Copper Chelate Affinity Chromatography of Human Fibroblast and Leucocyte Interferons

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SUMMARY

Human fibroblast and human leucocyte interferons display a strong affinity for the copper chelate of bis-carboxymethyl amino agarose, binding tenaciously over a wide pH range (7.4 to 4.0). Their binding is apparently irreversible on a sorbent saturated with copper (24.8 μmol of Cu²⁺/ml of column bed). However, both interferons can be partially recovered from sorbents of lower copper content, prepared by leaching the columns with sodium citrate at pH 9.0. The recovery of fibroblast interferon from a leached sorbent (5.8 μmol of Cu²⁺/ml of column bed) is about 30% and that of leucocyte interferon about 60%. Moreover, the strength of binding of leucocyte interferon can be modulated by leaching copper chelate-agarose with citrate of varying concentration.

Several proteins have an affinity for transition metal chelates, and this property has been utilized for their purification (Porath et al. 1975; Lönnnerdal et al. 1977; Lebreton, 1977). Recently, human fibroblast interferon was highly purified on Zn²⁺ chelate-agarose with a good (approx. 60%) recovery of activity (Edy et al. 1977). Syrian hamster interferon was chromatographed on Cu²⁺ chelate-agarose with excellent (about 90%) recovery of activity (Bollin & Sulkowski, 1978). Our preliminary attempts to chromatograph human fibroblast and human leucocyte interferons on Cu²⁺ chelate-agarose were unsuccessful due to their tenacious binding. A similar, apparently irreversible, binding of some other proteins may also occur. We now report that the binding strength of Cu²⁺ chelate-agarose can be modulated by varying its metal content. Both human interferons can be displaced from Cu²⁺ chelate-agarose columns which have been appropriately leached with citrate at pH 9.0.

Iminodiacetic Acid-/ /-Sepharose CL-4B (iminodiacetic acid coupled to Sepharose CL-4B matrix via a molecular arm) was purchased from Pierce Chemical Co. (Rockford, Ill., U.S.A.). Concanavalin (Con A)-Sepharose was obtained from Pharmacia Fine Chemicals (Piscataway, N.J., U.S.A.). Fluorescamine was purchased from Roche Diagnostics (Nutley, N.J.) and ethylene glycol from Baker Chemical Co. (Phillipsburg, N.J.). All other chemicals were of analytical grade. Human fibroblast interferon was prepared in human diploid fibroblasts, essentially according to Havell & Vilček (1972) and purified as previously described (Davey et al. 1976). Its specific activity was about 2.7 × 10⁶ reference units/mg protein. Human leucocyte interferon was prepared according to Pidot et al. (1972). Its specific activity was 5 to 6 × 10³ reference units/mg protein. Disc SDS-PAGE was performed as described by Summers et al. (1965). Interferon activity was recovered from gel slices as described elsewhere (Chadha et al. 1978). The columns of iminodiacetic acid-agarose were charged with copper as follows. Columns (9 × 50 to 100 mm) were equilibrated with 0.1 M-sodium acetate, 1 M-NaCl, pH 4.0 at 4°C. Volumes of 50 ml of CuSO₄, 5H₂O or ZnCl₂ at 5 mg/ml in the equilibrating buffer were applied and the columns were rinsed with 100 ml of the same buffer. The columns were charged with metal ions in the equilibrating buffer because the same buffer was used as the terminal eluant of all columns.
The copper content of Cu²⁺ chelate-agaroses was measured by displacing the Cu²⁺ ions with 2 mM-EDTA in 0.02 M-sodium phosphate, 1 M-NaCl, pH 7.4, and measuring the absorption at 675 nm. The calibration curve was prepared with CuSO₄·5H₂O in the same solvent. A solution of 3 mg of CuSO₄·5H₂O per ml gave a reading of 0.7 A₆₇₅ (1 cm light path) in a Gilford spectrophotometer 240. The copper content of a saturated sorbent prepared as described above, was approx. 24.8 µmol/ml of column bed. Less than saturated sorbents could be prepared by rapidly admixing the sorbent cake into a CuSO₄ solution of an appropriate concentration. However, partially charged sorbents, prepared in such a manner, gave rather poorly reproducible results. Partial saturation with Cu²⁺ ions was therefore achieved by leaching a column as follows. A column containing sorbent saturated with Cu²⁺ was sequentially washed with: (1) 0.02 M-sodium phosphate, 1 M-NaCl, pH 9.0, 100 ml; (2) 0.1 to 0.5 M-sodium citrate, 1 M-NaCl in 0.02 M-sodium phosphate, pH 9.0, 250 ml; (3) 0.02 M-sodium phosphate, 1 M-NaCl, pH 9.0, 100 ml and (4) 0.20 M-sodium phosphate, 1 M-NaCl, pH 7.4, 100 ml. All washings were performed at 4 °C. The copper contents of sorbents leached with citrate of different concentrations were as follows: 9.3 µmol of Cu²⁺/ml (0.1 M-citrate) and 5.8 µmol of Cu²⁺/ml (0.5 M-citrate).

All columns were equilibrated, charged and developed at 4 °C. Columns were eluted at a flow rate of approx. 30 ml/cm²/h using a peristaltic pump. When the interferon preparations were chromatographed, the eluate was divided by a stream splitting device. Larger fractions were used for protein determination; smaller fractions were collected into a 1% solution of bovine serum albumin in 0.02 M-sodium phosphate, pH 7.4 (0.15 M-NaCl), and used for interferon assays. Interferon activity was assayed on human fibroblasts by the colorimetric technique of Finter (1969) and titres are given in research reference units. Protein concentration was measured by fluorometric assay (Böhlen et al. 1973) with bovine serum albumin as a standard.

Human fibroblast interferon (HF-IF) preparations, obtained by chromatography on Con A-Sepharose, were dialysed and applied to Zn²⁺ chelate-agarose columns in 0.02 M-sodium phosphate, 1 M-NaCl, pH 7.4, or in 0.1 M-sodium acetate, 1 M-NaCl, pH 6.0. All HF-IF was retained and was subsequently eluted (70% recovery) from the columns at pH 5.0 (not illustrated). However, when an eluate from a Con A-Sepharose column containing 50% ethylene glycol was diluted twofold and applied on a Zn²⁺ chelate-agarose column, all HF-IF activity was found in the breakthrough fraction. Apparently, HF-IF does not bind to Zn²⁺ chelate-agarose in the presence of ethylene glycol. As dialysis can result in a partial loss of HF-IF activity, an attempt was made to chromatograph HF-IF preparations (containing 25% ethylene glycol) on copper chelate-agarose, which is a stronger sorbent than zinc chelate-agarose (Porath et al. 1975; Bollin & Sulkowski, 1978). All HF-IF was retained on a Cu²⁺ chelate-agarose column, regardless of the presence of ethylene glycol. However, the binding strength of copper chelate-agarose (saturated sorbent) was found to be excessive, as HF-IF could not be recovered even after a prolonged elution of the column at pH 4.0 (not illustrated).

Fig. 1 illustrates the chromatography of an HF-IF preparation, partially purified on Con A-Sepharose (containing 25% ethylene glycol), on a Cu²⁺ chelate-agarose column which was leached with 0.1 M-citrate, pH 9.0. The retention of HF-IF activity, despite a much lower copper content (5.8 µmol Cu²⁺/ml) was still complete, but only 30% of the activity was recovered by elution of the column at pH 4.0. There was also a concomitant purification of HF-IF to an extent which cannot reliably be estimated from such a small scale experiment but probably of at least tenfold. Chromatography on Cu²⁺ agarose can also eliminate traces of concanavalin A found as contaminant (Berthold et al. 1978) in Con A-Sepharose purified HF-IF (not illustrated).
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Fig. 1. Chromatography of human fibroblast interferon on copper chelate-agarose. An interferon preparation, purified on Con A-Sepharose, was dialysed against an equilibrating buffer (0.1 M-sodium acetate, 1 M-NaCl, pH 6.0, containing 25% ethylene glycol). A sample of 2 ml, containing \(1.2 \times 10^4\) units of activity and 0.45 mg of protein was applied to the column (9 x 100 mm) leached with 0.5 M-sodium citrate, pH 9.0, which was initially washed with the equilibrating buffer, and then with buffer at pH 4.0, as indicated by arrows. The recovery of interferon activity was about 30% and that of protein about 57%. ○—○, interferon; ■—■, protein.

Having shown that human fibroblast interferon can be chromatographed on Cu\(^{2+}\) chelate-agarose, we attempted to extend our observations to human leucocyte interferon (HL-IF). In a preliminary set of experiments, we observed that HL-IF did not bind to zinc chelate-agarose, thus confirming the earlier observations of Edy \textit{et al.} (1977). Copper chelate-agarose was then tested as a possible metal chelate sorbent. It was soon observed that HL-IF had a high affinity for this metal chelate. Fig. 2(a) illustrates the chromatography of HL-IF on a Cu\(^{2+}\) chelate-agarose column (saturated sorbent). HL-IF bound completely and only an insignificant amount, about 5%, of the applied activity could be recovered from the column with a falling pH gradient. By contrast, when the chromatography was performed on a leached sorbent (0.5 M-citrate, pH 9.0), as shown in Fig. 2(b), the recovery of HL-IF was approx. 60%. However, a major portion of HL-IF recovered from the column was found, together with other proteins in the breakthrough fraction. Moreover, the bound and then displaced portion of HL-IF was eluted from the column along with large amounts of other proteins, rendering this particular sorbent (leached with 0.5 M-citrate) valueless for the purification of HL-IF. Clearly, a sorbent with binding properties intermediate between fully charged and thoroughly leached could be more useful. To this end, we explored the sorptive properties of copper chelate-agarose leached with citrate of varying concentration. Copper chelate-agarose, prepared by leaching a fully charged column with 0.1 M-citrate, pH 9.0 (0.3 \(\mu\)mol/ml of column bed), proved to be a sorbent with the desired properties: there was a complete retention of HL-IF and its displacement, although partial (about 60% of the applied amount), was rather selective as shown in Fig. 2(c).

The experiments illustrated in Fig. 2 were performed in the presence of ethylene glycol (25%). When ethylene glycol was omitted from all solvents, the results were essentially the same (not illustrated).
Fig. 2. Modulation of the binding strength of copper chelate-agarose for human leucocyte interferon. (a) Copper chelate-agarose saturated with copper. An interferon preparation was dialysed against 0-02 M-sodium phosphate, 1 M-NaCl, pH 7-4, containing 25% ethylene glycol. A sample of 3 ml containing 40000 units of activity and 8-1 mg of protein was applied to a column (9 x 50 mm) equilibrated with the dialysis buffer. The column was initially washed with equilibrating buffer then with 0-1 M-sodium acetate, 1 M-NaCl, pH 6-0, 25% ethylene glycol. The column was then developed with a pH 6 to 4 gradient (E1). Finally, the column was developed with the terminal buffer (E2). The recovery of interferon was about 5% and that of protein about 10%. (b) Copper chelate-agarose leached with 0-5 M-sodium citrate, pH 9-0. A sample of 5 ml containing 70000 units of activity and 10-5 mg of protein was applied to the column. The development of the column was identical to that described in (a). The recovery of interferon was about 60% and that of protein about 55%. (c) Copper chelate-agarose leached with 0-1 M-sodium citrate, pH 9-0. A sample of 5 ml, containing 62 500 units of activity and 12 mg of protein was chromatographed as described in (a). The recovery of interferon was about 60% and that of protein about 48%. O—O, Interferon; ——, protein; ———, pH.
The partial retention of HL-IF on a column leached with 0.5 M-citrate (Fig. 2b) could reflect the molecular size heterogeneity of HL-IF (Stewart & Desmyter, 1975), or be due to other factors of trivial nature (column capacity, complexing with other proteins, etc.). In order to check whether the first alternative is true, we determined the mol. wt. of the HL-IF portion found in the breakthrough fraction and the HL-IF portion retained and subsequently displaced from a column (leached with 0.5 M-citrate, pH 9.0). SDS-PAGE analysis revealed that both molecular subcomponents of HL-IF, mol. wt. 21000 and 15000, were present not only in the breakthrough fraction but also in the fraction eluted by a falling pH gradient. The proportion of 21000 mol. wt. component to 15000 mol. wt. component was 3:7 in the breakthrough fraction and 6:4 in the retained and subsequently eluted fraction. Thus it seems that there is threefold enrichment rather than selective retention of the higher mol. wt. form of HL-IF on the Cu$^{2+}$ chelate-agarose column (leached with 0.5 M-citrate, pH 9.0).

Metal chelate affinity chromatography of proteins offers a novel purification principle which has already been successfully exploited with crude HF-IF (Edy et al. 1977). Our aim is to use this technique to follow the initial purification of HF-IF (Davey et al. 1976) and for the purification of HL-IF. Zinc chelate chromatography of HF-IF cannot be performed in the presence of ethylene glycol. As the dialysis of a Con A-Sepharose eluate, containing HF-IF and ethylene glycol, may result in some loss of activity, we tried to avoid it by employing copper chelate-agarose rather than zinc chelate-agarose. The data reported here show that copper chelate affinity chromatography can be performed with both HF-IF and HL-IF, although the recovery of the interferon activity, particularly in the case of HF-IF, is not completely satisfactory.

Excessive binding of both interferons to copper chelate-agarose, in comparison to that on zinc chelate-agarose, may be due to the formation of additional linkages; this would occur if some amino acid side chains on human interferons coordinate to Cu$^{2+}$ chelates but not to Zn$^{2+}$ chelates. Another explanation also has to be considered, namely that at a certain metal density of the sorbent, not all possible bonds are formed between percolating protein molecules and the sorbent. An increase in the metal density, above a certain critical level, would result in the formation of multiple coordination bonds. Whichever alternative is correct, and they are not mutually exclusive, a low metal density should result in a decrease in the number of attachment points and a fall in the strength of binding. The change in the sorptive properties of Cu$^{2+}$ chelate-agarose, as a result of its leaching with citrate, is congruent with the above interpretation. A further substantial contribution to the understanding of binding of both interferons to metal chelates could be made by preparing a series of sorbents with different degrees of substitution with iminodiacetate and evaluating their sorptive properties. The chelates of other transition metals (Co$^{3+}$, Ni$^{2+}$) should also be explored as potential ligands and such an investigation is under way.

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