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Chemical Studies of Tissue Polypeptide Antigen (TPA). III.* On the Nature of the Antigenic Determinant(s) of TPA Subfraction B_1

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Tissue Polypeptide Antigen (TPA)*** which is a protein isolated from *e.g.* human carcinoma cells, has previously been separated into subfractions and studied with biochemical methods. Gel diffusion studies show that the antigenic determinants are retained through the isolation and purification procedures. Specific modifications of the amino acid residues lysine, tyrosine, trytophan and arginine in subfraction B_1 have been related to the change in the capacity of the antigen to bind to horse anti-HeLa serum. Complete although reversible loss of binding capacity resulted from blocking of arginine and a minor loss was noted upon modification of tyrosine. No measurable influence was noted upon modification of lysine or tryptophan. No cysteine has been detected in subfraction B_1. Circular dichroism measurements show that TPA subfraction B_1 is largely α-helical in solution, and that no correlation could be detected between antigenic activity and conformation.

Studies on the biochemical properties of subfractions of tissue polypeptide antigen (TPA) were recently published.[1] The main subfraction B_1, with an apparent M_r of 4.3×10^4, was extensively studied. Sedimentation analysis showed that subfraction B_1 in 0.01 M NaOH appears as a dimer with an unusually high frictional ratio, indicating an elongated arrangement of the protein.

Attempts in relating antibody saturation to modification of particular amino acids as well as to changes in secondary and tertiary structure have been published for carcinoembryonic antigen (CEA).[2] A similar approach has been applied in the present investigation on tissue polypeptide antigen (TPA). Since only part of the primary structure of subfraction B_1 is known, attempts were made to induce quantitative modification in order to reveal if certain amino acids *per se* participate in any antigenically important sites.

Circular dichroism measurements were made to elucidate whether any gross conformational changes accompanied the chemically induced changes of the antigen-antibody reaction.

MATERIALS AND METHODS

All chemicals used were of analytical grade or better. Ultraviolet spectra were measured with a Beckman DB-G spectrophotometer, fluorescence was measured with an Aminco Bowman spectrofluorometer, model 4-8106, and the intensity of the emitted light given in arbitrary units. CD was measured with a Jasco Model J 41 A spectropolarimeter.

Antigen. Tissue polypeptide antigen was obtained from a pool of at least 30 different human carcinoma tumors of different sites and types and purified as described elsewhere.[3,4,1] In principle the homogenized tumor tissue after defatting with ether and removal of soluble proteins (Washed Tissue

* For Part II in this series, see Ref. 26.

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*** *Abbreviations used.* TPA, tissue polypeptide antigen; PAGE, polyacrylamidegelelectrophoresis; SDS sodium dodecyl sulfate; TPCK, L-1-tosylamido-2-phenylethylchloromethylketone; TAME, *p*-tosyl-L-argininemethylester. HCl.

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Powder, WTP) is extracted at pH 9.5 with water. The extract, adjusted to pH 7.5, is heated to 90 °C and then passed through a column of Biogel A-50 m. The protein mid fraction (M_r 15 × 10^6) is precipitated at pH 5 and the precipitate eluted with a downward pH gradient in a Sephadex G 15 column.[4] The fraction eluted between pH 4.5 and 3, containing 2 % of the protein and 20 – 50 % of the antigenic activity, was collected and denoted TPA. This TPA was separated in the presence of SDS on Sephadex G 200 into four fractions, designated A, B_1, B_2 and C. The latter three fractions gave the same slope ratio and minimum diameter in the hemagglutination inhibition test (see below).

In this investigation subfraction B_1 with an apparent M_r of 4.3 × 10^4 was used. This material has a specific TPA activity (see below) of 3 – 5 units per μg (U/μg). In a few experiments subfraction C with a specific activity of 2 – 3 U/μg was used.

Immune serum. Horse anti-HeLa serum was prepared by *i.v.* injections over 7 months of 1 g of insoluble residue of HeLa cells.[5] After 15 months 4 *i.m.* injections of 40 mg of the same antigen in Freunds adjuvant were given biweekly and one bleeding (Nov. 26, 1962) was used throughout.

The horse serum was absorbed with sheep red cells, then with 40 mg lyophilized human plasma that had previously been absorbed with sheep red cells. After removal of precipitates the serum was passed through a Biogel A-0.5m column. The antibody, agglutinating sheep red cells tanned and labeled with TPA, was recovered in the IgG fraction.

Rabbit anti-subfraction B_1 serum was prepared by three *i.m.* injections once a month. The first inoculate consisted of 130 μg subfraction B_1 in complete Freunds adjuvant and the following of 65 μg subfraction B_1 in incomplete Freunds adjuvant. Immune serum was prepared from a bleeding 12 days after the third inoculation.

Characterization of antigen and antibody by double immunodiffusion. Agarose gel (Biorad), 1 % in phosphate buffered saline with 0.01 % thimerosal (Elanco) pH 7.5, was layered on plastic plates (8 × 9 cm, LKB Multiphor). Antigens used: HeLa cells (3 × 10^7 cells) and homogenized tissues (0.37 g wet weight of liver met. from four individuals)* were washed five times in 1 ml distilled water and then extracted with 1 ml of 0.15 M NaOH each at 20 °C for one hour. After centrifugation (5000 × *g* at 5 °C for 20 min) the supernatants were collected, and pH adjusted to 7.5. WTP extract, before and after heat treatment, Biogel A-50m column inlay, TPA (after pH gradient elution) and TPA subfraction B_1, B_2 and C (SDS removed)[1] were also used.

Double diffusion patterns (Figs. 1a and 1b) against horse anti-HeLa serum (absorbed with 40 mg human plasma per ml serum) show a major precipitin line of identity through the tumor- and

* Liver metastases originating from the following primary carcinomas: mamma and ventricle, mamma, ventricle, and intestine.

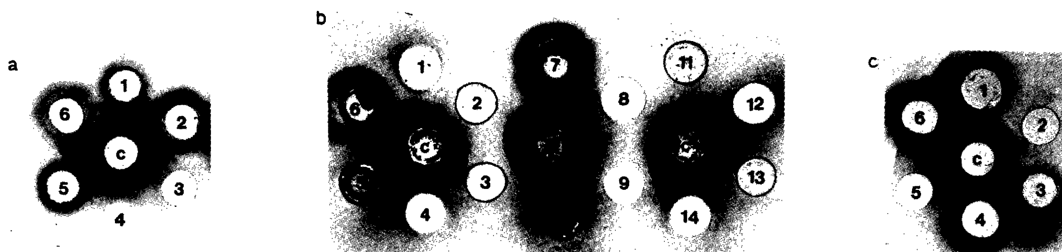

Fig. 1. Double diffusion analysis of TPA preparations. (a). *Central well:* 40 μl of horse anti-HeLa serum; *well 1 and 5:* 20 μl of tumor extract (from 0.37 g wet tumors/ml); *2:* 20 μl of HeLa cell extract (3 × 10^7 cells/ml); *3:* 20 μl of subfraction B_1 (1.2 mg/ml); *4:* 20 μl of WTP extract, Biogel A-50m inlay (10 mg/ml); *6:* 10 μl of normal human serum. (b). *Central wells:* 60 μl of horse anti-HeLa serum, absorbed with lyophilized human plasma (40 mg/ml) from several individuals; *wells 1 and 4:* 8 μl of normal human serum; *2, 3, 11 and 13:* 60 μl of subfraction B_1 (1 mg/ml); *5 and 10:* 60 μl of WTP extract, not heat treated (10 mg/ml); *6 and 7:* 60 μl of WTP extract, heat treated (10 mg/ml); *8 and 9:* 60 μl of WTP extract, Biogel A-50m inlay (10 mg/ml); *12 and 14:* 60 μl of TPA (after pH gradient elution) (1.3 mg/ml). (c). *Central well:* 40 μl of rabbit anti-subfraction B_1 serum; *well 1:* 60 μl of subfraction B_1 (1.2 mg/ml); *2:* 60 μl of subfraction B_2 (1.6 mg/ml); *3:* 60 μl of subfraction C (1.6 mg/ml); *4 and 6:* 10 μl of normal human serum.

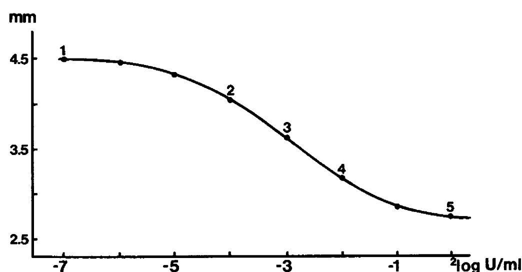

Fig. 2. Standard curve in hemagglutination inhibition test, diameter versus ^{2}log U/ml. Point 1 represents the max. diameter obtained at no inhibition of the antibody; points 2, 3 and 4 are situated at the linear part of the curve, used for determination of the activity; point 5 shows the level of complete specific inhibition.

HeLa-extracts, WTP-extracts, TPA and subfraction B_1. The heat treatment of the WTP extract does not alter the precipitin line. The tumor- and HeLa-extracts show precipitin lines that are not related to purified TPA. None of the precipitin lines shown by TPA active materials crossreact with normal human serum. Rabbit anti-subfraction B_1 serum gives one precipitin line of identity between subfractions B_1, B_2 and C (Fig. 1c).

The occasionally appearing double lines between purified TPA preparations and anti-HeLa serum could be assigned to aggregation of TPA molecules rather than to the presence of other antigens capable of precipitating anti-HeLa serum.

Determination of antigenic activity. The TPA-activity was estimated in a heterologous system by means of a standardized hemagglutination inhibition test,[3,6] labeling antigen: TPA obtained by pH gradient elution, antiserum: horse anti-HeLa serum treated as described above. The test was performed on two-step dilutions of the samples in micro titration plates (Linbro IS-MRC-96) with spherical bottoms. The diameters of the hemagglutination patterns for the series of dilutions of each sample were plotted against $^2\log c_{\text{antigen}}$ (Fig. 2), expressed in U/ml. Using the definition of a hemagglutination unit (U) given by Björklund [3] the point of 50 % inhibition is found to correspond to a concentration of 1/8 unit TPA per ml. The slope of the steepest part of the curve was assumed to be dependent on the affinity between antigen and antibody. Incomplete inhibition manifested by a higher minimum diameter of the hemagglutination pattern may reflect the lack of certain determinants in the antigen tested in relation to the antigen used as labeling antigen. The ratio between the slope for a sample and that of standard TPA in the same experiment was always determined. In intra- and interassay reproducibility determined by repeated testing of a solution of purified antigen is $\pm 10\%$ of the value.

Amino acid analysis. HCl hydrolysates were analyzed with the aid of an automatic amino acid analyzer (Jeol, JLC-6AH), using a 30 cm column of Durrum DC-6A and the Durrum Pico Buffer System II (both from Pierce, Rockford, Ill.). The buffers were supplied to the column through an ammonia filtration system. Hydrolysis of subfraction B_1 modified by 1,2-cyclohexanedione was performed in the presence of mercaptoacetic acid.[7]

p-Toluenesulfonic acid hydrolysis.[8] Subfraction B_1 was hydrolyzed for 18 h at 110 °C with 3 M p-toluenesulphonic acid in presence of 0.2 % tryptamine in evacuated ampoules.

Electrophoresis in polyacrylamide gel. Gels were 4.5 × 110 mm. Running gel: 7 %, 0.1 % crosslink, 0.75 M Tris-HCl, pH 8.6. Stacking gel: 2.5 %, 0.63 % crosslink, 0.12 M Tris-HCl, pH 6.8. Inlay: 30 μg protein in 25 μl of 0.01 M NH_4HCO_3, 8 M urea. Bromophenol Blue (BFB) was used as inner reference, staining with Coomassie Blue. For determination of TPA activity gels were cut into slices 1.3 and 5 mm thick, which were extracted with 0.3 and 1.0 ml 0.1 M NH_4HCO_3, respectively.

Reaction of TPA with anhydrides of succinic, maleic and citraconic acid.[9,10,11] Subfraction B_1 and C (0.2 – 1.0 mg) were dissolved in 1 ml each of 1 M sodium bicarbonate, 8 M urea and the pH adjusted to 9.0 (NaOH). Eight 25 μl portions of a 25 % anhydride (succinic, maleic or citraconic) solution in acetone were added under vigorous stirring over 10 min, keeping pH above 8.8. The reaction mixture was desalted with Sephadex G 25 (Pharmacia, Uppsala) in 0.1 M NH_4HCO_3 and lyophilized. Subfractions B_1 and C, treated with 1 % SDS in 0.1 M NH_4HCO_3 for 15 min at 100 °C, were acylated in the same way as described above.

De-acylation of citraconyl TPA.[11] Subfraction B_1 (100 μg) citraconylated in the presence of SDS was incubated at 20 °C for 2 h in 10 M urea, pH 2.5 (HCl), neutralized with solid NH_4HCO_3 and desalted with Sephadex G 25 in 0.1 M NH_4HCO_3.

Dansylation.[12] Subfraction B_1 (40 μg) was incubated in 1 ml 1 M Na_2CO_3 pH 9.0, 8 M urea, mixed with 200 μl of 10 % dansyl chloride in acetone,

at 20 °C for 1 h and 4 °C for another 18 h, and desalted with Sephadex G 25 in 0.1 M NH_4HCO_3.

Acylation of subfraction B_1 with N-succinimidyl-3[4-hydroxy-5(^{125}I)-iodophenyl] propionate (BHR).[13] Subfraction B_1 (200 μg, 4 nmol) was dissolved in 100 μl 0.01 M NaOH. After the addition of 2 mg $NaHCO_3$, the solution was transferred to a glass tube in which 1 mCi (0.5 nmol) of BHR had been distributed in a thin layer by evaporation of a benzene solution. The tube was stoppered and shaken gently for 25 min at 0 °C. After the addition of 3 mg glycine the reaction mixture was desalted in 0.2 M NH_4HCO_3 with Sephadex G 25.

Nitration.[14] Subfraction B_1 (35 μg) in 0.5 ml 0.1 M $NaHCO_3$ 8 M urea pH 8.4 was incubated with 25 μl of a 16% solution of tetranitromethane in ethanol for 85 min at 20 °C, and then desalted with Sephadex G 25 in 0.1 M NH_4HCO_3.

Iodination with KI_3.[15,16] Freshly prepared KI_3 solutions (2.0, 0.2, 0.02, 0.002 and 0 mM in 0.1 M Na_2CO_3 pH 9.2) were rapidly mixed with equal portions (50 μl) of a solution containing subfraction B_1 (15 μg in 0.1 M Na_2CO_3 pH 9.2). The mixtures were incubated for 1 h at 20 °C and were then immediately diluted in 1% human serum solution and tested for TPA activity.

Iodination with ^{125}I in presence of chloramine-T.[17] The antigen (90 μg) was dissolved in 100 μl 0.02 M NaOH followed by the addition of 2 mg $NaHCO_3$. This solution was divided into three aliquots, the pH values of which were adjusted to 7.4, 7.7 and 8.5, respectively. These solutions were radioiodinated by a standard method[17] using sequential addition of reagents. TPA was exposed to the oxidizing conditions for only 15 sec. The iodinated protein was desalted in 0.2 M NH_4HCO_3 with Sephadex G 25 within 5 min from the start of the reaction.

Reaction of subfraction B_1 with dimethyl-(2-hydroxy-5-nitrobenzyl)sulfonium bromide.[18] Subfraction B_1 (750 μg) and human serum albumin (HSA) (300 μg) were dissolved in 1 ml portions of 0.1 M HCOOH 8 M urea pH 3.5. To each protein solution, 10 mg of the alkylating reagent was added and reaction was allowed to proceed for 3 h at 20 °C. The alkylated proteins were immediately desalted with Sephadex G 25 (2.5 x 35 cm) in 0.1 M HCOOH and lyophilized.

BrCN-cleavage.[19] Aliquots of subfraction B_1 and alkylated subfraction B_1 (300 μg) were dissolved in 0.5 ml portions of 70% HCOOH, solid BrCN (5 mg) was added and the solutions incubated for 24 h at 20 °C. After lyophilization they were filtered through Sephadex G 75 in 0.1 M HCOOH.

Reaction with diazomethane.[20] To a solution of 75 μg of subfraction B_1 in 2 ml of 0.03 M HCl in 85% ethanol, 800 μl 3% solution of diazomethane in ether was added and the mixture kept for 4 h at −2 °C. The pH rose from 1.97 to 2.15. The solution was evaporated to 0.2 ml and diluted with 0.5 ml 0.1 M HCOOH, 8 M urea prior to activity measurement.

Transformation of arginine residues in subfraction B_1 to N^5-(4-oxo-1,3-diaspiro[4.4]non-2-ylidene)-L-ornithine[21] *(CHD-subfraction B_1).* To a solution of 50 μg subfraction B_1 in 0.5 ml 0.2 M NaOH, 10 mg of 1,2-cyclohexanedione was added and the mixture kept for 3 h at 20 °C, pH was adjusted to 8.4 ($NaHCO_3$) and the modified protein desalted with Sephadex G 25 in 0.1 M NH_4HCO_3.

Transformation of arginine residues in subfraction B_1 to N^7,N^8-(1,2-dihydroxycyclohex-1,2-ylene)-L-arginine[7] *(DHCH-subfraction B_1).* Four aliquots of subfraction B_1 (400 μg) were dissolved in 1.0 ml 0.25 M borate buffer pH 9.0. To these solutions, 1,2-cyclohexanedione in 2.0 ml portions of 0.25 M borate buffer pH 9.0 was added to give molar ratios between reagent and arginine residues, ranging from 40 to 4000. The reaction mixtures were incubated for 2 h at 36 °C and desalted with Sephadex G 25 in 0.2 M NH_4HCO_3.

Table 1. De-blocking of DHCH-subfraction B_1 at 36 °C.

Composition of deblocking solution in 8 M urea	Reaction time (h)	Per cent restored arginine	Per cent restored TPA-activity	Slope ratio
0.05 M NH_2OH in 0.2 M NH_4HCO_3	2	51	26	1.00
0.50 M NH_2OH in 0.2 M NH_4HCO_3	2	56	41	1.00
0.37 M NH_2OH in 0.1 M $NaHCO_3$ 7% HS $(CH_2)_2OH$	16	77	50	1.00

Table 2. Activity and slope ratio of acylated TPA subfractions.

Subfraction	Acyl	Specific activity %		Yield [a]	Slope ratio in activity measurement
SDS treated	–	3.1	100	100 [b]	1.00
Subfraction B_1	Succinyl	2.7	87	62	1.00
	Maleyl	2.6	84	59	1.00
	Citraconyl	2.7	87	62	1.00
Subfraction B_1	–	3.1		100 [b]	1.00
	Succinyl	n.d.		53	1.00
	Citraconyl	n.d.		53	1.00
SDS treated	–	2.5		100 [b]	1.00
Subfraction C	Succinyl	n.d.		59	0.93
	Citraconyl	n.d.		59	0.93
Subfraction C	–	2.5		100 [b]	1.00
	Succinyl	n.d.		42	1.00
	Citraconyl	n.d.		33	1.00

[a] % TPA activity after acylation. [b] By definition.

De-blocking of DHCH-subfraction B_1 with hydroxylamine.[7] Aliquots of subfraction B_1 (300 μg) were reacted to form DHCH-subfraction B_1 (88 % modification) as described above. The lyophilized completely inactive portions of DHCH-subfraction B_1 were dissolved in 0.5 ml portions of three different de-blocking solutions listed in Table 1, incubated at 36 °C and desalted with Sephadex G 25 in 0.2 M NH_4HCO_3.

Trypsine cleavage of DHCH-subfraction B_1. Aliquots (1.5 mg) of subfraction B_1 and DHCH-subfraction B_1 were dissolved in 3.5 ml each of 0.1 M $NaHCO_3$ with 0.01 M $CaCl_2$, pH 8.4. To each of the TPA preparations, 100 μl of TPCK-trypsine solution (0.6 mg/ml, 107 IU TAME/mg) was added and the mixtures were incubated for 1 h at 35 °C. Urea (1.8 g), mercaptoethanol (300 μl) and hydroxylammonium chloride (63 mg) were added successively, followed by an incubation for 16 h at 37 °C.

Table 3. Mobility in PAGE of acylated TPA-preparations.

BAND	Succinyl B_1	Citraconyl B_1	SDS Succinyl B_1	SDS Citraconyl B_1	Succinyl C	Citraconyl C	SDS-Citraconyl C
I	0.26	0.26	–	–	–	–	–
II	0.44	0.44	0.47	0.47	–	–	–
III	0.47	0.47	0.49	0.49	–	–	–
IV	0.49	0.49	0.52	0.52	–	–	–
V	0.52	0.52	0.57	0.57	–	–	–
VI	0.58	0.58	0.60	0.60	–	–	–
VII	0.62	0.62			–	–	–
VIII	0.67	0.67			–	–	–
IX	–	–	–	–	–	–	0.98
X	–	–	1.0	1.0	1.0	1.0	–

RESULTS

Modification of subfraction B_1 by acylation methods

Subfraction B_1 was acylated with succinic, maleic and citraconic anhydride. An approximate quantitation of the amount of maleylation and citraconylation was obtained by measuring the absorbance at 218 nm, subtracting that of the intact protein and using the absorbance of the corresponding acid as a reference. The protein, at this wavelength, has a low absorbance in comparison with the conjugated chromophores of the modifying groups. The absorbance of the derivatives does not differ markedly from that of the free acids. Portions of 100 μg subfraction B_1, containing approximately 60 nmol lysine, were acylated with maleic and citraconic anhydride yielding in both cases an incorporation of around 80 nmol acyl group. Assuming a quantitative acylation of the amino groups, hydroxyl groups were also acylated.[22] The TPA activity was largely retained as is evident from Table 2. The slope ratios and the minimum diameters were not changed. Electrophoresis of acylated subfraction B_1 was performed at pH 8.6 and the result is presented in Table 3. The distribution of TPA activity over several bands can be explained in view of the differential acylation of hydroxyl groups producing proteins with variation in charge.[22] Upon deacylation of citraconylated subfraction B_1, a 60 % yield of active antigen was separated from the citraconate ions.

After dansylation of subfraction B_1, the amino acid analysis showed a quantitative yield of protein in which the mol-% of lysine had decreased from 6.8 to 3.6 and that of tyrosine from 2.5 to 0.5 mol-%. Polyamide thin layer chromatography of acid hydrolysates[23] of dansylated subfraction B_1 showed massive spots of ε-dansylated lysine and *O*-dansylated tyrosine, without however, giving any evidence for an *N*-terminal amino acid. TPA activity of the dansylated protein was less than 3 %.

Table 4. Iodination of TPA in alkaline medium.

Molar ratio I_2/Tyrosines in TPA	Yield[a] of TPA-activity	Slope ratio
1000	17	0.83
100	54	1.00
10	84	1.17
1	109	1.07
0	100	1.00

[a] In % of starting material.

Subfraction B_1, acylated by Bolton-Hunter technique, contained 70 % of the original TPA activity and a radioactivity of 50 – 85 kBq per μg. The labeled antigen showed a complete inhibition and the same slope ratio as standard TPA in the TPA activity test. The labeled antigen has been used in the development of a radioimmunoassay technique for the determination of TPA activity in various solutions (*e.g.* human serum).[24,25] In that assay, radioiodinated TPA shows a maximum binding capacity of approximately 65 % (B/T) in the presence of excess antibody.

Nitration

After reaction of subfraction B_1 with tetranitromethane, the amino acid analysis showed a quantitative yield of nitrated antigen in which tyrosine alone could no longer be detected. The fluorescence (activation 288 nm, emission 350 nm) of subfraction B_1 decreased to 3 % after nitration and 40 % of the TPA activity was retained. Subfraction

Table 5. Results of iodination of TPA in the presence of chloramine-T.

	Data of ^{125}I–TPA			
Condition[a] of reaction	Yield[b] radioactivity	Yield[c] TPA activity	kBq/U	Slope ratio
pH 7.4	9	70	20	1.00
pH 7.7	9	83	15	1.00
pH 8.5	<1	63	2	1.00

[a] pH at the oxidative step. [b] In %, starting from approx. 40 MBq. [c] In %, starting from 90 U TPA determined in the hemagglutination test.

B_1 treated in the same way but without the addition of tetranitromethane showed no detectable change of its capacity to inhibit the antibody.

Iodination

Subfraction B_1 was iodinated at high pH and the results are summarized in Table 4. The radioactivity and TPA activity of subfraction B_1, radioiodinated in the presence of chloramine-T, are presented in Table 5. The slope ratio and the minimum diameter in the activity test was the same for the three ^{125}I-labeled antigen aliquots as for standard TPA.

Alkylation

Selective electrophilic alkylation of tryptophan in subfraction B_1 and HSA gave rise to proteins with extinction coefficients at 410 nm (pH 10.5)[18] for alkylated subfraction B_1 (M_r 4.3×10^4) and alkylated HSA (M_r 6.8×10^4) which were found to be 3.4×10^4 and 3.5×10^4, respectively, indicating an equal molecular amount of tryptophan alkylated in each protein (one residue per molecule). The TPA activity was not significantly altered by the alkylation. By amino acid analysis of subfraction B_1, hydrolyzed with *p*-toluenesulfonic acid in the presence of tryptamine, the mol-% of tryptophan was found to be 0.3 corresponding to the presence of one residue per molecule of subfraction B_1. Cyanogen bromide cleavage of subfraction B_1 and alkylated subfraction B_1, followed by separation of fragments, gave the result presented in Fig. 3 (*cf.* Ref. 26). The distribution of TPA active fragments is not changed by alkylation whereas the fluorescence of one of the inactive fragments is not detectable after alkylation.

After reaction of subfraction B_1 with diazomethane, the activity had decreased to about 50 % but was unchanged in a reference brough through the reaction steps in absence of diazomethane.

Table 6. Modification of arginine residues in subfraction B_1 with cyclohexanedione at pH 9.0.

Molar excess of cyclohexanedione over available arginines	Per cent[b] decrease of arginine	Per cent decrease of activity	Slope ratio
0[a]	0	0	1.00
40	12	17	1.00
400	54	49	1.00
4000	88	97	1.00

[a] These values apply to a sample which underwent all reaction steps without the addition of cyclohexanedione and are calculated on basis of untreated TPA subfraction B_1. [b] By amino acid analysis.

Reaction with 1,2-cyclohexanedione

The degree of modification of arginine residues in subfraction B_1 after reaction with 1,2-cyclohexanedione and its relation to the TPA

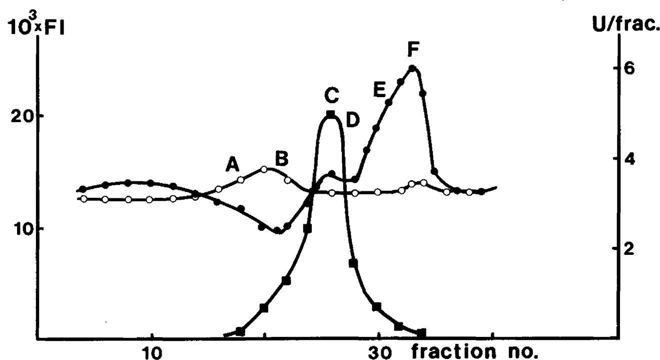

Fig. 3. Gel filtration of BrCN fragments of subfraction B_1 with Sephadex G 75 in 0.1 M formic acid; column: width 1 cm, length 82 cm, fraction volume 1.6 ml. ○, fluorescense, activation 288 nm, emission 350 nm of alkylated subfraction B_1; ●, fluorescense, activation 288 nm, emission 350 nm of non-alkylated subfraction B_1; ■, TPA-activity of alkylated and non-alkylated subfraction B_1 (identical curves).

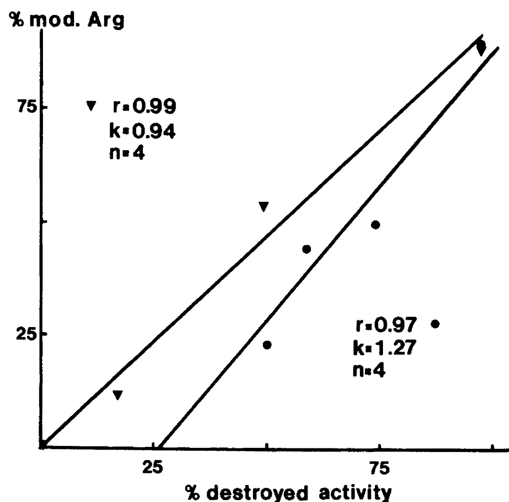

Fig. 4. Relation between % modified arginine in subfraction B_1 and perturbation of TPA activity. ▼, blocking of subfraction B_1; ●, de-blocking of DHCH-subfraction B_1.

activity are presented in Table 6 and Fig. 4. According to amino acid analysis the antigen lost 90% of detectable arginine and simultaneously the antigen lost 99 % of its capacity to react with the antibodies. An aliquot of subfraction B_1 which had undergone the same treatment without the addition of reagent, was largely unimpaired.

The reaction product between the guanidinyl groups and cyclohexandione could be cleaved by hydroxylamine and the yield of restored TPA activity and unblocked arginine residues are presented in Table 1 and Fig. 4. There is a linear ratio between TPA activity and degree of blocking of arginine residues in the blocking as well as in the de-blocking reaction (Fig. 4). The slope ratio and the minimum diameter in the TPA activity measurements were identical for all reaction products and standard TPA.

Subfraction B_1, untreated and blocked with cyclohexanedione, was cleaved with trypsine. After cleavage, attempts were made to restore the activity of the fragments by reaction with hydroxylamine. Most of the TPA activity was abolished in the solution of subfraction B_1, cleaved without preblocking, whereas 10% of the TPA activity was restored in the aliquot that had been blocked with DHCH groups during cleavage (Table 7). The two aliquots were filtered through Sephadex G 50 fine (Figs. 5a and 5b, Table 7). The TPA activity of low M_r fragments was measured after concentration of the solutions.

Circular dichroism measurements

CD was measured for solutions of widely distributed pH values and at different concentrations. The ellipticities are collected in Table 8 and Fig. 6. The data are expressed in mean residue ellipticities, taking 115 for the mean residue weight of subfraction B_1. The appearance of a strong positive band at 192 nm and negative bands at 208 and 222 nm (Figs. 6a and 6b) indicate the presence of a great deal of α-helix.[27,28] Estimates indicate an

Table 7. TPA activity of tryptic fragments of subfraction B_1 and DHCH-subfraction B_1.

Treated material[a]	Yield TPA activity[b]	Slope[c] ratio	M_r[d]
Subfraction B_1	100.0	1.00 C	–
T	<0.4	– –	–
T,S(P I a)	0.1	1.00 C	$2-5\times10^3$
T,S(P I b)	<0.1	0.78 –	2×10^3
DHCH-subfraction B_1	0	– –	–
D	50.0	0.93 C	–
D,T	10.0	1.02 C	–
D,T,S(P II a)	0.7	0.67 I	$10-12\times10^3$
D,T,S(P II b)	1.0	0.56 I	$6-8\times10^3$

[a] T = trypsine cleaved; D = de-blocked; P = pooled and concentrated (see Fig. 5); S = material filtered with Sephadex G 50 fine. [b] % based on starting material. [c] C = complete inhibition; I = incomplete inhibition. [d] Approx. determination by gelfiltration.

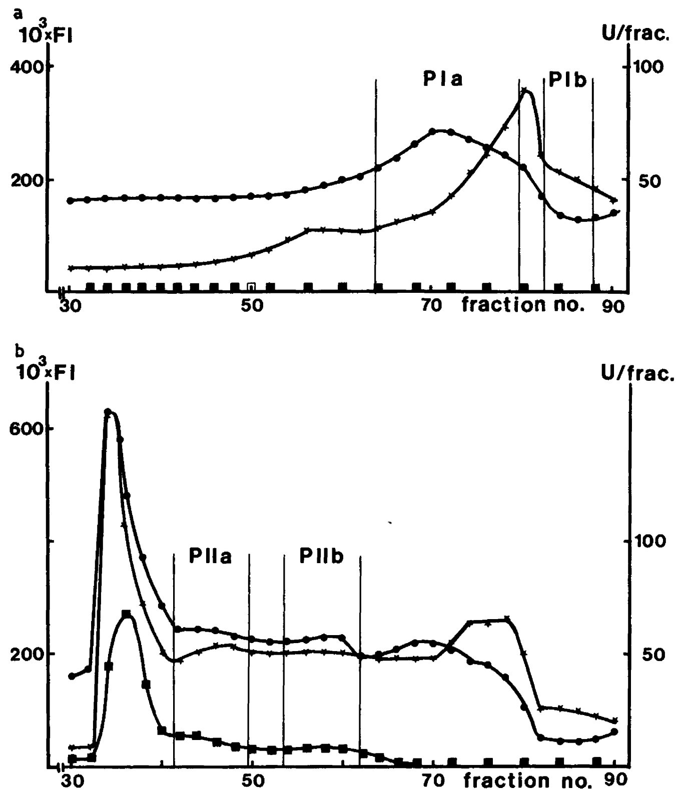

Fig. 5. Gel filtration of trypsine-cleaved subfraction B_1 (a) and DHCH-subfraction B_1 (b), with Sephadex G 50 fine in 0.2 M NH_4HCO_3 at 5 C; column: width 2.5 cm, length 140 cm, fraction volume 8.5 ml.★, fluorescence, activation 288 nm, emission 350 nm; ●, fluorescence, activation 288 nm, emission 312 nm; ■, TPA activity.

amount of around 80% α-helix at pH 2 and 12 in subfraction B_1 whereas only 60% α-helix is present at pH 8.4. Succinylation is accompanied by a rise in α-helix to 70% whereas the stabilization of α-helix is disturbed in the presence of SDS and in the DHCH-derivative. The high value of α-helix is well in agreement with calculations for secondary structure [29,30] made on hitherto sequenced parts of

Table 8. Molar residue ellipticities and α-helical content of TPA preparations.

TPA preparation	Dissolved in[a]	Conc. (μg/ml)	$10^{-3}\times\theta$ (208 nm)[b]	$10^{-3}\times\theta$ (217 nm)[b]	$10^{-3}\times\theta$ (222 nm)[b]	% α-helix
Subfraction B_1	0.01 M HCl	24	29.5	27.8	30.1	88
Subfraction B_1	0.01 M HCl	953	30.2	29.1	30.6	90
Subfraction B_1	0.01 M NaOH	28	24.5	22.2	24.5	71
Subfraction B_1	0.01 M NaOH	1100	27.0	26.1	27.7	79
SDS treated	0.1 M $NaHCO_3$ 1% SDS	31	15.8	11.8	13.0	41
Subfraction B_1	0.1 M $NaHCO_3$ 1% SDS	1235	16.9	13.8	14.1	45
Subfraction B_1	0.1 M $NaHCO_3$	28	20.7	18.0	19.9	58
Succinyl-subfr.B_1	0.1 M $NaHCO_3$	34	25.5	22.5	24.0	74
DHCH-subfraction B_1	0.1 M $NaHCO_3$	36	14.7	12.6	13.8	37

[a] Glass distilled water used. [b] deg. cm^2/d mol.

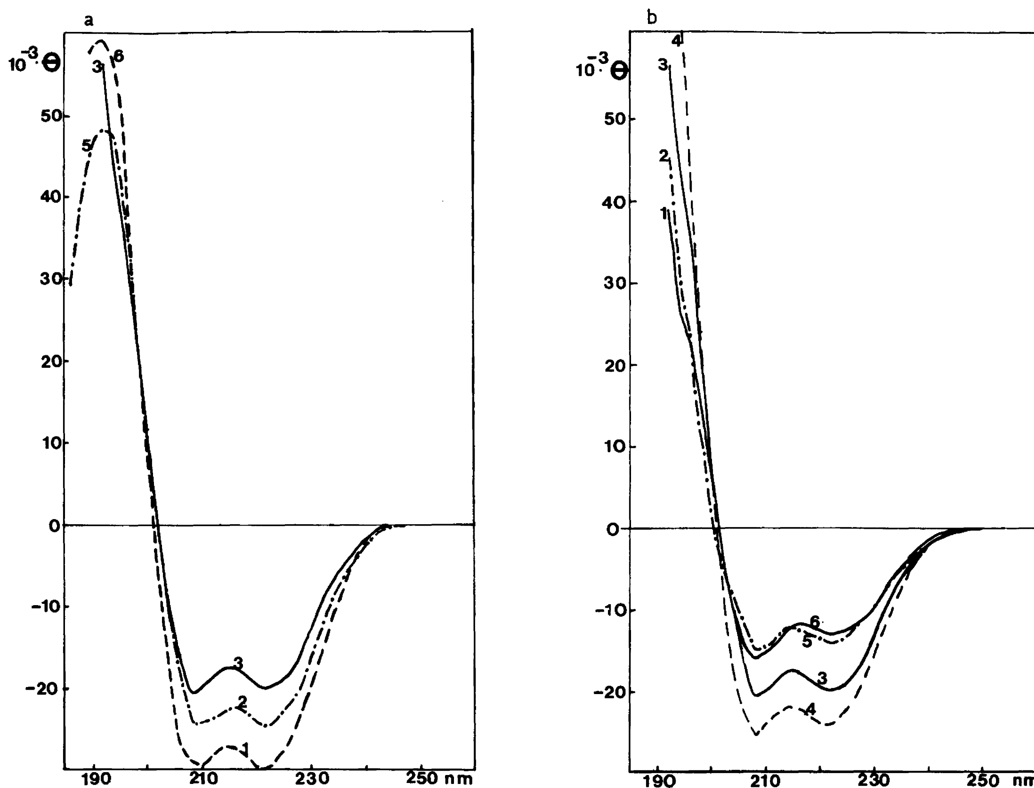

Fig. 6. Circular dichroism curves of TPA preparations: 1, Subfraction B_1, 24 μg/ml in 0.01 M HCl; 2, Subfraction B_1, 28 μg/ml in 0.01 M NaOH; 3, Subfraction B_1, 28 μg/ml in 0.1 M $NaHCO_3$; 4, Succinylated subfraction B_1, 34 μg/ml in 0.1 M $NaHCO_3$; 5, DHCH-subfraction B_1, 36 μg/ml in 0.1 M $NaHCO_3$; 6, SDS-subfraction B_1, 31 μg/ml in 0.1 M $NaHCO_3$, 1% SDS.

TPA [26] (150 amino acid residues) which indicate the presence of 70–80 % α-helix, only minor amounts of β-pleated sheet and 1–2 β-turns. This result is not surprising in view of the high molecular percentage of Glu (17.9), Ala (8.6) and Leu (10.8) in subfraction B_1. There is no clearcut correlation between secondary structure and TPA activity.

DISCUSSION

TPA subfraction B_1 has been found to give rise to antibodies in rabbit which produce a line of identity in gel diffusion between TPA subfractions B_1, B_2 and C. The rabbit anti TPA:B_1 serum gives only one weak line against normal human serum, thereby confirming the immunological purity of the antigen. The subfractions are also indistinguishable by hemagglutination inhibition. Since no differences were found between subfractions B_1, B_2 and C and B_1 is the major reasonably pure product, the latter was chosen for most subsequent work on the chemistry of TPA. The rabbit anti TPA:B_1 serum also has the the ability to detect TPA active structures by immunohistology in cancer cells of various locations [31] as well as in a few other cell types.[32] Therefore the purification procedure would not markedly change the immunological specificity of the protein. A precipitin line of identity (Fig. 1) is also found between products from various steps of preparation, including heat treatment. The finding that the protein is largely α-helical also indicates that the protein is not heavily denaturated. The elongated α-helical structure of TPA:B_1 [1] implies that all groups should be equally accessible for

chemical reactions. This is observed in the blocking of arginine with cyclohexanedione and de-blocking with hydroxylamine, where a linear relationship is obtained between TPA activity and free arginine. The complete inactivation and reactivation attainable, show that all antibody binding sites in TPA:B_1 are dependent, directly or indirectly, of arginine for their function. The excess of cyclohexanedione required for reaction with TPA:B_1, illustrated by a logarithmic relationship between the concentration of reagent and the extent of modification, can be explained in terms of an equilibrium between sterically hindered aggregates and oligomeric TPA:B_1. A hundredfold higher molar excess of reagent has to be used to obtain quantitative modification with TPA:B_1 than with other proteins.[7] The occurrence of polymeric states of TPA:B_1 can also be inferred from the results of sedimentation analysis.[1]

Acylation of lysine residues does not markedly change the activity of TPA:B_1 and the activity is also retained when de-acylating citraconylated TPA:B_1. Dansylation of TPA:B_1 completely removes its activity. This may be due to steric effects of the bulky dansyl group or to sulfonation of tyrosine, the dansyl ester of which constitutes a prominent spot in TLC after HCl hydrolysis. No *N*-terminal amino acid was, however, revealed by this method. The role of tyrosine for the activity is not clear since exhaustive nitration only slightly removed the activity.

Toluenesulfonic acid hydrolysis of TPA:B_1 in the presence of tryptamine showed the presence of one tryptophan residue per TPA:B_1 molecule and Trp was also found to be responsible for the characteristic fluorescence of TPA:B_1. Alkylation of the indole nucleus of Trp did not markedly decrease the activity of TPA:B_1 and subsequent cleavage of the alkylated product, compared with the corresponding cleavage of intact TPA:B_1, showed that a prominent active site in TPA:B_1 is located in a fragment differing from that containing Trp. For a globular protein, having an M_r of around 43 000, one can expect the occurrence of five immunoreactive sites.[33] In TPA:B_1, which has a fibrous character, three sites have so far been found,[26] one in the sequenced part of BrCN fragment B and one in the peptide remaining after sequencing of the same fragment. The third active site is located in BrCN fragment C between amino acids 20 and 50 in a strictly α-helical region (calculated).[29,30] Fragment peptides with partial TPA activity, showing up as a diminished slope ratio in the hemaglutination assay, are obtained by BrCN cleavage as well as in trypsine cleavage of TPA:B_1 previously reacted with cyclohexanedione. In the latter case the fragment peptides show their activity only after de-blocking with hydroxylamine.

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