed as described in the text, excluding the resonances from the proton on the δ-carbon in proline. The broken line in the region of 700–750Hz indicates the appearance of the spectrum if the resonances arising from δH in proline is included. The resonance position of a number of signals have been indicated. SS denotes spinning side bands. For details, see the text.

In the far-u.v. region in aqueous solution there is a minimum at 200nm. The mean residue ellipticity at this wavelength is 21×10^3 degrees·cm^2·dmol^{-1}. When protein C was dissolved in the various buffers outlined in the Experimental section there were no obvious changes in the spectra. The mean residue ellipticity at 200nm in these spectra varied from 18×10^3 to 21×10^3 degrees·cm^2·dmol^{-1}.

The spectra recorded in the far-u.v. region of protein A were very similar to the previously recorded spectrum of protein A in water (Bennick, 1975). The mean residue ellipticity at the minimum in the far-u.v.

region (198–200nm) varied from 24×10^3 to 27×10^3 degrees·cm^2·dmol^{-1}. These values do not appear to be different from the value of 28×10^3 degrees·cm^2·dmol^{-1} obtained for the minimum in the far-u.v. region for protein A in water (Bennick, 1975).

N.m.r. spectroscopy

A spectrum of protein C dissolved in 5mM-$C^2H_3CO_2{}^2H$/NaO^2H buffer, nominal pH6.0, is illustrated in Fig. 5. This spectrum was very similar to a previously recorded spectrum of protein A (Bennick, 1975), except that the intensities of the resonances corresponding to protons in proline, glutamic acid and glycine relative to the resonances of protons in the hydrophobic residues were different in proteins A and C. This variation in relative intensities can be explained by the differences in amino acid composition of the two proteins. The recorded spectrum also agrees well with a spectrum computed as described by McDonald & Phillips (1969), although the same discrepancies were seen as observed with protein A. Thus the intensity of the resonance at 444Hz in the recorded spectrum is considerably lower than predicted. This may be explained by small changes in the position of the overlapping resonances from the protons on the γ- and β-carbon atoms in proline and the protons on the β-carbon in glutamic acid. As has been observed in protein A (Bennick, 1975) no resonance corresponding to δ-protons in proline could be seen, as predicted by McDonald & Phillips (1969), at 725Hz.

When 1mM-$CaCl_2$ or 1M-NaCl was added to the buffer, no changes could be observed in the spectrum.

When protein C was dissolved in 10mM-$C^2H_3CO_2{}^2H$ the signal at 595Hz was shifted downfield to 615Hz. No other changes were seen in the spectrum.

Spectra recorded of protein A in 5mM-$C^2H_3CO_2{}^2H$/NaO^2H, pH6.0, before and after addition of 1mM-$CaCl_2$ or 1M-NaCl did not show any differences from the spectrum of protein A dissolved in ^2H_2O, except that the resonance at 7Hz was not present (Bennick, 1975). Since this resonance could not be demonstrated reproducibly, it appears that it was due to a contaminant of the solution used to record the previously published spectrum.

When protein A was dissolved in 10mM-$C^2H_3CO_2{}^2H$ the resonance at 595Hz had shifted downfield to 615Hz.

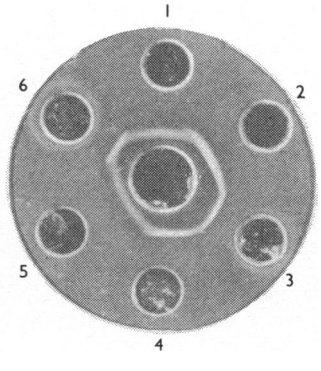

Fig. 6. *Double immunodiffusion of salivary proteins against antiserum to protein C*

A 1% agar gel containing diethylbarbituric acid/sodium diethylbarbiturate buffer, pH8.6, *I* 0.02, was used. The contents of the wells are as follows: centre well, antiserum to protein C; well 1, protein A; well 2, protein C; well 3, parotid saliva; well 4, submandibular saliva; well 5, protein A; well 6, parotid saliva.

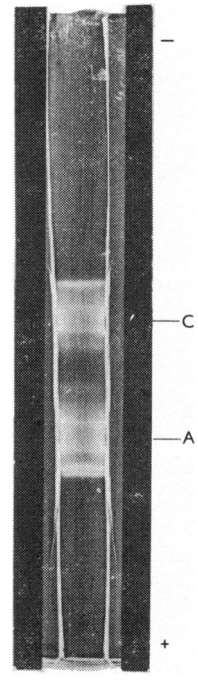

Fig. 7. *Polyacrylamide-gel electrophoresis of stimulated parotid saliva followed by immunodiffusion*

Electrophoresis of 100μl of parotid saliva was performed on polyacrylamide gels polymerized between concentrically placed cylindrical glass tubes as described in the text. Proteins migrating towards the anode in 0.1M-Tris/HCl buffer, pH9.2, have been separated. After electrophoresis the inner glass tube was removed and replaced by an agar gel containing antiserum to protein C. The Figure illustrates the appearance of the gel after immunodiffusion at 20°C. A and C indicate the location of precipitated bands corresponding to the location of proteins A and C. Note that in addition to these major bands of immunoprecipitate, minor bands are also present.

Reactivity with antiserum to protein C

Double immunodiffusion in Ouchterlony plates in which antiserum to protein C was placed in the centre well (Fig. 6) gave a single precipitation line with protein C, which was continuous with precipitation lines with unfractionated parotid and submandibular saliva. A precipitation line also formed with protein A, but this precipitation line only showed reaction of partial identity with protein C, since the line formed with protein C showed spurring over the line formed with protein A.

When polyacrylamide-gel electrophoresis of unfractionated stimulated and unstimulated parotid and submandibular saliva was followed by immunodiffusion in the second dimension, discrete bands of immunoprecipitate were seen in the agar gel corresponding to the location of proteins A and C in the polyacrylamide gels (Fig. 7). The number of bands observed depends on the amount of saliva electrophoresed. Precipitation reactions could not be observed in regions of the gel apart from the one containing proteins A and C, and in double immunodiffusion no reactivity of proteins remaining at the origin could be observed with antiserum to protein C.

Discussion

A previously published method for purification of protein C made it possible to prepare about 5mg of protein C at a time (Bennick & Connell, 1971). The present method allows preparation of about 50mg of protein C at a time, and it should be possible to scale up the present method to allow purification of larger quantities.

The amino acid composition of protein C is similar to previously published results (Bennick & Connell, 1971) and the contents of most of the amino acids are integral values, although there are some exceptions, notably in the amounts of arginine, aspartic acid and glycine. Although other criteria such as polyacrylamide-gel electrophoresis and ultracentrifugation indicate a high degree of purity, the amino acid analysis suggests the possibility that the preparation may contain two or more proteins that are extremely similar.

If the amino acid compositions of proteins A and C are based on the minimum molecular weight of the proteins, it is apparent that both proteins lack threonine, tyrosine, tryptophan and sulphur-containing amino acids. Moreover, both proteins contain the same amount of alanine, valine, isoleucine, leucine and phenylalanine. The main difference in the composition is due to an additional amount of glutamic acid, proline and glycine in protein C. The *N*-terminal residues on proteins A and C are both blocked, but whereas a *C*-terminal sequence of -Ser-Gly-Pro-Gln is proposed for protein C it was found to be -Gln-Gly-Arg-Arg for protein A. The similarity in amino acid composition of proteins A and C suggest that there are stretches of identical sequences in proteins A and C; the higher molecular weight of protein C and the differences in the *C*-terminal sequences of proteins A and C indicate that the difference between the proteins is at least partly due to the presence of an additional length of polypeptide chain at the *C*-terminus of protein C.

The similarity of proteins A and C is also apparent from the identical immunological reaction of these proteins with antiserum to protein A (Bennick, 1976). The immunological reaction of proteins A and C with antiserum to protein C indicates the presence of antigenic determinants on protein C not present in protein A. This is in agreement with the higher molecular weight of protein C.

Levine *et al.* (1973) have demonstrated the presence of the polypeptide precursor of a glycoprotein in stimulated human parotid saliva. In unstimulated parotid saliva they demonstrated the presence of the corresponding glycoprotein. The glycosylation thus appeared to be dependent on the flow rate of the secretion. Since no difference could be found between the immunoelectrophoretic patterns developed with antiserum to protein C of unstimulated and stimulated parotid saliva, there is no indication that proteins A and C are polypeptide precursors of glycoproteins which would have glycosylation rates depending on flow rates of the saliva.

The small amount of glucose found in proteins A and C, and the absence of hexosamine, suggest that the carbohydrate is a contaminant, although it is possible that protein C could be an unusual glycoprotein.

The 2:1 molar ratio of phosphorus/protein in protein C is the same as previously found in protein A (Bennick, 1975). The release of phosphorus from protein C on digestion with NaOH or alkaline phosphatase indicates that phosphorus is present as phosphate linked in an ester linkage to the protein. The phosphate is probably present as phosphoserine. Experiments (A. Bennick, unpublished work) have shown that proteins A and C have high tendencies to be adsorbed on hydroxyapatite. This may be due to the phosphate, since phosphoproteins have high tendencies to be adsorbed on hydroxyapatite.

The minimum mol.wt. of 15460 calculated from the chemical analyses is close to the value of 16340 obtained from ultracentrifugation.

The c.d. spectrum of protein C in a solution with neutral pH is very similar to that of protein A (Bennick, 1975), except that the mean residue ellipticity at the minimum in the far-u.v. region is of somewhat smaller magnitude than that obtained for protein A. As previously discussed for protein A (Bennick, 1975), the spectrum does not give any evidence for the presence of secondary structure in

protein C, including polyproline and collagen-like structure. This does not exclude the possibility that such conformations are present in the protein, since a spectrum such as that recorded for protein C could be obtained from a protein in which part of the polypeptide chain was folded into a polyproline helix whereas other parts lacked secondary structure.

Since proteins A and C are acidic proteins, it might be expected that a conformational change would occur in these proteins when the pH of the solutions was lowered, owing to protonation of negative charges on the proteins. No conformational changes could be seen in solutions of proteins A and C at pH 3.5. Proteins A and C have been shown to be Ca^{2+}-binding proteins (Bennick, 1975, 1977), but no conformational changes could be observed in proteins A and C at concentrations of Ca^{2+} that would ensure saturation of the proteins with Ca^{2+}. Similarly there were no conformational changes in solutions containing univalent ions with ionic strength similar to that of saliva. These results suggest that the negative charges in proteins A and C are not necessary to maintain the conformation of the proteins.

The n.m.r. spectrum of protein C recorded at neutral pH is very similar to that obtained previously for protein A and as discussed does not indicate the presence of polyproline (Bennick, 1975). It is suggested that the resonance at 791 Hz is due to the δ-protons in proline because of the position of this resonance between 760 and 875 Hz in low-molecular-weight derivatives of proline (Madison & Schellman, 1970). The lack of spectral changes in the protein owing to the presence of Ca^{2+} or high concentrations of NaCl also suggests that there are no conformational changes in the protein under these conditions. The shift in the position of one resonance from 595 Hz to 615 Hz on lowering of the pH of the solution suggests that this resonance is due to β-protons in aspartic acid, particularly since the resonance position for β-protons in aspartic acid is predicted to be 590 Hz (McDonald & Phillips, 1969).

The analysis for γ-carboxyglutamic acid was undertaken because of the importance of this residue in the Ca^{2+} binding in prothrombin (Magnusson *et al.*, 1974; Stenflo *et al.*, 1974; Nelsestuen *et al.*, 1974) and the demonstration of γ-carboxyglutamic acid in proteins from bone which bind strongly to hydroxyapatite (Price *et al.*, 1976). The absence of γ-carboxyglutamic acid from proteins A and C indicates that the binding sites in these proteins are different from the Ca^{2+}-binding site in prothrombin.

The chemical and physical data obtained for proteins A and C indicate that the two proteins are closely related to each other. Protein A is very similar and probably identical with either protein III or IV isolated by Oppenheim *et al.* (1971), and protein C is probably identical with either protein I or II also isolated by Oppenheim *et al.* (1971). These proteins have been shown to belong to a group of genetically related proteins (Azen & Denniston, 1974) which apparently were present in parotid as well as submandibular saliva. This suggestion was based on the presence of proteins with similar and unusual staining characteristics in both secretions. The results of the present immunoelectrophoresis experiments clearly demonstrate the presence of protein C in both submandibular and parotid secretions, as found previously for protein A (Bennick, 1976). The present experiments also demonstrate that there are a number of minor proteins migrating close to proteins A and C which also react with the antiserum, although it is difficult to say how many immunoreactive proteins there are present. Hay & Oppenheim (1974) have observed the presence of a number of minor proline-rich proteins in addition to proteins I, II, III and IV isolated by Oppenheim *et al.* (1971).

Rat salivary secretions and salivary-gland secretory granules contain proteins remarkably similar in compositions to proteins A and C (Keller *et al.*, 1975; Robinovitch *et al.*, 1975; Wallach *et al.*, 1975; Muenzer *et al.*, 1976).

The compositions of proteins A and C are also in many respects similar to that of enamel protein and collagen. This is further borne out by the presence of a collagen sequence -Gly-Pro-Gln at the *C*-terminus of protein C and the digestibility of both proteins by collagenase (Bennick, 1976, 1977). It is therefore desirable to establish the primary structure of proteins A and C.

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