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Purification of human hemoglobin valence intermediates by preparative immobilized pH gradients

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Summary

We compare three separation techniques for preparative purposes, i.e. ion-exchange chromatography on CM-cellulose, conventional isoelectric focusing in polyacrylamide gel slabs and immobilized pH gradients. The biological system used to test the three methods is a solution containing four hemoglobin (Hb) valence intermediates, i.e. metHb, oxyHb, $(\alpha^{+}\beta^{O_2})_2$ and $(\alpha^{O_2}\beta^{+})_2$. The ΔpI between the two valence intermediates is 0.04 pH units. Immobilized pH gradients give the best performance in terms of resolving power, total amount of protein which can be loaded and retention of biological activity by the protein (the latter assessed by determination of CO dissociation rates).

Key words: Immobilized pH gradients; Isoelectric focusing; Preparative electrophoresis; Hemoglobin valence intermediates

Introduction

The symmetrical valence intermediates of human hemoglobin (Hb), $(\alpha^{L}\beta^{+})_2$ and $(\alpha^{+}\beta^{L})_2$, where L represents a heme ligand such as oxygen, CO and NO, are of great interest when investigating the functional properties of hemoglobin. In fact, the other six theoretically possible valence intermediates can be obtained by

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hybridizing the symmetrical valence intermediates with HbL4 or metHb [1], and the fast reduction of oxidized hemes with sodium dithionite gives the intermediates of the reaction of hemoglobin with heme ligands [2]. In addition, the symmetrical valence intermediates are stable with respect to the ligand and dimer exchange, and can be obtained in pure form [1,2].

Several methods have been reported for obtaining the symmetrical intermediates, including chain reconstitution [3], ion-exchange chromatography [4] (IEC), isoelectric focusing [5] and chromatofocusing [6]. If we exclude the first method (a rather unphysiological one) and the last (used up to now for analytical purposes only), the remaining methods are not quite satisfactory, because the separation of the intermediates is very poor. In this work we test an alternative method, i.e. immobilized pH gradients (IPG) [7], for separation of valence intermediates of hemoglobin for preparative purposes. We show in this report that this method is preferable to the others both in terms of total amount tolerated per unit volume in the fractionation chamber and in terms of resolving power. In addition, the valence intermediates obtained by using IPGs are shown to be functionally similar to those obtained by traditional methods, implying that the IPG purification procedure does not alter the tertiary and quaternary structure nor the chemical and functional properties of the hemoglobin molecule.

Materials and Methods

Hemoglobin

Stock oxyHb is obtained from fresh heparinized blood by washing cells with isotonic saline, hemolyzing them with water and CCl_4 in the ratio 1 : 1 : 0.4 and filtering the solution through Sephadex G-25 equilibrated with 0.1 M KCl to remove organic phosphates. Methemoglobin (metHb) is obtained by reacting oxyHb with 20% molar excess potassium ferricyanide at pH 6.8 at room temperature for 60 min. Partially oxidized solutions are obtained by reacting oxyHb with half-saturating ferricyanide either at room temperature for 30 min (to favor oxidation of alpha-hemes) or at 0 °C for 1 min (to favor formation of beta-oxidized tetramers). Both solutions are then filtered through Sephadex G-25 in a 5 mM potassium phosphate buffer (pH 6.7 at room temperature).

Ion-exchange chromatography (IEC)

One of the methods used to obtain the symmetrical valence intermediates is ion-exchange chromatography [4]. Briefly, the partially oxidized solution (50 mg) is loaded to a 4 × 32 cm CM-52 (Whatman Ltd., England) column previously equilibrated with 5 mM potassium phosphate, 1 mM EDTA, pH 6.8 at 25 °C. An ionic strength gradient (5–15 mM potassium phosphate, 1 mM EDTA, pH 7.5 at 25 °C) is then applied at a flow rate of 60 ml/h. All operations are performed at 2 °C.

Conventional isoelectric focusing (IEF)

IEF on polyacrylamide gel plates (5%T) is performed in 0.5 mm thin slabs, containing 2% Ampholine pH 6–8 (LKB, Bromma, Sweden). The partially oxidized

solution is loaded in slots precast in the gel on the cathodic side (150 μg/track) and focused at 4° C for 4 h with 1500 V at equilibrium [8].

Immobilized pH gradients (IPG)

Preparative IPGs are run as previously described [9,10]. Briefly, an IPG pH 7.15–7.75 interval is made in a dilute polyacrylamide matrix (3%T). The gel is 11×12 cm in size, 2.0 mm thick. A total of 120 mg Hb is loaded in a trench pre-cast on the cathodic side, after pre-running the gel for 2 h. Running conditions: overnight at 10° C with 2000 V at equilibrium (5 W maximum power setting). The protein was recovered by cutting the IPG strips, embedding them in 1% agarose and eluting them electrophoretically into hydroxyapatite beads [9].

Isoelectric focusing at −25° C

The composition of the partially oxidized solution and of the purified valence intermediates is assayed by IEF at −25° C, a temperature at which exchange of dimers is virtually blocked during the time necessary for the separation [1]. Load: 50–200 μg protein per gel tube; focusing: 22–24 h at −25° C at 2000 V.

CO dissociation from the intermediates

The intermediates half-saturated with CO, $(\alpha^{+}\beta^{CO})2$ and $(\alpha^{CO}\beta^{+})2$ are obtained by flushing the valence intermediates (separated by any of the methods described above) with CO at 0° C for 15 min. The rate of CO dissociation is obtained by

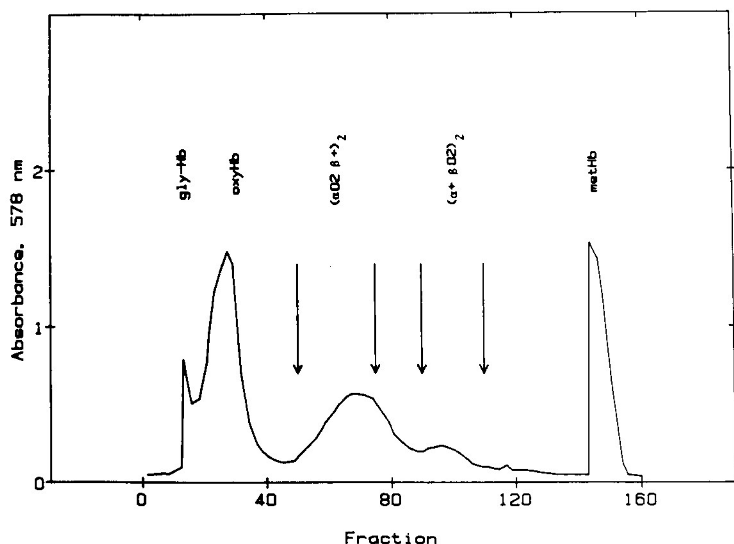

Fig. 1. Ion-exchange chromatography of the partially oxidized solution, obtained by oxidizing stock oxyHb for 1 min at 0° C. The arrows indicate the boundaries of the fractions that were collected for functional and purity studies. 50 mg Hb were loaded to a 4×32 cm CM-52 column and eluted with a 5–15 mM phosphate buffer gradient, pH 7.5, in 1 mM EDTA, at a 60 ml/h flow rate.

mixing anaerobically at 20 °C 0.4 mmol of the intermediate with 0.6 mmol of microperoxidase (Sigma, St. Louis, U.S.A.) in the presence of 70 mg/ml sodium dithionite. Both solutions are previously degassed and titrated to pH 7.0. The reaction is followed at 592 nm (wavelength at which the absorbance change is maximal) in a stopped flow apparatus equipped with a 2 cm path length cuvette (Dionex, Palo Alto, CA). Two thousand data points are recorded on a Nicolet digital oscilloscope and transmitted to a PC-350 personal computer (Digital, Maynard, MA, U.S.A.) for storage, reduction and computation.

Results

Fig. 1 shows the elution profile from a CM-Sephadex 52 of a 50 mg Hb solution enriched in the β-oxidized form: it can be seen that while the two extreme species (oxyHb and metHb) are well separated, the two valence intermediates severely

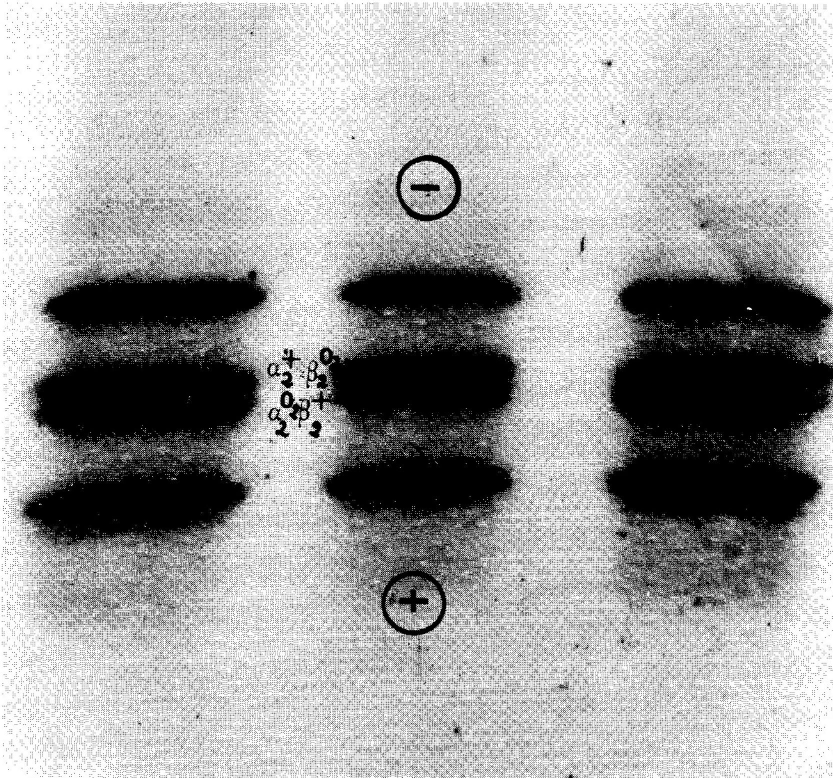

Fig. 2. Conventional IEF of a 50% oxidized Hb solution. 5%T polyacrylamide gel, 0.5 mm thick, containing 2% Ampholine pH 6–8. Focusing: 1500 V at equilibrium at 4 °C. The cathode is uppermost. Staining: Coomassie Brilliant Blue R-250.

intersect each other, so that at most only the outer shoulders of the two peaks contain pure species. Even Hb A1c (a glycosylated form of adult Hb) is not well separated from the parent oxyHb species. In both instances the ΔpI is ca. 0.04 of a pH unit: this confirms previous findings that in ion-exchange chromatography no better resolution than $\Delta pI = 0.2$ can be obtained [11].

Fig. 2 shows a similar separation by conventional IEF (in carrier ampholytes) of a 50% oxidized sample in an analytical gel, overloaded so as to mimick preparative conditions (150 μg sample/track, in a 0.5 mm thin gel would be a load comparable to the column size of Fig. 1). The two intermediate species can be seen stacked one next to the other, but with very little space in between. This is in agreement with Vesterberg and Svensson [12], who have given the resolution limit of conventional IEF as $\Delta pI = 0.02$ pH unit. Thus, while conventional IEF allows a resolution ca. one order of magnitude greater than ion-exchange chromatography at the analytical level ($\Delta pI = 0.02$ vs 0.2 with the latter), in the preparative scale this resolving power is reduced by a factor of two to three, depending on the total amount of protein applied.

Fig. 3 shows a preparative run of 50% oxidized Hb (α-oxidized form prevalent) in a preparative IPG gel, spanning a 0.6 pH unit interval: even though the gel was

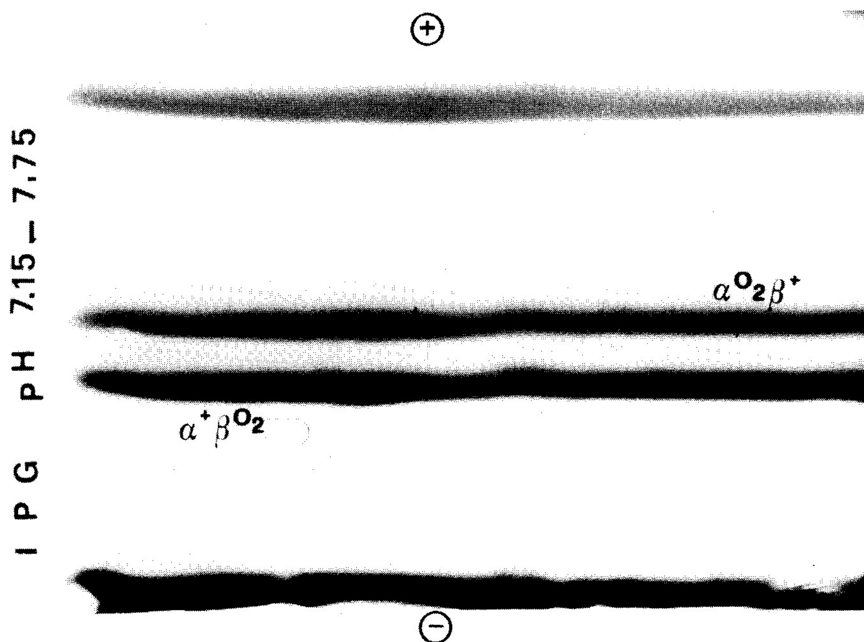

Fig. 3. Preparative IPG fractionation of partially oxidized Hb. Sample: 120 mg Hb oxidized for 30 min at room temperature. Gel: 3%T polyacrylamide gel, 12×11 cm, 2 mm thick with an Immobiline pH 7.15–7.75 interval. Run: overnight at 10 °C, 2000 V. The anode is uppermost. The ΔpI between the two intermediates has been estimated to be 0.04 pH units.

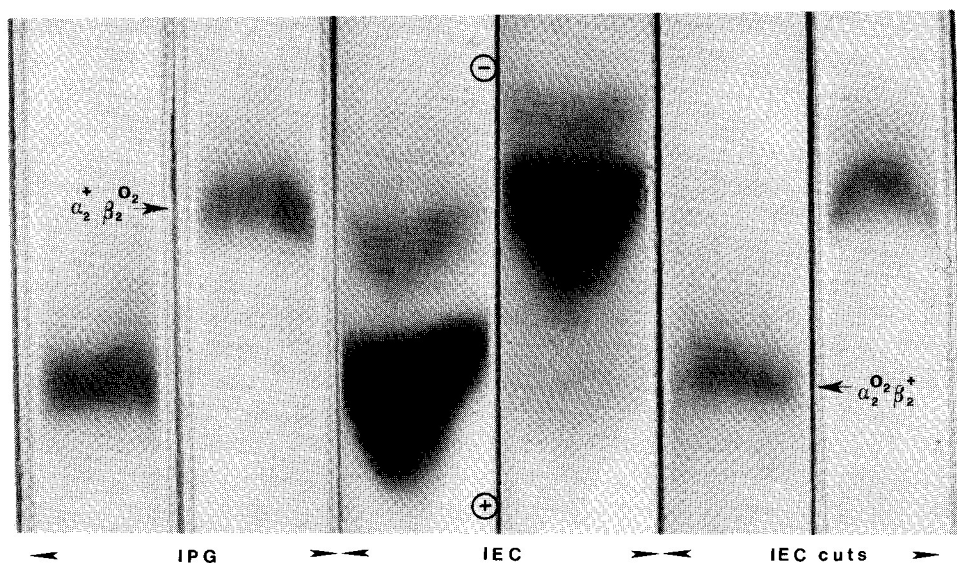

Fig. 4. Sub-zero IEF of the intermediates obtained by IPG, by IEC and by narrow elution cuts from IEC (from left to right). The left and the right tubes in each panel contain the β-oxidized vs the α-oxidized tetramers, respectively. Left set of two tubes: fractions recovered from an IPG run; central set: fractions eluted from an IEC column; right set: narrow cuts (outer shoulders) from an IEC eluate (see arrows in Fig. 1). Focusing: 5%T, 1 mm diam. gel tube containing 3% Ampholine pH 7–9. Run: 22–24 h at $-25\,^{\circ}$C and 2000 V. The cathode is uppermost.

barely 11×12 cm in size and 2.0 mm thick, and a total of 120 mg protein was applied, ample resolution was obtained between the two intermediate species (8 mm empty space inbetween). What is even more striking is that a preparative run maintains essentially the same resolution as an analytical run.

In order to check the purity of the valence intermediates purified by the various methods, we have taken the fractions eluted from the CM column and extracted from the IPG gel and analyzed them by conventional IEF at $-25\,^{\circ}$C (i.e. under conditions stabilizing the tetramer against the dimer exchange). As shown in Fig. 4 (set of two tubes to the left) the intermediates purified by the IPG technique appear as virtually 100% pure species, with no cross contamination, while the same tetramers purified in a CM column are cross contaminated (set of two tubes to the center). If, however, only part of the eluted peak (see arrows in Fig. 1) is collected, then the purity again approaches 100%, but with a protein loss of more than one half of each peak (set of two tubes to the right).

The last question to be addressed was whether the functional properties of the tetramers purified by IPGs are the same as with other purification protocols. We have thus taken the α- and β-oxidized intermediates purified by CM cellulose and from an IPG bed and measured the functionality by following the rate of release of carbon monoxide from the CO-saturated species: as shown in Fig. 5, the two types of valence intermediates purified with the two methods dissociate CO from either α

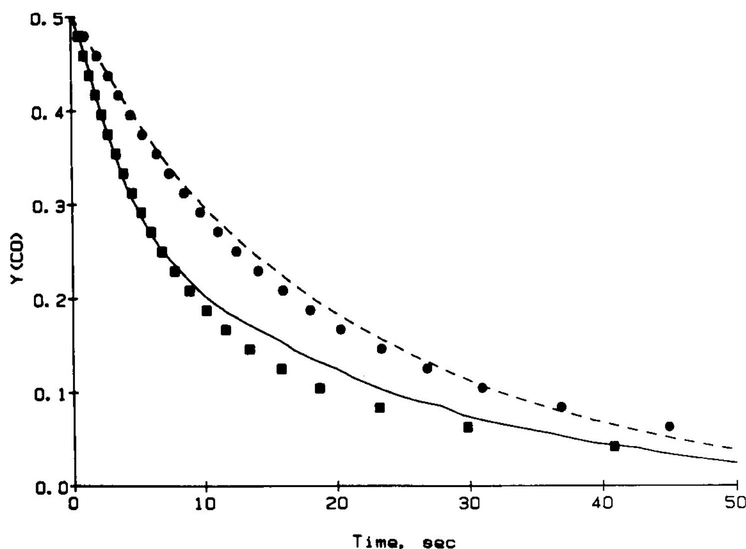

Fig. 5. CO dissociation curves from β-chains (●) and α-chains (■) obtained from intermediates purified either by IEC (●, ■) or by IPG (———, - - - - - -). For experimental details, see Materials and Methods. The ordinate represents the fractional saturation for CO (i.e. the ratio between CO-bound hemes and total hemes).

or β chains with almost coincident decay profiles, suggesting no apparent functional differences between the species obtained with totally different purification protocols.

Discussion

The resolving power and the total amount of protein tolerated per unit volume in the separation chamber are the two main criteria in the selection of a suitable protein separation technique [13]. The former can be expressed, in the case of charge dependent separations, like the methods here described, in terms of ΔpI units, i.e. the difference in isoelectric point between the protein of interest and the nearest resolved contaminant. The ΔpI values have been found to be 0.2, 0.02 and 0.001 for IEC, IEF and IPGs, respectively. In this work we show that IPG is not only the most advanced tool in protein separation technology, but can also be used successfully for preparative separations, while retaining the resolving power typical of analytical runs. It is in fact evident that the resolution of the valence intermediates of human hemoglobin by IPG is by far better than that of IEC and IEF. This is a very unique feature of the IPG technology which sets it apart from other preparative methodologies. It is in fact clear (see Figs. 1 and 2) that, even when the resolving power is acceptable at the analytical level (as in the case of conventional IEF) it is completely lost when attempting to scale the method up at the preparative level. In

the case of conventional IEF, moreover, preparative fractionations are often ruined by near-isoelectric precipitations of the major components, due to the extremely low ionic strength environment [14]. Conversely, in preparative IPGs, this is an extremely rare event, as the purified protein zone is isoelectric but not isoionic (i.e. it forms a salt with the surrounding Immobiline anions and cations grafted onto the polyacrylamide strings) [15]. The high ionic strength environment thus prevents near-isoelectric smears at high protein loads and possibly also stabilizes the tertiary and quaternary structures of the protein during the fractionation process.

The CO dissociation from the intermediates, used here as a probe for testing possible alteration of functional properties of the protein after the separation process, is virtually the same for the intermediates prepared by IEC and IPG, indicating that the tertiary and quaternary structures of the protein are not affected by the separation procedure. We conclude that IPG represents the most advanced protein fractionation technique now available, since it combines high loading capacity (up to 90 mg protein/ml gel), high resolution ($\Delta pI = 0.001$ pH unit) and full retention of the biological activity after the fractionation process.

Simplified description of the method and its application

The valence intermediates of human adult hemoglobin, oxidized either on the α- or β-chains, are purified in the preparative scale either by ion-exchange chromatography, conventional isoelectric focusing or immobilized pH gradients (IPG). The distance in isoelectric points between the two intermediates is barely 0.04 pH units. Of the three techniques used, only IPGs allow full resolution between the two species, high sample loads (in the hundreds of milligrams range) and complete retention of biological activity (as checked from the rate of release of CO from alpha and beta globin chains in the tetramer). IPGs should thus become the technique of choice when difficult separation problems are encountered (e.g. purification of valuable products from recombinant DNA technology).

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