HEAVY AND LIGHT CHAINS OF A HOMOGENEOUS IMMUNOGLOBULIN-G

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1. Introduction

The extent of heterogeneity of populations of immunoglobulin molecules is too great to be resolved by conventional electrophoretic methods. Light and heavy chains isolated from a heterogeneous immunoglobulin population can however be resolved by electrophoresis at alkaline pH in gels containing urea, into a series of discrete bands [1,2]. It has been pointed out that for light chains prepared from highly heterogeneous immunoglobulin populations, bands of single mobility are still highly heterogeneous (see discussion by Cohen, [1]). However, for immunoglobulin molecules of limited heterogeneity, a relationship was expected to exist between the number of light and heavy chain bands, and the heterogeneity of the original immunoglobulin preparations. Consequently, the electrophoretic analysis of light and heavy chain sub-units is now employed as a criterion in the search for a homogeneous antibody [3].

We have isolated a homogeneous G-myeloma protein in order to evaluate this type of analysis. The pattern of light and heavy chains resulting from reduction of this homogeneous IgG was examined, by electrophoresis and isoelectric focusing, as a function of the conditions of reduction and subsequent handling of the chains. Under carefully controlled conditions and with minimal manipulation of the reduction mixture a single light chain and a single heavy chain species are observed; under the usual conditions used for reduction of IgG and isolation of light and heavy chains a more heterogeneous pattern results.

2. Experimental procedures

G-myeloma protein 5563 was purified from ascitic fluid of tumour-bearing mice by chromatography on DEAE-cellulose [4]. Component *a* was isolated by fractionation of myeloma protein (50 mg) on a column of DEAE-cellulose (10 g - 30 cm X 2 cm) with a shallow linear gradient (500 ml each of 0.01 M and 0.05 M potassium phosphate buffer pH 6.5).

Myeloma protein in 0.1 M tris pH 8.2 was reduced by treatment with 0.1 M β-mercaptoethanol for two hr at 4°C and alkylated with 1.5 fold excess iodoacetamide. Heavy and light chains were separated by gel filtration on Sephadex G-100 in 1 M acetic acid [7] and freeze dried. Heavy chains were re-dissolved in 5 M urea.

Electrophoresis was carried out at pH 8.9, 0.1 M tris glycine buffer, 8 M urea, in 5% polyacrylamide gel at 4°C. After 16 hr at 1 mA/cm proteins were stained with amido black.

Isoelectric focusing was performed in thin layer 5% polyacrylamide gels containing 2% Ampholine carrier ampholytes (pH range 5–8) [5]. After focusing for 24 hr the pH gradient was measured [5] and proteins stained with bromophenol blue [6].

3. Results

The heterogeneity of 5563 myeloma protein as found in the serum of tumour-bearing mice is illustrated in fig. 1Ai. Four myeloma protein bands are identified as *a*, *b*, *c* and *d* [8] but only two of these are major components in this freshly prepared sample. The relative homogeneity of isolated component *a* is illustrated in fig. 1Aii. Reduction and alkylation was car-

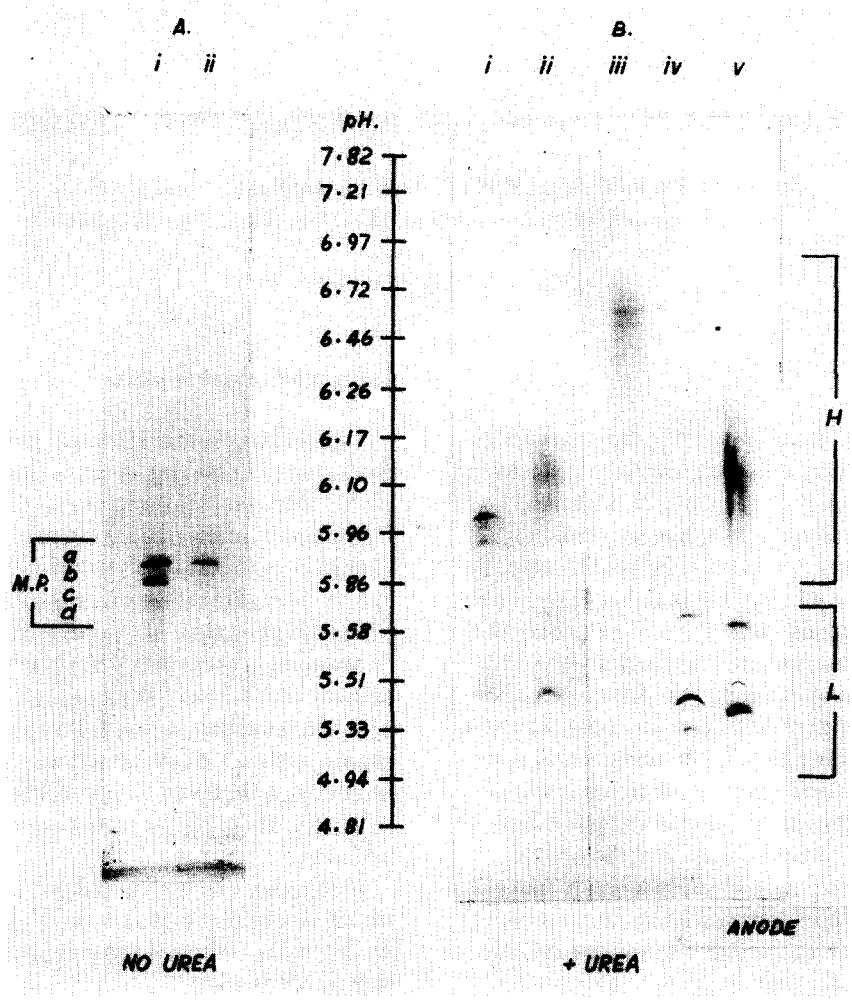

Fig. 1. Heterogeneity of 5563 myeloma protein and its heavy and light chains examined by isoeletric focusing in polyacrylamide gel. A: *i*, freshly isolated 5563 myeloma protein (M.P.); *ii*, purified band *a*. Ampholine pH range 5–8 used in absence of urea. B: *i*, freshly reduced and alkylated band *a*; *ii*, as *i* but stored at 4°C overnight; *iii*, isolated heavy chain of 5563 myeloma protein; *iv*, isolated light chain of 5563 myeloma protein; *v*, reduced and alkylated 5563 myeloma protein stored at 4°C overnight. Ampholine pH range 5–8 used in presence of 6 M urea. Potential gradient 500 V for 24 hr.

ried out at 4°C and the samples immediately subjected to polyacrylamide gel electrophoresis at pH 8.9 in the presence of 8 M urea. The protein bands were stained and scanned with a microdensitometer; the tracing (fig. 2) shows that the single homogeneous myeloma component gives rise to predominantly a single heavy chain and a single light chain band (fig. 2, upper) whereas the total 5563 myeloma protein resulted in multiple light and multiple heavy chain bands (fig. 2, lower).

Another fresh reduction mixture of component *a* was also examined by isoelectric focusing in polyacrylamide gel (fig. 1B i). Only a single light chain band, estimated pI 5.45 and mainly a single heavy chain band, pI 6.00, were observed, but this technique does resolve minor heavy chain bands of lower pI. When the reduction mixture of the homogeneous myeloma component was stored overnight at 4°C the pattern of light and heavy chain bands (fig. 1B ii) appeared similar to the heterogeneous pattern obtained by reduction

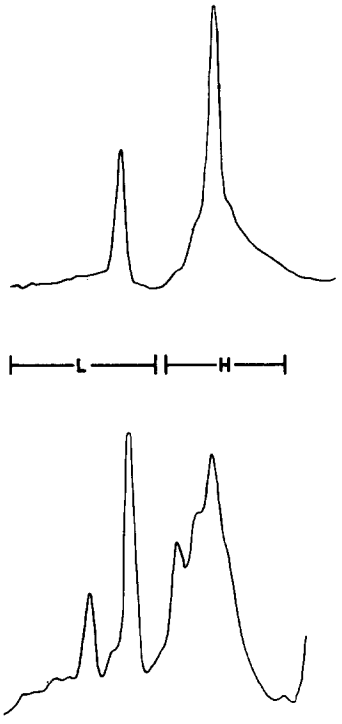

Fig. 2. Microdensitometer tracing of the heavy and light chains of 5563 myeloma protein separated by electrophoresis on polyacrylamide gel in 0.1 M tris glycine buffer pH 8.9 containing 8 M urea. Reduction and alkylation was performed as described in Experimental Procedure. Protein bands stained with amido black were traced using a Joyce-Lobel microdensitometer. Upper: reducation products of component *a*. Lower: reduction products of total 5563 myeloma protein.

of the total myeloma proteins and storage overnight (fig. 1B v). The multiple heavy chain bands are found in a higher pH range (6.0–6.2) and individual bands are poorly resolved; a more basic light chain band also appears on storage. Reduction of the homogeneous myeloma protein at room temperature (not illustrated) gives a less homogeneous banding pattern of heavy and light chains compared to reduction at 4°C.

In studies on the heterogeneity of immunoglobulin subunits it is frequent practice to separate light and heavy chains by gel filtration prior to their electrophoretic examination. When we isolated the light and heavy chains of 5563 myeloma protein the heterogeneity of both chains was found to be increased over that observed either in a fresh or a stored reduction mixture prior to acid separation (fig. 1B iii and iv). A series of light chains is seen with isoelectric points estimated as 5.67, 5.55 (faint), 5.45, 5.30 and 5.20; within the errors of measurement these can be considered as equally spaced with about 0.12 pH units separating each band. Isolated heavy chain gives a pattern of bands in the pH range 6.3–6.8 with an average separation of about 0.07 pH units between bands.

4. Discussion

The experiments described here show that the basic assumption that a homogeneous immunoglobulin molecule should give rise to a single species of heavy chain and a single species of light chain is indeed correct, but that in practice this simple theoretical result can only be obtained under very carefully controlled conditions. It is essential that the immunoglobulin is reduced and alkylated in the cold and that electrophoresis of the resultant mixture of heavy and light chains be carried out immediately without prior separation of the chains and without storage. Following reduction of IgG into its subunits, heavy and light chains become even more susceptible to changes in charge properties than the original molecule. This lability would explain recent reports that isolated light and heavy chains of a homogeneous immunoglobulin are heterogeneous [9, 10]. Even employing the precautions councilled above, interpretations of multiple light and heavy chain bands must be only a tentative guide to the extent of heterogeneity of the original immunoglobulin preparations. Examination of isolated heavy and light chain preparations could in fact be extremely misleading. Recent application of the equilibrium method of isoelectric focusing in polyacrylamide gels has made it possible to achieve resolution of unreduced immunoglobulin molecules directly [5].

We have not established the chemical nature of the groups which are being altered following reduction but there appear to be at least two types of change involved. The evidence is consistent with some of these groups being labile amide groups, probably glutamine residues; a carefully documented precedent can be found for this in the case of myoglobin [11,12]. The existence of certain labile groupings of this type in

5563 myeloma protein is indicated by the slow in heterogeity (seen at pH 8.9, or by the shift in pI of myeloma protein bands to lower values) either in serum or upon prolonged storage [8]. Reisfeld [13] has also shown that different immunoglobulin light chain bands isolated after polyacrylamide gel electrophoresis at alkaline pH contain decreasing numbers of amide groups with increasing mobility at pH 8.9, i.e. increasing negative charge.

Apart from changes which appear consistent with the idea of labile amide groupings it was also found that the isolated heavy and light chains of 5563 myeloma protein undergo another type of charge alteration which leads to the production of many components with higher pI values. In the case of heavy chains especially this increase in pI is extensive. This effect is as yet not understood.

Acknowledgements

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