

THE EFFECTS OF CHEMICAL MODIFICATION ON THE ANTIGENICITY OF A HUMAN KAPPA BENCE JONES PROTEIN

J. B. MATTHEWS and R. JEFFERIS

Department of Experimental Pathology, The Medical School, Vincent Drive, Birmingham B15 2TJ, England

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Abstract—The effects of a number of chemical modifications on the expression of V_{κ} and C_{κ} antigenic determinants of a human kappa (subgroup I) Bence Jones protein was investigated. Modification of ca. 88% of the ϵ -amino groups with citraconic anhydride or potassium cyanate was accompanied by a decrease in C_{κ} antigenicity with retention of complete idiotypic and subgroup reactivity. Gel filtration analysis of the modified proteins indicated extensive molecular expansion, limited to the C_{κ} domain, upon citraconylation but not carbamylation. These results were confirmed by studies of isolated V_{κ} and C_{κ} domains. Cleavage of the intrachain disulphide bonds by reduction and alkylation under dissociating conditions allowed retention of a large proportion of native V_{κ} antigenicity. In contrast, native C_{κ} antigenicity was virtually abolished with the revelation of a new set of 'denatured' C_{κ} determinants suggesting the relative instability of C_{κ} conformation as compared with that of the V_{κ} domain. Cleavage of the methionine (47)–isoleucine (48) bond with cyanogen bromide had no effect upon native V_{κ} or C_{κ} antigens.

INTRODUCTION

Specific chemical modification of amino acid side chains within proteins has yielded extensive information about the stability of protein molecules and the role of particular amino acid residues in maintenance of native structure and antigenicity (Nakagawa *et al.*, 1972; Ansari *et al.*, 1975; Lee *et al.*, 1975; Iseman *et al.*, 1975; Hunneyball and Stanworth, 1976). Recently, analysis of a variety of chemical derivatives of sperm whale myoglobin by Atassi and co-workers has played an important role in the elucidation of the complete antigenic structure of the protein (see Atassi, 1975, for review).

Human kappa Bence Jones proteins have been shown to express a large number of antigenic specificities associated with variable (V_{κ}) sequences in addition to the constant region (C_{κ}) determinants (Solomon and McLaughlin, 1969*a, b*, 1971; McLaughlin and Solomon, 1971, 1972; Kunkel, 1970). With the exception of the InV allotypic marker of the C_{κ} (Steinberg *et al.*, 1974) these antigenic determinants have not been structurally defined although serological analysis of a large number of sequenced kappa chains has indicated several sequences which correlate with antigenic reactivity (McLaughlin and Solomon, 1972).

This paper describes the use of a variety of chemical modification procedures to structurally locate some of the antigenic determinants expressed by human kappa Bence Jones protein NE. Partial sequence data for the variable region of this protein has been reported previously (Matthews and Jefferis, 1977). Antisera specific for the idiotypic, subgroup and C_{κ} antigenic determinants were raised and used to monitor the effects of substitution of lysine, cysteine and methionine residues on the expression of antigenicity.

MATERIALS AND METHODS

Kappa chains and sub-fragments

Kappa Bence Jones proteins were isolated by gel filtration of urines from patients with multiple myeloma on Sephadex G-75 equilibrated and eluted with 0.01 M phosphate buffer pH 8.0. The monomer light chain peaks were used throughout and were pure by immunoelectrophoresis, diffusion analysis and N-terminal amino acid analysis.

The trypsin variable region was isolated as described previously (Matthews and Jefferis, 1977). C_{κ} region was isolated from Bence Jones urine KE by gel filtration on Sephadex G-75. Approximately 30% of the urinary protein had an elution volume consistent with the presence of half molecule (mol. wt ca. 12,500). Amino acid analysis and N-terminal analysis gave results consistent with the known composition of C_{κ} region (residues 109–214; N-terminal threonine).

Subgrouped proteins

Kappa Bence Jones proteins CLA, LAY, McK and LEN, representative of the four V_{κ} serological subgroups, were a gift from Dr. A. Solomon (University of Tennessee Memorial Research Centre and Hospital, Knoxville, U.S.A.).

Purified myeloma proteins CAC, FAV, FRI, JAV, KLE, LEG, ROB, SOD, TRO and ZEG, with kappa chains of known subgroup, were a gift from Dr. A. Moulin (Centre de biochimie et de Biologie Moléculaire, CNRS, Marseille, France). These proteins were subgrouped serologically by Drs. Rivat and Ropartz and chemically by Moulin and Fougereau (1973).

Kappa chains of unknown subgroup were chemically typed by limited N-terminal sequence analysis and the method of Moulin and Fougereau (1973).

N-terminal analysis and sequence analysis

N-terminal amino acid analysis and manual sequence analysis was performed as described by Hartley (1970) employing dansyl chloride.

Citraconylation of proteins

Lyophilised light chain (100 mg) was dissolved in distilled water (5 ml) and the pH adjusted to 8.5 with 0.1 M NaOH. Citraconic anhydride (250 mg) in 1:4 dioxan (1.6 ml) was added dropwise to the stirred solution, cooled in an ice bath, whilst the pH was maintained between 8 and 8.5 with addition of 2.5 M NaOH. On cessation of base uptake the mixture was dialysed against phosphate buffered saline pH 7.2 (PBS). Solutions of citraconylated protein were stored at -20°C and used as soon as possible after modification. For removal of citraconyl groups, modified protein (5–10 mg/ml) was adjusted to pH 3.8 with 1 M acetic acid and incubated at 37°C for 24 hr with continuous stirring. Removal of citraconyl groups under these conditions caused irreversible precipitation of approx 30% of the protein treated.

Quantitation of citraconyl groups

The number of citraconyl groups present in modified protein was determined by the spectroscopic assay described by Vitale (1974). Molar extinction coefficients for protein NE (mol. wt 23,500) were $28,300\text{ M}^{-1}$ at 241 nm and $24,000\text{ M}^{-1}$ at 280 nm.

Quantitation of amino groups

The number of free amino groups in chemically modified proteins was determined by the method described by Habeeb (1966), employing 2,4,6-trinitrobenzenesulphonic acid (TNBS).

Carbamylation of proteins

Carbamylation of light chains was performed as described by Nakagawa *et al.* (1972). Solid potassium cyanate was added to a solution of light chain in 0.1 M sodium phosphate buffer, pH 7.7. (5 mg/ml) in small portions to give a final concentration of 0.5 M. The pH was maintained at 8.0–8.5 during the addition of cyanate by adjustment with 1 M acetic acid. The reaction was allowed to continue for 72 hr at room temperature, the pH being maintained between 8.0 and 9.0. Modified protein was dialysed against PBS and stored at -20°C .

Complete reduction and alkylation

Kappa chain (40 mg) was dissolved in 8 M urea, 0.35 M Tris, 0.15 M NaCl, 2×10^{-3} M EDTA buffer pH 8.2 (8 ml) and reduced with dithiothreitol (DTT, 11.25 mg) for 1 hr at 37°C . Free sulphhydryl groups were subsequently blocked with a solution of iodoacetic acid (140 mg) in the urea-Tris buffer (2 ml) which had been adjusted to pH 8 by addition of solid Tris. Alkylation was allowed to proceed for 40 min at 37°C in the dark. Fully reduced and alkylated proteins were renatured by dialysis (24 hr periods) against PBS containing decreasing amounts of urea (4, 2, 1, 0.5, 0.1 M). The final dialysis against PBS alone was for 24 hr. Analyses were performed immediately after renaturation because of the instability of the final product.

Each reduction and alkylation experiment was accompanied by control preparations that were treated in an identical manner except that DTT was not added in one case and DTT, but not iodoacetic acid, added in the other. Controls were renatured in an identical manner to that described above. The reduced sample was dialysed against buffer which had been saturated with oxygen to promote reformation of disulphide bonds.

Cyanogen bromide cleavage

Kappa chain (50 mg) was dissolved in 70% formic acid (5 ml) and solid cyanogen bromide (22 mg) was added (100:1 molar excess of CNBr:Met). The tube was flushed with nitrogen, tightly stoppered and reaction allowed to continue at room temperature for 30 hr. The reaction mixture was finally diluted 100-fold with distilled water and

lyophilised. A control preparation was treated in an identical manner without addition of cyanogen bromide.

Gel-filtration

A Sephadex G-75 column (120 \times 1.6 cm), equilibrated and eluted with phosphate buffered saline, pH 7.2, was used to characterise modified proteins. The column was calibrated using Blue Dextran 2000 (Pharmacia Ltd.), ovalbumin, kappa Bence Jones protein (NE) monomer and dimer, sperm whale myoglobin and cytochrome c. The elution volumes (V_e) and partition coefficients (K_{av}) were obtained according to standard procedures.

SDS polyacrylamide disc electrophoresis

SDS polyacrylamide electrophoresis was performed as described by Weber and Osborne (1969) in 0.1 M phosphate buffer pH 7.2 with 0.1% sodium lauryl sulphate.

Antigenic analysis

Immunodiffusion was performed in 1.0% agarose in barbitalone buffer ($I = 0.05$, pH 8.6) and allowed to proceed for 24 hr at 4°C .

Passive sensitisation of sheep red cells (SRC) using glutaraldehyde was performed by a modification of the method described by Onkelinx *et al.* (1969). To a solution of protein (5 mg) in PBS (5 ml) was added 50% SRC (0.2 ml) and 2.5% glutaraldehyde in PBS (0.4 ml). The mixture was stirred gently for 2 hr at room temperature and reaction continued overnight at 4°C . The washed, sensitised cells were resuspended in diluent (0.1% ovalbumin PBS) to give a 1% suspension. The method of Butler *et al.* (1967) was used for bisdiazotised benzidine (BDB) sensitisation of SRC with kappa chains. Stock BDB solution was prepared as described by Gordon *et al.* (1958). Sensitised cells were used as a 1% suspension in diluent.

Antigenic differences between modified and native proteins were quantitated by haemagglutination inhibition. A fixed dilution of antiserum was added to serial two-fold dilutions of protein (inhibitor) and incubated at 37°C for 1 hr. Sensitised cells were then added and titres read at 2 hr. The results are expressed as the highest dilution of inhibitor capable of inhibition of agglutination (in nanomoles). All inhibitions were accompanied by the appropriate serum, cell and inhibitor controls.

Inhibition System	Antiserum	Dilution	Red Cell Coat
Idiotypic (NE)	S117A	1/500	NE (BJK _I)
Subgroup (K1)	R9871A	1/50	TA (BJK _I)
C _κ region	Z275A	1/1000	BO (BJK _{III})

Quantitative precipitin analysis was performed using the method of Heidelberger and Kendall (1935).

Antisera

Antisera to kappa chains were raised in rabbits, guinea pigs (Dunkin-Hartley) and chickens (White Leghorn) using the immunisation schedule of Solomon and McLaughlin (1969a) with antigen emulsified incomplete Freund's adjuvant. Sheep antisera were raised by intramuscular injection of antigen (1 mg) emulsified in complete Freund's adjuvant (1 ml). Injections were made at monthly intervals and blood samples taken 7 days after each injection.

RESULTS

A group of ten Bence Jones proteins were studied which on chemical typing by limited amino acid

Table 1. Subgrouping of kappa chains

Protein	N-terminal sequence	Sequence	Subgroup Moulin-Fougereau	Antigenic ^a
BE	Asp-	I or II	I	I
BO	Glu Ile Val Leu	III	III	III
HO	Asp Ile Val	II	(II)	II
KE	Asp-	I or II	I	I
LA	Glu Ile Val Leu	III	III	III
NA	Asp Ile Val Met	II	II	II
NE	Asp Ile His Leu	(I)	(I)	I
SO	Asp-	I or II	I	I
TA	Asp Ile Glx Met	I	I	I
WI	Asp-	I or II	I	I

^a Antigenic typing performed with anti-K_I and anti-K_{III} antisera only. Non-reacting proteins were classified as KII (HO and NA).

Results in parentheses indicate atypical sequence or peptide patterns.

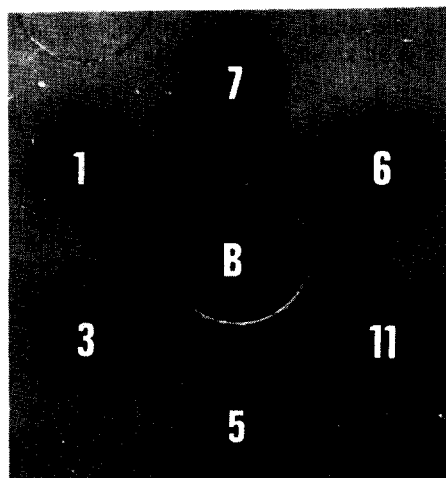
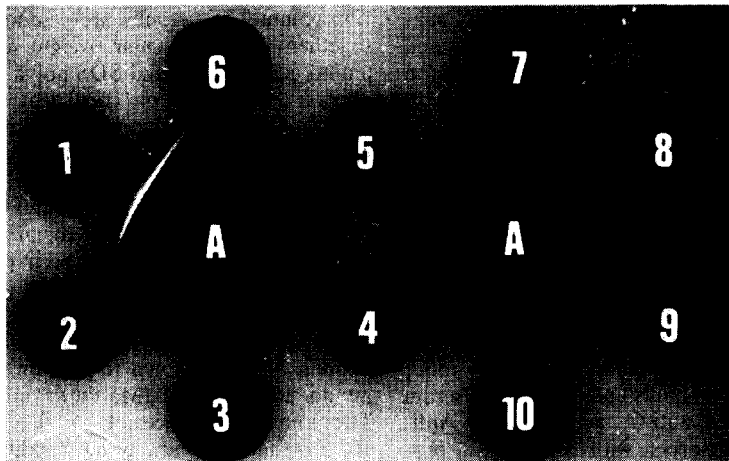


Fig. 1. Gel diffusion analysis of subgroup specific antisera (3-fold concentrated) in 3% PEG-agarose medium. Antisera: A = Anti-K_I; rabbit anti-NE absorbed with BO (R9871A); B = Anti-K_{III}; guinea-pig anti-LA absorbed with NA (G210A). Kappa I proteins: 1 = NE, 2 = TA, 4 = CLA, 9 = BE; Kappa II proteins: 6 = NA, 8 = LAY, 10 = HO; Kappa III proteins: 3 = BO, 7 = LA, 11 = McK; Kappa IV proteins: 5 = LEN.

sequence analysis and the technique of Moulin and Fougereau (1973) were found to represent examples of K_I , K_{II} and K_{III} proteins (Table 1).

In attempts to obtain antisera having idiotypic and subgroup specificity, 62 animals (10 rabbits, 2 sheep, 6 chickens, 44 guinea pigs) were immunised with these proteins. All antisera were absorbed with insolubilised kappa Bence Jones proteins shown to be of a different subgroup, by chemical typing, to the immunogen and screened for their ability to agglutinate sheep red cells sensitised with immunogen, the protein used to absorb the antiserum, or kappa chain of the same chemical subgroup as the immunogen. Of the 62 sera tested only 7 demonstrated subgroup reactivity (2 rabbits, 1 sheep, 1 chicken, 3 guinea pigs) which were directed against K_I and K_{III} subgroups. None of the 10 animals (2 rabbits, 2 chickens, 6 guinea pigs) immunised with K_{II} proteins produced a detectable response to K_{II} subgroup determinants. Two of the anti-subgroup antisera were strong enough to give precipitin reactions on gel diffusion (Fig. 1). Results obtained using these antisera and Bence Jones proteins subgrouped by other workers (see Materials and Methods; McLaughlin and Solomon, 1972; Moulin and Fougereau, 1973) were in complete agreement with the published findings.

Antisera were similarly tested for idiotypic activity by agglutination of red cells coated with immunogen after absorption with insolubilised proteins of the same subgroup and normal light chain. For the detailed study of the expression of constant region (C_k), subgroup and idiotypic activity of protein NE, three antisera were used; a rabbit antiserum (R9871A) having strong anti-subgroup activity (Fig. 1); a sheep antiserum (S117) having precipitating antibody to C_k and NE idiotypic determinants and a sheep antiserum (Z275A) having strong anti- C_k activity only. Antiserum R9871A was absorbed with K_{II} and K_{III} proteins and used in a haemagglutination system rendered specific for the subgroup (K_I) determinants of protein NE by employing cells coated with an unrelated K_I protein (TA). The idiotypic antiserum (S117A) was rendered specific by absorption with Bence Jones proteins representative of all kappa sub-

groups and normal light chain to remove C_k region, subgroup and 'subgroup related' (McLaughlin and Solomon, 1972) antibodies.

Effects of citraconylation

In these experiments native NE, citraconylated NE, citraconylated NE which had the blocking groups removed and protein NE which had been exposed to the acid conditions used to deblock the substituted lysines was employed. The extent of substitution was determined both by a colourimetric assay for free amino groups using trinitrobenzenesulphonic acid and the spectroscopic method of Vitale (1974). The results indicated 10.6 and 10.7 blocked amino groups respectively compared to 12 free amino groups present in the native protein (including the α -amino group at the N-terminus).

Gel filtration analysis of the four protein preparations on a calibrated column of Sephadex G-75 demonstrated that the citraconylated sample behaved anomalously eluting with a significantly reduced elution volume. The results are summarised in Table 2 together with molecular weight estimations from amino acid analysis and SDS polyacrylamide electrophoresis. The partition coefficient of citraconylated NE was lower than that of native, deblocked, or acid treated (control) molecule and therefore appeared to have a higher molecular weight. This apparent increase in molecular weight probably results from conformational changes leading to an expansion of the molecule. Such effects have been described for immunoglobulins by several workers (Nakagawa *et al.*, 1972; Hunneyball and Stanworth, 1976). All samples exhibited identical mobilities on SDS electrophoresis. Protein NE was shown to contain eleven lysines by amino acid analysis (Matthews and Jefferis, 1977). The known sequence of the C_k region (residues 107-214) includes nine lysine residues and hence the variable region of protein NE (residues 1-106) contains only two lysines. It would be expected therefore that substitution at lysine residues would have a greater effect upon the conformation and antigenicity of the C_k region domain than on the V_k region domain.

Table 2. Physico-chemical properties of native, citraconylated and carbamylated protein NE and isolated variable and constant regions

Sample	% modification ^d	Kav ^a	Molecular weight		SDS Electrophoresis ^c
			Sephadex ^a	Amino acid analysis ^b	
Native NE	0	0.32	25,000	23,500	25,000
Control NE ^c	0	0.32	25,000	23,500	25,000
De-citraconylated NE	2	0.31	26,000	23,500	25,000
Citraconylated NE	88	0.15	47,000	25,000	25,000
Carbamylated NE	90	0.30	27,000	24,000	25,000
Native V_{NE}	0	0.52	12,500	12,000	ND
Citraconylated V_{NE}	95	0.50	13,500	12,000	ND
Native C_k	0	0.52	12,500	12,000	ND
Citraconylated C_k	94	0.41	18,500	13,000	ND

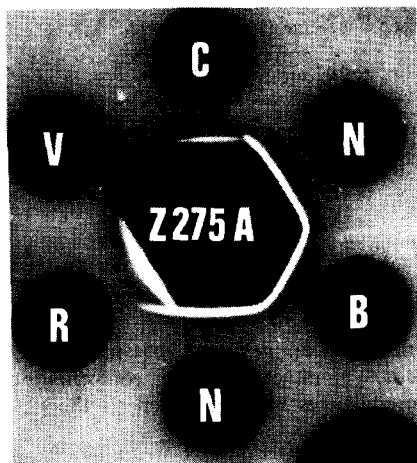
^a Gel filtration on Sephadex G-75.

^b Molecular weight based on 214 amino acid residues per intact molecule and including contribution of substituent groups. Values given to the nearest 500 daltons.

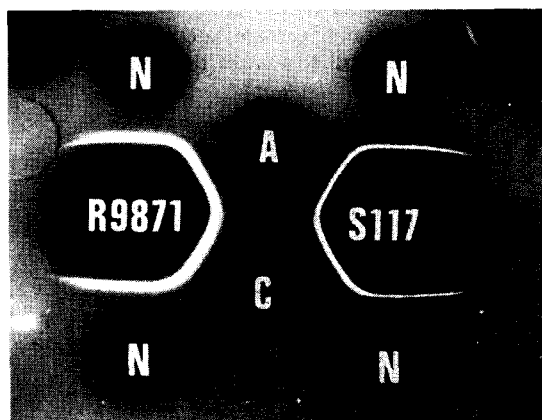
^c Control NE is native protein which has been subjected to the de-citraconylation conditions.

^d Based on results from TNBS analyses.

^e Standard proteins included BSA, ovalbumin, sperm whale myoglobin and cytochrome C.
ND Not determined.



(a)



(b)

Fig. 2. Gel-diffusion analysis of citraconylated (C), carbamylated (A), reduced and alkylated (R) and native protein NE (N) compared with isolated variable region (V), native (B) and citraconylated protein BO (D) using sheep anti-C_κ (Z275A), unabsorbed sheep anti-NE (S117) and rabbit anti-NE (R9871) antisera.

Gel diffusion analysis with sheep anti-C_κ antiserum demonstrated that the citraconylated protein was more weakly reactive and antigenically deficient to native molecule (Fig. 2a). Similar losses of C_κ antigenicity were shown by haemagglutination inhibition (Table 3). The loss of antigenicity in this system varied with the antiserum employed but the increase in the amount of citraconylated protein required for inhibition was in the range of 8–32 fold over that of native molecule. This antigenic loss was completely reversed upon regeneration of the amino groups by acid treatment.

In contrast to the above observation, lines of identity were obtained between citraconylated and native NE when tested in gel diffusion with homologous antiserum possessing both V_κ and C_κ antibody specificities (Fig. 2b). However, loss of antigenicity was confirmed by quantitative precipitin analysis with modified protein yielding, at the optimum proportions point, only 60% of the precipitate obtained with native protein. The antigenic valency of native molecule was calculated as 6 and the valency decreased to 4 in the citraconylated sample.

Haemagglutination inhibition analysis (Table 3) demonstrated that citraconylation had no detectable effect on the expression of idiotype or subgroup determinants. A clear differential loss of antigenicity between C_κ and V_κ region determinants was observed which accords with the expected greater loss of conformation within the C_κ domain due to the high lysine content. Since glutaraldehyde sensitised cells were used in these analyses (which links proteins via free amino and sulphhydryl groups) it may be argued that the sensitising system itself affects the expression of antigenicity. An alternative sensitisation system using bis-diazotised benzidine, which is relatively specific for the phenolic hydroxyl groups of tyrosine (Bozsoky and Franklin, 1966), was therefore employed. The distribution of tyrosines in NE was shown to be 4 residues in the C_κ and 5 residues in the V_κ region (Matthews and Jefferis, 1977). Results from analyses using bis-diazotised benzidine cells are summarised in Table 4. As previously shown, no loss

Table 3. Antigenic analysis of citraconylated and carbamylated derivatives by haemagglutination inhibition^a

Sample	Idiotype	Subgroup	C region
Native NE	0.04	0.08	0.08
Control NE	0.04	0.08	0.08
De-citraconylated NE	0.08	0.08	0.08
Citraconylated NE	0.04	0.08	0.64
Carbamylated	0.04	0.08	0.64
Native V _{NE}	0.08	0.08	> 1000
Citraconylated V _{NE}	0.08	0.16	> 400
Native C _κ	> 1000	> 1000	0.08
Citraconylated C _κ	> 1000	> 400	1.28
BJK _I ^b	> 2000	0.08	0.08
BJK _{II} or _{III}	> 2000	> 2000	0.08
Normal L chain	> 2000	ND	ND

^a Haemagglutination using glutaraldehyde as the linking agent. Results are expressed as the amount of protein (in nanomoles) to give inhibition in each test system.

^b Indicates inhibition with kappa Bence Jones proteins belonging to the various kappa subgroups.

> Indicates that no inhibition was observed at the highest concentration tested.

Table 4. Antigenic analysis of citraconylated protein by haemagglutination inhibition using red cells sensitised by the bis-diazotised benzidine method

Sample	Idiotype	Subgroup	C _κ region
Native NE	0.005	0.3	0.3
Control NE	0.005	0.3	0.3
De-citraconylated NE	0.005	0.3	0.6
Citraconylated NE	0.005	0.3	9.6
BJK _I	>2000	0.3	0.3
BJK _{II} or III	>2000	>2000	0.3
Normal L chain	>2000	ND	ND

of idiotypic or subgroup antigenicity was detectable on citraconylation whilst the loss of C_κ antigenicity appeared more marked, which was reflected in the increased amount of modified protein required for inhibition.

These observations were verified by modification studies on the variable region, isolated by trypsin digestion of protein NE, and C_κ region, isolated from a second Bence Jones urine (KE; Matthews and Jeffers, 1977). The physicochemical data are presented

in Table 2 and antigenic analysis in Table 3. As in the studies of intact molecule, conformational and antigenic changes were restricted to the C_κ region.

Effects of carbamylation

Parallel experiments to those reported above were performed on a sample of protein NE after carbamylation of 90% of free amino groups. The ultraviolet absorption spectrum of carbamylated protein NE was found to be identical to that of the native molecule

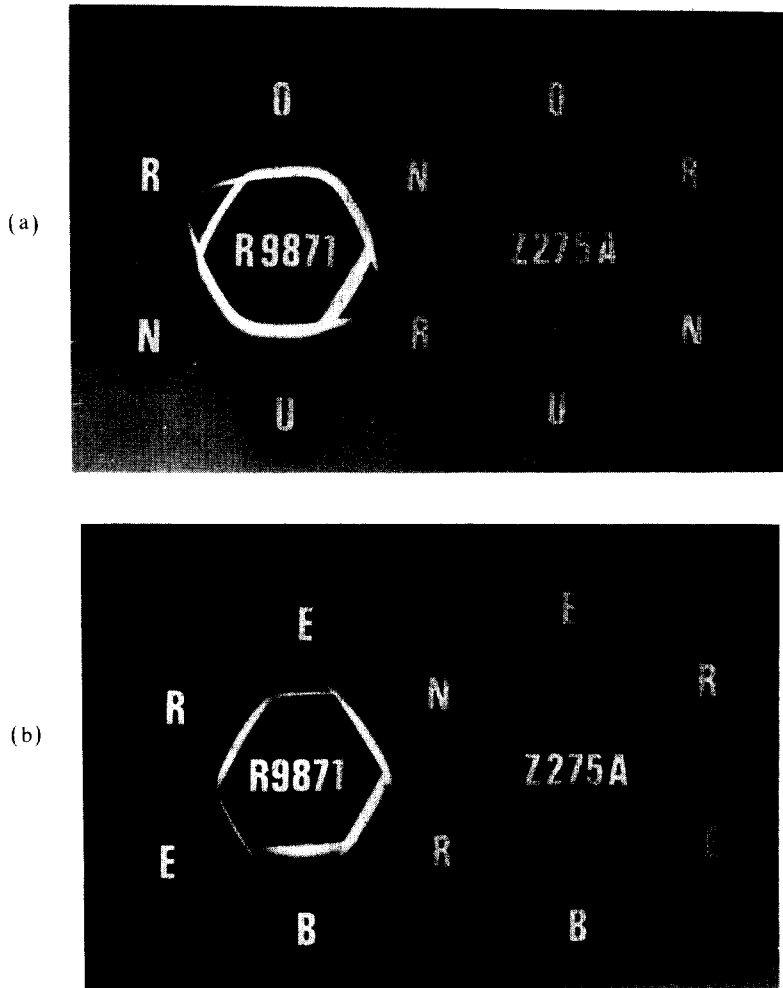


Fig. 3. (a) Gel-diffusion analysis of native (N), iodoacetate/urea-treated (U), reduced/oxidised (O) and reduced and alkylated (R) protein NE with sheep anti-C_κ (Z275A) and unabsorbed rabbit anti-NE (R9871) antisera. (b) Comparison of reduced and alkylated protein NE and native (B) and reduced and alkylated (E) protein BO (BJK_{III}).

indicating that no substitution of aromatic residues had occurred. Carbamylated protein exhibited elution characteristics on Sephadex G-75 indistinguishable from the native protein (Table 2) indicating that the molecular expansion found on citraconylation had not occurred. Antigenic testing in haemagglutination inhibition systems demonstrated retention of idiotypic and subgroup antigens but a loss of C_k antigenicity (Table 3) similar to that observed after citraconylation. Lines of identity between native and carbamylated protein were obtained with antisera possessing both V_k and C_k specificity (Fig. 2b) and all antisera showed identity between citraconylated and carbamylated derivatives.

Complete reduction and alkylation

The intra-chain disulphide bonds of protein NE were cleaved by reduction and alkylation under denaturing conditions (8 M urea). Control experiments with the omission of DTT or in which DTT but no alkylating agent was added were performed to yield blocking and reducing controls respectively. Gel diffusion analysis showed these control preparations to have antigenic identity with native protein (Fig. 3a). The fully reduced and alkylated protein was antigenically deficient to native protein when tested with homologous antisera having V_k and C_k specificity. Lines of non-identity between native and modified protein were detected with antisera specific for the C_k determinants (Fig. 3a and b) and occasionally with homologous antisera. Thus it would appear that disulphide cleavage destroys native C_k antigenicity whilst revealing a new set of 'denatured' C_k determinants. A similar phenomenon has been reported (McLaughlin and Solomon, 1973; Solomon, 1976) where 'new' C_L determinants were revealed as a result of cleavage of light chains into half molecules. Modification of a second kappa chain (K_{III} BO) caused antigenic changes parallel to those reported for protein

NE. Figure 3b demonstrates that at least some variable region antigenicity is retained by protein NE after disulphide cleavage. Using antisera raised to protein NE spurs indicate that another reduced and alkylated kappa chain (BO, K_{III}) is antigenically deficient to modified protein NE. The effects of reduction and alkylation on V_{NE} antigenic determinants can be seen in Fig. 4. Unabsorbed antiserum (R9871) demonstrated a strong spur of native over modified NE and a weak spur of native variable region over modified NE suggesting that some native variable region antigens were retained. Absorption of this antiserum with the fully reduced and alkylated molecule abolished reactivity with variable region but retained strong anti- C_k activity. Control absorption with native molecule abolished all precipitin reactions.

Haemagglutination inhibition analyses were per-

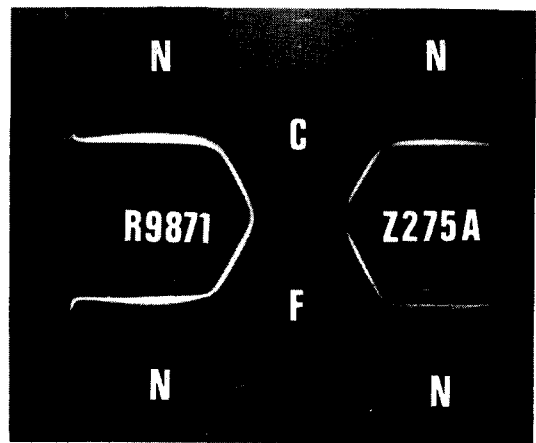


Fig. 5. Gel diffusion analysis of native (N), acid treated (F) and CNBr cleaved (C) protein NE with unabsorbed rabbit anti-NE (R9871) and sheep anti- C_k (Z275A) antisera.

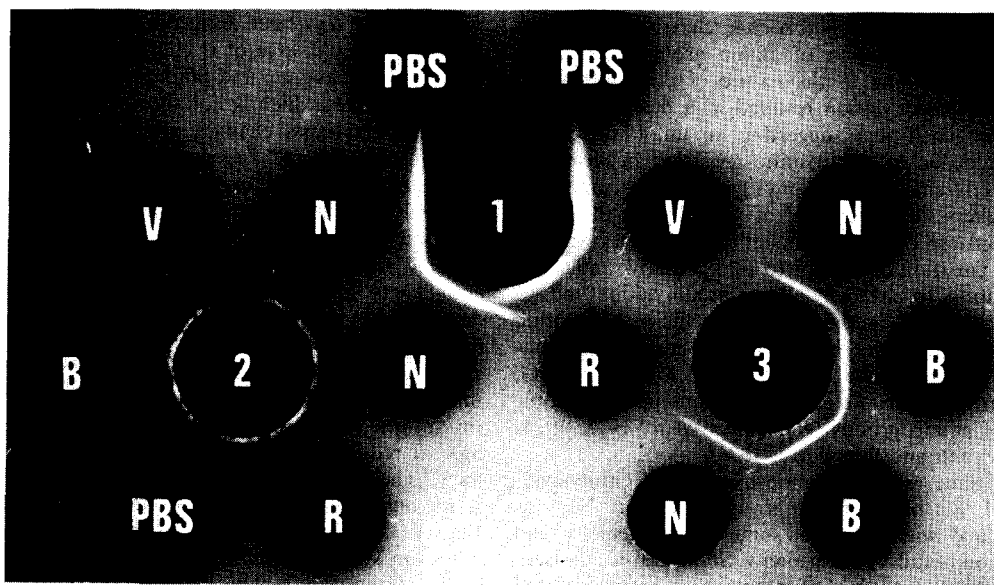


Fig. 4. Gel diffusion analysis of fully reduced and alkylated protein NE demonstrating the differential retention of V_k and C_k antigenic determinants. Antigens as in Fig. 3. Antiserum wells contain R9871 anti-NE before (1) and after absorption with native (2) or reduced and alkylated (3) protein NE.

Table 5. Antigenic analysis of cyanogen bromide cleaved protein by haemagglutination inhibition^a

Sample	Idiotype	Subgroup	C _κ region
Native NE	0.005	0.01	0.08
Control NE ^b	0.01	0.01	0.16
CNBr cleaved NE	0.01	0.02	0.16
BJK _I	>2000	0.01	0.08
BJK _{II} or _{III}	>2000	>2000	0.08
Normal L chain	>2000	ND	ND

^a Haemagglutination using glutaraldehyde as the linking agent.

^b Control NE is native protein NE subjected to cleavage conditions in the absence of cyanogen bromide.

formed but the results were not reproducible presumably because of the unstable nature of the modified proteins in aqueous buffers at near neutral pH. Quantitative precipitin tests were attempted but the reduced and alkylated molecules failed to precipitate in free solution.

Cleavage at methionine 47

In a previous paper we showed that protein NE has a methionine residue at position 47 within the disulphide loop of the variable region (Matthews and Jefferis, 1977). Native protein was reacted with cyanogen bromide in 70% formic acid and by amino acid analysis cleavage was greater than 98%. Control preparations exposed to 70% formic acid only, the cleaved protein, and native protein demonstrated antigenic identity in diffusion and haemagglutination inhibition analyses (Fig. 5 and Table 5). Thus methionine 47 and the integrity of the methionine (47)-isoleucine (48) bond have no detectable role in the expression of idiotypic, subgroup or C_κ antigenicity of protein NE.

DISCUSSION

The gel filtration elution characteristics of protein NE after modification of amino groups was found to vary according to the nature of the substituting group. Citraconylation resulted in a decrease in the partition coefficient suggesting an unfolding of the molecule due to electrostatic repulsion between the negatively charged citraconyl groups. This unfolding was reversible as removal of virtually all the citraconyl groups by acid treatment resulted in a reversion of the partition coefficient to that of native molecule. In contrast to citraconylation, carbamylation had no significant effect on the partition coefficient suggesting retention of native conformation. Similar conformational changes have been reported for rabbit IgG, human IgG, Fab, Fc and light chains upon substitution of amino groups (Nakagawa *et al.*, 1972, 1974; Hunneyball and Stanworth, 1976).

Antigenic analysis demonstrated that citraconylation of 88% of the amino groups of protein NE was associated with a loss of some C_κ antigenicity and retention of full V_κ antigenicity. Some anti-C_κ antisera demonstrated a spur of native over citraconylated kappa chain whereas others identity. In both cases the line produced by modified protein was weaker than that of native molecule. A similar phenomenon

was reported as a result of amidation of an unspecified number of amino groups in kappa chains by Messner *et al.* (1971). In contrast to the present study Nakagawa *et al.* (1974) have reported complete loss of C_κ antigenicity upon citraconylation. This conclusion was drawn from a simple gel diffusion test using a single goat anti-C_κ antiserum. Recent evidence has indicated large differences in the ability of potent anti-C_κ antisera to react with certain groups of C_κ antigens (Solomon, 1976) which emphasises the need to test a battery of antisera when assessing the effects of chemical modification on antigenicity. All antisera raised to protein NE (i.e. possessing antibodies to both variable and constant regions) gave lines of identity between citraconylated and native molecule. Haemagglutination inhibition analyses indicated loss of some C_κ antigenicity but retention of full idiotypic and subgroup reactivity. Carbamylation of 90% of the amino groups of protein NE resulted in similar changes in antigenicity. This finding contrasts with the work performed on human and rabbit IgG (Nakagawa *et al.*, 1972; Hunneyball and Stanworth, 1976) where citraconylation destroyed virtually all antigenicity and carbamylation had little effect. In addition removal of over 90% of the substituent groups resulted in only partial recovery of native antigenicity suggesting an irreversible conformational change not detectable by physical techniques. The loss of C_κ antigenicity in protein NE was completely reversed when the citraconyl groups were removed by acid treatment. Similar reversibility was shown for C_κ antigens by Nakagawa *et al.* (1974) by simple gel diffusion tests.

From the distribution of lysine residues within protein NE (two V_κ lysines at positions 39 and 45 with nine in the C_κ; Matthews and Jefferis, 1977) it would seem probable that substitution of the amino groups of intact protein NE would have a greater effect on the conformation and antigenicity of the constant region than the variable region. This was verified by analysis of the isolated variable and constant regions.

The ability to detect antigenic changes in the C_I as a result of cleavage of L chains into their V and C halves (McLaughlin and Solomon, 1973; Solomon, 1976), a process which is not accompanied by extensive conformational changes as measured by ORD and CD (Ghose and Jirgensons, 1971; Bjork *et al.*, 1971) suggests that the expression of C_I determinants is very dependent upon conformation. Cleavage of the disulphide bonds of protein NE resulted in retention of some native V_κ antigenicity with loss of native C_κ antigenicity and revelation of new 'denatured' C_κ determinants. As these 'denatured' determinants were recognised by anti-native antisera it is probable that some denatured molecules were present in the immunogen preparations. The ability of 'native' protein (in antigen excess) to absorb all antibody reactivity against reduced and alkylated protein supports this view. These antigenic analyses indicate that C_I is more unstable and liable to conformational change than V_I. Such a conclusion is supported by enzyme digestion studies (Seon *et al.*, 1972; Solomon *et al.*, 1976) and physical measurements of L chains and their isolated domains (Ghose, 1972; Karlsson *et al.*, 1972; Longworth *et al.*, 1976). The conditions used for cleavage of the intrachain disulphide bonds had

no detectable effect upon antigenicity. Reduction followed by oxidation and reformation of disulphide bonds allowed retention of full native antigenicity. Similar recovery of native antigenicity and conformation has been reported previously for kappa and lambda chains (Karlsson *et al.*, 1972; Solomon, 1976).

Treatment of protein NE with 70% formic acid for 30 hr at room temperature did not result in any detectable changes in antigenicity or non-specific cleavage of the molecule. Cyanogen bromide cleavage of the methionine (47)–isoleucine (48) bond, together with the conversion of methionine to a mixture of homoserine and homoserine lactone, had no detectable effect on antigenicity. This result, in addition to that obtained from the lysine substitution studies, suggests that the area of protein NE incorporating lysines 39, 45 and methionine 47 is not important for the expression of the antigenic determinants recognised by our antisera. This area of the V region includes the sequence correlated 'site 2' associated with inter-subgroup V_{κ} antigenic cross-reactions (lysine 45; McLaughlin and Solomon, 1972).

Our studies thus indicate independence of the V_{κ} and C_{κ} antigenic determinants (as recognised by our antisera) within the intact kappa chain. Losses of C_{κ} antigenicity upon citraconylation, carbamylation and reduction and alkylation were not accompanied by similar changes in V_{κ} antigens. The C_{κ} antigenic determinants were found to be particularly susceptible to chemical modification which was probably a reflection of changes in native conformation. Furthermore, the integrity of the intrachain disulphide bond would seem to be essential for the expression of native C_{κ} but not V_{κ} antigenicity and conformation. Loss of C_{κ} antigenicity as a result of a variety of denaturing treatments (e.g. 70% formic acid, 8 M urea, citraconylation, reduction and reformation of disulphide bonds) was completely reversible indicating a refolding of the molecule upon adjustment to physiological conditions. However, complete loss of native C_{κ} antigenicity and the revelation of a new set of 'denatured' determinants resulted upon irreversible cleavage of the intrachain disulphide bonds suggesting an inability to refold and attain native antigenicity and conformation. Recently Isenman *et al.* (1975) have suggested a model for the folding of β 2-microglobulin (and immunoglobulin domains) in which one of the early folding events results in disulphide bond formation, this being an essential step for continued folding to the native state. Although our results on C_{κ} are in agreement with this model our observations indicate a different sequence of events in the folding of the V_{κ} domain with disulphide bond formation not playing an early and essential role.

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