Journal of Chromatography, 105 (1975) 353–358

CHROM. 8004

EVIDENCE AGAINST THE OCCURRENCE OF ARTIFACTS DUE TO CARRIER AMPHOLYTE–PROTEIN BINDING DURING ISOELECTRIC FOCUSING

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(Received September 2nd, 1974)

SUMMARY

The formation of irreversible complexes between carrier ampholyte components and proteins was investigated by gel filtration of mixtures of proteins and radioactively labelled ampholytes. Experiments were performed both with purified proteins (albumin, ferritin, β-glucuronidase) and with a complex mixture of proteins (serum); in no case was binding of ampholytes to proteins detected. Thus the results argue against the occurrence in isoelectric focusing of proteins of artifacts due to such complex formation.

INTRODUCTION

The finding that a number of proteins which are homogeneous in gel electrophoresis, exhibit multiple forms during isoelectric focusing[1], has led some authors to suggest that this might be due to the binding to proteins of Ampholine (carrier ampholyte) components, giving rise to complexes differing in isoelectric point from the free proteins. Usually, this explanation of protein heterogeneity is dismissed by demonstrating that the focusing pattern is unchanged by varying the Ampholine concentration and mode of sample application, and that single components retain their isoelectric points on re-focusing without giving rise to other bands, *e.g.*, ref. 2. However, such experiments do not constitute rigorous proof that the observed multiple forms are genuine.

There is some evidence, though none conclusive, for the formation of Ampholine–protein complexes. The binding of 1.5 moles of an Ampholine component per mole of L-amino acid oxidase, was demonstrated by gel filtration of mixtures of tritiated Ampholine and the purified enzyme[3]. As free amino acids were detected in the batch of Ampholine used, this may merely have represented the formation of enzyme–substrate complexes. However, this binding was reversible, since 87% of the bound radioactivity was removed during a subsequent 44-h dialysis. In a study of an acidic wool protein[4], Frater, using a flat-slab polyacrylamide focusing system,

found that the protein banding pattern depended on the sample loading position, the extent of focusing before addition of sample, and on Ampholine concentration. Inconsistencies in the focusing pattern of bovine plasma albumin[5] have been attributed to an irreversible interaction between the protein and a minor constituent of the Ampholine, producing an artifactual component of p*I* 4.8. Anomalous behaviour of yeast isocitrate dehydrogenase during isoelectric focusing[6] was similarly attributed to protein–Ampholine interaction. Finally, in the case of ferritin inconsistencies in focusing patterns have been claimed[7,8] and Bryce and Chrichton[9] have argued that the multiple forms observed by other authors[2,10–13] are artifacts, their main reason being that with purified horse and human apoferritin[14], only one, or sometimes two, bands were seen in polyacrylamide gel focusing in the ranges pH 3–10 or pH 3–5. However, the sensitivity of their protein staining method was such that minor bands may well have escaped detection.

If, after mixing carrier ampholytes with a variety of proteins, complete separation of the components under conditions similar to those applying during focusing could be achieved within a short time, *i.e.*, considerably less than the duration of focusing runs, this would constitute strong evidence that ampholyte–protein binding could not be responsible for the generation of multiple forms, as the only carrier ampholyte which could remain bound to a protein, at the end of the focusing, would be one of identical isoelectric point.

A number of methods for removing the carrier ampholytes from proteins after focusing are available. It has been shown that 99.99 % of ^{14}C-labelled ampholytes mixed with either bovine serum albumin, α-haemolysin or egg white lysozyme can be removed by repeated ammonium sulphate precipitation, but this procedure is rather slow and tedious[15]. Removal of ampholytes by dialysis[16] is even slower. Separation by ion-exchange chromatography can be relatively fast, and a rapid and nearly complete separation of human plasminogen from carrier ampholytes using DEAE–Sephadex has been described[17]. However the most generally applicable method is gel filtration, and complete separations of carrier ampholytes from human serum albumin, human γ-globulin, egg white lysozyme and α-haemolysin have been obtained in brief filtrations on Sephadex G-50[15,16].

In order to obtain further evidence on the possibility of ampholyte–protein binding, these gel filtration experiments have been extended to a wider variety of proteins, including complex mixtures. In contrast to the experiments of Vesterberg and co-workers[15,16] we performed gel filtration at low ionic strength, *i.e.*, under conditions which more nearly resemble those of isoelectric focusing.

MATERIALS AND METHODS

A new ^{14}C-labelled ampholyte mixture (pH 3.5–10) of slightly smaller molecular size than the normal Ampholine (average apparent molecular weight less than 300) was used[18] (LKB Produkter, Bromma, Sweden). Heterogeneity of the proteins tested was confirmed by focusing in polyacrylamide gels as described by Barrett[19] with the radioactive ampholytes or normal (pH 3–10) Ampholine, both at 1 % w/v. Polyacrylamide gel electrophoresis was done as described previous[20], with 7-% gels in a buffer at pH 8.1.

Rabbit liver β-glucuronidase was purified by a recent procedure[2] and rabbit

liver apoferritin monomer by a previously published method[14]. Horse apoferritin monomer was obtained from five-times crystallised horse spleen ferritin (Miles-Seravac, Maidenhead, Great Britain) by the later stages of the same procedure (from gel filtration on Sepharose 6B onwards[14]). A mixture of rat proalbumin and albumin was kindly given by Dr. J. D. Judah[21]. Rat serum was obtained from fresh rat blood. Bovine serum albumin, Cohn Fraction V, was purchased from Sigma (St. Louis, Mo., U.S.A.).

β-Glucuronidase activity in gels was detected as previously[2]. Protein was stained with Coomassie Brilliant Blue R250[22] but the trichloroacetic acid (TCA)-washing step was usually omitted as normal Ampholine did not stain significantly with this method. However, the radioactive ampholytes exhibited a number of stained bands in control gels without protein, and so for staining gels containing these ampholytes, the TCA-washing step was included since this prevented staining of the radioactive ampholytes.

For the binding experiments, proteins, in distilled water, were incubated for 30 min at room temperature with 0.1 μCi (20 mg) of ^{14}C-labelled ampholytes. Mixtures were made up to a volume of 1 ml with distilled water (final concentration of ampholytes 2%, w/v). Amounts of proteins used are shown in Table I. Gel filtration of the mixtures was performed on a 21 × 1.5 cm I.D. Sephadex G-75 column. The column was equilibrated with either 0.01 *M* Tris–HCl (pH 7.8) or 0.01 *M* sodium acetate buffer (pH 5.0) or distilled water and eluted with the equilibrating solution at 40 ml/h; complete separations were thus achieved in approximately 1 h. All mixtures were gel filtered at pH 7.8 and in distilled water. In addition β-glucuronidase, bovine serum albumin and horse and rabbit apoferritins were separated at pH 5.0. Recoveries of radioactivity in every experiment exceeded 93%.

A mixture of serum proteins for binding studies was prepared by applying 2 ml of normal serum to the column. Material recovered at effluent volumes completely separated from the elution position of the radioactive ampholytes —a very slightly retarded symmetrical peak— was pooled, reconcentrated to 2 ml and transferred to distilled water by ultrafiltration using a PM-10 membrane.

A Packard 3375 scintillation counter was used for determination of radioactivity, with Aquasol (New England Nuclear, Boston, Mass., U.S.A.) as scintillant. The manufacturer's information on the characteristics of the radioactive ampholytes[18]

TABLE I

AMOUNTS OF PROTEINS USED IN BINDING EXPERIMENTS

Protein	*Amount used (μg)*	*Amount present (nmoles)*	*Number of subunits*	*Subunit present (nmoles)*
Rabbit β-glucuronidase	500	1.6	4 (ref. 2)	6.4
Horse spleen apoferritin monomer	500	1.1	24 (ref. 26)	26.4
Rabbit apoferritin	1000	2.2	24 (ref. 26)	52.8
Rat proalbumin and albumin	approx. 50 and 100, resp.	approx. 0.75 and 1.5, resp.	1 (ref. 21)	0.75 and 1.5, resp.
Bovine serum albumin	20,000	300	1	300
Fractionated normal rat serum	20,000	—	—	—

does not permit precise calculation of the specific radioactivities of any individual ampholyte. However, it is stated that more than 98 % of the radioactivity is associated with components of apparent molecular weights lower than 600 daltons, and in gel filtration on Sephadex G-25, radioactivity is eluted in a nearly symmetrical peak centred on a molecular weight of 300. Thus a crude estimate of average specific radioactivity may be made by assuming all the radioactivity to be associated with a molecule of molecular weight 300: the figure so deduced is 1.5 μCi/mmole. On this assumption the counting system is capable of detecting a minimum of about 3 nmoles of such a component.

As the purified proteins used in the binding studies, with the exception of albumin, are polymers (Table I), probably each of a single kind of subunit, it may be expected that any ampholyte binding will bear a stoichiometric relationship to subunit concentration. Even with the relatively small amounts of purified proteins available binding of a single ampholyte per subunit should be detectable. However, it must be pointed out that there may well be components of the ampholyte mixture which are of too low specific radioactivity or concentration to allow detection.

RESULTS AND DISCUSSION

The isoelectric focusing pattern of rabbit β-glucuronidase in the radioactive ampholytes was very similar to that obtained with normal Ampholine[2,23], and the albumin/proalbumin mixture gave similar patterns of six bands with both ampholyte mixtures. While the horse apoferritin monomer from gel filtration on Sepharose 6B[14] was quite homogeneous in polyacrylamide gel electrophoresis at loadings of 100 μg per gel, the rabbit apoferritin monomer —unlike a previous preparation by a similar method[2]— was clearly contaminated with two proteins migrating faster than the apoferritin (employing loadings of up to 50 μg per gel). This impure preparation was used for binding studies, but in order to obtain a satisfactory preparation of rabbit apoferritin for focusing, the monomer bands from a number of gels were cut out, homogenised with an Ultraturrax homogeniser, sonicated in a MSE 150-W ultrasonic disintegrater, and extracted into 5 ml of distilled water by shaking overnight in a water bath at 37°. After concentration by ultrafiltration on an XM-50 membrane, the protein was re-run in gel electrophoresis at 100 μg per gel and its homogeneity confirmed. Lyophilisation of ferritin was avoided[9]. The focusing pattern of horse apoferritin monomer is shown in Fig. 1. Both rabbit and horse apoferritin were heterogenous, and each gave a similar compact group of bands. The patterns were unaffected by the inclusion in the gel of 0.01 *M* dithiothreitol which tends to disaggregate ferritin and apoferritin polymers[24].

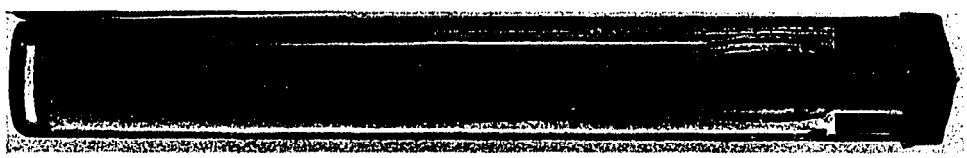

Fig. 1. Isoelectric focusing of purified horse spleen apoferritin monomer in the range pH 3 (towards top of gel) to pH 10 (towards bottom of gel). Focusing was performed as described in the text, but was continued for only 3 h at 250 V. Sample loading, 300 μg.

All the binding experiments gave similar results. Protein and ampholyte peaks were completely separated by Sephadex gel filtration in each case and no detectable radioactivity was eluted with any of the protein peaks. These experiments thus show that any ampholyte–protein complex formation that might occur during 30-min incubation of the various proteins in ampholyte solution —a period similar to that taken for ampholytes to stack during focusing[25]— must be rapidly reversible both in 0.01 *M* Tris–HCl buffer at pH 7.8 and in distilled water, *i.e.* in media which, like those used in focusing, have low ionic strength. Since for β-glucuronidase, ferritin and bovine serum albumin, gel filtration was also done at pH 5.0, any complex formation which might occur with these proteins would be similarly reversible at a pH isoelectric for some form of each protein and near that of all forms[2,10–13,21,23]. These conditions are similar to those achieved at the end of focusing.

One remaining possible type of ampholyte–protein interaction is that which might occur some time after commencement of focusing when the ampholytes have partly stacked and the protein is at a pH distinct from its isoelectric point. At this stage a protein might irreversibly bind an ampholyte component which it does not bind at the pH of the unfractionated ampholyte mixture. This possibility was tested in the cases of the commercial horse spleen ferritin, the impure rabbit apoferritin and the unfractionated normal rat serum by focusing pairs of protein samples and staining one of each pair for protein, in each case using a loading of 100 μg per gel. The zones in the remaining gels (loading 500 μg per gel) corresponding to the stained zones of the replicate gels were cut out, homogenised and sonicated as for ferritin but with cooling (to avoid heat denaturation) and extracted in distilled water (final volume, 3 ml) for 18 h at 37° in a shaking water-bath. Subsequently, acrylamide was removed by centrifugation. Although extraction of protein by this procedure was inefficient (recovery, 50–60%), extraction of radioactivity was satisfactory (75–85%). The extracts were then passed through the Sephadex G-75 column in 0.01 *M* sodium acetate buffer, pH 5.0. In no case was radioactivity greater than background observed in the fractions containing protein and, therefore, no irreversible binding of ampholytes of p*I* distinct from the final protein p*I* was detectable.

In conclusion, it is evident that rat albumin, proalbumin and bovine serum albumin do not give rise to ampholyte–protein complexes on exposure to these ampholytes. Therefore alternative explanations for the previous anomalous or artifactual results obtained during isoelectric focusing of bovine plasma albumin[5], may have to be sought. On the other hand, the heterogeneity of horse spleen and rabbit liver apoferritin and of rabbit β-glucuronidase seems to be non-artifactual in agreement with the results of most workers[2,10–13,23].

In view of the large variety of proteins used in the present experiments, the lack of detectable irreversible binding of ampholytes by any of the proteins, and the similarity of focusing patterns with normal Ampholine and the labelled ampholytes, our results constitute strong evidence that artifacts in focusing due to ampholyte–protein interactions are at worst a rare occurrence[3,4,6].

ACKNOWLEDGEMENT

One of us (R.T.D.) thanks the Medical Research Council for support.

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