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Journal of Chromatography A, 767 (1997) 255–262

JOURNAL OF CHROMATOGRAPHY A

Study of haptoglobin–hemoglobin complexes by titration curves, capillary electrophoresis and capillary isoelectric focusing

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Received 8 October 1996; revised 3 December 1996; accepted 3 December 1996

Abstract

A novel method is described for monitoring complex formation between macromolecules, based on combined isoelectric focusing–electrophoresis in capillaries. The example studied is the binding of serum haptoglobin (Hp) to hemoglobin (Hb). A known amount of Hb is focused in a capillary in a pH 6–8 range (p*I* of Hb=7.0) and thus kept temporarily "immobilized" in the electrophoretic chamber. Subsequently, increasing amounts of ligand (Hp) are loaded cathodically and allowed to sweep past the focused Hb zone. As the complex formed has a p*I* value well-outside the bounds of such a pH gradient (the 1:1 molar Hb–Hp complex has a p*I* of 5.5; the 1 to 1/2 molar Hp–Hb complex has a p*I* of 5.0) it escapes immobilization and moves past the detector window, where it is monitored and quantified. Since the detector is set at 416 nm, where only Hb absorbs, and since the molar extinction coefficient of Hb is well known, it is quite easy to calculate the molar amount of Hb bound to the complex. As an additional check, the amount of unreacted Hb can now be mobilized by disrupting the pH gradient and allowing this residual free Hb to also reach the detector and be quantified. The method is easy, fast, simple and fully automated and thus could represent a valid alternative to existing methods in clinical chemistry for quantifying the amount of Hp in human sera in pathological conditions, such as hemolytic anemias and transfusion reactions.

Keywords: Isoelectric focusing; Complexation; Haptoglobin; Haemoglobin; Proteins; Glycoproteins

1. Introduction

Capillary zone electrophoresis (CZE) [1] is gaining increasing importance also as a tool for measuring physico-chemical parameters, such as binding constants of proteins for ligands, enzyme reaction rates and macromolecule–macromolecule interactions. An interesting example is the electrophoretically mediated micro-analysis (EMMA) of enzymes [2–5] and substrates [6] by CZE. EMMA is based on the electrophoretic mixing of two substances in an electrophoretic system utilizing differences in the electrophoretic mobilities of the analytes and reagents. The capillary is filled with appropriate substrate solution and, upon injection of a zone of enzyme, product will form upon electrophoretic mixing of enzyme and substrate. At zero potential, one can let a zone of reaction product accumulate and then transport it past the detector by re-applying the current. By this technique, Harmon et al. have studied the reaction kinetics of a number of enzymes,

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PII S0021-9673(97)00011-3

including alkaline phosphatase, β-galactosidase, leucine amino peptidase (the latter by exploiting a variant of EMMA, called moving boundary electrophoretically mediated microanalysis) [7]. As demonstrated by Emmer and Roeraade [8], it is also possible to perform such analyses by adding to standard CZE equipment a micro-post-column reactor coupled with a second detector: this requires addition of a flow of substrate in the post-column section. Additional examples regard measurement of carboxypeptidase A activity [9] and analysis of ligase chain reaction products by silicon glass chip electrophoresis [10].

In another variant, affinity capillary electrophoresis (ACE), it is possible, for instance, to measure the initial velocity of an enzyme reaction, as well as to calculate its Michaelis constant [11]. ACE can also be used in a multiple plug binding assay: multiple plugs of proteins, and non-interacting neutral and protein standards, are injected in a capillary. Analysis of the electrophoretic mobilities of the individual protein plugs, relative to the non-interacting neutral standard, as a function of the concentration of ligand, yield values for their binding constants to the protein [12].

CZE can efficiently be adopted also for measuring binding constants (K_a) of ligands to proteins. As an example, Sun et al. [13] have measured the K_a values of the association of two inflammatory drugs (ibuprofen and flurbiprofen) to bovine and human serum albumins. Pèdron et al. [14] have determined the molar coupling ratio between abscisic-acid and bovine serum albumin. Other examples of ACE have been reported by Gomez and co-workers [15,16].

In the present report, we describe the possibility of studying the haptoglobin–hemoglobin complex formation by combined capillary zone electrophoresis and isoelectric focusing. Haptoglobin (Hp) is a serum glycoprotein which exhibits a strong affinity for hemoglobin (Hb). Hp appears to conserve and foster the recycling of heme iron by forming an essentially irreversible but non-covalent complex with Hb, when released from erythrocytes into the plasma by lysis. The complex is readily absorbed into the liver, where it is catabolized. Three major types or groups of Hp, now designed as Hp 1-1, Hp 2-1 and Hp 2-2 have been described. Hp 1-1 is a tetra-chain structure formulated as $(\alpha^1\beta)_2$, where α^1 has a M_r of 8900 and β of 40 000 Da; Hp 2-1 forms a linear polymeric series of $\alpha^2\beta$ (where α^2=17 300); this series can be represented as $(\alpha^1\beta)_2$ $(\alpha^2\beta)_n$, where n=0, 1, 2,... The formula for Hp 2-2 polymeric series is $(\alpha^2\beta)_m$, where m=3, 4, 5,... [17]. This extreme affinity occurs between Hp and the α-chain of the globin portions of Hb A, F, S or C; Hp does not bind methemoglobin, heme or unusual form of Hb in which the α-chain is missing. Hp thus acts to prevent loss of Hb to urine and to conserve iron. In acute episodes of intravascular hemolysis, the total circulating Hp pool has been shown to be capable of binding about 3 g of Hb [18]. An easy and reliable assay for measuring serum Hp level would thus be a welcome addition to present methods of analysis.

The paper is organized as follows: first titration curve analysis is performed in gel slabs in excess and defect of Hb, so as to evaluate the stoichiometry of the complex. Subsequently, in a capillary electrophoresis unit, Hb is immobilized by isoelectric focusing (IEF) in a pH 6–8 gradient. Hp is then electrophoretically migrated through the focused Hb zone and the Hp–Hb complex (which has a p*I* value well below the pH 6 lower bounds of the pH gradient) mobilized and quantified past the detector window (set at 416 nm, so as to detect the red Hb colour). Finally, the residual, excess free Hb focused into the capillary is mobilized electrophoretically and also quantified by passage through the detector window. This allows us to double check, directly and indirectly, the complex formation.

2. Materials and methods

2.1. Chemicals

Ampholine carrier ampholytes, pH 6–8, were from Pharmacia LKB (Uppsala, Sweden). Acrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED) and persulphate were from Bio-Rad Labs. (Hercules, USA). Short-chain liquid polyacrylamide was prepared and purified according to Gelfi et al. [19]. The novel monomer, N-acryloyl amino propanol (AAP), was synthesized and characterized according to Simò-Alfonso et al. [20,21]. Purified Hp 1-1 was a kind gift from Dr. D. Labie, Hop. Cochin, Paris, France. Purified human serum haptoglobin (a mixture

of 1-1, 2-1 and 2-2) was purchased from Sigma (St. Louis, MO, USA).

2.2. Biological samples

Adult red blood cells (RBC) were taken at the Ospedale Uboldo, Cernusco sul Naviglio (Milan, Italy). RBCs were washed three times with isotonic saline and lysed in 5% KCl. Then the hemolysate was centrifuged (ca. 18 000 *g*) for 12 min and the clear supernatant gassed for 1 min with carbon monoxide, so as to prevent auto-oxidation. Hemolysates had a concentration of ca. 2 m*M* in Hb. For capillary IEF, the Hb samples were diluted with carrier ampholytes (200 fold, corresponding to ca. 10 μ*M* Hb). Standards of pure Hb A were prepared by small-scale preparative immobilized pH gradients, in the pH 6.8–7.8 range, according to Righetti and Gelfi [22].

2.3. Capillary isoelectric focusing

Capillary IEF was performed on a Bio Focus 2000 (Bio-Rad) instrument, equipped with fused-silica capillaries of 75 μm I.D. and 24/19.4 cm total/effective length (Polymicro Technologies, Phoenix, AZ, USA). The capillary inner wall was deactivated by a poly(AAP) coating, as described [23]. Isoelectric Lys (50 m*M*, pH 9.7) and acetic acid (50 m*M*, pH 3.5) were used as catholyte and anolyte, respectively. The carrier ampholyte consisted of 5% Ampholine pH 6–8, added with 0.5% pH 3–10 range. Samples were injected already premixed to the focusing solution, containing all the ingredients for focusing. In most experiments, both the sample and the background electrolyte filling the capillary contained 3% short-chain liquid polyacrylamide, which ameliorates the separation probably by two mechanisms: by being adsorbed to potentially uncoated patches on the silica surface and by increasing the viscosity, which minimizes peak distortion during elution. Focusing was typically performed at 10 kV constant, with an initial current of 13 μA (3 μA at steady state) and a total focusing time of 600 s. For mobilizing the residual, focused Hb, un-complexed with Hp, 20 m*M* NaCl was added to the anodic compartment [24]. This was coupled to a syphoning effect, obtained by having a higher catholyte level (650 μl) and a lower anolyte level (450 μl). During mobilization, 10 kV (constant voltage) were applied. In all experiments, the capillary was thermostated at 15°C and the sample vial at 6°C. Hbs were detected at 416 nm. Every fourth run, the capillary was washed with 0.1% 3-[(cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS) in water for 120 s, followed by a rinse with distilled water for 120 s.

2.4. Gel slab isoelectric focusing

IEF with carrier ampholyte buffers was performed in 5% T, 4% C polyacrylamide gels[1], reswollen in 3% pH 3.5–10 Ampholine, 20% ethylene glycol and 10% glycerol. The anodic filter paper strip was soaked in 40 m*M* acetic acid and the cathodic one in 50 m*M* Tris free base. The gel was pre-focused for 1 h at 500 V, then pure Hb was applied anodically and forced to migrate first for 30 min at 500 V and then focused for 1 h at 1000 V. The Hp ligand was then applied at the cathode and focusing continued for 1 h at 500 V, 1 h at 1000 V and 1 h at 2000 V. The gel was then stained with Coomassie Brilliant Blue R-250 in presence of Cu^{2+} [25].

2.5. Electrophoretic titration curves

These curves are generated in square-size gel slabs (12.5×12.5 cm, 1 mm thick), supported by a Gel Bond PAG foil [26]. The slab contains a long trench in the middle, spanning the anode to cathode distance, for seeding the sample. The gel is made to contain 5% T, 4% C polyacrylamide, 2% Ampholine pH 3.5–10, 0.2% Ampholine pH 6–8, 5 m*M* Asp, 5 m*M* Glu and 5 m*M* Lys. Anodic and cathodic filter paper strips are impregnated as in Section 2.4. Running conditions: LKB Multiphor 2117 chamber, run at 10 W (600 V at equilibrium) with a constant wattage power supply, for 90 min at 4°C in the first dimension (IEF). After that, the anodic and cathodic filter paper strips are eliminated, the gel is turned 90°, the trench is filled with sample, new electrodic filter paper strips are placed (perpendicular to the first dimension run) and the second dimension run

[1] T=[g acrylamide+g *N,N′*-methylenebisacrylamide (Bis)]/100 ml solution; C=g Bis/% T.

(electrophoresis against a stationary pH gradient) is performed for 15 min at 600 V (constant voltage) at 4°C. The gel slab is then stained as described [25].

3. Results

3.1. Gel slab studies

Fig. 1A shows the titration curves of interacting Hb–Hp, run in an excess of Hp. The gel position where the sample crosses the application trench represents the zero-mobility plane of each macromolecule, i.e., its isoelectric point value (as marked by an arrow-head). Three curves can be seen, which by elution, denaturation in sodium dodecyl sulfate (SDS) and SDS–polyacrylamide gel electrophoresis (PAGE) could be identified as follows: curve 1 (to the left), with a p*I* of 5.5, represents the 1:1 molar complex Hp 1-1/Hb; curve 2 (middle, pI=5.0) is the complex between 1 mol of Hp and 1/2 mol of Hb (an αβ dimer); curve 3 (to the right) representing free, excess Hp (p*I* 4.5). The existence of the complexes throughout the wide pH gradient explored (pH 3.5–10) suggests that the Hp–Hb complex is not ionically bound, as it would otherwise split in an electric field. This is in agreement with the well-know extreme stability of this complex and with the fact that hydrophobic interactions with the Hb α-chains are most probably involved [18]. Conversely, Fig. 1B shows the electrophoretic titration curves of interacting Hb–Hp run in an excess of Hb: this time only two major curves can be distinguished: one (No. 1), with p*I* of 7.0, representing the free excess Hb, the other (No. 2), with a p*I* of 5.5, representing the fully saturated 1:1 molar complex Hb–Hp. These data clearly suggest that it should be possible to form a complex in an electric field and transport it electrophoretically outside the p*I* zone of either one of the interacting species.

That this could indeed be accomplished is shown in Fig. 2: free Hb was first focused in a gel slab, in a pH 4–8 gradient, to a steady-state (lane 3). Simultaneously, free Hp was focused in another control lane (No. 1), giving the p*I* positions of the two free species. After that, half-saturating amount of Hp were swept through the focused zone, by cathodic application. As shown in lane 4, 1/2 amount of free Hb remains at its original p*I* 7.0 zone, the remaining focuses as a number of band with intermediate p*I* values between free Hb and free Hp. When a slightly higher molar excess of Hp is run through the focused Hb zone (see track No. 2), essentially all Hb is swept away from its p*I* and the zones of the various

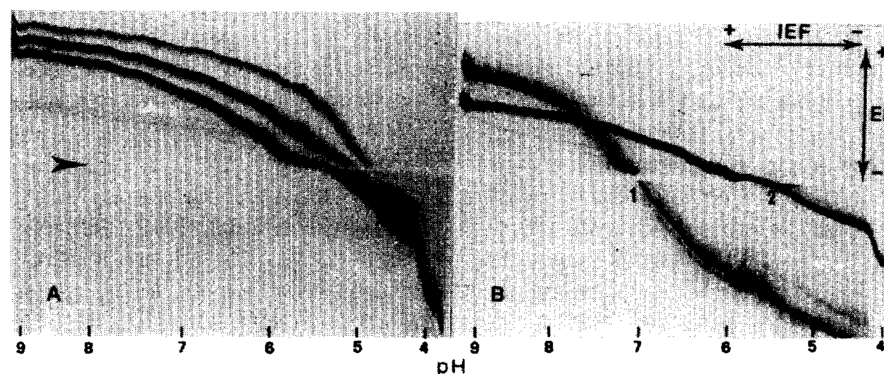

Fig. 1. Electrophoretic titration curves of interacting Hp–Hb. Gel: 5% T, 4% C polyacrylamide, reswollen in 2% Ampholine pH 3.5–10, 0.2% Ampholine pH 6–8, 5 m*M* Asp, 5 m*M* Glu and 5 m*M* Lys. First dimension run: 600 V for 90 min at 4°C. Second dimension run: 600 V for 15 min. (A) 2:1 molar excess of haptoglobin. Curve 1: 1:1 molar complex Hp–Hb; curve 2: 1 molar complex of Hp with an αβ dimer of Hb; curve 3: free excess Hp. (B) 1.5 molar excess of hemoglobin; curve 1: free excess Hb; curve 2: 1:1 molar complex Hp–Hb. Staining with Coomassie Brilliant Blue R-250 added with copper sulphate.

Fig. 2. Gel slab isoelectric focusing. A 5% T, 4% C polyacrylamide gel was reswollen in 3% pH 3.5–10 Ampholine, 20% ethylene glycol and 10% glycerol. Free Hb (lane 3) and free Hp (lane 1) were focused for 2 h at 2000 V. Lane 2: after focusing free Hb, a 1.2 molar excess of haptoglobin was applied at the cathode and focusing of the complex continued for 1 h at 500 V, 1 h at 1000 V and 1 h at 2000 V (note the complete removal of free Hb from its p*I* position). Lane 4: same as lane 2, except that 1/2 molar amount of Hp was added to the focused Hb zone (note in this last case that 1/2 free Hb remains in its p*I* zone). The cathode is uppermost. Staining with Coomassie Brilliant Blue R-250 added with copper sulphate.

Hp–Hb complexes are now seen to increase in intensity, accompanied by some amount of free, excess Hp focusing around the p*I* 4.5 position.

3.2. CZE studies

Fig. 3A shows the spectrum of Hp bands (pooled human sera, containing the 1-1, 2-1 and 2-2 forms) resolved by CZE, against a background electrolyte consisting of focused carrier ampholytes in the pH 6–8 range (control run, in the absence of Hb ligand). Since Hp has an average p*I* (4.5) well below the

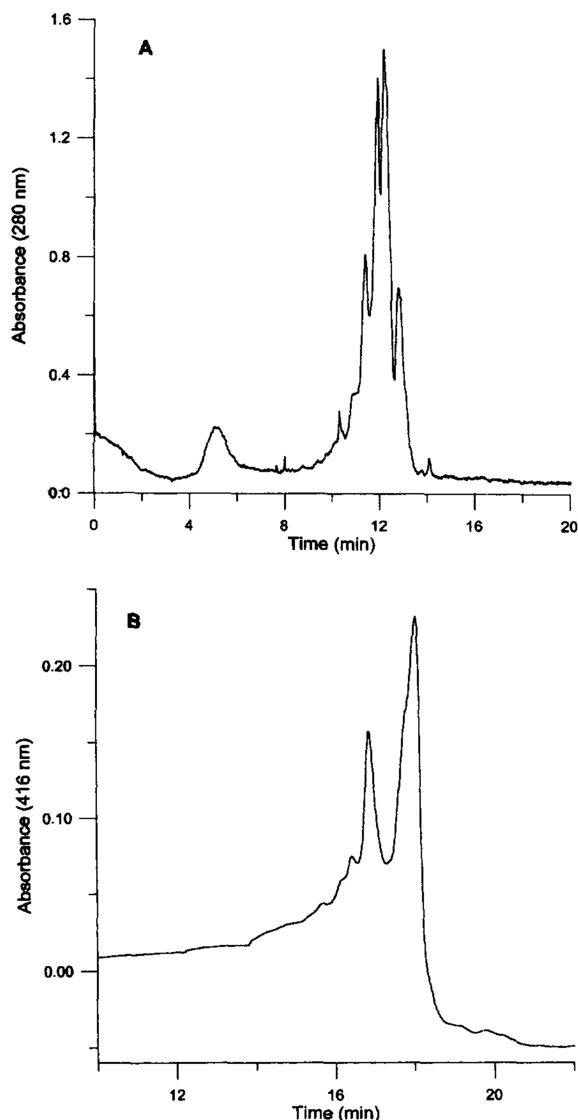

Fig. 3. CZE patterns of free Hp and free Hb. IEF was performed in 75 μm I.D. and 24/19.4 cm total/effective length capillaries, coated with poly(AAP). Isoelectric Lys (50 m*M*, pH 9.7) and acetic acid (50 m*M*, pH 3.5) were used as catholyte and anolyte, respectively. The carrier ampholyte consisted of 5% Ampholine pH 6–8, added with 0.5% pH 3–10 range. Focusing was performed for 600 s at 10 kV (13 μA initial, 3 μA at steady state). (A) Electrophoretic migration of free Hp through the pH 6–8 gradient, in the absence of any focused ligand Hb (detection at 280 nm). (B) Mobilization (at 10 kV, with a syphoning effect) of focused free Hb after addition of 20 m*M* NaCl to the anodic compartment (detection at 416 nm).

lower bounds of the pH gradient, it escapes such gradient and runs through the detector (set at 280 nm). Fig. 3B shows a control run of Hb, focused in the same pH 6–8 gradient. Hb had to be mobilized, of course, after the focusing step, and moved electrophoretic away from its p*I* zone past the detector (set at 416 nm).

Fig. 4 shows the Hb–Hp complex formation. First, an excess of Hb was focused in the above pH 6–8 gradient. After that, increasing amounts of Hp were loaded cathodically and transported, by zone electrophoresis, through the focused Hb zone. Any amount of bound Hb complexed to the Hp zone could be detected during this electrophoretic transport as it passed through the detector, set at 416 nm, so as to monitor only the complexed Hb. It is seen that, as progressively higher amounts of Hp are loaded at the cathode, the eluted complex increases in area. This can be better appreciated in the various curves, which represent individual elution profiles (after addition of a given amount of Hp to a prefocused Hb zone): it is seen that the absorbance of the peaks at the lowest Hp level (0.015, curve D) is only one fourth of the absorbance of the complex obtained by injecting a four times higher amount of Hp, thus able to complex a proportionally four times higher level of Hb (0.06, curve A).

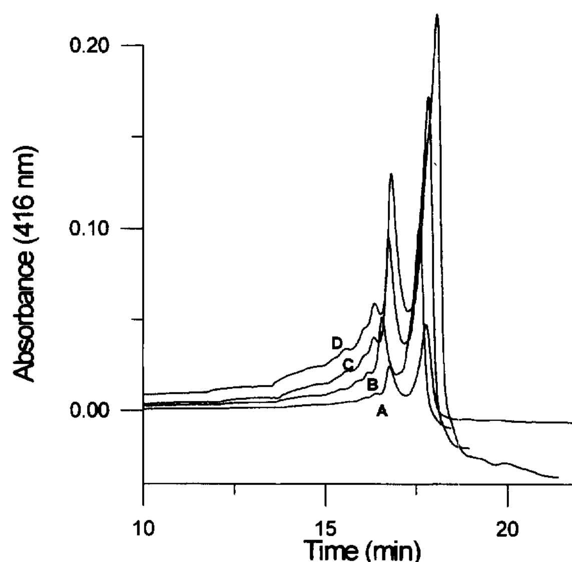

Fig. 5. IEF patterns of uncomplexed free Hb. After adding increasing amounts of haptoglobin to the focused Hb zone (see the relevant tracings in Fig. 4), the excess free Hb was mobilized by salt addition and forced to move past the detector at 416 nm. Note that, after adding equivalent molar amount of haptoglobin (curve A in Fig. 4, with highest absorbance) the remaining free Hb drops to a very small level (ca. 10%) (lowest absorbance curve A in this Fig.). Curves A–D of residual, free Hb, correspond to curves A–D in Fig. 4 of complexed Hb.

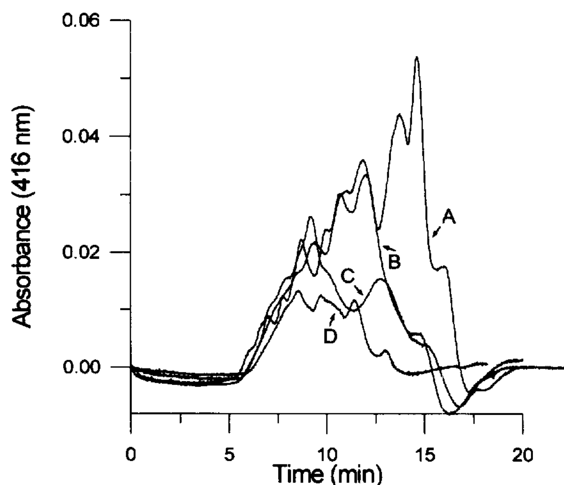

Fig. 4. CZE pattern of the Hp–Hb complex. After focusing free Hb (conditions as in Fig. 3) free haptoglobin was added to the cathodic capillary end and swept through the focused ligand zone (at 14 kV). Lowest curve (D) addition of 1/4 molar amount of Hp; uppermost curve (A) addition of a 1:1 molar amount of Hp; (B) addition of 1/3 molar amount of Hp; (C) addition of 1/2 molar amount of Hp to the Hb zone. Detection at 416 nm.

As an additional check for complex formation, the amount of free, excess Hb can be monitored as well, by mobilizing the pH gradient after the first electrophoretic passage of the complex. This is shown in Fig. 5: here the upper curve (D) represents the residual amount of focused Hb after injecting the lowest amount of Hp in Fig. 4 (see the corresponding curve D); the lowest curve (A) shows that indeed, after injecting a stoichiometric amount of Hp, the residual amount of focused Hb is extremely small.

These results can be summarized in Fig. 6, which shows the Hb–Hp complex formation as a function of added Hp to a known amount of focused Hb. The right ordinate gives the amount of residual free Hb after every Hp addition.

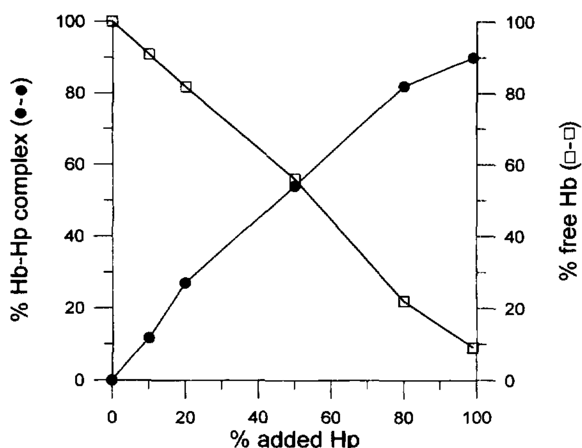

Fig. 6. Estimation of the amount of Hp–Hb complex formation as a function of addition of free Hp to a pre-focused Hb zone. The right ordinate gives the amount of free Hb remaining in the focused Hb zone after each Hp addition. The relevant values have been estimated from Figs. 4 and 5, respectively.

4. Discussion

The study of complex formation reported here represents a different way of exploring ligand binding, via a combination of isoelectric focusing and zone electrophoresis. One of the main problems in such studies is, in fact, on how to "immobilize" one of the two (or multiple) reacting species, so as to allow only the reaction product(s) to go past the detector window and be quantified. In general, such "immobilization" is achieved by chemical means, like covalently attaching an enzyme to a solid support. As an example, Guzman [27] has described a method for performing consecutive on-line protein digestion followed by derivatization of the resulting peptides with a fluorescent dye and mapping by CZE of the reaction products. Two tandem microreactors had to be fabricated to achieve this purpose. The first microreactor, or cartridge, contained *Staphylococcus aureus* V8 protease covalently bound to a porous glass support. This cartridge was then attached to a second microreactor containing fluorescein isothiocyanate bound to anti-fluorescein isothioacyanate monoclonal antibody that was in turn covalently linked to a porous glass support. The whole assembly was finally connected, through sleeve connectors, to the capillary column for on-line mapping of generated peptides. As seen from this description, such a methodology requires quite an extraordinary amount of work and skills. In our case, "immobilization" of one of the reactants is a temporal event, obtained by a continuous titration process in presence of a pH gradient, as customary in isoelectric focusing, a now well-established and user-friendly technique even in the capillary format. The only other requirement is that the complex formed upon passage of the other macromolecule through the focused zone has a p*I* value outside the bounds of the stationary pH gradient, a condition well satisfied by our Hb–Hp complex. In most cases, this can be simply achieved by selecting progressively narrow pH gradients, in case the variation of p*I* of the complex, as compared with the free species, is not so pronounced. Although routinely 2-pH unit wide carrier ampholytes are supplied, we have shown that it is quite easy to prepare narrow pH cuts of, e.g., only 0.5 pH unit span [28]. Our method offers an additional bonus: complex formation and measurement of the stoichiometry of the complex can be obtained by both, direct and indirect means. In fact, after passage of the complex, one can easily "mobilize" the excess of reactant still blocked in the focused zone (in our case Hb) and thus quantify additionally also the amount of unreacted species. Our job has also been facilitated by the fact that we can monitor the complex by setting the wavelength at 416 nm, were only Hb absorbs. By knowing the molar extinction coefficient of Hb, it is then easy to calculate the amount of Hb bound to the complex.

The present method could additionally have interesting applications in clinical chemistry (work in progress). As with many serum proteins, Hp level determination is used to monitor healthy vs. pathological conditions. In the case of Hp, its serial determination is used for detecting and monitoring acute-phase reactions and hemolytic states. In the first case, increments of plasma Hp are expected. On the contrary, low serum Hp levels are most frequently associated with conditions of increased intravascular hemolysis or hemoglobin turnover, such as occur in hemolytic anemias, transfusion reactions and malaria. Present-day assays for Hp in clinical chemistry comprise immunochemical methods and, to a lesser extent, nephelometry. It will be of interest

to see whether the present IEF–CZE method could offer a valid alternative.

Acknowledgments

P.G.R. is supported by grants from Agenzia Spaziale Italiana (ASI, Roma), by Progetto Strategico Tecnologie Chimiche Innovative, Comitato Chimica CNR (No. 96.05076.ST74) and by the Comitato Tecnologico (CNR, Roma).

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