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HYDROPHOBIC INTERACTION BETWEEN ALKALINE IMMOBILINES AND FERRITIN DURING ISOELECTRIC FOCUSING IN IMMOBILIZED pH GRADIENTS

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SUMMARY

It has been found that three alkaline Immobilines (out of seven weak acids and bases used to generate immobilized pH gradients), having p*K* values of 6.2, 7.0 and 9.3, act as cross-linking agents, aggregating and precipitating out of solution ferritin and other large macromolecules (*e.g.*, from serum and tissue extracts) present in body fluids and human biopsies. All the acidic Immobilines (p*K* 3.6, 4.4 and 4.6) and the basic species of p*K* 8.5 appear to be unreactive. The three precipitin Immobilines mimic cationic detergents, acting on the basis of two different principles at the opposite extremes, by ionic interaction at one end and by hydrophobic bonding at the other end of the molecule. The ionic type of interaction was clearly demonstrated, owing to its sensitivity to pH extremes and to progressively increasing ionic strength. The hydrophobic interaction in the region of the double bond (Immobilines are N-substituted acrylamido acids and bases) was deduced on the basis of the following observations: (a) oxidation of the double bond with introduction of a vicinal diol totally inhibited ferritin aggregation; (b) addition of SH groups to the double bond increased protein precipitation and (c) the protein–Immobiline aggregates were found to be sensitive to alkyl-substituted ureas (especially ethyl- and propylurea), which are known to bind to hydrophobic regions of proteins, and insensitive to urea, which is known to split only hydrogen bonds. Interestingly, neutral and zwitterionic detergents were unable to split the Immobiline–ferritin complexes, suggesting that their large micelles could not have access to the tightly packed Immobiline cross-linking region.

INTRODUCTION

Immobilines are seven, non-amphoteric weak acids and bases with the general formula $CH_2=CHC(O)NHR$, where R denotes either three weak carboxyls (having p*K* 3.6, 4.4 and 4.6) or four weak bases (with p*K* 6.2, 7.0, 8.5 and 9.3)[1]. They are used, mixed with appropriate amounts of acrylamide and bisacrylamide, to create any desired pH gradient, from as narrow as 0.1 up to 7 pH units (pH 3–10)[2], for

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isoelectric focusing (IEF) separations. In contrast to conventional IEF, where the amphoteric buffers (carrier ampholytes, CA) are moved electrophoretically in the field and are able to diffuse in the absence of an applied voltage, the Immobilines are covalently affixed to the polyacrylamide gel, producing indefinitely stable and well defined and reproducible pH gradients[3]. Immobilized pH gradients (IPGs) have solved all the problems of CA-IEF and have proved useful in genetic analysis, clinical chemistry, preparative protein purification and two-dimensionl (2-D) mapping[4].

However, as is common in biochemical separation techniques, new methodologies which solve existing problems often generate new, unexpected problems, and IPGs are no exception. We had found that membrane-bound proteins often tended to precipitate in the gel at the application point or produce a smear along the separation track. We therefore resorted to a mixed-bed technique, by which the primary, Immobiline-based pH gradient was overlayered with a secondary, carrier ampholyte-driven pH gradient[5,6]. Possibly the CA buffers, during the transient state, coat the membrane proteins, thus improving their solubility and shielding them temporarily from the surrounding IPG matrix, until attainment of a steady state. However, even with this mixed-bed method, a substantial number of separations were improved, but there still remained some hidden, unresolved problems. For example, in 2-D maps of complex biological samples (*e.g.*, sera and tissue extracts) we had noticed the disappearance of a number of large proteins (M_r >100000 daltons) and severe streaking of other macromolecules, suggesting a time-dependent precipitation–solubilization cycle during the IPG step. The key to this phenomenon was recently found when focusing ferritin: this large macromolecule (M_r 450000) constantly precipitated at the application point and did not even enter the IPG gel. It was observed that three alkaline Immobilines (pK 6.2, 7.0 and 9.3) specifically bind and aggregate ferritin in solution. It was hypothesized that these three chemicals act as cross-linkers, forming an ionic bond at the charged extremity and a hydrophobic bond in the double bond region, thus binding ferritin in a large lattice, resembling antigen–antibody complexes, which would then precipitate out of solution. Whereas the ionic ferritin–Immobiline interaction was easily demonstrated[7], the hydrophobic linkage remained in our model as only a working hypothesis. In this paper we give direct evidence of this hydrophobic interaction and suggest remedies, the long-term goal being the synthesis of three new, more hydrophilic Immobilines.

EXPERIMENTAL

Materials

Immobilines, Ampholines of pH 4–6, acrylamide, N,N′-methylenebisacrylamide, ammonium persulphate, N,N,N′,N′-tetramethylethylendiamine (TEMED), Gel Bond PAG, the Multiphor 2 chamber, Multitemp thermostat and the Macrodrive 5 power supply were obtained from LKB (Bromma, Sweden). Four-times crystallized horse spleen ferritins were a gift from Dr. P. Arosio, University of Milan. Nonidet P-40, sulfobetaine 3-12 (SB_{12}), ethylurea and sodium dodecyl sulphate (SDS) were obtained from Sigma (St. Louis, MO, U.S.A.), propylurea from Eastman Kodak (Rochester, NY, U.S.A.) and tetramethylurea from Carlo Erba (Milan, Italy).

Conventional IEF

IEF was performed in 0.5 mm thin gels, made of a 4%T, 4%C polyacrylamide matrix impregnated with 2% carrier ampholytes, pH 4–6. Runs were carried out at 4°C for 5 with 1500 V at equilibrium, 5 W limiting. Staining was with Coomassie Briliant Blue R-250[8].

IEF in IPGs

This was run in 0.5 mm thin gels, with two kinds of recipes: (a) a pH 4–6 Immobiline gel, made according to the formulation given in LKB Application Note 324 (1984) (containing the p*K* 3.6, 4.6, 6.2 and 9.3 Immobilines); (b) a pH 4–5 Immobiline gel, again with the formulation of LKB Application note 324, in which, however, the p*K* 9.3 was substituted with p*K* 8.5 Immobiline. Both kinds of gels were made to contain polyacrylamide (4% T, 4% C) and were run in either the absence or presence of various amounts of CAs (1–4%)[5]. With recipe (a), the gels were made to contain either the standard molarity of Immobiline or progressive dilutions up to 100-fold. The runs were usually performed overnight at 10°C and 2000 V.

Turbidimetry

Turbidity was read at 600 nm with a Varian (Palo Alto, CA, U.S.A.) spectrophotometer by correcting for the volume changes after addition of either salts or titrants. Complexing between ferritin and Immobilines in solution was studied as follows: each Immobiline species was dissolved (10 m*M* concentration) and titrated to pH 6.0 with hydrochloric acid or sodium hydroxide solution. To this a constant amount of ferritin (500 μg) was added and the turbid (or clear) solution was read at 600 nm. Disaggregation of the complex was studied as a function of ionic strength (by adding sodium chloride), as a function of pH (by titrating the solution to pH 4 or 10), as a function of addition of detergents (neutral, zwitterionic and anionic) or of alkylureas. With the p*K* 6.2 Immobiline, the disaggregation experiments were repeated in a solution titrated to pH 5.0 instead of 6.0.

Modification of the double bond in Immobilines

Two reactions were performed on the double bond of free Immobilines (p*K* 6.2, 7.0 and 9.3) in solution: (a) oxidation and (b) addition of an SH group. In the former instances to a 10 m*M* solution of each Immobiline, titrated to pH 5.0, was added potassium permanganate until the appearance of the first stable violet colour in the supernatant[9]. At the end of the reaction, the brown manganese(IV) oxide precipitate was eliminated by filtration on Millipore membranes and the modified Immobiline was tested for its ability to precipitate ferritin by the above turbidimetric assay. In this reaction two *cis*-OH groups should be added as follows:

$$CH_2=CH-\underset{\underset{O}{\|}}{C}-NHR \xrightarrow[KMnO_4]{H^+} CH_2-\underset{OH}{\underset{|}{CH}}-\underset{OH}{\underset{|}{C}}-\underset{O}{\underset{\|}{C}}-NHR$$

thus rendering the Immobilines strongly hydrophilic.

In the latter instance, to a 10 m*M* solution of each Immobiline, titrated to pH

9.0, was added 20 m*M* 2-mercaptoethanol and the reaction was continued for 1 h at 50°C. The course of the addition of SH to the double bond was followed spectrophotometrically at 280 nm. The modified Immobilines were tested for their ability to precipitate ferritin by the above turbidimetric assay. The reaction proceeds as follows:

$$CH_2=CH-\underset{\underset{O}{\|}}{C}-NHR \xrightarrow[HOCH_2CH_2SH]{OH^-} OHCH_2CH_2SCH_2CH_2-\underset{\underset{O}{\|}}{C}-NHR$$

thus rendering the Immobilines more hydrophobic.

RESULTS

Fig. 1A shows the impossibility of focusing ferritin in an IPG range containing the two "precipitin" p*K* 6.2 and 9.3 Immobilines: the protein barely moves out of the pocket as a smear and will not focus into well separated bands even at very high voltages (*e.g.*, 400–500 V/cm) or for extended periods of time (more than 2 days under a voltage gradient). Having found that, among the alkaline Immobilines, only the p*K* 8.5 species had a favourable, non-precipitin structure, a new IPG pH 4–5 range was prepared, using only the p*K* 4.6 as buffering and the p*K* 8.5 as titrant ions. In this modified IPG recipe, a sharp array of ferritin bands was obtained (Fig. 1B) with a much superior resolution than that obtainable in conventional IEF in am-

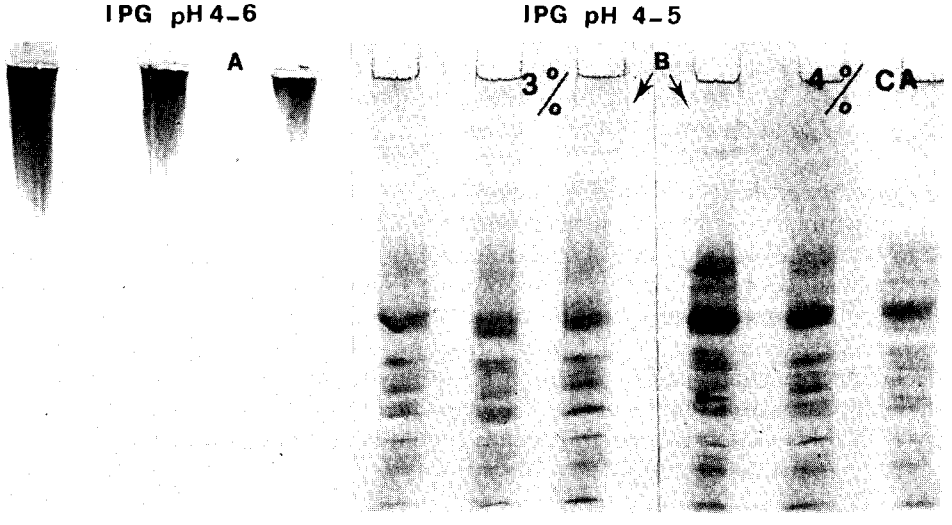
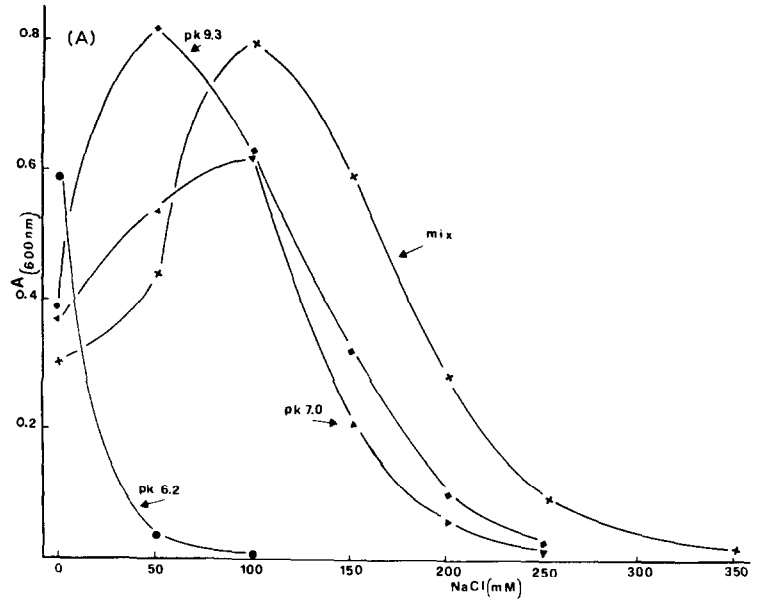


Fig. 1. Isoelectric focusing of ferritin in immobilized pH gradients. (A) 4%T polyacrylamide gels containing a pH 4–6 immobilized gradient (formed with p*K* 3.6, 4.6, 6.2 and 9.3 Immobilines); (B) 4%T polyacrylamide gel containing a pH 4–5 immobilized gradient (formed with only p*K* 4.6 and 8.5 Immobilines). This gel was cut into two halves and impregnated with 3% and 4% carrier ampholytes (CA) in the pH 4–6 range. Sample load (from left to right in each gel): 200, 150 and 100 μg horse spleen ferritin. Focusing overnight at 2000 V (final) and 10°C. Staining with Coomassie Blue R-250.



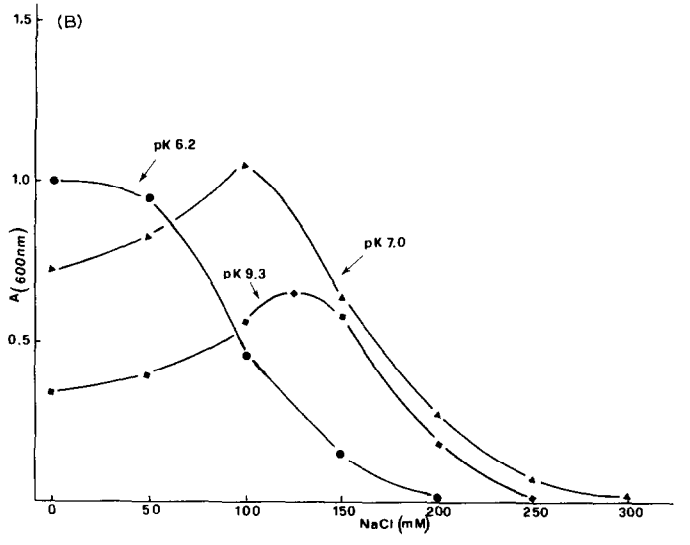


Fig. 2. Disaggregation of the ferritin–Immobiline complexes as a function of environmental ionic strength. The complexes among ferritin (100 μg) and the pK 6.2, 7.0 and 9.3 Immobilines (10 mM each) were made in solutions titrated to pH 6.0. Turbidity readings were performed at 600 nm in a Varian spectrophotometer as a function of increasing sodium chloride concentration, added as solid crystals. (A) Control, unreacted Immobilines; (B) Immobilines reacted with 2-mercaptoethanol. Note the biphasic shape of the dissociation curves, suggesting a concomitant hydrophobic interaction.

photeric buffers[10]. In previous work[7] we had hypothesized that the unfavourable Immobilines (p*K* 6.2, 7.0 and 9.3) would act as cross-linkers between two ferritin molecules, bridging them with two unlike interactions, ionic at one extreme and hydrophobic at the opposite extreme (the double bond region of the acrylamide moiety of the Immobiline molecule). As shown in Fig. 2A, the ionic type of interaction is easily demonstrated by the dissolution of all complexes in sodium chloride solution of increasing molarity: at sodium chloride levels between 250 and 300 m*M* even the sturdiest lattices are fully disaggregated. A similar behaviour was demonstrated when titrating the complexes to pH 4 or 10, in full agreement with an ionic interaction between a cluster of negative charges on the ferritin surface and a stack of positively charged Immobiline molecules clinging to it[7].

It remained to demonstrate the hydrophobic interaction at the opposite extreme of the bridge. When adding to the double bond of Immobilines the SH group of 2-mercaptoethanol (2-ME), the results in Fig. 2B were obtained: the turbidity maxima of the three Immobilines were in general increased (up to 1.1 absorbance) and shifted towards higher sodium chloride molarity, this being consistent with a stronger hydrophobic interaction occurring in the aggregates (the SH-modified Immobilines have substantially higher hydrophobicities). Conversely, when the double bond region was rendered more hydrophilic, by addition of a vicinal diol (permanganate oxidation), no precipitate was formed with any of the three Immobilines (Table I).

In principle, hydrophobic interactions should also be destroyed by detergents, *e.g.*, as used in membrane protein solubilization[11]. Surprisingly, however, when neutral surfactants (*e.g.* Nonidet P-40) are added to pre-formed Immobiline–ferritin complexes, they are essentially unable to disaggregate them (Table I). When using sulphobetaines (like SB_{12}, known to have the highest solubilizing power among detergents)[12], the results are even worse: if they are added to a pre-formed complex, they seem to induce aggregation even further; if they are present in solution before the addition of the two complexing species, they are completely unable to prevent

TABLE I

STABILITY OF THE IMMOBILINE–FERRITIN COMPLEXES AS A FUNCTION OF DETERGENT ADDITION AND OF MODIFICATION OF THE ACRYLIC DOUBLE BOND

Immobiline	*Absorbance at 600 nm**						
	Control	*20 mM 2-ME***	*$KMnO_4$****	*2% SB_{12}*§	*2% SB_{12}*§§	*2% NP-40*	*2% SDS*
p*K* 6.2	0.600	1.00	0.00	0.948	0.756	0.540	0.00
p*K* 7.0	0.375	0.720	0.00	0.492	0.540	0.330	0.00
p*K* 9.3	0.390	0.400	0.00	0.557	0.730	0.400	0.00

* Abbreviations: NP-40 = Nonidet P-40; SDS = sodium dodecyl sulphate; SB_{12} = sulphobetaine with a 12 carbon tail.

** Addition of 2-mercaptoethanol (2-ME) to the double bond.

*** Addition of a vicinal diol to the double bond.

§ Addition of zwitterionic detergent (sulphobetaine SB_{12}) to a pre-formed Immobiline–ferritin precipitate.

§§ Zwitterionic detergent present in solution prior to the formation of the complex.

TABLE II

BEHAVIOUR OF THE IMMOBILINE–FERRITIN COMPLEXES IN THE PRESENCE OF ETHYLUREA

Immobiline	*Absorbance at 600 nm*						
	Control	*7 M EtU**	*4 M EtU**	*2 M EtU**	*7 M EtU***	*4 M EtU***	*2 M EtU***
p*K* 6.2	1.458	0.160	1.020	1.403	0.003	0.380	1.367
p*K* 7.0	1.134	0.600	1.140	1.460	0.013	1.073	1.145
p*K* 9.3	0.570	0.292	0.390	0.749	0.078	0.764	0.766

* Addition of ethylurea (EtU) to a pre-formed Immobiline–ferritin complex.
** Ethylurea present in solution prior to the formation of the complex.

lattice formation (Table I). This is interpreted (see Discussion) as an inability of the large detergent micelles to penetrate the narrow gap between two ferritin macroions bridged by the Immobiline cross-linker. The only detergent able to disrupt all complexes is SDS (but probably because of its simultaneous action on the ionic side of the bridge), which, however, is incompatible with IEF runs[13].

In a search for agents able to disrupt such hydrophobic interactions and compatible with an isoelectric focusing separation, we recalled that in the 1970s alkyl-substituted ureas were used extensively for this purpose[14]. When a range of molarities of ethylurea was explored, the results in Table II were obtained: at low concentrations (2 *M*) the aggregates were favoured, at intermediate concentrations (4 *M*) they began to be disrupted and at high concentrations (7 *M*) they were almost completely redissolved. The last effect, however, was strongly dependent on the mode of mixing the reactants: if ethylurea was present in solution before addition of the complexing species, essentially complete inhibition was achieved. However, if the ethylurea was added to the pre-formed complexes, only the Immobiline p*K* 6.2–ferritin aggregate was split, the other two being inhibited by only 50%. In this instance, no better solubilizing power was obtained if, in addition to 7 *M* ethylurea, 2% neutral (Nonidet P-40) or zwitterionic (SB_{12}) detergents were admixed in solution (Table III, last two

TABLE III

EFFECT OF ALKYLUREAS ALONE AND IN THE PRESENCE OF DETERGENTS ON THE IMMOBILINE–FERRITIN AGGREGATES

Immobiline	*Absorbance at 600 nm**						
	Control	*5 M PrU***	*5 M PrU****	*7 M EtU***	*6 M TMU****	*7 M EtU + 2% SB_{12}*	*7 M EtU + 2% NP-40*
p*K* 6.2	1.458	0.000	0.000	0.163	1.100	0.160	0.320
p*K* 7.0	1.134	0.024	0.000	0.600	0.800	0.600	0.630
p*K* 9.3	0.570	0.048	0.000	0.292	0.450	0.290	0.330

* Abbreviations: PrU = propylurea; EtU = ethylurea; TMU = tetramethylurea; SB_{12} = sulphobetaine with a 12-carbon tail; NP-40 = Nonidet P-40.
** Alkylureas added to a pre-formed Immobiline–ferritin precipitate.
*** Alkylureas added before forming the precipitate.

columns). Even better results were obtained if a longer chain alkylurea was added: as shown in Table III, 5 *M* propylurea has a complete disaggregating power on all types of Immobiline–ferritin complexes, even when added to a pre-formed precipitate. Butyl urea and higher substituted ureas were not tried, as their solubility in water decreases sharply with increasing size (butylurea has a solubility limit of only 0.6 *M* at 25°C in water).

DISCUSSION

It seems clear that the three precipitin Immobilines (p*K* 6.2, 7.0 and 9.3) act by bridging macromolecules by two completely different mechanisms of interaction: ionic and hydrophobic on each extremity, respectively. In a previous paper[7] we provided ample evidence for the ionic type of interaction. We feel that the data in this paper demonstrate also the other, missing link, the hydrophobic interaction. This latter type of bond, in fact, could have devastating consequences for IPG technology, as no easy remedy is available. From our results, it appears that the double bond environment of the acrylamido extremity of the Immobilines is hydrophobic and is responsible for aggregating proteins that have hydrophobic patches on the surface. In fact, if this character is reversed to hydrophilic, by adding a vicinal diol to the double bond, the Immobilines lose completely their ability to precipitate proteins. This remarkable effect will have to be taken into consideration when synthesizing three new Immobilines with a "favourable" structure, *i.e.*, unable to precipitate and aggregate proteins. Conversely, when rendering the double bond moiety more hydrophobic, by addition of 2-ME, the situation is aggravated and proteins are more extensively precipitated. Note that the 2-ME–Immobiline adducts in reality resemble more closely the behaviour of these chemicals when grafted into the polyacrylamide matrix as, once reacted, each Immobiline molecule will be flanked, on either side, by a $-CH_2CH<$ moiety, belonging to the extended polyacrylamide coils. Thus, the over all hydrophobic behaviour of these species will be manifested in an IPG matrix. It is clear that our long-term strategies with IPG technology will have to change; selection of more hydrophilic gels might be beneficial. In fact, in previous work[7], the use of a polytrisacryl instead of a polyacrylamide gel was found to improve the separation, owing to the higher hydrophilicity of the former matrix. Use of a more hydrophilic cross-linker (*e.g.*, DHEBA, N,N′-1,2-dihydroxethylenebisacrylamide) could also be beneficial, owing to the vicinal diol in the middle of the molecule. However, it is also clear that the major obstacle to good IPG separations is the overall hydrophobicity of the three "unfavourable' Immobilines.

In the field of 2-D maps, in which the introduction of IPGs appeared to be a major innovation[15], we have noticed recently that various large proteins from biological fluids (*e.g.*, sera or cerebrospinal fluid) and tissue biopsies had disappeared from the map, no doubt owing to the same precipitin mechanism described here for ferritin[7]. Some relief appears to be in sight: owing to the strong disaggregating power of alkylureas, it is to be expected that a major improvement could be obtained by adding to the inefficient detergents the much more efficient propylurea. In membrane analysis, detergents will still have to be added for membrane disaggregation, as they are efficient in sweeping away the membrane lipids, but during the run in the IPG matrix propylurea will have to be present to shield the protein moiety from hydrophobic interaction with the IPG gel.

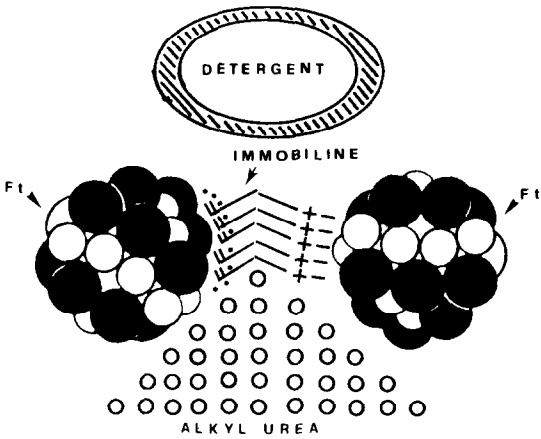


Fig. 3. Model of the ferritin–Immobiline complexes and of the effect of detergents and alkylureas on them. It is hypothesized that Immobiline act as bifunctional bridges, by forming an ionic linkage at one extreme (containing the ionizable group) and a hydrophobic interaction at the double bond opposite extreme (note the pedunculate double bond region flattened against the ferritin shell and the electron cloud surrounding it, represented by small black dots). For thermodynamic stability, a stack of at least 4–5 Immobiline molecules has to form in the cross-linking region. It is hypothesized that the complex forms with two unlike regions on the ferritin surface, one containing a cluster of negative charges and one containing an hydrophobic region. The detergent micelle, owing to its large size, does not have access to the narrow gap between the two cross-linked ferritin macromolecules. Conversely, the small alkylurea molecules can converge into the bonding region and split the cross-link on the side of the hydrophobic linkage.

We interpret the contrasting behaviour of detergents (aggregate stabilizers) and of propylurea (disaggregating agents) with the model in Fig. 3: here two ferritin macroions are shown bridged by a stack of five Immobiline molecules, neutralizing an equal number of negative charges on the ferritin surface on one side and hooking-up to a hydrophobic patch on the opposite side. Owing to the small size of the Immobilines (average M_r 150–200 daltons), the gap in between is in reality fairly narrow. When the large detergent micelles are added in solution, they can only circumnavigate the precipitated lattice and, owing to steric hindrance, they cannot penetrate and act in proximity to the hydrophobic Immobiline–protein contact. Even if the micelles were small (*e.g.*, octyl glucoside), it is doubtful that they could be active in this instance, as in general detergent micelles are spatially oriented so as to have the hydrophobic segments buried within and the hydrophilic portions coating the surface. The only exception is SDS, which probably acts simultaneously on both sides of the bridge, weakening both the hydrophobic and the ionic interactions, the latter owing to its strong negative charge density. The fact that it can disrupt these complexes could mean additionally that SDS is also active in the monomeric state, whereas the neutral and zwitterionic detergents act preferentially in the micellar form. Conversely, the small propylurea molecules can converge and penetrate inside the cross-linking region and effectively compete with the bound Immobilines, thus disrupting the complex (the turbid solution is immediately clarified). Asymmetric alkylureas have an added advantage: once they have displaced the Immobiline, they are able to shield effectively any hydrophobic patch on the protein molecule and coat

it with the free $-CONH_2$ extremity, still able to be extensively hydrogen bonded with water and thus solvate the macromolecule. It is also possible that this shielding effect could also be operative on the IPG matrix, thus keeping the two interacting species well apart for the duration of an IPG run. If this hypothesis is correct, asymmetric ureas should be much more effective than symmetrically substituted ureas. In fact, as shown in Table III, tetramethylurea has little disaggregating power on ferritin–Immobiline complexes.

CONCLUSIONS

The mechanism of the precipitin power of alkaline Immobilines on ferritin and other large macro-ions is now understood and remedies are in sight. When focusing acidic proteins in IPG gels, good patterns will be obtained by substituting the alkaline titrant (in general the precipitin p*K* 9.3 species) with the "favourable", non-precipitin p*K* 8.5 Immobiline. When running 2-D maps, where proteins are analysed under denaturing conditions, a judicious blend of propyl- and ethylureas (*e.g.*, 4 *M* and 2 *M*, respectively) should allow good sample penetration and full solubilization (experiments in progress with Dr. T. Rabilloud). The long-term goal will be the synthesis of three new Immobilines having the same p*K* values (6.2, 7.0 and 9.3) but with more hydrophilic substituents.

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REFERENCES

1 B. Bjellqvist, K. Ek, P. G. Righetti, E. Gianazza , A. Görg, W. Postel ad R. Westermeier, *J. Biochem. Biophys. Methods*, 6 (1982) 317–339.
2 E. Gianazza, F. Celetano, G. Dossi, B. Bjellqvist and P. G. Righetti, *Electrophoresis*, 5 (1984) 88–97.
3 P. G. Righetti, *J. Chromatogr.*, 300 (1984) 165–223.
4 P. G. Righetti ad E. Gianazza, *Methods Biochem. Anal.*, 32 (1986) 215–278.
5 M. Rimpiläinen and P. G. Righetti, *Electrophoresis*, 6 (1985) 419–422.
6 P. K. Sinha and P. G. Righetti, *J. Biochem. Biophys. Methods*, 12 (1986) 289–297.
7 P. G. Righetti, C. Gelfi, M. L. Bossi and E. Boschetti, *Electrophoresis*, 8 (1987) 62–70.
8 P. G. Righetti, *Isoelectric Focusing: Theory, Methodology and Applications*, Elsevier, Amsterdam, 1983, pp. 256–259.
9 A. Bianchi-Bosisio, C. Loeherlein, R. S. Snyder and P. G. Righetti, *J. Chromatogr.*, 189 (1980) 317–330.
10 J. W. Drysdale, *Biochim. Biophys. Acta*, 207 (1970) 256–258.
11 A. Helenius and K. Simons, *Biochim. Biophys. Acta*, 415 (1975) 29–79.
12 D. Satta, G. Schapira, P. Chafey, P. G. Righetti and J. P. Wahrmann, *J. Chromatogr.*, 299 (1984) 57–72.
13 P. G. Righetti, *Isoelectric Focusing: Theory, Methodology and Applications*, Elsevier, Amsterdam, 1983, pp. 273–279.
14 T. T. Herskovits, H. Jaillet and B. Gadegbeku, *J. Biol. Chem.*, 245 (1970) 4544–4550.
15 E. Gianazza, S. Astrua-Testori, P. Caccia, P. Giacon, L. Quaglia and P. G. Righetti, *Electrophoresis*, 7 (1986) 76–83.