Journal of Chromatography, 177 (1979) 219–225

CHROM. 12,007

TITRATION CURVES OF POLYPEPTIDE CHAINS BY COMBINED ISOELECTRIC FOCUSING–ELECTROPHORESIS IN 8 *M* UREA

PIER GIORGIO RIGHETTI

Department of Biochemistry, University of Milano, Via Celoria 2, Milan 20133 (Italy)

and

RAJAGOPAL KRISHNAMOORTHY, CLAUDINE LAPOUMEROULIE and DOMINIQUE LABIE

Institut de Pathologie Moléculaire, Groupe INSERM U.15, 24 Rue du Faubourg Saint-Jacques, 75014 Paris (France)

(Received May 21st, 1979)

SUMMARY

Titration curves of reduced and alkylated polypeptide chains can be successfully performed in 8 *M* urea–polyacrylamide gel plates by electrophoresis perpendicular to a stationary stack of focused carrier ampholytes. All buffers and thiol reagents with p*K* values in the range pH 3–10 should be removed, since their pH-dependent ionization affects the migration and apparent p*I* values of the protein chains. No blurring of the patterns below pH 4.5 is observed, as usually found in titration curves in the absence of urea, thus allowing the direct titration of Glu and Asp residues. It is not possible by the present technique to titrate any group below pH *ca.* 3 and above pH *ca.* 10, due to the lack of suitable carrier ampholytes and to a "flooding" phenomenon, with concomitant identical electrophoretic mobility for all protein species, irrespective of their relative p*I* values and amino acid composition. The "electrophoretic titration curves" thus obtained are well correlated with the overall amino acid composition of the polypeptide chains analyzed.

INTRODUCTION

In 1976, in Hamburg, Rosengren *et al.*[1] presented "a simple method of choosing optimum pH conditions for electrophoresis", which was a sort of two-dimensional technique consisting of electrophoresis performed perpendicular to an Ampholine pH gradient, focused in a flat bed of polyacrylamide gel. Their data clearly demonstrated that the pH–mobility curves thus obtained were indeed related to titration curves. In view of the enormous importance of these findings, it is astonishing that they were not followed up. Recently, by further exploiting this idea, we have demonstrated that, by running a protein and its genetic mutants in a mixture, it was possible to reveal, from the analysis of the shape of the pH–mobility curves thus generated, which charged amino acid had been substituted in the mutant phenotype[2].

Moreover, within a given family of proteins of very similar size and shape, it was possible to correlate the electrophoretic mobility at a given pH with the number of protons bound or released by the macromolecule[2]. This investigation was then extended to liganded states of proteins, in order to see whether the bound species could be resolved from unliganded ones, and their physico-chemical properties studied. We were in fact able to isolate complexes of hemoglobin with organic phosphates, such as inositol hexaphosphate and inositol hexasulphate, and to measure their half-life, the pH range of stability as well as the stoichiometry of the protein–ligand complex[3]. The technique was then applied to the isolation and characterization of interacting protein species. We were thus able to demonstrate a direct binding of cytochrome b_5 to methemoglobin, to determine the half-life of the complex, its pH range of maximum stability and the amino acids involved in the binding in each protein molecule[4].

By use of the present technique, it is possible to "titrate electrophoretically" a protein in the range pH 3.5–10. However, we have noticed that below pH 4.5 many proteins tend to be unstable and thus give blurred patterns, rendering rather difficult the direct titration of Glu and Asp residues. Instability of proteins at acidic pH values seems to be a rather general phenomenon, well documented in the 1950s by moving boundary electrophoresis[5], and is probably due to the beginning of a denaturation process in the macromolecule. In fact, Tanford *et al.*[6] have concluded from titration and viscosity data that below pH 4.5 bovine serum albumin undergoes a change from a compact to an expansible form. Moreover, "electrophoretic titration" of macromolecules in their native configuration does not allow direct titration of all ionizable groups, but only of surface groups directly accessible to the solvent and of groups not engaged in subunit contacts, hydrogen bonding, etc. All these considerations have led us to the conclusions that a useful addition to pH–mobility curves of native macromolecules would be "electrophoretic titration curves" in 8 *M* urea of denatured structures, since many proteins will exist in this solvent mostly as random coils, subunits will be split apart, buried groups will be exposed to the solvent and the macromolecule will be stripped free of non-covalently bound ligands or cofactors.

MATERIALS AND METHODS

Ampholine carrier ampholytes in the range pH 3.5–10 were obtained from LKB (Bromma, Sweden), acrylamide, N,N'-methylenebisacrylamide, ammonium persulphate, N,N,N',N'-tetramethylethylenediamine and urea (ultrapure) from Bio-Rad Labs. (Richmond, Calif., U.S.A.), Coomassie Brilliant Blue G-250 from Serva (Heidelberg, G.F.R.) and 2-mercaptoethanol (2-Me), dithiothreitol (DTT), iodoacetamide, Arg, Lys, Glu and Asp (free bases and acids) and β-lactoglobulins A and B from Sigma (St. Louis, Mo., U.S.A.).

The preparation of heme-free α and β chains[7] and their alkylation with iodoacetamide[8], the two-dimensional isoelectric focusing (IEF)–electrophoresis technique[2], pH measurements[9] and gel staining[10] were performed as described previously. For pH gradient measurements, the gel slices were eluted with 8 *M* urea–10 m*M* KCl and a correction factor of 0.42 pH units was subtracted[11] from the pH readings.

RESULTS

Several factors affect the mobility of polypeptide chains in 8 *M* urea gels. In denaturing solvents, proteins are usually reduced by thiols since reduction not only dissociates aggregates which are held together by disulphide bonds, but also allows polypeptide chains to completely unfold by breaking intramolecular disulphide bonds[12]. In constant pH electrophoresis, the presence of thiol reagents in the sample usually does not have any deleterious effect upon its migration. However, as shown in Fig. 1A, the presence of 2-Me when developing pH–mobility curves has a disastrous and pH-dependent effect on the sample migration. Whereas at pH < 7.5 the α- and β-globin chains seem to migrate unperturbed to the cathode, above pH 8 their migration to the anode is severely quenched for β chains (p*I* 7.1) and almost entirely suppressed for α chains (p*I* 7.8). This is no doubt due to the weak –SH group in 2-Me (p*K* 8.9) which is fully protonated below pH 7 but progressively ionized above pH 8, with the effect that the sample is not at constant ionic strength, being in an ion-free environment at acidic pH but at progressively increasing ionic strength at alkaline pH. The situation is much improved when the high levels of 2-Me in the sample are replaced by 0.5% DTT. As shown in Fig. 1B, the migration of globin chain appears to be normal even above their respective isoelectric points. However, these low thiol levels still affect the migration to the anode in a pH-dependent fashion, since the apparent p*I* of α chains (the crossing point of the protein titration curve and the application trough) is too high (pH 8.7 instead of 7.8) while the β chain behaves normally. This suggests that the ionization of DTT at alkaline pH hampers the migration of α-globin.

Not only thiol reagents, but also buffer salts can strongly influence the pH–

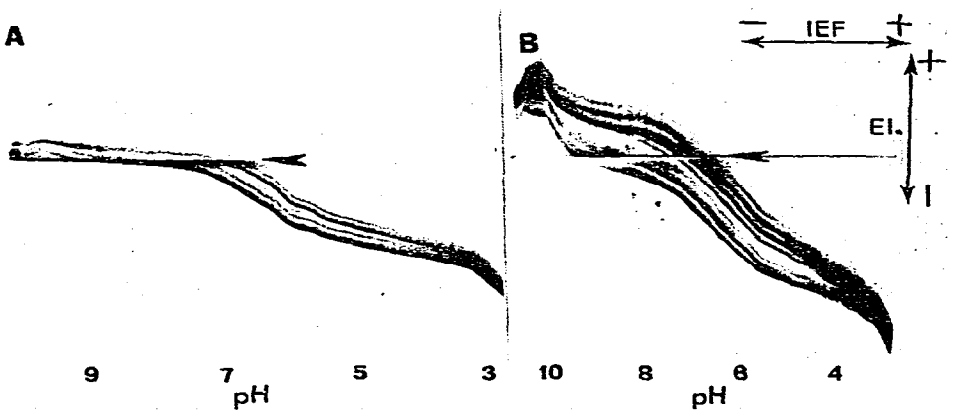

Fig. 1. Titration curves of the acid–acetone powder of a total red blood cell lysate. Gel slab (2 mm thick): 6% acrylamide, 2% Ampholine pH 3.5–10, 5 m*M* Asp, 5 m*M* Glu, 5 m*M* Lys, 5 m*M* Arg and 8 *M* urea. First dimension (IEF): 90 min run in the LKB 2117 Multiphor chamber by delivering 15 W (constant). Coolant temperature: 8°. Second dimension (El.): 40 min run at 800 V (constant), 10 mA with a sample load of *ca.* 200 μg protein. Staining: colloidal dispersion of 0.1% Coomassie Brilliant Blue G-250 in 12% TCA and 1 *N* H_2SO_4, as in ref. 10. The two double-arrows and positive and negative symbols represent the direction and polarity of isoelectric focusing (IEF) and electrophoresis (El.). The arrowheads indicate the sample application trench (*i.e.*, the zero-mobility or isoelectric plane). In A the sample was dissolved in 8 *M* urea and 10% 2-Me, while in B the sample was in 8 *M* urea and 0.5% DTT.

mobility curves of denatured polypeptide chains. Fig. 2A shows the "electrophoretic titration curves" of α- and β-globins dissolved in 10 m*M* Tris·HCl, pH 8.8, 8 *M* urea and 5 m*M* DTT and subsequently alkylated in 15 m*M* iodoacetamide. The sample was run as such, without desalting. Again, the migration toward the anode is strongly affected, with erroneous apparent p*I* values for both chains (p*I* 9.1 for α and p*I* 7.5 for β chains). This is due both to the pH-dependent ionization of DTT and to the presence of a weak base (Tris, p*K* 8.3) in the buffer system. Moreover, the overall ionic strength is also increased upon reaction of iodoacetamide with –SH groups, since free I^- is generated. Only when the sample, after –SH group reduction and alkylation with iodoacetamide, is stripped free of salts by gel filtration, do the pH–mobility curves behave as a unique function of the total amino acid composition of each polypeptide chain (see Fig. 2B).

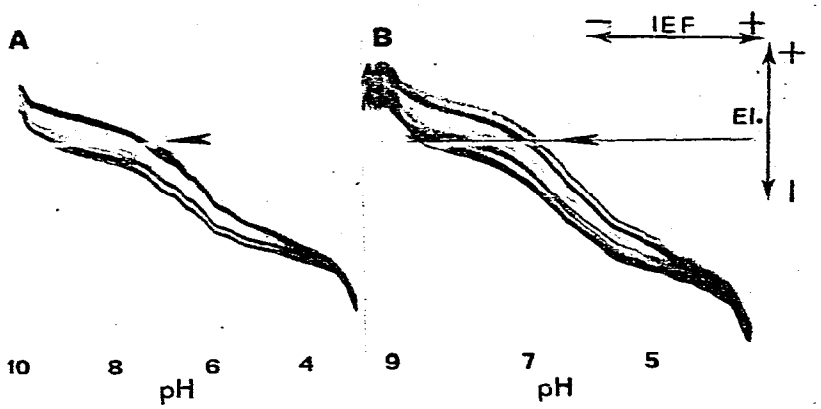

Fig. 2. Titration curves of reduced and alkylated α- and β-globin chains. The sample (heme-free α and β chains from purified normal human hemoglobin) was dissolved in 10 m*M* Tris–HCl, pH 8.8, 8 *M* urea, 5 m*M* DTT and then alkylated by adding iodoacetamide to 15 m*M* concentration. In A the sample was run in this buffer, while in B it was desalted through Sephadex G-25 equilibrated with 8 *M* urea. Here, as well as in Fig. 1, the extensive heterogeneity of both α and β chains is probably due to an "ageing" (deamidation) phenomenon, possibly coupled to carbamylation due to cyanate formation in urea solutions. All other conditions as in Fig. 1.

As shown in the figures, the preparations of globin chains exhibit an extensive heterogeneity, even when freshly dissolved in urea and immediately analyzed. The samples titrated were a few weeks or few months old, lyophilized and stored as a dry powder in a desiccator either at room temperature or at +4°. The sample "ageing" is probably due to a slow deamidation process, as demonstrated by the fact that all minor components join with the respective α or β chains at acidic pH, where Glu and Asp residues are protonated. This deamidation is a spontaneous chemical process and is probably favoured by traces of water in the sample, which cannot be removed even upon exhaustive lyophilization. Ageing by deamidation seems to be one of the most common cellular events, occurring *in vivo*, and probably transforming the deamidated protein into a configuration more susceptible to proteolytic attack[13,14]. This process is slowed down, but not completely abolished, when the same lyophilized samples are stored desiccated at −20°. The only time in which we could observe a "single" titration curve from a chromatographically pure sample was when the globin

chain was run as soon as it was eluted from the CM-cellulose column, or when the same sample was immediately frozen in liquid nitrogen after elution and analyzed as soon as it had thawed (Fig. 3A). In Fig. 3B are shown the titration curves of an equimolar mixture of β-lactoglobulins A and B, reduced, alkylated and desalted. The two proteins cross over the trench at their respective p*I* values (5.15 and 5.30) and are seen to join together below pH 4.0, as expected from their amino acid composition[15] (the A form is the deamidation product of the B form, probably the conversion of a Gln into Glu, as suggested by the titration curve).

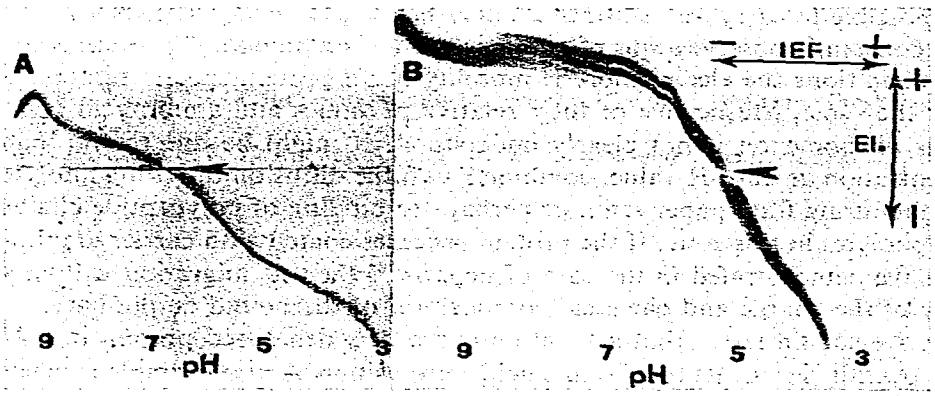

Fig. 3. A, Titration curve of a freshly-prepared, CM-cellulose purified β-globin chain, reduced, alkylated and desalted (sample load, *ca.* 50 μg). B, Titration curves of a 1:1 mixture of commercial β-lactoglobulins A and B, reduced, alkylated and desalted (sample load, *ca.* 150 μg). All other conditions and symbols as in Fig. 1.

DISCUSSION

Our data suggest that "electrophoretic titration" of denatured polypeptide chains in 8 *M* urea can be successfully performed and can be used to yield complementary information to that obtained with titration curves of native species. However, care should be taken in avoiding several pitfalls. Since the protein chains are titrated over a continuous pH 3–10 gradient, all weak buffer electrolytes or thiol compounds with p*K* values in this pH range should be avoided, as their pH-dependent ionization will force the protein chains to move in regions of different ionic strength, thus altering their true electrophoretic mobility. This means that practically all buffer systems will have to be eliminated. The best results are thus obtained when the proteins are reduced and alkylated at appropriate pH, and then desalted through Sephadex G-25 equilibrated with 8 *M* urea. The denatured chains should then be analyzed within a short time, so as to avoid carbamylation reactions. Our data show that "microheterogeneity" seems to be a common status of proteins and that "homogeneous" proteins only exist for a short time at the end of a purification process or when the purified sample is stored in liquid nitrogen. Lyophilization and subsequent storage at room temperature or at −20° does not prevent "ageing" of proteins *in vitro*.

It is odd that the presence of ionizable buffers and thiols should alter so markedly not only the electrophoretic mobility, but also the apparent isoelectric points of the globin chains investigated. We can only suggest that, in the presence of

salts, we are measuring isoelectric points, while in equilibrium focusing or in the absence of salts in titration curves we are dealing with isoionic points (for an explanation of these terms, see Rilbe[16,17]). However, this would imply some degree of binding, *i.e.*, a direct interaction, of these ions to the protein chain, which is hard to imagine in the presence of 8 *M* urea. Alternatively, the ions could have an indirect effect by altering the degree of ionization of ionizable groups in the protein chains.

Our data show that titration curves in 8 *M* urea can be performed down to pH *ca.* 3, with no blurring of protein patterns, as previously observed[1–4]. However, even though carrier ampholytes buffering down to pH 2 are available, we believe it would be impossible to carry out a titration curve below pH *ca.* 3, since, below this pH value, we constantly observe a sort of "sweeping away" or "flooding" phenomenon. Under these conditions the electrophoretic mobility of all protein species present in the system is the same, irrespective of their relative p*I* values and amino acid composition. This phenomenon is not clearly understood. It might be due to the high proton concentration at this pH value, combined with the diffusion of strong anolyte from the neighbouring filter-paper strip, or perhaps to binding of polypeptide chains to carrier ampholytes in this zone. If the protein becomes coated with carrier ampholytes (as we have demonstrated in the case of heparin[18]) then its migration will be a function only of the charge and chemical composition of the bound ampholytes.

Lastly, we should stress that a titration curve of a denatured protein in 8 *M* urea gives an overall view of its total amino acid composition. A nice example is given by the shape of the titration curve of α and β chains. These two species differ mostly in their acidic residues (a total of 12 Glu + Asp in α against 15 in β chains) while their total sum of basic amino acids is almost identical (3 Arg, 10 His and 11 Lys in α and 3 Arg, 9 His and 11 Lys in β chains). Accordingly, these two chains almost meet at pH 3.5, where most of the charge difference is abolished due to the protonation of Glu and Asp residues.

ACKNOWLEDGEMENTS

This work was supported in part by grants 76.01488.04 and CT77.01471.04 from Consiglio Nazionale delle Ricerche (CNR, Roma) and by I.N.S.E.R.M. grants No. 14.75.37 and 41.76.73. P.G.R. thanks I.N.S.E.R.M. for a visiting professorship to the Institut de Pathologie Moléculaire (Paris), and Professor G. Schapira for the hospitality received at this Institute.

REFERENCES

1 A. Rosengren, B. Bjellqvist and V. Gasparic, in B. J. Radola and D. Graesslin (Editors), *Electrofocusing and Isotachophoresis*, De Gruyter, Berlin, 1977, pp. 165–171.
2 P. G. Righetti, R. Krishnamoorthy, E. Gianazza and D. Labie, *J. Chromatogr.*, 166 (1978) 455–460.
3 R. Krishnamoorthy, A. Bianchi Bosisio, D. Labie and P. G. Righetti, *FEBS Lett.*, 94 (1978) 319–323.
4 P. G. Righetti, G. Gacon, E. Gianazza, D. Lostanlen and J. C. Kaplan, *Biochem. Biophys. Res. Commun.*, 85 (1978) 1575–1581.
5 R. A. Brown and S. N. Timasheff, in M. Bier (Editor), *Electrophoresis*, Academic Press, New York, 1959, pp. 317–367.

6 C. Tanford, J. G. Buzzel, D. G. Rands and S. A. Swanson, *J. Amer. Chem. Soc.*, 77 (1955) 6421–6425.
7 J. B. Clegg, M. A. Naughton and D. J. Weatherall, *J. Mol. Biol.*, 19 (1966) 91–108.
8 L. C. Lane, *Anal. Biochem.*, 86 (1978) 655–664.
9 P. G. Righetti and T. Caravaggio, *J. Chromatogr.*, 127 (1976) 1–28.
10 P. G. Righetti and F. Chillemi, *J. Chromatogr.*, 157 (1978) 243–251.
11 E. Gianazza, P. G. Righetti, S. Bordi and G. Papeschi, in B. J. Radola and D. Graesslin (Editors), *Electrofocusing and Isotachophoresis*, De Gruyter, Berlin, 1977, pp. 173–179.
12 R. Pitt-Rivers and F. S. A. Impiombato, *Biochem. J.*, 109 (1968) 825–830.
13 R. Krishnamoorthy, G. Gacon and D. Labie, *FEBS Lett.*, 77 (1977) 99–102.
14 R. Krishnamoorthy, G. Gacon and D. Labie, in *Molecular Interactions of Hemoglobin*, Vol. 70, I.N.S.E.R.M., Paris, 1977, pp. 309–318.
15 S. Fredriksson, *Anal. Biochem.*, 50 (1972) 575–582.
16 H. Rilbe, *Ann. N.Y. Acad. Sci.*, 209 (1973) 11–22.
17 H. Rilbe, in N. Catsimpoolas (Editor), *Isoelectric Focusing*, Academic Press, New York, 1976, pp. 14–52.
18 P. G. Righetti, R. Brown and A. L. Stone, *Biochim. Biophys. Acta*, 542 (1978) 232–244.