Affinity Electrophoresis in Gels Containing Hydrophobic Substituents*

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The electrophoresis of a number of proteins was studied in poly(N,N-dimethylacrylamide) gels containing entrapped linear copolymers of N-dimethylacrylamide with N-alkyl-substituted acrylamides. The mobility of soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, and myoglobin were unaffected by the hydrophobic residues in the gel. The mobilities of bovine serum albumin and β-lactoglobulin A were sharply reduced, with the effect increasing as the alkyl side chain on the acrylamide residue was extended from dodecyl to octadecyl. The effect of the octadecyl ligand could be further increased by interposing a spacer between it and the polymer chain backbone. The retardation of the mobilities was used to obtain protein association constants with the alkyl residues. Interaction with the hydrophobic ligand produced a separation of β-lactoglobulin A into two fractions.

Gel electrophoresis has found a number of important applications in biochemistry. It is a powerful tool for the characterization of mixtures of native proteins, being particularly useful in distinguishing between closely related species (1, 2). The electrophoretic mobility depends here on the charge and the size of the particle. In the presence of anionic detergents, proteins unfold, and since the charge of the adsorbed anions seems to be proportional to the length of the chain molecule, the electrophoretic mobility may be used to estimate the molecular weight (3, 4). Such estimates seem to be generally fairly reliable, although lactose permease exhibits a mobility which is much higher than expected, presumably because of unusually strong binding of the detergent to hydrophobic sequences of the polypeptide chain (5). Electrophoresis carried out in two mutually perpendicular directions under different conditions constitutes a powerful method to characterize complex protein mixtures (6). When the electrophoresis of a protein is observed in a gel with a gradient of urea concentration perpendicular to the electrical field, the result may be interpreted in terms of the kinetics of protein unfolding (7).

Finally, gel electrophoresis has been of inestimable value in the development of procedures for the sequencing of DNA (8, 9).

Relatively few studies have attempted to introduce groups into the gel which would interact specifically with a protein to be studied by gel electrophoresis. Takeo and Nakamura (10) observed that glycogen entrapped in polyacrylamide gel reduced the electrophoretic mobility of glucan phosphorylase, and they showed how the data may be interpreted in terms of the association constant of the enzyme with its substrate. Most of the work utilizing this technique was concerned with the study of lectins. It used either agarose gels with covalently attached or entrapped lectins which interacted specifically with glycoproteins (11) or polyacrylamide gels with entrapped linear copolymers of acrylamide with allyl glycosides (12, 13) which slowed the electrophoresis of lectins.

It has long been known that serum albumin exhibits a high affinity for long chain fatty acids (14). This has been interpreted in terms of clefts in the surface of the globular protein which have hydrophobic character and contain a cationic site (15). In this investigation, we used the technique of affinity electrophoresis to study the interaction of bovine serum albumin with paraffinic chains. More limited data were obtained for β-lactoglobulin A, which is also known to bind aliphatic hydrocarbons (16).

MATERIALS AND METHODS

Proteins—BSA1 (essentially fatty acid-free), carbonic anhydrase (from bovine erythrocytes, dialyzed and lyophilized), soybean trypsin inhibitor (chromatographically prepared), ovalbumin (crystallized and lyophilized), myoglobin (from sperm whale skeletal muscle, crystallized and lyophilized), and β-LG-A were purchased from Sigma.

Monomers—N,N-Dimethylacrylamide, donated by Alcolac Inc. (Baltimore, Md.), was purified by distillation under reduced pressure. Aliphatic amines, 6-aminoacapric acid, 11-aminooundecanoic acid, acryloyl chloride, benzyl chloroformate, and N,N’-dicyclohexylcarbodiimide were purchased from Aldrich.

To prepare N-acrylyl 6-amidocaproic acid, N-acrylyl amino acids were prepared by acylation of the corresponding amine or amino acid under Schotten-Baumann conditions: N-dodecylacrylamide, m.p. 54–55 °C, yield 78%; N-tetradecylacrylamide, m.p. 61–62 °C, yield 82.5%; N-hexadecylacrylamide, m.p. 65.5–67 °C, yield 68%; N-octadecylacrylamide, m.p. 72.3–73 °C, yield 57%; N-acrylyl-6-amidocaproic acid, m.p. 88–88.5 °C, yield 72%; N-acrylyl-11-amidooundecanoic acid, m.p. 90.5–91.6 °C, yield 65%.

To prepare N-acrylyl 6-amidocaproic acid, 11-aminooundecanoic acid, 6-aminoacaproic acid was converted by benzyl chloride to N-benzoylcarbonyl 6-amidoacaproyl-11-amidooundecanoic acid. The protecting amino acid was treated with thionyl chloride and condensed with 11-aminooundecanoic acid to N-benzoyloxycarbonyl 6-amidoacaproyl-11-amidooundecanoic acid. The protecting group was subsequently removed, and the N-6-aminoacaproic acid by acylation to N-acrylyl-6-amidoacaproyl-11-amidooundecanoic acid, yield 17%, based on the 6-aminoacaproic acid.

N-Acrylyl 6-amidoacaproic acid N-octadecylamide was prepared by coupling N-acrylyl 6-amidoacaproic acid and n-octadecylamine in alcohol-free chloroform using N,N’-dicyclohexylcarbodiimide as condensing reagent, m.p. 117–118 °C, yield 29%.

The monomers were characterized by NMR spectra recorded on a Varian model A-60 spectrometer, and IR spectra recorded on a Perkin-Elmer model 457 spectrometer. N-Tetradecyl-N, N-hexadecyl- and N-octadecylacrylamide were also characterized by chemical ionization mass spectra and found essentially free of their homologs.

Linear Copolymers—N,N-Dimethylacrylamide was copolymerized

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1 The abbreviations used are: BSA, bovine serum albumin; β-LG-A, β-lactoglobulin A.
for 24 h with acrylamide derivatives carrying hydrophobic side chains in methanol solution (containing 25%, w/w, of the mixed monomers) at 60 °C using 0.1 mol % (based on monomer concentration) of azobisisobutyronitrile initiator. Complete monomer conversion was obtained so that the copolymers had the same composition as the mixed monomers. The copolymers were three times dissolved in acetone and reprecipitated in anhydrous ether before drying in a vacuum oven at 50 °C.

Electrophoresis—The disc electrophoresis procedure of Davis (17) was used with the multiphasic buffer systems A of Rodbard and Chrambach (18). Separation gels were prepared by dissolving linear copolymers, N,N-dimethylacrylamide and methylene-bis-acrylamide (3%, w/w, based on monomers and linear copolymer) in pH 8.79 buffer so as to obtain a 7% (w/v) gel. After exposing the solution at room temperature to reduced pressure for 5 min to eliminate atmospheric oxygen, 0.02 ml of 1% ammonium persulfate and 1 µl of N,N,N',N'-tetramethylethylenediamine were added per ml of the solution. Polymerization to a gel was carried out in glass tubes (0.5-cm inner diameter) at 25 °C for 1 h. The spacer gel and the sample gel (pH 7.18, containing 30 µg protein/ml) were prepared as described by Davis (17). Electrophoresis was performed at 25 °C and a current of 3 mA/tube. After electrophoresis, the gel was stained with Coomassie blue, rinsed with water, and destained. The destaining was found to be much less efficient in gels containing hydrophobic substituents. The position of the protein zone was generally determined by inspection with a Manostat caliper (±0.05 mm). For some gels, a densitometer trace was obtained.

RESULTS AND DISCUSSION

Polyacrylamide is insoluble in organic solvents, and it is therefore difficult to prepare acrylamide copolymers with hydrophobic monomers. We employed, therefore, N,N-dimethylacrylamide as the main monomer constituent, since its polymers are soluble both in water and in organic media. Thus, it was possible to copolymerize in methanol or in benzene dimethylacrylamide with various acrylamides N-substituted with long chain alkyl groups, purify the copolymer, and entrapt it in a poly(N,N-dimethylacrylamide) gel prepared from an aqueous solution of the monomer and a cross-linking reagent.

Table I lists the compositions of the various linear copolymers used in this study. The electrophoretic mobilities of BSA and β-LG-A were greatly reduced by the introduction of even very small concentrations of hydrophobic groups into the gel, while the mobility of soybean trypsin inhibitor, carboxy anhydrase, ovalbumin, and myoglobin were not affected and entrap it in a gel, while the mobility of soybean trypsin inhibitor, carboxy anhydrase, ovalbumin, and myoglobin were not affected and entrap it in a gel. In addition, lectoglobulin is split into two peaks.

Table 1: Association constants of BSA and β-LG-A with hydrophobic residues in poly(N,N-dimethylacrylamide) gel

| Hydrophobic comonomer | Hydrophobic comonomer | Association constant |
|-----------------------|-----------------------|----------------------|
|                       |                       | BSA | β-LG-A |
| 12                    | 4.82                  | 1.00 | 1.100 |
| 14A                   | 1.57                  | 1,100 | 1,100 |
| 14B                   | 2.49                  | 480  | 480   |
| 14C                   | 4.40                  | 260  | 400   |
| 16                    | 1.70                  | 2,000 | 2,000 |
| 18                    | 1.70                  | 2,000 | 2,000 |

* Am-14 stands for an acrylamide substituted with C14H30O2. Other abbreviations used are: Am-10-COOH, N-acryloyl-11-amido-decanolic acid; Am-05-10-COOH, N-acryloyl-6-amido-caproyl-11-amidodecanolic acid; Am-05-18, N-acryloyl-6-amido-caproyl-11-amidodecanolic acid. Am-05-18, N-acryloyl-6-amido-decanolic acid N-octadeacylamide.

a Fraction with the higher electrophoretic mobility.

b No significant association was observed.

denotes the presence and the absence of the hydrophobic ligand. It may be seen that the protein peak is greatly broadened by the introduction of associating groups into the gel. In addition, lectoglobulin is split into two peaks.

In Fig. 3, we have plotted the ratio of the BSA mobilities ut and uo, in the absence and presence of the hydrophobic groups in the gel, against the concentration c of the hydrophobe. Theory predicts (10)

\[ \frac{u_t}{u_o} = 1 + cK \]

where \( K \) is the association constant of the protein with the ligand group. It should be noted that this formulation is based on the assumption that the protein has a single binding site for the ligand; in the case of BSA, we are dealing with a protein with a number of sites exhibiting varying affinities for, for example, fatty acids. Nevertheless, we have obtained the linear plots consistent with the simple model, and the apparent association constants derived from their slopes characterize, presumably, the strongest binding site. Table I lists association constants obtained in this manner for BSA with our various gel substituents. The following points may be noted.

1) When comparing the binding to alkyl substituents of varying length, using entrapped linear copolymers with a similar content of the hydrophobic comonomer, \( K \) is found to increase sharply when the alkyl group is extended from tetradecyl to octadecyl. By contrast, Reynolds et al. (15) reported that the binding of alkyl sulfates to BSA is very similar for all ligands whose alkyl group is longer than decyl, while Spector et al. (14) reported binding of a 16-carbon fatty acid to be stronger than that of either a 14- or 18-carbon homolog. In our case, steric hindrance may restrict the approach of the protein to the backbone of the polymer chain. To check on...
this possibility, we prepared a copolymer of N-acryloyl-6-amidocaproyl-11-amidoundecanoic acid N-octadecylamide in which a spacer group separates the octadecyl group from the chain backbone. Table 1 shows that the use of this spacer increased the apparent association constant 7-fold, approaching association constants observed for the binding of long alkyl chain anions (14, 15).

2) When linear copolymers of dimethylacrylamide with different contents of the same hydrophobic comonomer, N-tetradecylacrylamide, were entrapped in the cross-linked gel, the efficiency with which the hydrophobe reduced the serum albumin mobility was found to decrease with an increase of the hydrophobe content in the linear copolymer. We believe that this effect is due to an increasing tendency of the hydrophobic side chains to associate with each other, so that they become unavailable for protein binding.

3) When the alkyl side chain was terminated by a carboxyl group in copolymers of N-acrylyl-11-aminoundecanoic acid and N-acryloyl-6-amidocaproyl-11-amidoundecanoic acid, no significant retardation of BSA was observed. This is in striking contrast with the binding of fatty acids. It may be concluded that in fatty acid binding, the alkyl group is placed at the bottom of the cleft in the globular protein while the carboxyl lies close to the protein surface. Thus, a polymer side chain in which the carboxyl is at the end cannot be accommodated in the binding site.

The most striking result in our study of β-lactoglobulin A was the observation that this protein, which migrated as a single sharp peak in the control gel, separated in the presence of hydrophobes into a very slowly migrating fraction with a relatively sharp peak and a fraction with a higher mobility characterized by a diffuse peak. We found no previous record of such a fractionation. Affinity constants for the faster fraction were slightly higher than those for BSA when a tetradecyl group was the ligand while the longer ligands bound more weakly to β-LG-A. This protein was reported to have a single hydrophobic binding site which can accommodate only relatively small ligands (16).

The effect of hydrophobic substituents on the electrophoretic mobility of proteins is analogous to the effect of such substituents on protein behavior in chromatography. Phenomena of this type have been studied using agarose modified with a variety of hydrophobic substituents (19, 20). Protein-ligand association constants have also been obtained from the retardation of a protein on a chromatographic column with the ligand attached to the stationary phase (21), and spacers are widely used to facilitate protein-ligand interaction in affinity chromatography (22). Both affinity electrophoresis (23) and affinity chromatography (24, 25) can resolve mixtures of similar proteins with slightly different ligand affinities. We see then that the two techniques have many similar characteristics. The advantage of affinity electrophoresis is in the very small sample size required and the wide variation of well defined continuous media which may be employed.

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