Journal of Chromatography, 377 (1986) 361—367
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3019

Note

Effect of intramolecular S—S bond cleavage upon the mobility of proteins in sodium dodecyl sulphate polyacrylamide gel electrophoresis

J. HAUSMANN, J. HORÁČKOVÁ and Z. DEYL*

Institute of Physiology, Vídeňská 1083, Prague - 4, Krč (Czechoslovakia)

(First received September 30th, 1985; revised manuscript received November 26th, 1985)

Molecular weight determination of proteins by polyacrylamide gel electrophoresis (PAGE) became a widely applied method in biomedical research. In the vast majority of situations, this technique is based on a simple comparison of the mobility of an unknown protein with a series of standards and the actual properties of the protein investigated are not taken into consideration. This may lead to erroneous molecular weight estimates and hence to inadequate interpretation of results. It is necessary to realize that the mobility of protein molecules in polyacrylamide gel depends on the effective radius of the molecule and that the molecular weight estimation is only indirect. This is particularly important with proteins containing intramolecular disulphide bonds. There are reports that such molecules exhibit a higher relative molecular mass on sodium dodecyl sulphate (SDS)-PAGE after reduction of the disulphide bonds with β-mercaptoethanol [1]. For example, bovine serum albumin, which contains seventeen disulphide bonds per molecule, appears in its non-reduced form as a molecule with a relative molecular mass of 55 000 while after reduction it exhibits a relative molecular mass of 67 000 [2]. This effect is explained by a conformational change leading to an increased Stokes' radius and consequently to an increased friction between the molecule and the gel. Schach [3] observed a similar effect in the case of jojoba proteins subjected to electrophoresis in the presence of urea and mercaptoethanol and he suggests that the increase in relative molecular mass estimates results from random polymerization of the reduced form, which represents another point of view on this problem.

In the present investigation, we attempted to reveal whether the observed changes in protein mobility after S—S bond reduction are due solely to the

0378-4347/86/$03.50

conformational change or whether the decreased mobility observed after splitting of the disulphide bridges is also affected by the effective charge of the molecule. We approached this problem by using the relation between the mobility of the macromolecule (U) at a given gel concentration (T), described by the Fergusson equation [4]:

$$\log U = \log U_o - K_r \cdot T \quad (1)$$

In this equation, the intercept on the ordinate gives the free mobility (U_o) of a given macromolecule whereas the retardation coefficient (K_r) is given by its slope.

EXPERIMENTAL

The electrophoresis procedure followed that described by Laemmli [5]. The amount of acrylamide used was such to ensure the desired gel concentration and the ratio of acrylamide to bisacrylamide was kept constant (36.5:1, w/w). A vertical slab gel electrophoresis cell (Model 220, Bio-Rad Labs., Richmond, CA, U.S.A.) was used. Acrylamide and bisacrylamide were the products of Bio-Rad Labs. N,N,N′,N′-Tetramethyldiamine (TEMED), SDS and β-mercaptoethanol were purchased from Serva (Heidelberg, F.R.G.).

The nature and sources of protein standards are summarized in Table I. All other chemicals were of the highest available purity and were the products of Lachema (Brno, Czechoslovakia).

An electrode buffer consisting of 0.025 mol/l Tris, 0.192 mol/l glycine and 2% SDS was used. Of the marker dye (bromphenol blue) 1% (w/v) was added to the cathode buffer to mark the front of the electrophoreogram. If reduction of disulphide bonds was desired, the samples were dissolved in the sample buffer [5] containing 5% β-mercaptoethanol and boiled for 5 min. In each lane, 30–50 μl of the sample buffer containing 0.8—1.2 mg of protein were loaded. A constant-voltage source (Tesla Bs 275, Czechoslovakia) was used to supply 20 mA per slab (180 V) and the front line of electrophoresis migrated for 3—4 h depending on the concentration of the gel. The gels were stained in 0.1%

TABLE I

SPECIFICATION OF PROTEINS USED

Protein	Relative molecular mass	Number of disulphide bonds	Source*
Bovine serum albumin (BSA) monomer	67 000	17	Serva
BSA trimer	201 000	51	Serva
Pepsin	36 000	3	Serva
Growth hormone (bovine) monomer	25 000	2	Calbiochem
Growth hormone (bovine) dimer	50 000	4	Calbiochem
Growth hormone (bovine) trimer	75 000	6	Calbiochem
Elastase	24 000	4	Serva
Myoglobin	17 900	0	Serva

*Serva (Heidelberg, F.R.G.); Calbiochem (Lucerne, Switzerland).

Coomassie Brilliant Blue dissolved in 50% trichloroacetic acid and the rest of the dye was washed out using 7% acetic acid.

The values of relative migration of protein bands (R_F) were obtained by measuring the ratio between the front (migration distance of the marking dye) and the distance migrated by the protein zone. The Fergusson relationship was obtained by plotting log R_F versus the concentration of the polyacrylamide gel.

RESULTS AND DISCUSSION

The differences in mobility of individual protein standards resulting from the reduction of the disulphide bonds are shown in Fig. 1A—C. The corresponding Fergusson plots are presented in Fig. 2A–C. The most obvious observation here is the fact that the Fergusson plots corresponding to the reduced and non-reduced form of a particular protein are practically parallel. It follows from the Fergusson equation (eqn. 1) that two lines representing two molecules with different relative molecular mass (different Stokes' radius) exhibit different slopes (K_r) and that two parallel lines represent molecules of the same relative molecular mass (Stokes' radius) but differ in the charge-to-mass ratio (and thus in the U_o values). When SDS is added to a protein solution, the charge-to-mass ratio is practically constant and the above-mentioned lines intersect at the same point. In our experiments, however, the series of lines representing the reduced and non-reduced form of the protein intersect at two different points (Fig. 2); this suggests that the charge-to-mass ratio is different and because the relative molecular mass is identical there should be a charge difference between the reduced and non-reduced forms. The most obvious explanation would be that the reduced and non-reduced form of the same protein bind different amounts of SDS, this being lower with the reduced form. Consequently, no difference in mobility should be seen in proteins in which S—S bonds are absent. This latter fact was experimentally verified (Fig. 3 and ref. 6). One may speculate that unfolding of the protein molecule after reduction may result in a change in hydrophobic region distribution that does not favour SDS binding.

The above explanation, however, contradicts the results obtained by equilibrium dialysis. As shown by Pitt-Rivers and Impiombato [6], the reduced forms of protein bind ca. 1.4 g of SDS per g of protein as compared to the non-reduced form in which ca. 1 g of SDS per g of protein are bound. Since the quoted authors have used a different series of proteins, we have verified their data by using our samples. In accord with the data of Pitt-Rivers and Impiombato [6], it turned out that their conclusion is also valid for all proteins used in the reported experiments.

Another possible explanation may be that the retardation of electrophoretic mobility caused by unfolding of the molecule after S—S bond cleavage predominates over the charge increase (and hence increased mobility) caused by the increased binding of SDS by the unfolded molecule. From this point of view, the idea that the changes in the friction coefficient due to the change in the molecular shape compensate for most of the charge difference [6] appears incorrect.

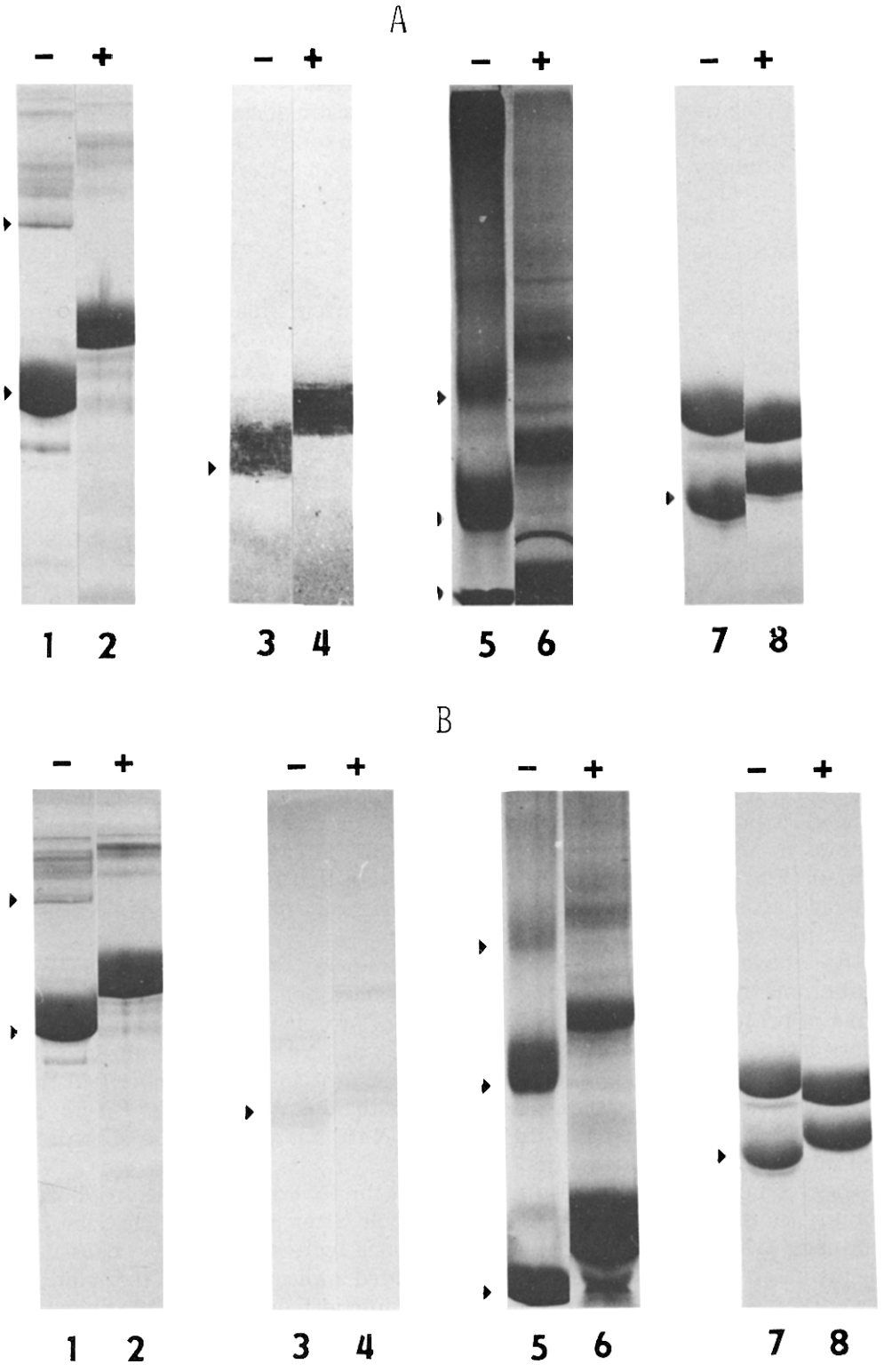

Fig. 1.

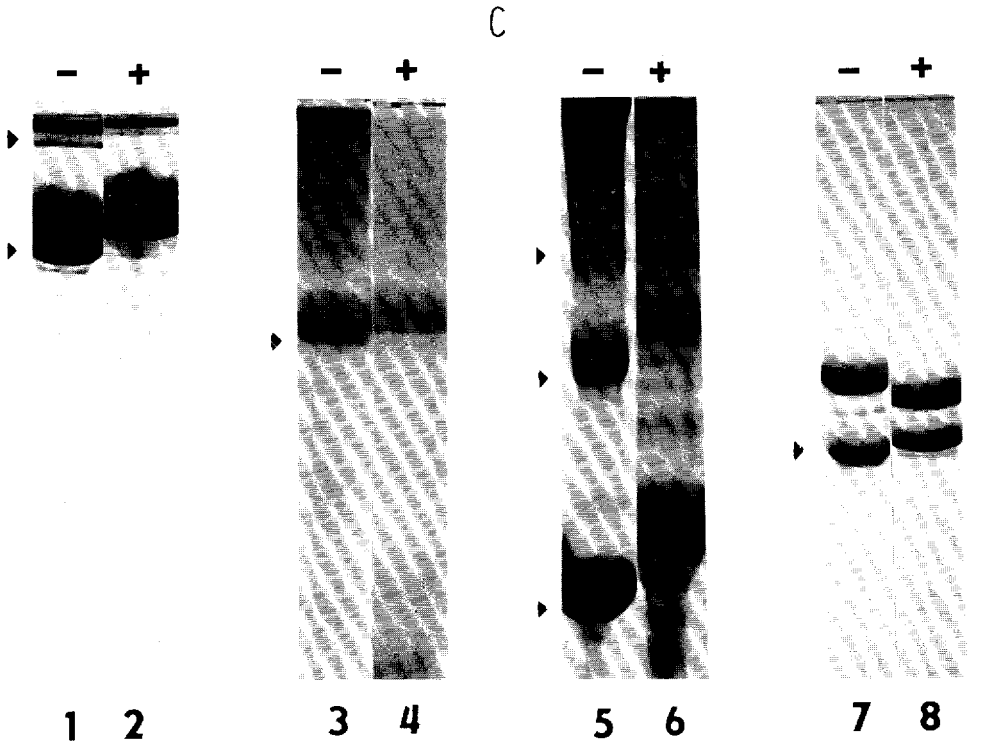

Fig. 1. Typical SDS-PAGE of reduced (+) and non-reduced (−) proteins: (A) 8% gel; (B) 10% gel; (C) 13% gel. Lanes 1 and 2: BSA; lanes 3 and 4: pepsin; lanes 5 and 6: growth hormone; lanes 7 and 8: elastase (lower of the bands). Arrows indicate the position of bands under investigation. The second more-slowly-moving band in the elastase preparation represents breakdown products, always observed in this enzyme.

Finally, one may speculate that the amount of SDS bound to the protein as revealed by equilibrium dialysis may not be identical with that bound to the protein backbone during electrophoresis. It seems well established (see ref. 6 and other references therein) that SDS is bound to proteins in several micellar forms, some of which are broken down during the electrophoretic separation. Putnam and Neurath [7] found that with horse serum albumin three types of SDS–protein complexes are formed, which contain 55, 110 and 220 SDS molecules per molecule of albumin, respectively. Of these complexes, the largest is unstable upon electrophoresis and breaks down under formation of the complex containing the lowest amount of SDS.

Generally, the difference in mobility between the reduced and non-reduced form of proteins with similar molecular mass increases with increasing number of disulphide bonds per unit mass. Thus, the reduction of seventeen disulphide bonds in serum albumin causes a higher retardation than the three or four disulphide bonds in other molecules. This relation is, however, not strictly quantitative. Small differences resulting from the presence of one or two bonds may not be detected at all, particularly if such a bond has only a minimum effect on the steric conformation being, for example, terminally located.

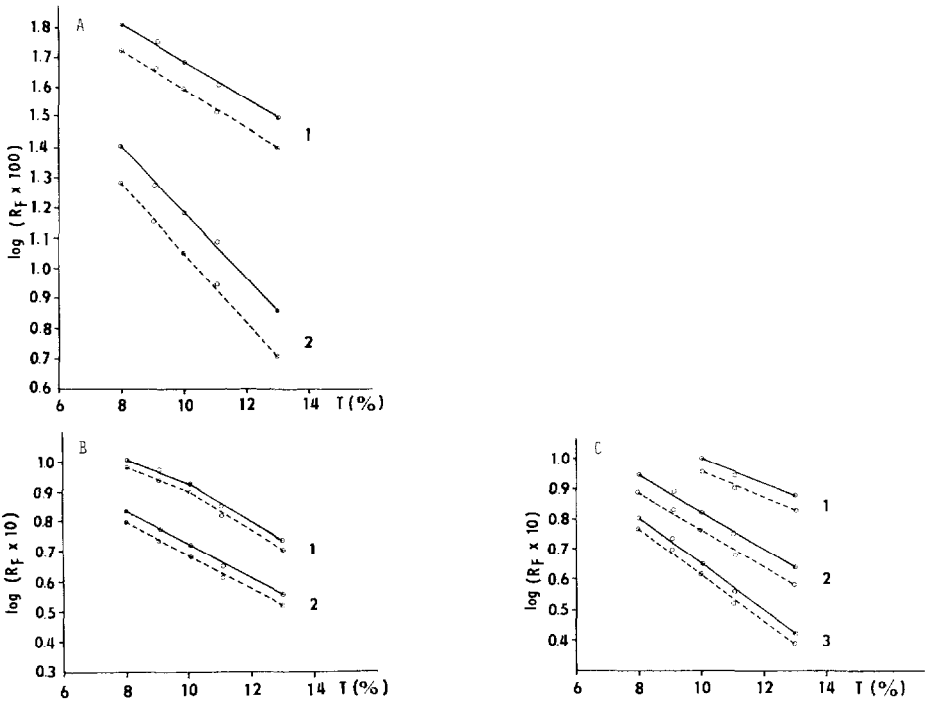

Fig. 2. Plot of the relative mobility (R_F) versus gel concentration (T) for different reduced (dashed line) and non-reduced (full line) protein—SDS complexes. (A) BSA monomer (1) and dimer (2); (B) elastase (1) and pepsin (2); (C) growth hormone monomer (1), dimer (2) and trimer (3). Note the convergence of the lines representing the non-reduced proteins and the parallel relation between the reduced and non-reduced form of the same molecule.

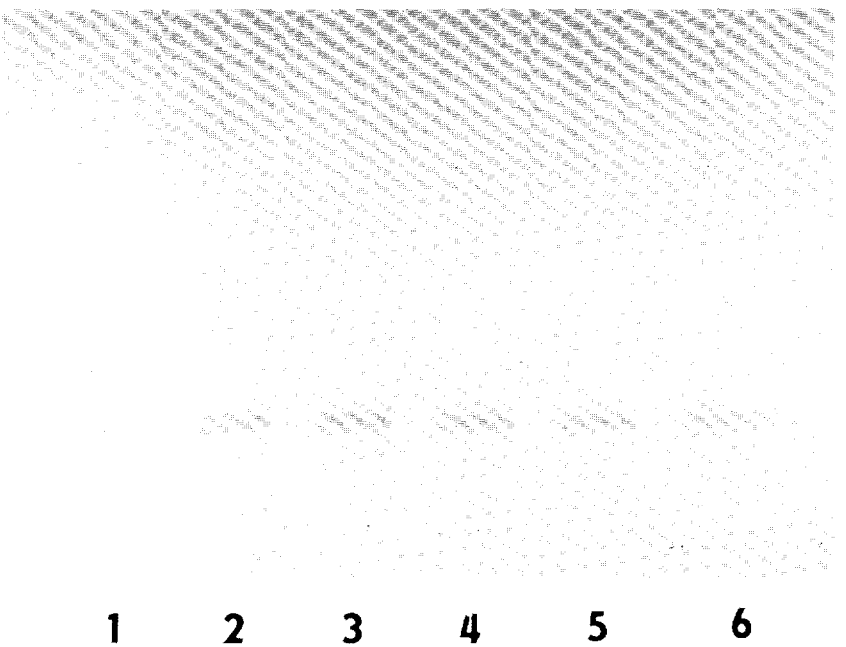

Fig. 3. SDS-PAGE of myoglobin. All samples were incubated at 100° C with various concentrations of β-mercaptoethanol. Lanes 1—6: increasing concentrations of β-mercaptoethanol: 0, 0.5, 1, 2.5, 6 and 10% in the sample.

REFERENCES

1 R.L. Allore and B.H. Barber, Anal. Biochem., 137 (1984) 523.
2 M.J. Owen, B.H. Barber, R.A. Faulkes and M.J. Crumpton, Biochem. J., 192 (1980) 49.
3 A.A. Schach, Electrophoresis, 5 (1984) 180.
4 O. Gaâl, G.A. Medgyesi and L. Vereczkey, Electrophoresis in the Separation of Biological Macromolecules, Wiley, Chichester, 1980, p. 206.
5 U.K. Laemmli, Nature, 227 (1970) 680.
6 R. Pitt-Rivers and F.S.A. Impiombato, Biochem., J., 109 (1968) 825.
7 F.W. Putnam and H. Neurath, J. Biol. Chem., 159 (1945) 195.