Mobility of Sodium Dodecyl Sulphate–Protein Complexes

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Reduced and unreduced lysozyme aggregates formed by formaldehyde cross-linking comprise a set of model compounds for studying the effects of protein conformation on the electrophoretic mobilities of sodium dodecyl sulphate–protein complexes. The reduced aggregates were indistinguishable from normal proteins, but the unreduced aggregates migrated anomalously fast by about 14%. Contrary to expectations, plots of logarithm R_F versus T (acrylamide concentration) failed to detect an anomalous surface-charge density for the unreduced aggregates. Equally unexpected, plots of logarithm R_F versus K_R (retardation coefficient) failed to reveal an unusual conformation for the unreduced aggregates. Thus the anomalous mobility caused by several intramolecular disulphide bonds escaped detection by the above two diagnostic plots. Also included in this paper is a discussion of the implications of these results with regard to current models for sodium dodecyl sulphate–protein complexes.

The use of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis to estimate protein molecular weights (Shapiro *et al.,* 1967) was found empirically to be reliable to within 5–10% (Dunker & Rueckert, 1969; Weber & Osborn, 1969). Investigations on the nature of the sodium dodecyl sulphate complex (Reynolds & Tanford, 1970; Fish *et al.,* 1970; Shirahama *et al.,* 1974), extension of Ogston's (1958) theory of gel sieving (Chrambach & Rodbard, 1971; Rodbard & Chrambach, 1970) to explain Ferguson's (1964) equation, and finally application of all of the above to the particular case of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Neville, 1971) have shifted sodium dodecyl sulphate/polyacrylamide-gel molecular-weight determinations from a firmly empirical to a more nearly theoretically based analytical technique.

Use of bifunctional reagents to probe the neighbourhoods of the various polypeptide components of complex structures (Wold, 1967) has been combined with sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Davies & Stark, 1970; Carpenter & Harrington, 1972). Thus the mobility of cross-linked proteins on sodium dodecyl sulphate/polyacrylamide gels is of considerable interest. In some studies, cross-linked proteins were found to be indistinguishable from similar-sized continuous proteins (Carpenter & Harrington, 1972; Payne, 1973); in other studies, anomalous behaviour for cross-linked proteins was reported (Davies & Stark, 1970; Griffith, 1972). Although this discrepancy is almost certainly due to the extent of cross-linking (Payne, 1973) it would be

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useful to verify this interpretation by determining the number of cross-links introduced by a given procedure.

Dunker & Rueckert (1969) pointed out that the effects of intramolecular (S–S) cross-links on the electrophoretic mobility are unexpectedly small, perhaps owing to a compensating interplay between sodium dodecyl sulphate binding and conformation; yet the relative contributions of these two parameters have not been determined for any cross-linked protein. By using methodology developed by Neville (1971), the present paper has the primary aim of determining the relative contributions of sodium dodecyl sulphate binding and conformation to the mobility of sodium dodecyl sulphate–protein complexes. (Throughout this paper, sodium dodecyl sulphate–protein complex refers to the 1.4g of sodium dodecyl sulphate/1 g of protein complex.)

Materials and Methods

Materials

Lysozyme, chymotrypsinogen A, ovalbumin and chymotrypsin were obtained from Worthington Biochemical Corp. (Freehold, N.J. 07728, U.S.A.), and bovine serum albumin was obtained from Calbiochem (La Jolla, Calif. 92037, U.S.A.). [^{14}C]-Formaldehyde was obtained from New England Nuclear (Boston, Mass. 02118, U.S.A.). The [^{14}C]-formaldehyde, which is provided as a 1% (w/w) solution, was diluted 1:20 with 37% (w/w) formaldehyde, giving a final specific radioactivity of approx. 13 μCi/mmol.

Polyacrylamide-gel electrophoresis

Preparation of the polyacrylamide gels was as described by Dunker & Rueckert (1969). Gels contained acrylamide and bisacrylamide at a ratio of 29:1 (w/w); polymerization was promoted by 0.5% (w/v) ammonium persulphate. Time of electrophoresis, voltage, etc. are given in the text.

Staining

The polyacrylamide gels were soaked for 2–6 h in a solution containing 0.5–1% (w/v) Coomassie Brilliant Blue in methanol/acetic acid/water (5:1:5, by vol.) (Weber & Osborn, 1969). De-staining was accomplished by soaking in methanol/acetic acid/water for 1–2 h and then in 7% (v/v) acetic acid until contrast developed. Gels were photographed with a Polaroid MP3 camera.

Cross-linking

Freeze-dried protein samples (1–1.2 mg) were spread over the bottoms of 10 ml beakers (total area approx. 4 cm²/beaker). The beakers were exposed to formaldehyde vapour, issuing from 37% (w/w) formaldehyde, in a closed 1000 ml container at room temperature (approx. 22°C). Reaction times up to 30 min were found to be effective. Prolonged exposure led to insoluble proteins. The cross-linked lysozyme was first soaked in water for 10–20 min, and then made to 2% (w/v) sodium dodecyl sulphate, 5% (v/v) mercaptoethanol, 5% (v/v) glycerol and 0.05 M-Tris/HCl, pH 7.6, with the protein at 1–2 mg/ml. The prior soaking step was absolutely necessary as cross-linked lysozyme was insoluble when a sodium dodecyl sulphate solution was added directly. Mercaptoethanol was omitted when reduction was to be avoided. Samples were immersed in boiling water for 1–3 min just before electrophoresis.

Results

Formation of formaldehyde cross-linked proteins

Exposure of protein powder to formaldehyde vapour was found to be a fast, simple and reliable method for obtaining cross-linked polypeptide chains. In a typical experiment, various exposure times up to 30 min resulted in the electrophoretic patterns shown in Plate 1.

Determination of the number of cross-links

To estimate the effect of cross-linking on electrophoretic mobility, it is necessary to determine the number of cross-links introduced. The vapour-solid-state technique was used with [^{14}C]formaldehyde, except that the surface area of the formaldehyde
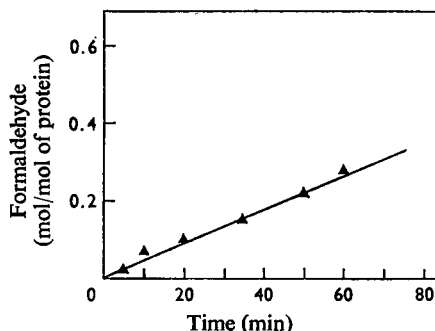

Fig. 1. [^{14}C]Formaldehyde quantitation of cross-linking

Freeze-dried lysozyme was exposed to [^{14}C]formaldehyde vapour; using the specific radioactivity of the [^{14}C]-formaldehyde, dialysis-resistant radioactivity counts at each time-point gave mol of formaldehyde/mol of protein incorporated as a function of time.

was about one-tenth of that for the experiments leading to Plate 1 owing to the limited amount of [^{14}C]formaldehyde available. In an attempt to remove exchangeable (hemiacetal) formaldehyde, after the reaction the proteins were dialysed against several changes of 2% sodium dodecyl sulphate/5% mercaptoethanol/5% glycerol/0.05 M-Tris/HCl, pH 7.6, until the bound radioactivity remained stable (Fig. 1). The 60 min time-point sample, in which 0.28 mol of formaldehyde had reacted with each mol of lysozyme, gave a gel-electrophoresis pattern intermediate between the 10 and 20 min time-point gels of Plate 1. The decreased reaction rate was probably due to the decreased surface area of the formaldehyde solution, although this point was not tested critically.

The distribution of protein in the gel from the 60 min time-point was determined and is presented in Table 1. For a cross-linked molecule with n lysozyme units there must be at least $n-1$ formaldehyde cross-links. Thus an aggregate with n units has a molar ratio of formaldehyde/lysozyme of at least $(n-1)/n$. If an aggregate with n units comprises a fraction, fn, of the total protein, the formaldehyde contribution from that aggregate is $[(n-1)/n]fn$ and the expected average ratio of formaldehyde to protein is simply $\sum_{n=1}^{n}[(n-1)/n]fn$. These values are presented in Table 1 and it can be seen that the formaldehyde due to cross-linking must be at least 0.2 mol of formaldehyde/mol of protein. Since the observed value was approx. 0.28, there is an excess of 0.08 mol of formaldehyde/mol of protein over that required for the intramolecular formaldehyde cross-links. This 0.08 mol may be as extra cross-links or as hemiacetal groups, thus the total number of cross-links for a reduced aggregate of n molecules of lysozyme is

Table 1. *Number of intrachain cross-links per molecule*

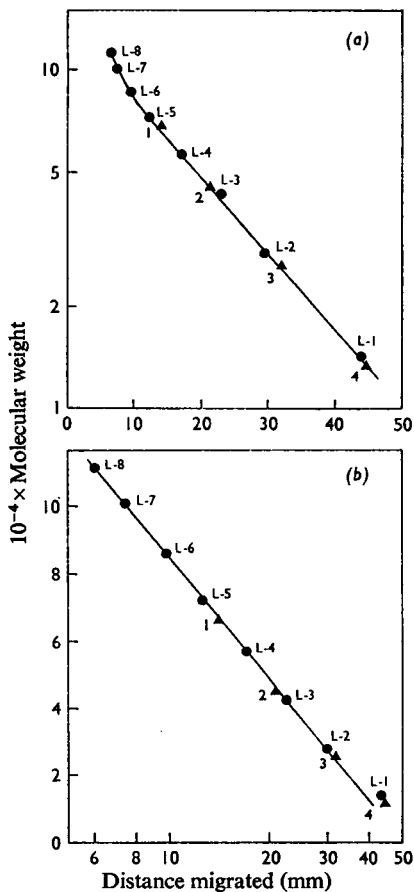| No. of lysozyme units (n)	Fraction of total stain (fn)	Minimum intermolecular formaldehyde cross-links (mol of formaldehyde/ mol of protein)	No. of cross-links	
			Reduced	Unreduced
1	0.64	0	0–0.08	4–4.08
2	0.23	0.115	1–1.16	9–9.16
3	0.09	0.060	2–2.24	14–14.24
4	0.03	0.022	3–3.32	19–19.32
5	0.01	0.008	4–4.40	24–24.40
	Total 0.205			

Fig. 2. *Molecular weight versus relative mobility for cross-linked lysozyme*

The mobilities (migration from left to right) of the aggregated lysozyme molecules are plotted against their calculated molecular weights (●). L-1, L-2, etc., refer to the number of the protein band, and it is assumed that this is the same as the number of lysozyme molecules in the aggregate. A set of markers (▲) was run in parallel: 1, bovine serum albumin; 2, ovalbumin; 3, chymotrypsinogen A; 4, the B-chain of chymotrypsin. The plots are logarithm of molecular weight versus mobility (a) and molecular weight versus logarithm (mobility) (b).

between $n-1$ and $n-1+0.08n = 1.08n-1$. These values are also given in Table 1. In addition to formaldehyde cross-links, unreduced lysozyme has four S–S cross-links per lysozyme molecule. Thus the number of intramolecular cross-links should be between $n-1+4n = 5n-1$ and $1.08n-1+4n = 5.08n-1$. These values are also listed in Table 1.

Mobility of reduced cross-linked lysoyzme

The molecular-weight versus electrophoretic-mobility curves derived from the cross-linked lysozyme are indistinguishable from curves defined by marker proteins (Fig. 2), whether the data are plotted as logarithm molecular weight versus relative mobility (Fig. 2a) or as logarithm (relative mobility) versus molecular weight (Fig. 2b). A 1–2% systematic deviation between the standards and the cross-linked lysozyme cannot be ruled out. It is of historical interest that Shapiro et al. (1967) used the functional relationship in Fig. 2(a), whereas that of Fig. 2(b) is on a more sound theoretical basis (Neville, 1971). [See eqn. (1) below; since it has been found that molecular weight is proportional to K_R for sodium dodecyl sulphate–protein complexes (Neville, 1971), plotting logarithm of mobility against molecular weight should give straight lines.] Owing to individual differences between different proteins, the resulting scatter of the data obscures the correct functional relationship, thus accounting for the incorrect choice in the earlier work (Shapiro et al., 1967; Dunker & Rueckert, 1969; Weber & Osborn, 1969). However, the cross-linked molecules, which comprise identical repeating units, have the advantage of lessening data scatter, thus revealing the advantage of Fig. 2(b) over Fig. 2(a).

Mobility of unreduced cross-linked lysozyme

To test the effects of conformation on migration of proteins in sodium dodecyl sulphate/polyacrylamide gels, formaldehyde-cross-linked lysozyme was electrophoresed with and without prior reduction. The presence of four intramolecular S–S bridges/14400

daltons has an obvious, though slight, effect on protein mobilities (Plate 2). On the basis of these gels and the ones used for Fig. 3 (see below) unreduced proteins had apparent molecular weights about $14 \pm 3\%$ less than those of their reduced counterparts. In the 50 or so determinations for the decrease in apparent molecular weight due to cystine bonds, the percentage decrease was found to be about 14% irrespective of the size of the aggregate. Such a dependence of mobility on the state of oxidation of intramolecular disulphide bridges is of the same order of magnitude as those previously reported (Dunker & Rueckert, 1969; Griffith, 1972).

Molecular basis of anomolous mobility

Anomalously migrating proteins can be detected by comparing electrophoretic mobilities at a variety of gel concentrations (Neville, 1971). The relative mobilities at the different concentrations are found to obey the relationship (Ferguson, 1964):

$$\log R_F = \log Y_0 - K_R T \qquad (1)$$

where R_F is the relative electrophoretic mobility of a protein in a gel of concentration T [the notation is from Hjerten (1962) where T denotes the total weight in grams of monomer acrylamide plus bisacrylamide per 100 ml of gel solution], Y_0 is the limiting relative mobility [this name, rather than 'mobility in free solution' was suggested for Y_0 by Shirahama *et al.* (1974)], and K_R is the retardation coefficient (Neville, 1971).

The results of such an experiment with reduced and unreduced cross-linked lysozyme are shown in Fig. 3. These lines are considerably steeper than those reported for similar-sized proteins (Neville, 1971; Banker & Cotman, 1972); the reason for this steepness is our increased bisacrylamide concentration; that is, Weber & Osborn (1969) showed that for gels of $T = 10$, increasing bisacrylamide concentration dramatically decreases protein mobilities. This effect accounts for the steep slopes of Fig. 3. With regard to the cross-linked proteins, the main finding is that unreduced lysozyme aggregates have free mobilities essentially indistinguishable from those observed for the reduced aggregates.

From eqn. (1) it is evident that plots of logarithm of R_F versus K_R should also yield straight lines (of slope $-T$ and intercept Y_0). Whereas plots of logarithm R_F versus T connect data points from the same protein and by extrapolation allow the limiting relative mobilities of the different proteins to be compared, the plots of logarithm R_F versus K_R connect data points from different proteins, and thus proteins with unusual K_R values are observed to lie off the line set by normal proteins (Neville, 1971). Obtaining the various K_R values from the data of Fig. 3, plots of logarithm R_F versus K_R for $T = 14$, 10, and 6 are given
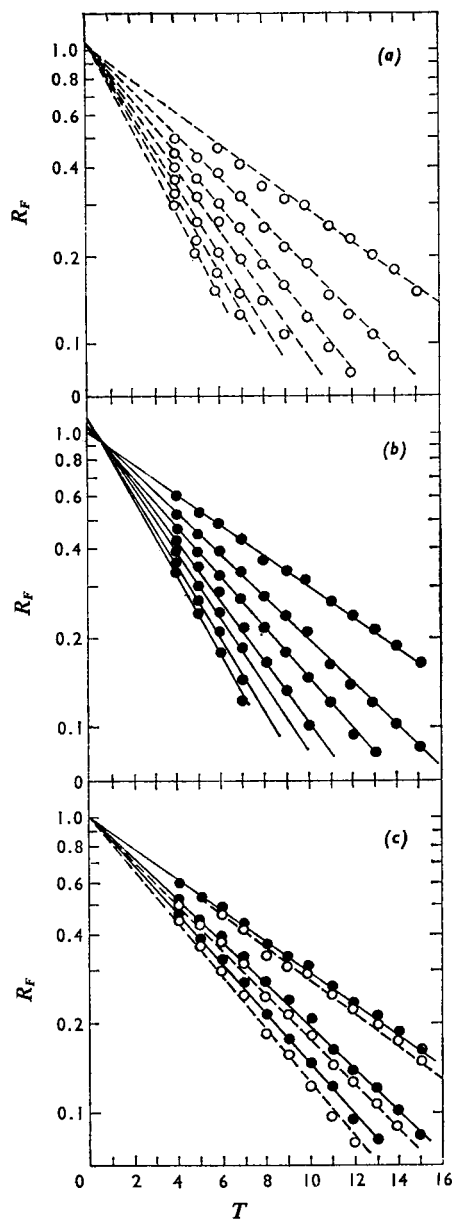

Fig. 3. *Plots of logarithm R_F versus T for reduced and unreduced lysozyme*

Polyacrylamide gels from $T = 15$ to 4 were prepared by dilution from a common stock. Reduced and unreduced lysozyme were electrophoresed by the split-gel technique. From top to bottom in a given panel, the lines represent the monomer, dimer, trimer, etc. The data for the reduced aggregates (○) is plotted in (*a*), that for the unreduced aggregates (●) in (*b*). The first three lines for the reduced (○) and unreduced (●) aggregates are plotted in (*c*).
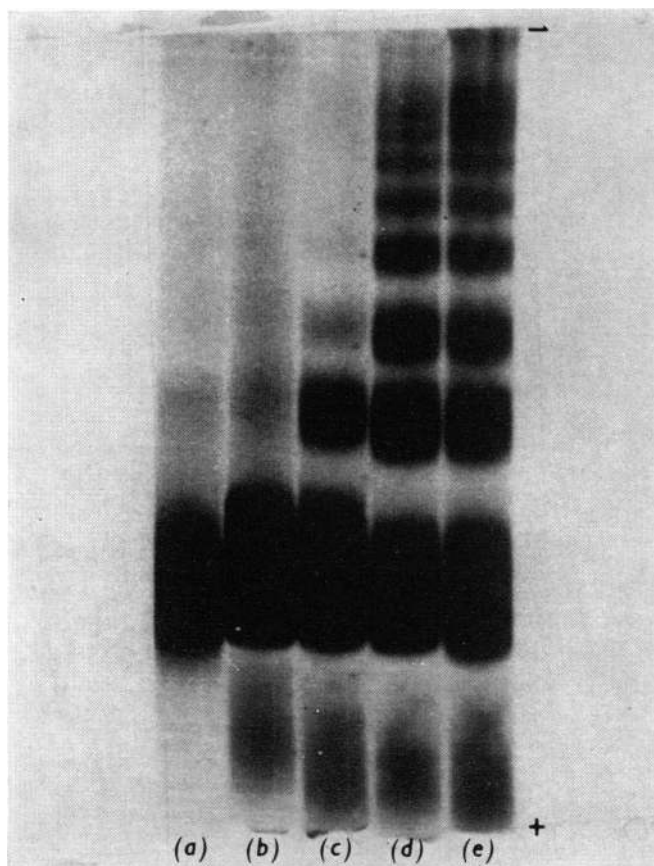
EXPLANATION OF PLATE I

Cross-linking of lysozyme by formaldehyde

Lysozyme powder was exposed to formaldehyde vapour and the resulting protein aggregates (approx. $100\,\mu g$/gel) were electrophoresed on $T = 10$ polyacrylamide gels for 4h at about 2V/cm. The times of exposure to formaldehyde vapour were $0(a)$, $5(b)$, $10(c)$, $20(d)$ and $30(e)$min. A small proportion of the lysozyme was cross-linked into a dimer even at zero time, presumably due to disulphide interchange. The material below lysozyme in gels (b)–(e) (and also in the gels of Plate 2) co-migrates with Coomassie Brilliant Blue tracking dye. The composition and origin of the material below lysozyme is uncertain, although it has been subsequently removed by raising the temperature and prolonging the time of the methanol/acetic acid/water soaking step, which suggests that the material is not protein.

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(a) (b) (c)   (d) (e) (f)
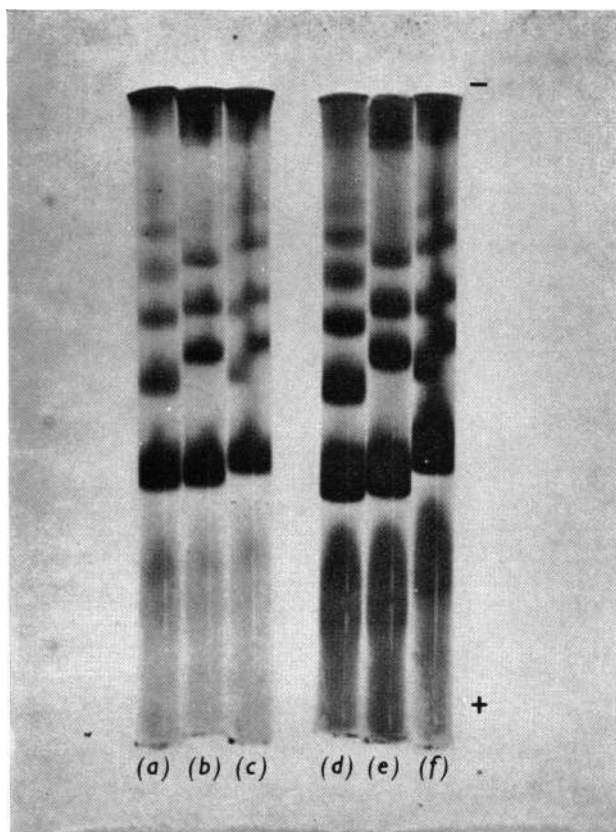
EXPLANATION OF PLATE 2

Effects of intramolecular covalent bonds on electrophoretic mobility of sodium dodecyl sulphate–protein complexes

Lysozyme was cross-linked by the vapour technique, soaked in water and then adjusted to 2% (w/v) sodium dodecyl sulphate, 5% (v/v) glycerol, 0.05 M-Tris/HCl, pH 7.6, with a twofold concentrated solution. The sample was divided into two; mercaptoethanol was added to 5% (v/v) to one half and both samples were immersed in boiling water for 3 min. Two sets of samples were prepared independently The resulting $T = 10$ gels after 4 h at about 2 V/cm are unreduced lysozyme (*a* and *d*), reduced lysozyme (*b* and *e*), and split gels (*c* and *f*) with unreduced lysozyme on the left in the split gels.

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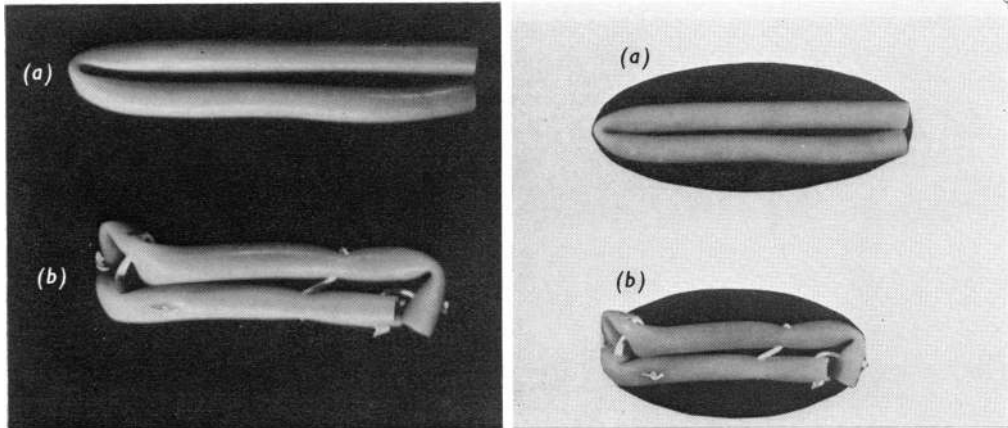
EXPLANATION OF PLATE 3

Simplified scale models comparing reduced and unreduced lysozyme

Rubber tubing models without (*a*) and with (*b*) connecting wires representing cystine bonds. The positions and lengths of the simulated cystine bonds are correctly scaled. The outlines of prolate ellipsoids of appropriate semi-major and semi-minor axes are indicated in the models on the right.
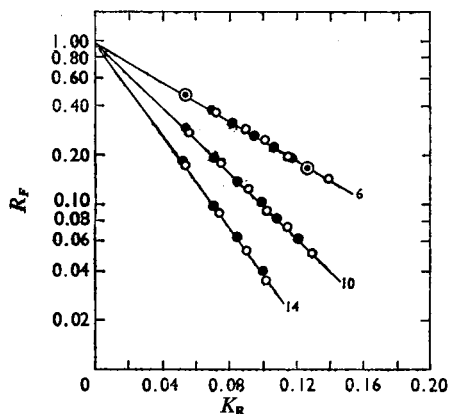
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Fig. 4. *Plots of logarithm R_F versus K_R for unreduced lysozyme*

The data of Fig. 3 were used to determine the K_R values for the various reduced (○) and unreduced (●) lysozyme aggregates. From top to bottom the data are from $T = 6$, 10 and 14 gels.

in Fig. 4. The K_R values for the reduced and unreduced lysozyme aggregates appear to be members of a common set.

Discussion

Equilibrium dialysis has shown that certain unreduced proteins bind substantially less sodium dodecyl sulphate than do the reduced proteins (Pitt-Rivers & Impiombato, 1968). A significant decrease in sodium dodecyl sulphate binding would lead to a substantial lessening in the surface-charge density of the unreduced proteins; a reduced surface-charge density would be expected to decrease the limiting relative mobility (Neville, 1971; Banker & Cotman, 1972), since free mobilities should be very sensitive to surface-charge densities and less sensitive to overall changes in shape. The anomalous mobility of certain membrane glycoproteins has been attributed in part to their unusual limiting relative mobilities, evidently due to anomalous sodium dodecyl sulphate binding (Banker & Cotman, 1972), showing that the plot of logarithm R_F versus T is indeed useful as a test to reveal unusual free mobilities. Further, the predicted decrease in free electrophoretic mobility of unreduced as compared with reduced proteins has been experimentally verified by free-boundary electrophoresis (Shirahama et al., 1974). Therefore we expected that the substantially lessened sodium dodecyl sulphate binding would lead to measurably decreased limiting relative mobilities for the unreduced lysozyme aggregates. Our results (Fig. 3) are exactly in contrast with this expectation. The most likely

explanation is that the high (approx. 2%) sodium dodecyl sulphate concentrations and the high (100°C) temperature induce binding of sodium dodecyl sulphate to regions of the unreduced protein that are refractory to the low (0.1%) sodium dodecyl sulphate and moderate (23°C) temperatures used in the equilibrium-dialysis and free-boundary experiments. This explanation is supported by the following observations: both Dunker & Rueckert (1969) and Shirahama et al. (1974) showed that ribonuclease migrates anomalously slowly, but that the anomalous mobility depends on the methods used to reduce and block the thiols, thus suggesting that an unusually stable S–S bond is the cause of the diminished electrophoretic mobility. Shirahama et al. (1974) went on to show that the effects of the presumed S–S bond could be reversed by raising the sodium dodecyl sulphate concentration.

Plots of logarithm R_F versus K_R have been used to demonstrate that certain anomalous proteins have unusual K_R values, even though their limiting relative mobilities are not unusual (Neville, 1971; Banker & Cotman, 1972). The interpretation of such results is that the retardation suffered by a protein during passage through acrylamide is very sensitive to the shape of the protein, and thus atypical shapes are revealed as atypical K_R values. It is evident that aggregates of reduced lysozyme would be expected to have significantly different overall shapes from aggregates of unreduced lysozyme. In this event, plots of logarithm R_F versus K_R should give one set of lines for the reduced aggregates and a separate set of lines for the unreduced aggregates. Thus the plot of logarithm R_F versus K_R shows that reduced and unreduced lysozyme have unexpectedly similar overall conformations.

Thus Figs. 3 and 4 together imply that the reduced and unreduced lysozyme have essentially identical overall surface-charge densities and gross conformations, yet it should be kept in mind that the unreduced proteins migrate unmistakably faster than their reduced counterparts (Plate 2). This anomolous mobility, if not known beforehand, would have been totally concealed from detection by the techniques currently used to reveal unusual proteins. Therefore caution should be exercised when evaluating cross-linked aggregates, especially if the structure under analysis has similar-sized proteins.

Two models have been proposed for the gross conformation of sodium dodecyl sulphate–protein complexes: (1) the prolate ellipsoid model (Reynolds & Tanford, 1970); and (2) the 'necklace' model, in which micelle-like clusters of sodium dodecyl sulphate are scattered along a flexible polypeptide backbone like the beads of a necklace (Shirahama et al., 1974).

For prolate ellipsoid-shaped beads, the 'necklace' model converges with the prolate ellipsoid model as the beads become contiguous. Thus it could be

argued that the difference between the two models lies not primarily with the gross conformation of the complex, but rather with the detailed conformation of the polypeptide backbone within the complex. The prolate ellipsoid would seem to require that the structure of the polypeptide backbone is continuous along the length of the complex. [One possibility consistent with the overall length is that the polypeptide is entirely helical and folded back along itself; however, there is no compelling reason to believe that this is the actual structure of the complex (Reynolds & Tanford, 1970).] On the other hand, the 'necklace' model would permit the polypeptide backbone to be organized discontinuously in discrete lumps along the length of the structure. A sodium dodecyl sulphate micelle would contain about 80–100 molecules under the conditions used for electrophoresis [estimated from the ionic strength of the electrophoresis conditions and from the data in Table 5 of Helenius & Simons (1975)]. There is about 0.5 molecule of sodium dodecyl sulphate per amino acid residue (Shirahama *et al.*, 1974); thus each bead in the 'neck-

lace' model could contain roughly $90 \times 2 = 180$ amino acid residues. Since lysozyme contains 129 residues, a bead would contain approximately one lysozyme molecule.

Suppose the prolate ellipsoid model were correct. Then cross-linked aggregates and especially unreduced cross-linked aggregates would not be expected to be able to form a regular structure along the length of the complex. Thus, compared with regular polypeptides, cross-linked aggregates and especially unreduced cross-linked aggregates would be expected to have unusual K_R values.

On the other hand, suppose the 'necklace' model were correct. Then continuous polypeptides would be arranged in a set of discontinuous lysozyme-sized lumps on the order of the size of lysozyme. Both reduced and unreduced lysozyme aggregates would also be like a string of beads and so cross-linked aggregates would have normal K_R values.

The K_R values of reduced and unreduced lysozyme aggregates are members of a common set and are indistinguishable from the K_R values of normal proteins. Thus, on the face of it, the data in this paper would seem to strongly support the 'necklace' model over the prolate ellipsoid model.

To further test this tentative conclusion, we have constructed simplified scale models of reduced and unreduced lysozyme by ignoring side chains, assuming 1.0 nm for the (presumed to be helical) polypeptide backbone, and assuming 0.14 nm/residue along the backbone [calculated from Table 2 of Reynolds & Tanford (1970), by assuming a folded structure]. The wire cross-links lead to a convoluted structure that surprisingly can be folded to achieve a final shape very similar to that for the uncross-linked model (Plate 3). The model with wire cross-links is about 12% shorter than the un-cross-linked model in apparent agreement with the 14% reduction in molecular weight for the unreduced lysozyme aggregates. Finally, since there would be formaldehyde-reactive groups at both ends of lysozyme molecules folded as shown in the models in Plate 3, end-to-end aggregation is chemically possible. As illustrated in Fig. 5, end-to-end aggregation of such molecules would yield a set of molecules consistent with the data of this paper.

Owing to the fortuitous loci of the S–S bonds, lysozyme cannot be used to distinguish the prolate ellipsoid model from the 'necklace' model. However, it is evident that the methodology developed in this paper could be used to distinguish the two proposed models for sodium dodecyl sulphate–protein complexes, provided that an appropriate monomer protein could be found. Such a protein should have a number of S–S bonds distributed in a manner that would prevent deformation of the protein into a prolate ellipsoid. The recently characterized protein neurophysin is apparently a suitable protein.
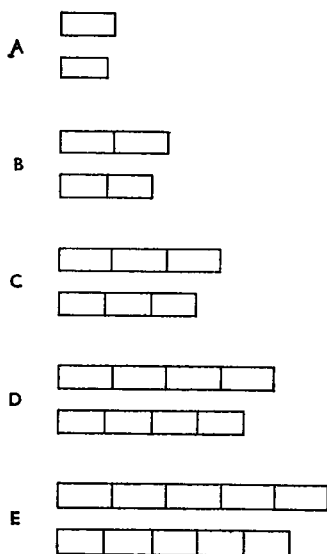

Fig. 5. *Schematic diagram for formaldehyde-cross-linked lysozyme*

Prolate ellipsoids are approximated by appropriately scaled rectangles. Assuming that the effect of the four cystine bonds is to shorten the length of the monomer by 14%, the upper rectangle in each pair represents reduced lysozyme and the lower, 14% shorter, rectangle in each pair represents unreduced lysozyme. End-to-end aggregation then generates the set of molecules depicted in this Figure; such a set of molecules is consistent with the data of this paper.

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