Moving Boundary Electrophoresis Behavior and Acid Isomerization of Human Mercaptalbumin*

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The behavior of bovine plasma albumin in moving boundary electrophoresis has been studied in detail, especially by Aoki and Foster (1–6) and by Cann (7–10). In general terms, it has been found that this protein yields a single moving boundary at pH values above the isoelectric point (approximately pH 4.5) but two, and in some cases three, boundaries between pH 3.5 and 4.5. These results have been interpreted by Aoki and Foster (1–6) in terms of a cooperative alteration in the protein conformation, which takes place near pH 4. Some independent evidence for this "isomerization" has been obtained from solubility studies (11) and, more recently, from studies of optical rotation and rotary dispersion (12, 13). It has also been suggested that the well known anomaly in the acid titration curve of this protein can be explained, in the main, as due to this phenomenon (6).

At the time of initiation of the experiments reported here, no evidence existed for the isomerization of plasma albumins of any species other than bovine. It was shown earlier by Tanford, Swanson, and Shore (14) that the titration curves of human and bovine albumins are essentially identical. This would imply that if indeed the titration anomaly in bovine plasma albumin is due to the isomerization reaction, the same reaction must take place in the human protein. However, Jirgensons (15) suggested that this may not be the case. We considered it of some importance, therefore, to examine the electrophoretic behavior of the human protein. Furthermore, since the plasma albumins are patently heterogeneous, in that some of the molecules contain free sulphydryl groups and others do not, we were led to utilize the mercaptalbumin fraction of the protein by the thought that it might be more homogeneous.

In the interim since this program was initiated, ample evidence has appeared that human albumin exhibits much the same type of isomerization behavior as bovine (12, 16–19). However, we know of no published electrophoretic studies that demonstrate the isomerization equilibrium in human mercaptalbumin nor, in fact, in the mercaptalbumin fraction of any albumin. A more important reason for presenting these results at this time is that they will be utilized in a new treatment of the low pH titration behavior of human mercaptalbumin and also will be of interest for comparison with studies of human mercaptalbumin by zone electrophoresis. These subjects will be treated in two forthcoming papers from this laboratory.

EXPERIMENTAL PROCEDURE

Materials—HMA1 was prepared from Fraction V of human plasma2 through the mercury dimer by a modification of the procedure of Dintzis (20), which was suggested to us by Saroff.3 The mercury dimer was recrystallized three times, lyophilized, and stored in the dry state at –4°. Isoionic stock solutions of the monomer were prepared as needed by passage of concentrated solutions of the dimer through the thioglycolate-containing mixed bed ion exchange column as described by Dintzis (20). The monomer was then delattted by lowering the pH (HCI) to 2.5 to 3.0 and holding the solution in the cold room for approximately 48 hours. Under such conditions, all, or certainly most, of the residual lipid impurities are released from the protein (21). The insoluble material was removed by centrifugation or by filtration through sintered glass filters. Finally, the solution was again deionized by passage through the mixed bed column.

Each stock solution of monomer was checked in the ultracentrifuge (Spinco model E) to determine the dimer content. This was generally found to be in the range of 5 to 10%, in agreement with usual experience. In addition, each stock was checked by moving boundary electrophoresis at pH 4.0 (0.2% protein concentration and 0.02 M chloride) to verify that the protein showed the normal behavior. This precaution was adopted after it was observed that occasional preparations yielded electrophoretic results which were out of line with that normally observed; in such cases the pH region of coexistence of the N and F boundaries appeared to have been shifted upwards by several tenths of a pH unit. These anomalies have been tentatively, but not conclusively, traced to contamination of solutions by heavy metal ions. It has been shown that traces of cupric ion have a profound effect on the N-F equilibrium.4

Solutions were prepared from the concentrated stock solutions by dilution with deionized water and the required reagent grade chemicals.

Procedures—Three supporting media were employed. These

1 The abbreviations used are: HMA, human mercaptalbumin; BPA, bovine plasma albumin.

2 Supplied by the American Red Cross through the courtesy of Dr. J. N. Ashworth.

3 Dr. H. Saroff, personal communication.

4 W. W. Everett, personal communication.

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were (a) mixtures of hydrochloric acid and sodium chloride; (b) mixtures of hydrochloric acid or potassium hydroxide and potassium chloride; and (c) mixtures of acetic acid and sodium acetate. The total ionic strength of the media was kept constant, either 0.02 or 0.10. Dialysis of the protein solution against the supporting electrolyte was carried out in the cold room (1-2°) for at least 15 hours with continuous mechanical agitation. The protein concentration used was 0.2% when the ionic strength was 0.02, and 0.4% when the ionic strength was 0.1.

Electrophoresis was conducted at 0° with a Perkin-Elmer electrophoresis apparatus, model 38, equipped with schlieren scanning optical system. The pH values of solutions and supporting media were measured with a Beckman model G glass electrode pH meter. Protein concentrations were determined by measuring the optical density with a Beckman model C glass cell electrophotometer. For this purpose $E_{1\%}$ was taken as 5.30 at 278 m$\mu$, which value was established by Kjeldahl analysis of HMA solutions if the accepted value of 15.95% is assumed as the percentage of nitrogen in the protein.

For analysis of the boundary areas, the patterns were first enlarged approximately 20-fold by projection and tracing. Resolution was then made into peaks, assuming Gaussian shape in regions of overlap of boundaries. The areas under the resulting peaks were measured by means of a compensating polar planimeter. The percentage compositions reported are mean values of the respective ascending and descending patterns, the areas of which were rarely significantly different.

The mobilities were calculated in the customary manner from the distances of migration of the maximal positions of the individual peaks. The mobilities reported are the average values of the mobilities obtained from ascending and descending boundaries.

**RESULTS**

The compositions of the systems as function of pH are given in terms of the percentage of the N form in Figs. 1 and 2. Fig. 1 shows results in 0.02 M chloride; Fig. 2, in 0.1 M chloride. It is clear that alteration in ionic strength has little effect on the results. The range over which the isomerization occurs is approximately 3.5 to 4.5 at either 0.02 ionic strength or 0.1 ionic strength. The mid-points of the curves (pH at which N and F occur in equal concentration) are, respectively, 3.95 and 4.05 at the two values of ionic strength. This small shift of 0.1 pH unit is quite comparable to that observed in the case of BPA (2).

Figs. 3 and 4 show representative tracings of the schlieren patterns at various pH values as obtained from experiments at 0.02 and 0.1 ionic strength, respectively. Clearly the patterns are relatively enantiographic. The patterns obtained in 0.02 M chloride show at most only two boundaries. In 0.1 M chloride (Fig. 4), definite evidence for a separation of the F boundary into two subcomponents is seen in several of the patterns obtained close to pH 4. This is also in good agreement with the earlier results on BPA (2-3).

Results of two experiments to test the pH reversal of the transformation are shown in Fig. 2 as half-shaded circles. In one of these, the protein was dialyzed extensively at pH 3.81. The solution was then brought to room temperature and quickly titrated to a pH of 4.20, and electrophoresis was run against a supporting electrolyte of this latter pH. In the other case, the protein was first dialyzed at pH 4.12, then adjusted to pH 3.83 and run at this pH. In both of these cases, electrophoresis was initiated as soon as the cell could be filled and equilibrated (1 to 2 hours). The electrophoretic patterns were highly enantiographic, and no differences could be detected between these results and those obtained on samples dialyzed extensively at the pH of the final experiment.

Figs. 5 and 6 show resultant mobilities of both N and F forms as function of pH in 0.02 and 0.1 M chloride, respectively.

A limited number of experiments were conducted in 0.1 M acetate systems but are not reported here because they were insufficient to establish the full pH profiles. It can be stated, however, that the resulting patterns were of the same general character as those in chloride and clearly exhibited the same resolution into two peaks, with appearance of a third peak close to pH 4.

The results of two special experiments designed to demonstrate that the N and F boundaries do, indeed, correspond to interconvertible forms should be mentioned briefly. In the first of
these, prolonged electrophoresis of a 0.4% solution of HMA was carried out in 0.02 M chloride at pH 3.91. This separation was carried out in a 6.0-ml electrophoresis cell with compensation of the boundaries, so that it was possible to isolate a small portion of solution ahead of the ascending N boundary, presumed to contain only the F form. This sample was diluted with fresh 0.02 M chloride and rerun in a 2.0-ml cell. Unfortunately, the pH of the second run (4.05) was somewhat different from that of the first; furthermore, there was a delay of several hours between the runs, and the concentration of the rerun solution was too low for accurate quantitative evaluation of the percentage composition. However, the solution clearly yielded two components, and the areas under the two boundaries were approximately equal. It can be concluded that the isolated F form does re-equilibrate under such conditions to give a mixture of the two forms.

In another experiment, conducted in 0.02 M chloride at pH 4.09, electrophoresis of a 0.2% HMA solution was carried out for 8200 seconds at 3.56 volts per cm. At the end of this time, the power was turned off for a period of 9000 seconds. Electrophoresis was then reinstated for a period of 6000 seconds. The final pattern showed only two peaks, which were quite widely separated with, however, a non-zero plateau region in the schlieren patterns between the two peaks. There was no evidence of any resolution of the two peaks into new peaks. This result suggests that re-equilibration is reasonably slow, the half-time being at least of the order of several hours. The character of the gradient curves, however, suggests that some slow equilibration might be taking place during the prolonged electrophoresis. Alternatively, the plateau region might result from a third, poorly resolved, component which is seen in the conventional experiments at 0.1 ionic strength but not at 0.02 ionic strength.

**DISCUSSION**

The present results on HMA qualitatively are identical with the previously published results on BPA. Even quantitative differences are minor. The results also agree well with those of Hori and Aoki on human serum albumin (16). This shows the similarity of both the bovine and human proteins, on the one hand, and of the mercaptalbumin and nonmercaptalbumin components, on the other, at least insofar as electrophoretic behavior is concerned.

Further proof that the N and F peaks correspond to interconvertible forms is seen in the two special experiments reported under “Results.” Again, however, the evidence strongly implies that these forms are not in rapid equilibrium. It would appear that the half-time for interconversion must be at least of the order of several hours. It may be that the rate of interconversion of N and F forms is somewhat faster in the case of HMA than in BPA. Thus, as pointed out above, prolonged electrophoresis of HMA yields a pronounced non-zero plateau region between N and F components, whereas in case of BPA we have frequently observed the gradient pattern to go to zero between the peaks. We believe that, at least to a first approximation, the observed boundaries may be treated as representing stable, essentially frozen components and that the relative areas under the boundaries can be taken as proportional to the relative concentrations of the two forms.

Cann (7-10) has emphasized the thesis that such boundaries...
represent reaction boundaries and cannot be treated in this fashion. It is important to emphasize again that most of his experiments have been carried out at significantly higher protein concentrations than ours. Under such conditions, the patterns deviate seriously from ideal enantiography, and it may well be that they should be treated as reaction boundaries. The relatively enantiographic patterns such as reported herein and found also by Aoki and Foster (1-5) and Hori and Aoki (16) do not appear to be explicable as reaction boundaries.

Two other arguments may be given to support our contention that the boundaries can be treated as representing essentially stable forms of the protein. In this study, as previously for BPA (4), a careful examination was made of the displacement of the boundary positions as function of time. Again it was found that, with very few exceptions, the displacements are linear in the time of electrophoresis. A representative example is shown in Fig. 7. Such behavior is not to be expected if significant interconversion is taking place (22). Secondly, the excellent agreement between the percentage of N and F forms as function of pH, on the one hand, and the dependence of the specific optical rotation on pH, on the other, has been given in another paper by Leonard and Foster (12). It will be seen in Fig. 6 of that paper that the agreement is particularly good in the case of HMA.

Although it does not seem appropriate to belabor the question of the kinetics of the N-F transformation at this time, it should be pointed out that recent publications of Cann (9, 10) also point to the necessity of assuming the N and F forms not to be in rapid equilibrium. Thus, in one paper (9), he calls attention to the fact that the gradient patterns do in fact become zero between the two main peaks in some instances. This he suggests as due to convection. In another case (10), he concludes that there is a form, which he terms a, that converts slowly and, he thinks, irreversibly, to faster migrating forms. By careful comparison of his patterns with ours, we are forced to conclude that his a boundary corresponds to the one we have called N.

In any event, it seems clear that the low pH electrophoretic results cannot be explained purely on the basis of reaction boundaries, but that some slow transformation of the protein must be invoked. We believe that the most reasonable explanation for what is unquestionably a very difficult problem is that the kinetics of the transformation are complex, there being a smaller ionic strength dependence than, the corresponding transformation is obviously a more sensitive function of pH above pH 4 than below. This is not unexpected, and indeed it would in general be anticipated that q would be constant, since, if equilibrium is assumed, q would represent the difference in the mean number of hydrogen ions bound by N and F forms, and this difference would in general be pH-dependent.

A further difference in electrophoretic behavior between HMA and BPA should be pointed out. In the case of BPA, the difference in mobilities between N and F forms was found to correspond, on the basis of Henry's equation and the assumption that the protein is a prolate ellipsoid of axial ratio 4.0 and volume equivalent to a 30-A sphere, to a charge difference of 3 protone units (2). This result, taken in conjunction with the observed value of 3, suggested no difference in chloride binding between N and F forms. In the case of HMA, the difference in mobilities between the two forms (approximately 2.2 and 1.6 mobility units, respectively, at 0.02 and 0.1 ionic strength) corresponds, on the basis of the same assumptions, to a charge difference of approximately 6 protonic units. One obvious explanation is that in this case F actually has a lower binding affinity for chloride ions than has N. This point does not seem worth pressing, however, since if N and F are frozen and not in true equilibrium, it follows that q can have no thermodynamic significance.

Again, as in the case of BPA, the isoelectric point is difficult to define, since both N and F coexist (and have opposite sign of charge) at the mean isoelectric point. With weighted average mobility curves, the isoelectric pH values calculated are 4.42 ± 0.03 in 0.02 M chloride and 4.38 ± 0.03 in 0.1 M chloride. These values differ significantly from, and in particular manifest a smaller ionic strength dependence than, the corresponding values (4.52 and 4.22) found for BPA (2).

It is interesting to note further that Cann (10) mentions the unusual kinetics of the transformation of his a component to the faster moving forms. He states that "the transformation reaction proceeds rapidly to about 55 to 75% of completion after which it proceeds at a much slower rate..." This is precisely the type of kinetic behavior that is predicted by the kinetic mechanism we have proposed (23).

In the same paper, Cann presents essentially two experimental results in defense of his thesis that the patterns must be considered as reaction boundaries. The first of these relates to the fact that the measured pH or conductivity gradients across the boundaries cannot be explained in terms of the ideal Dole theory. Since these experiments were conducted at 0.4% protein and ionic strength 0.02, conditions that we (1-5) have previously concluded to yield nonideal electrophoresis, these results are to be expected even for electrophoresis of stable protein forms. The other argument is based on the dependence of the patterns on protein concentration in 0.1 M acetate, which results he concedes (10, footnote 18) could equally well be explained in terms of Dole theory for a mixture of two stable proteins.
As is indicated by Fig. 2, only a single moving boundary was found in the pH range from 4.5 to 9. It should be noted, however, that between pH 7 and 8 the patterns were found to be nonenantiographic. It may be that some structural transformation is taking place in this pH range but that the interconversions in this case are too rapid to permit resolution. Indeed, some evidence does exist that a transformation analogous to the N-F transition takes place between pH 7 and 9.6

**SUMMARY**

Results of an extensive investigation of the moving boundary electrophoretic behavior of human mercaptalbumin are summarized. In 0.02 M chloride, two boundaries exist between approximately pH 3.5 and 4.5. In 0.1 M chloride, the same situation prevails, except that a third boundary appears over a narrow pH range near 4.0. A few additional experiments in acetate buffer are reported. In all essential features, the behavior of this protein is the same as previously found for bovine plasma albumin, although a few minor quantitative differences are pointed out. Evidence is presented that these boundaries correspond to interconvertible forms (N and F) of the protein and that interconversion of the forms under conditions of electrophoresis must be very slow indeed. It would appear that these forms can best be regarded as conformational isomers in a frozen pseudoequilibrium state.

**REFERENCES**

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