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Separation of cobalt binding proteins by immobilized metal affinity chromatography

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Abstract

Cobalt binding proteins from mouse liver, which were expressed in response to $CoCl_2$ poisoning, were separated using gel permeation chromatography and then immobilised metal ion affinity chromatography (IMAC) with immobilized cobalt ions. Conditions used in IMAC-Co^{2+} were optimised. The fractions eluted with 60 mM imidazole were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Differences between the samples were also evaluated by a two-dimensional electrophoresis. Samples from the Co^{2+}-treated mice provided higher number of electrophoretic spots than those from the untreated mice. Relative molecular masses of these proteins are appropriately 37,000; 32,000 and 26,000 and their isoelectric points (p*I*) are 6.5–7.5.

Keywords: Immobilization; Cobalt-binding proteins

1. Introduction

Hypoxia, a principal consequence of ischemic or some other diseases, means an insufficient supply of oxygen for tissues or for a whole organism. The expression of some proteins, especially enzymes, growth and transcriptional factors, is enhanced in a cell during hypoxia [1]. They include erythropoietin, vascular endotheliar growth factor, hypoxia inducible factor-1, glucose transporters and some glycolytic enzymes. They are induced by presence of cobalt ions as well [2,3]. Cobalt ions mimic hypoxia in this respect, but the mechanism of this effect of cobalt is not clear yet [4–8].

IMAC represents an affinity separation technique that is based on specific interactions between molecules in solution and metal ions fixed to a solid support [9–11]. Metal ions are immobilized by means of chelating ligands. Since metal ions are electron acceptors, they interact with the electron-donor groups of biomolecules. In biomolecules, electrons can be donated by surface-exposed atoms of nitrogen, sulphur and oxygen, potentially phosphorus [12,13]. Just as biomolecules (e.g. proteins) are bound to metal chelate complex, weakly bound molecules of ligands (e.g. water) are displaced from the metal chelate complex.

IMAC-Co^{2+} relies on the formation of weak coordinate bonds between cobalt ions and basic groups on proteins, histidyl residues at the first place. Fig. 1 shows protein binding to a cobalt-chelated affinity support. Elution of the target protein is achieved mostly by protonation (lower pH) or by addition of a competing agent (e.g. imidazole) [14].

There are clear benefits of IMAC-Co^{2+}: stability of separated biomolecules, high loading, mild elution conditions, simple regeneration and low cost. The technique is therefore used for various purposes, including preparative and analytical purification of proteins and study of surface topography [15]. An efficient purification of recombinant proteins with engineered histidine affinity tags attached to the N- or C-terminus is another important application of this method [16–21].

Our goal was to separate proteins that are induced by the presence of Co^{2+} in the liver and exert an affinity to cobalt ions simultaneously. These proteins were separated by the immobilized metal affinity chromatography. As a transition metal to prepare an affinity column we used Co^{2+} ions.

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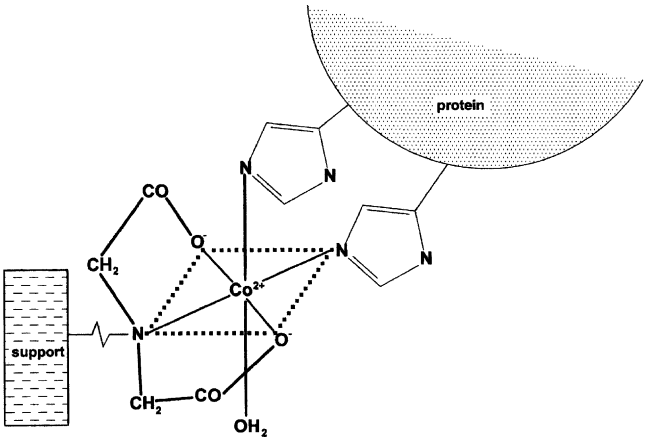

Fig. 1. Schematic illustration of the protein binding to a metal-chelated affinity support.

2. Experimental

2.1. Materials

Iminodiacetic acid Sepharose 6B (IDA-Sepharose), Sephadex G-25, acrylamide, dithiothreitol (DTT), ampholyte pH 3–10, cobalt chloride, imidazole, iodoacetamide and chromatographic columns—Sigma (Prague, Czech Republic).

Immobilized pH gradient (IPG) gel, power supply, Multiphor II and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS)-Ammersham Biosciences (Uppsala, Sweden).

Flow-through UV detector LCD 2082-Ecom (Prague, Czech Republic).

Ultrafiltration cell-Vivascience (Ústí nad Labem, Czech republic).

Programme ElfoMan 2.5 for densitometric evaluation of electrophoretic gels-ing. Semecký (Prague, Czech Republic).

2.2. Samples

C57Bl/6 mice (females, 8–12 weeks old, from Anlab, Prague, Czech Republic) were treated with $CoCl_2$ (10 mg/ml, 250 μl applied, 5 h before sacrificing). 1 g of liver tissue was homogenised in 2 ml of 0.1 M sodium phosphate buffer, pH 7.3, and centrifuged (15,000 × *g*, 60 min, 4 °C).

2.3. Chromatography

2.3.1. Gel permeation chromatography

Supernatant was prepurified by gel permeation chromatography on a column (50 mm × 10 mm i.d.) filled with Sephadex G-25. Proteins were eluted with the equilibration solution (0.01 M sodium phosphate, pH 6.2) at a flow rate 0.25 ml/min and 1-ml fractions were collected.

2.3.2. Metal ions removal

Samples were applied to a column (65 mm × 10 mm i.d.) filled with iminodiacetic acid Sepharose 6B after gel permeation chromatography. Before the proteins were applied, the column was washed with 0.2 M EDTA and then equilibrated with 0.1 M sodium phosphate buffer, pH 7.3. Proteins were eluted with the equilibration buffer (flow rate: 0.25 ml/min, 2-ml fractions). The absorbance at 280 nm was measured using a flow-through detector.

2.3.3. Co^{2+}-immobilized metal ion affinity chromatography

A column of IDA-Sepharose (65 mm × 10 mm i.d.) was charged with 0.2 M $CoCl_2$, washed with distilled water, equilibrated with 0.1 M sodium phosphate buffer, pH 7.3. The sample eluted from IDA-Sepharose was applied and after washing with the equilibration buffer, adsorbed proteins were eluted with 60 mM imidazole in the equilibration buffer (flow rate: 0.25 ml/min, 1-ml fractions). The absorbance at 280 nm was measured using a flow-through detector. The sample was concentrated in an ultrafiltration cell.

2.4. SDS-electrophoresis

Samples were analysed by the SDS–PAGE according to [22]. Following conditions were used: 12.8% T, 2.6% C running and 3.1% T, 2.6% C stacking gels; sample buffer: 0.063 M Tris–HCl, 2% (m/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.003% (m/v) bromphenol blue, pH 6.8; electrophoresis buffer: 0.025 M Tris–HCl, 0.192 M glycin, 0.1% (m/v) SDS, pH 8.3; 100 V; 2.5 h. Proteins were stained with Coomasie Brilliant Blue R-250 and electrophoretic bands were evaluated using the programme ElfoMan 2.5.

2.5. Two-dimensional electrophoresis [23,24]

Concentrated and dialyzed (5 mM sodium phosphate buffer, pH 7.3) samples (100 μg of proteins) were mixed with a rehydration solution (8 M urea, 3% (m/v) CHAPS, 0.2% (m/v) DTT, 2% (v/v) ampholyte pH 3–10 and 0.002% (m/v) bromphenol blue) to the final volume of 350 μl. IPG strips (pH 3–10, NL, 18 cm) were allowed to rehydrate at room temperature overnight. Then the IPG strips were placed onto a cooling plate of Multiphor II and the anodic electrode was positioned at the acidic end of the IPG strips. Isoelectric focusing (IEF) was run at 20 °C in four steps (350 V for 60 min, 500 V for 60 min, 1000 V for 60 min, 3500 V for 21 h), the current was always lower than 1 mA. Next, the IPG strips were equilibrated for 15 min (2% (m/v) SDS, 50 mM Tris–HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 0.002% (m/v) bromphenol blue) in two steps (10 ml of equilibration solution + 100 mg dithiothreitol; 10 ml of equilibration solution + 250 mg iodoacetamide). After the equilibration, the SDS–PAGE was performed (12.8% T, 2.6% C running and 3.1% T, 2.6% C stacking gels;

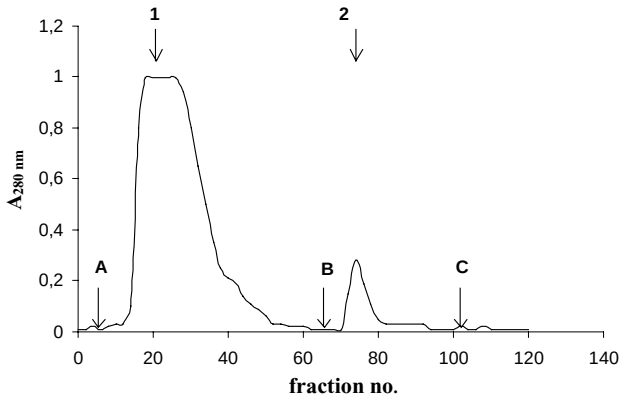

Fig. 2. Co^{2+}-immobilized metal ion affinity chromatography of prepurified samples. (A) Sample application followed by washing with 0.1 M sodium phosphate buffer, pH 7.3; (B) elution with 60 mM imidazole in the sodium phosphate buffer, (C) elution with 1 M NaCl in the sodium phosphate buffer. (1) Proteins unbound to the Co^{2+}-charged resin and (2) proteins eluted with 60 mM imidazole from the Co^{2+}-charged resin.

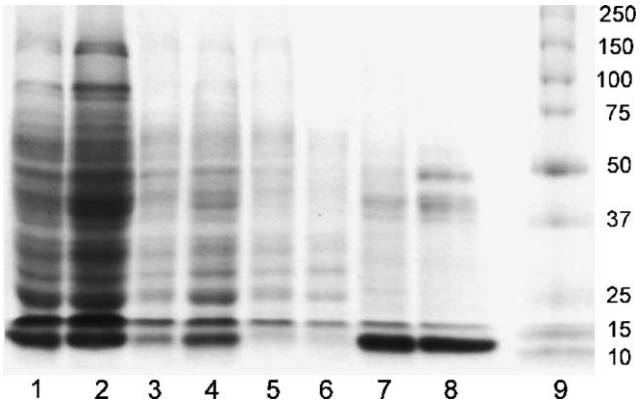

Fig. 3. Separation of cobalt binding proteins. SDS–PAGE (12.8% T, 2.6% C) stained with Coomassie Brilliant Blue. Comparison of the Co^{2+}-treated (+) and the control (−) samples. Lane 1 (+) and 2 (−)—desalted samples, lane 3 (+) and 4 (−)—samples after removal of metal ions, lane 5 (+) and 6 (−)—proteins unbound to the Co^{2+}-charged resin, lane 7 (+) and 8 (−)—proteins eluted with 60 mM imidazole from the Co^{2+}-charged resin, lane 9—relative molecular mass standard (10,000–250,000).

electrophoresis buffer: 0.025 M Tris–HCl, 0.192 M glycin, 0.1% (m/v) SDS, pH 8.3; 15 mA/gel for 60 min; 45mA/gel for 4–5 h). Samples, not treated by the IEF, were applied to the side of the gels next to the IPG strips. Protein spots were visualised by silver staining [23].

3. Results and discussion

3.1. Sample prepurification

Liver extract was prepurified by gel permeation chromatography on Sephadex G-25 to remove low-molecular mass compounds. Mouse liver treated with $CoCl_2$ can contain Co^{2+} ions bound to some proteins. To remove these ions desalted samples were applied to a column filled with iminodiacetic acid-Sepharose. Metal ions removal makes possible the use of chelating properties of IDA-Sepharose.

3.2. Co^{2+}-immobilized metal ion affinity chromatography

Affinity chromatography of prepurified samples on the IDA-Co^{2+} column is shown in Fig. 2. Proteins adsorbed to affinity carrier at pH 7.3 and ionic strength 0.1 M. Cobalt binding proteins represented only a minority portion of all

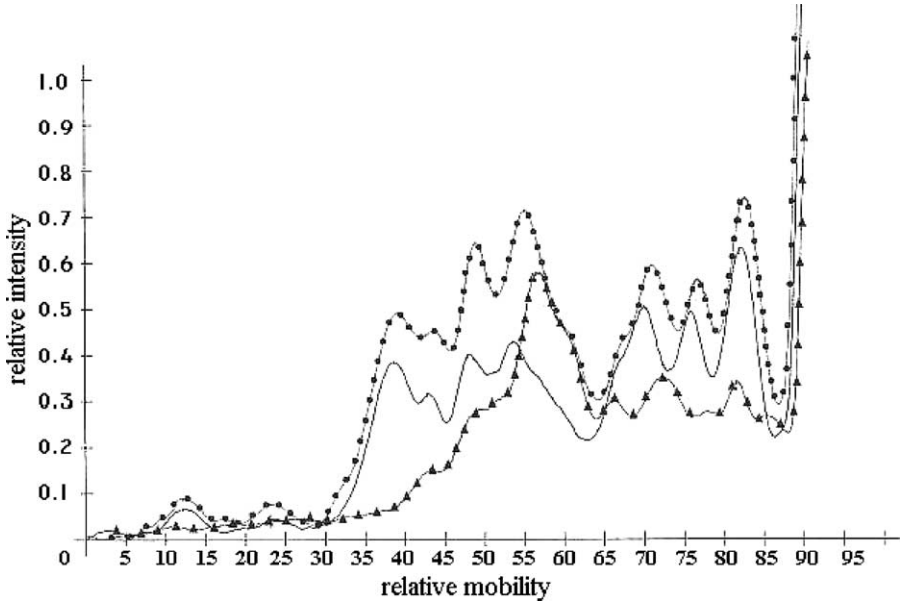

Fig. 4. Densitometric evaluation of SDS–PAGE. Particular steps of Co^{2+}-immobilized metal ion affinity chromatography: (●) all proteins applied to the column, (—) proteins unbound to the Co^{2+}-charged resin, (▲) proteins bound to the Co^{2+}-charged resin.

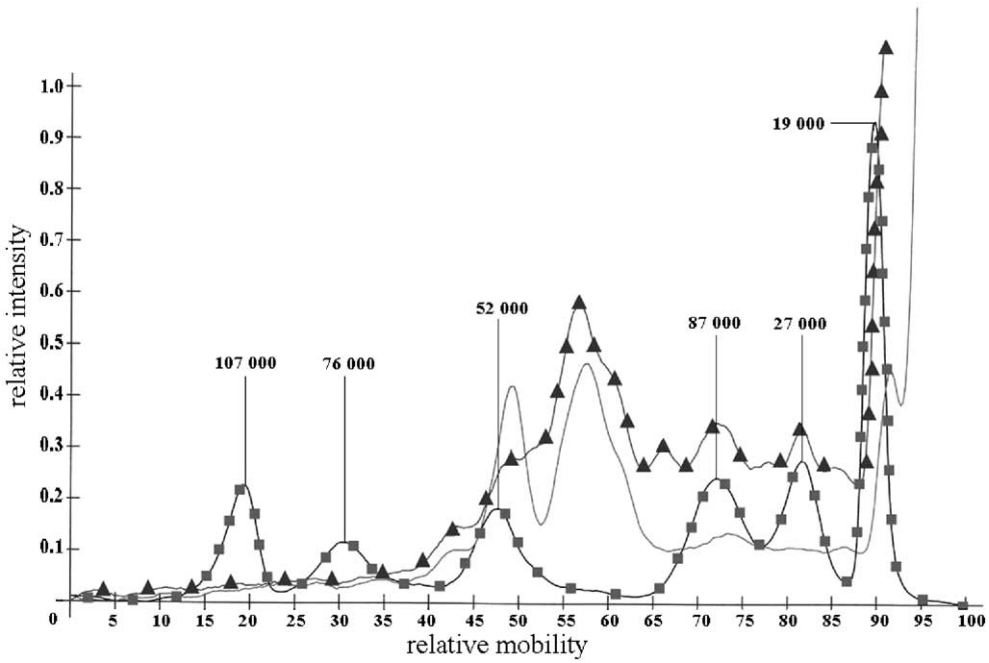

Fig. 5. Densitometric evaluation of SDS–PAGE. Comparison of the Co^{2+}-treated (▲) and the control (—) samples. Proteins bound to the Co^{2+}-charged resin were eluted with 60 mM imidazole in the sodium phosphate buffer. (■) Relative molecular mass standard (107,000; 76,000; 52,000; 37,000; 27,000 and 19,000).

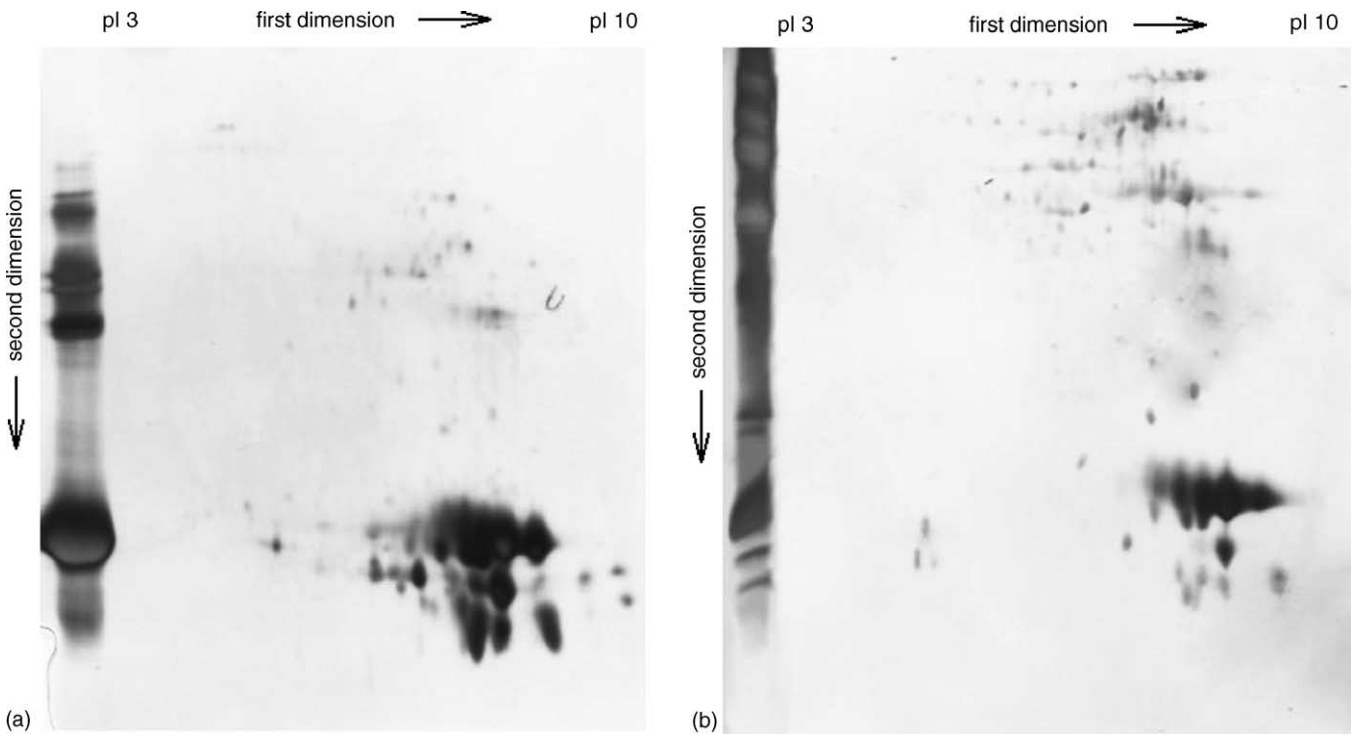

Fig. 6. Two-dimensional electrophoresis. Comparison of the control (a) and the Co^{2+}-treated (b) mouse liver proteins. Proteins bound to the Co^{2+}-charged resin were eluted with 60 mM imidazole in the sodium phosphate buffer. First dimension: IEF in an immobilized pH gradient pH 3–10 in a 18 cm long gel strip. Second dimension: SDS–PAGE in a 12.8% T, 2.6% C gel (sample, not treated by the IEF, was applied to the side of the gel). Silver stained.

proteins in the sample. Decreased pH or ionic strength was not sufficient for the elution of cobalt binding proteins and the elution buffer containing 60 mM imidazole was necessary. A gradient of imidazole (50 mM–1 M) was used to optimise elution conditions and the imidazole concentration of 60 mM was chosen.

3.3. SDS-electrophoresis and two-dimensional electrophoresis

Electrophoretic pattern of individual fractions is shown on Fig. 3. Samples from the Co^{2+}-treated and the control mice were compared. Numbers 1 and 2 denote crude extract, numbers 3 and 4 denote samples after removal of metal ions, 5 and 6 denote unbound proteins and 7 and 8 denote proteins bound to the IDA-Co^{2+} column. Very interesting is an area of relative molecular mass (Mr) 26,000–37,000 where additional bands of proteins were found in sample from the Co^{2+}-treated mice in comparison to the untreated one.

Electrophoretic bands were evaluated densitometricaly by the program ElfoMan 2.5. Fig. 4 shows proteins (from the Co^{2+}-treated mice) with mobilities 43, 49, 57, 66, 72, 81 and 91 units that were bound to the IDA-Co^{2+} carrier. Relative molecular masses of these proteins are approximately 61,000; 53,000; 45,000; 37,000; 32,000; 26,000 and 21,000.

Fig. 5 shows different adsorption of proteins from the Co^{2+}-treated and the control mice. The bands with mobilities 66, 72 and 81 units appear only in the sample treated with $CoCl_2$. Relative molecular masses of these proteins are approximately 37,000; 32,000 and 26,000.

The differences between the samples were also evaluated by two-dimensional electrophoresis (Fig. 6). Samples from the Co^{2+}-treated mice show higher number of electrophoretic spots in the area of Mr about 30,000 and p*I* 6.5–7.5.

4. Concluding remarks

Co^{2+}-immobilized metal ion affinity chromatography was used to separate cobalt binding proteins from mouse liver.

Purified fractions of proteins were analyzed by SDS–PAGE. Electrophoretic gels were evaluated densitometricaly by the program ElfoMan 2.5. The differences between the samples were also evaluated by the two-dimensional electrophoresis. The major difference, we found in the sample from the Co^{2+}-treated mice, was presence of some additional proteins in the area of Mr about 30,000.

Acknowledgements

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