THE RELATIONSHIP OF MOLECULAR WEIGHT TO ELECTROPHORETIC MOBILITY OF FLUORESCAMINE-LABELED PROTEINS IN POLYACRYLAMIDE GELS

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SUMMARY

Proteins of known molecular weights were labeled with fluorescamine and then subjected to electrophoresis through polyacrylamide gels. The electrophoretic mobilities of the fluorescamine-labeled proteins were dependent upon their respective molecular weights over a range of 17,000 to 70,000 daltons. The correlation of electrophoretic mobility of fluorescamine-labeled protein to molecular weight was similar to results obtained in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The speed with which data can be obtained with the described procedure is a definite advantage over currently employed procedures. These findings encourage the use of fluorescamine for rapid, sensitive determinations of molecular weights of proteins in polyacrylamide gels.

Fluorescamine is a new, novel reagent with broad applications in fluorescence analysis of proteins and other compounds which have primary amino groups (1,2). Recently, we have developed a method for fluorescence gel scanning of fluorescamine-labeled proteins in polyacrylamide gels (3). This procedure offers a rapid, simple, sensitive and quantitative method for the analysis of proteins in gels.

In the course of our studies, we observed that the electrophoretic mobilities of fluorescamine-labeled proteins were altered from the mobilities of unlabeled proteins in the same system. Fluorescamine reacts with the ε-amino groups of lysine, as well as the N-terminal amino groups, with the concomitant generation of a negative charge on the fluorophor (2) as illustrated as in Fig. 1. Thus, the net charge of a given protein would be drastically altered in favor of the formation of highly negatively charged molecules whose electrophoretic mobilities could be expected to be analogous to protein-SDS[1] complexes. Indeed, in early studies we found that the electrophoretic mobilities of fluorescamine-labeled proteins were very similar to protein-SDS complexes. These observations prompted us to examine the possibility of estimating the molecular weights of proteins in non-detergent, polyacrylamide gel systems.

The determination of molecular weight by SDS-polyacrylamide gel electro-

[1] SDS; sodium lauryl sulfate

phoresis is a well-documented and generally accepted technique. The method was used first in neutral polyacrylamide gel electrophoresis by Shapiro et al. (4) and later verified for many proteins by Weber and Osborn (5). Neville (6) showed that molecular weights of protein-SDS complexes also could be determined in alkaline, discontinuous buffer systems. While the theoretical aspects of the method remain obscure and complex, it generally has been agreed that the separation of highly negatively charged protein-SDS complexes is dependent primarily upon the sieving effect of the polyacrylamide gel while the charge differences remain relatively unimportant (5-7).

MATERIALS AND METHODS

Purified proteins of known molecular weights were obtained from commercial sources: γ-globulin (human), albumin (bovine), chymotrypsinogen A from Schwarz/Mann, Orangeburg, New York; catalase and glutamate dehydrogenase from Miles Seravac, Kankakee, Illinois. Tris(hydroxymethyl)aminomethane, Coomassie Brilliant Blue R250 and 2-mercaptoethanol were supplied by Sigma Chemical Company, St. Louis, Missouri; dimethyl sulfoxide[2], boric acid, hydrochloric acid, sulfuric acid, acetic acid and methanol by J. T. Baker Chemical Co., Phillipsburg, New Jersey; sodium lauryl sulfate by Fisher Scientific Co., Fair Lawn, New Jersey; ammonium persulfate by Matheson, Coleman and Bell, Norwood, Ohio; sucrose, density gradient grade, by Schwarz/Mann; acrylamide, N,N'-methylene-bisacrylamide and N,N,N',N'-tetramethylethylenediamine by Eastman Kodak Co., Rochester, New York. Fluorescamine (Fluram™) was a gift from Roche Diagnostics, Nutley, New Jersey.

Conventional electrophoresis of protein-SDS complexes was performed using one of the discontinuous buffer systems described by Neville (6). The same electrophoretic system was used for electrophoresis of fluorescamine-labeled proteins with the exception that SDS was omitted. The upper reservoir buffer consisted of 0.04 M boric acid-0.041 M Tris, pH 8.64; the upper gel buffer was 0.0267 M H_2SO_4-0.0541 M Tris, pH 6.1 (running pH 8.64); lower gel buffer and lower reservoir buffer were 0.0308 N HCl-0.4244 M Tris, pH 9.18 (running pH 9.5). When electrophoresis of protein-SDS complexes was performed, 0.1% SDS was added to the upper reservoir buffer. The gels were polymerized in 0.6 x 10 cm glass tubes. The upper gel contained 3.2% acrylamide with 6.25% bisacrylamide; the lower gel contained 11.1% acrylamide with 0.9% bisacrylamide.

Proteins were labeled with fluorescamine by a procedure similar to the one described by Böhlen et al. (8). One milliliter aliquots of fluorescamine (0.5 mg/ml in DMSO) were added with vigorous mixing to 1 ml-samples of protein (0.4 mg/ml in 0.04 M borate buffer, pH 9.0). After 10 minutes, 2 ml sample buffer (0.08 M boric acid, 0.082 M Tris and 20% sucrose, pH 8.64) and 10 µl

[2]DMSO; dimethyl sulfoxide

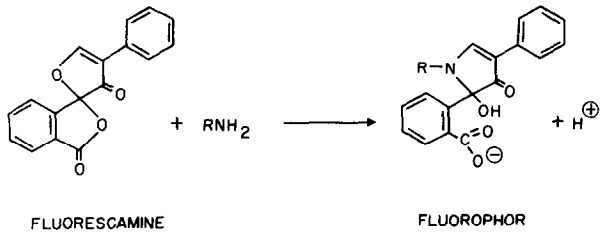

Figure 1. The reaction of fluorescamine with proteins.

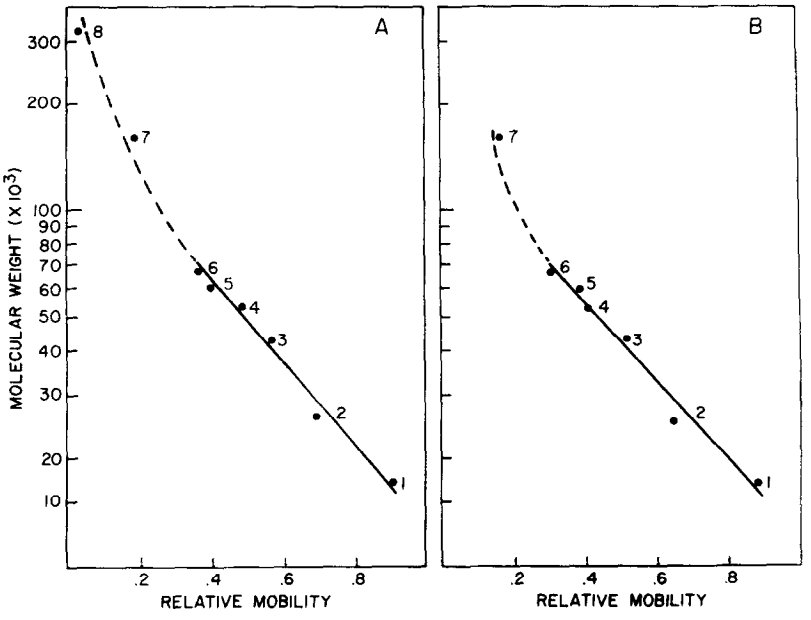

Figure 2. Relationship of molecular weights of proteins to their relative mobilities on separate polyacrylamide gels. A. Electrophoresis of fluorescamine-labeled proteins. B. Electrophoresis of protein-SDS complexes. Proteins: (1) myoglobin, (2) chymotrypsinogen A, (3) ovalbumin, (4) glutamate dehydrogenase (subunit), (5) catalase, (6) albumin, (7) γ-globulin (monomer), (8) glutamate dehydrogenase (undissociated). Each point represents the mean of 3 separate runs, s.e.m. < 3% of mean.

bromphenol blue were added. Fifty microliters of each sample (5 µg protein) were applied per gel and electrophoresis was performed for about 2 hours at 1.5 ma per tube until the marker dye had migrated about 6 cm into the lower gel. The gels were removed from the tubes and scanned for fluorescence using a modified Gilford 2410-S linear transport (Gilford Instrument Laboratory, Oberlin, Ohio) (3).

The fluorescence signal was observed as a negative absorbance and appeared

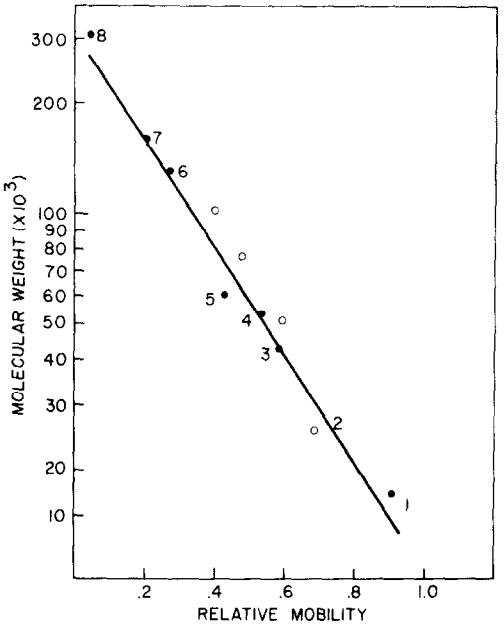

Figure 3. Relationship of molecular weights of 2-mercaptoethanol-treated, fluorescamine-labeled proteins to their relative mobilities. Proteins: (1) myoglobin, (2) chymotrypsinogen A, (3) ovalbumin, (4) glutamate dehydrogenase (subunit), (5) catalase, (6) albumin, (7) γ-globulin (monomer), (8) glutamate dehydrogenase (undissociated). The open circles indicate polymeric forms of chymotrypsinogen A. Each point represents the mean of 3 separate runs, s.e.m. <3% of mean.

as an inverted peak on the chart recorder (3). By this procedure the protein discs could be detected easily and their mobilities accurately measured immediately following electrophoresis. Fluorescence also was detected at the buffer front in all gels, even in those containing no labeled protein. This fluorescent material was inherent in the system and had the same mobility as bromphenol blue. For this reason relative mobilities were calculated as the distance of migration of fluorescamine-labeled protein divided by the distance of migration of the fluorescent contaminant.

When the electrophoresis of non-labeled, protein-SDS complexes was desired, samples were treated with 1% SDS and 5% 2-mercaptoethanol prior to the addition of sample buffer. Following electrophoresis, the gels were removed from the tubes, cut at the bromphenol blue band and stained with Coomassie Blue (5). The gels were destained (5), scanned at 620 nm and the relative mobilities were calculated.

RESULTS & DISCUSSION

During electrophoresis, fluorescamine-labeled proteins migrated as sharp, well-defined discs which could be seen by holding a UV hand lamp close to the

apparatus. Following fluorescence gel scanning, symmetrical peaks were observed on the chart recorder. These peaks corresponded to the position of the discs in the gels. The fluorescent discs were confirmed as protein by positive staining with Coomassie Blue.

The relative mobilities of fluorescamine-labeled proteins were dependent upon their molecular weights as shown in Fig. 2A. An apparent linear relationship existed in the molecular weight range between 17,000 and 70,000 daltons and, as judged from the mobility of γ-globulin, a gradual hyperbolic curve could be expected above 70,000 daltons.

Fluorescamine-labeled glutamate dehydrogenase migrated as two bands. The majority of the protein migrated as the undissociated form running just into the lower gel. A second distinct band migrated as the polypeptide subunit having a molecular weight of 53,000. Fluorescamine-labeled catalase migrated as one band, having a molecular weight corresponding to the 60,000 subunit.

The curve obtained when fluorescamine-labeled proteins were run on polyacrylamide gels was comparable to the curve for the electrophoresis of protein-SDS complexes (Fig. 2B). Fluorescamine-labeled proteins migrated slightly faster than the corresponding protein-SDS complexes.

Protein-SDS complexes of all of the proteins except γ-globulin were treated with 2-mercaptoethanol which is used in the conventional procedures to break disulfide bonds so that the proteins will migrate only as single polypeptide chains. It is obvious in Fig. 3 that 2-mercaptoethanol-treatment of fluorescamine-labeled proteins had entirely different results and did not produce the desired effects. In fact, it appeared that 2-mercaptoethanol-treatment of fluorescamine-labeled proteins encouraged the development of polymeric forms of certain proteins, the basis for which is not known. Albumin migrated as a dimer and chymotripsinogen A as mono-, di-, tri- and tetrameric forms.

Although a linear relationship was observed with 2-mercaptoethanol-treated, fluorescamine-labeled proteins, the points were scattered more widely than with the conventional SDS or the fluorescamine procedures. The latter two procedures yielded almost equivalent data, that is the relationship of molecular weight to mobility was linear up to about 70,000 daltons. The relationship of molecular weight to mobility of 2-mercaptoethanol-treated, fluorescamine-labeled protein was linear up to about 160,000 daltons but less accurate estimates of molecular weight will be obtained with this technique.

Molecular weight estimation of fluorescamine-labeled proteins has several advantages over the conventional SDS systems. The fluorescent technique is more sensitive, rapid and does not require the use of detergent or 2-mercaptoethanol. The major advantage is the ability to analyze gels immediately after

electrophoresis. Some proteins may behave abnormally in this system, a phenomenon also observed with the conventional SDS systems, and it might be advisable to employ both techniques. If they are not in agreement, other physiochemical methods such as gel filtration and ultracentrifugation should be considered.

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