Evidence of soluble proteins binding adriamycin by affinity chromatography

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Summary. The presence in various tissues of soluble proteins binding adriamycin is evidenced by affinity chromatography.

Affinity chromatography, which uses a specific ligand coupled to a solid matrix, has been extensively utilized in the purification of soluble proteins and in the purification of cells or cellular membrane fragments containing surface receptors. In a recent work[1], we suggested the application of this technique to study binding of drugs to plasma proteins. In particular, using specific adsorbents containing cardenolides, covalently bound to the matrix, we were able to isolate 3 proteins (an α_2-globulin, an haptoglobin and albumin) as carriers. Moreover, a relationship between aglycone polarity and type of protein acting as carrier was suggested. In order to extend this technique to study the binding of drugs to tissue proteins, we covalently bound adriamycin to agarose beads. Adriamycin, a powerful cancer chemotherapeutic agent, forms a complex with DNA, which is referred to as the basis of its action of mechanism. However, this drug shows a remarkable cardiotoxicity and a variable distribution in different tissues. Therefore, we looked for the presence of proteins in the soluble tissue fractions capable of binding the drug.

Materials and methods. In this study, adriamycin (kindly given by Farmitalia) was covalently bound to Sepharose 4B. By means of this adsorbent, the presence of soluble protein fractions specifically capable of binding adriamycin was shown in liver, kidney, thymus, spleen and heart. Drug linkage to Sepharose was accomplished in the following ways: 1. Adriamycin was oxidized with sodium periodate, as described by Hurwitz et al.[2], then allowed to react with adipic acid hydrazide-Sepharose in 0.1 M sodium acetate at pH 5 for 12–15 h, or allowed to react with 1-aminohexane-Sepharose in the same buffer for 10–12 h. 2. The amino group of the sugar unit (daunosamine) of adriamycin was bound to adipic acid azide-Sepharose at pH 8 in 0.1 M Tris-HCl for 0.5–1 h. By these techniques 4–8 µmoles of adriamycin per ml of packed Sepharose were coupled. The best yield of adriamycin bound to the resin was obtained with adipic acid hydrazide-Sepharose. Thus, we preferentially used this adsorbent, especially because it did not show any ion-exchange behaviour at pH 7.4. Tissues from calf were homogenized in a mechanical blender with 3 vol. of 10 mM Tris-HCl at pH 2.4 and then centrifuged at 12,000 × g. Supernatants were chromatographed on adriamycin-Sepharose. The resin was exhaustively washed with Tris buffer until the absorbance at 280 nm was negligible, and then washed again with the Tris buffer containing 0.5 NaCl until it approached to zero. The proteins specifically adsorbed on the resin were eluted with 5 mM adriamycin or alternatively with 8 M urea and 1 mM 2-mercaptoethanol. The supernatants were also chromatographed on a column containing adipic hydrazide-Sepharose to check the existence of nonspecific binding. In

Amount of proteins recovered from the examined tissues by the affinity column

Tissues	Proteins recovered (mg)
Liver	2.5
Kidney	2.2
Spleen	1.8
Lung	1.5
Thymus	1.4
Heart	0.8
Plasma	0.3

1. The 12,000 × g supernatants from the tissues examined were diluted to obtain a protein concentration of 10 mg/ml and dialyzed overnight against 10 mM Tris-HCl at pH 7.4. 2. 10 ml of supernatants were chromatographed on a 1 × 2 cm column of adriamycin. Sepharose protein content of eluates was determined by microbiuret method.

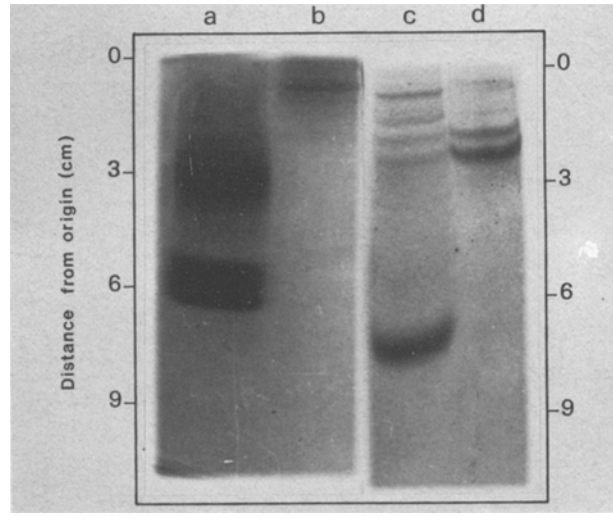

Fig. 1. SDS-urea polyacrylamide gel electrophoresis of heart and spleen soluble proteins eluted from the affinity column. Polyacrylamide gels (10%) were prepared according to Sender[5]. Samples of 0.1 mg of the proteins eluted from the affinity column were applied to each channel and run at 9 V/cm. Slabs were stained with Coomassie brilliant blue and destained the normal way. a and c crude extract of heart and spleen, respectively; b and d corresponding proteins eluted from the affinity column.

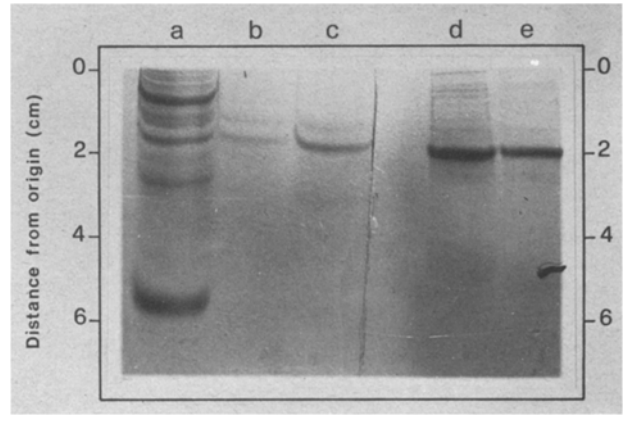

Fig. 2. SDS-urea polyacrylamide gel electrophoresis of lung and thymus soluble proteins eluted from the affinity column. Electrophoresis was carried out as described under figure 1. a and d crude extract of lung and thymus, respectively; b and c lung proteins and e thymus proteins, respectively, eluted from the affinity column.

this connection no protein was observed. Experiments to determine the binding capacity of the column were carried out. Increasing amounts of supernatant tissue proteins were chromatographed on the specific adsorbent column. Quantities of proteins recovered in the eluate were in agreement with a typical saturation curve. The experiments reported below were carried out in non-saturating conditions.

Results and discussion. In the table are reported the amounts of proteins of various tissues specifically eluted from the resin. It is remarkable that liver, kidney and spleen contain higher amount of proteins specifically adsorbed on the adriamycin-Sepharose. A small fraction of plasma proteins was retained by the resin. These data are in fairly good agreement with previous results of Kimura et al.[3], on blood levels and tissue distribution of adriamycin in rats, and of Lenaz et al.[4], who studied its distribution in mouse tissues.

Use of affinity chromatography to study the binding of drugs to proteins not only gives an indication of the presence of tissue proteins capable of binding, but also allows one to isolate and compare the proteins which bind drugs. The proteins specifically adsorbed by adriamycin and eluted were examined by electrophoresis on polyacrylamide gel in the presence of urea and SDS. (For experimental details see figures 1 and 2.) From this study, the following conclusions are drawn: 1. In the tissues examined various proteins capable of binding to adriamycin are present. 2. The pattern is characteristic for every tissue. In fact, differences are pointed out between spleen, thymus and heart, as shown in figure 1 and 2. 3. In spleen and heart eluates, some proteins are concentrated by the column; in particular, in the heart eluate the presence of a protein, not detectable in the supernatant, is shown. The presence of this protein, highly concentrated by the adriamycin-Sepharose column, indicates that in the heart 'in vivo', the binding of the drug to this protein could be one of the reasons for the particular cardiotoxicity of the drug. Studies are in progress to identify this protein. 4. Albumin appears as the only plasma protein, responsible of the slight binding to adriamycin.

1 A. Lucacchini, R. Barsacchi and C. Martinelli, J. Solid-Phase Biochem. *2*, 79 (1977).
2 E. Hurwitz, R. Levy, R. Maron, M. Wilchek, R. Arnon and M. Sela, Cancer Res. *35*, 1175 (1975).
3 K. Kimura, H. Fujita and Y. Saki, in: International Symposium on Adriamycin, p. 124, Milan, Sept. 1971. Springer, Berlin 1972.
4 L. Lenaz, G. Di Fronzo and R. Molinari, in: EORTC International Symposium on Adriamycin Review, p. 89. Brussels, May 1974. European Press, Ghent, 1975.
5 P.M. Sender, FEBS Lett. *17*, 106 (1971).

The amino acid composition of histidine ammonialyase from *Pseudomonas putida* NCIB 10807

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Summary. The amino acid composition of histidine ammonia-lyase from *Pseudomonas putida* NCIB 10807 suggests that this enzyme may be different from the *Pseudomonas testosteroni* NCIB 10808 histidine ammonia-lyase, whose amino acid composition is known[2].

Amino acid composition and sequence can provide information on regions of homology between related proteins. *Pseudomonas putida* NCIB 10807 belongs to the fluorescent group of Pseudomonas[3]. The aerobic *Pseudomonas* species constitute a large and fairly diverse array of bacteria. Histidine ammonia-lyase has been isolated and purified from *P. putida* NCIB 10807[4], but the amino acid composition was not determined. The histidine ammonia-lyase from *P. putida* NCIB 10807 does not form multiple polymers[4], while the histidine ammonia-lyase from *P. testosteroni* NCIB 10808, a nonfluorescent *Pseudomonad* forms multiple polymers[5]. It is quite likely that the ability of histidine ammonia-lyase to form multiple polymers or not may be dependent on the nature of its amino acid composition.

Materials and methods. P. putida NCIB 10807 was grown on a medium containing (g/l), L-histidine-HCl, 3; sodium succinate, 3; and KH_2PO_4, 5; with 0.02% (w/v) $MgSO_4$, and pH adjusted to 7.2 with 5 M NaOH as previously described[6]. Cells were harvested in the late exponential phase. Histidine ammonia-lyase was isolated from the cells of *P. putida* NCIB 10807 by the method described previously[6]. The procedure used for the purification of histidine ammonia-lyase was based on the method[7], and was similar to that employed by other workers for the purification of the enzyme[4,6]. Histidine ammonia-lyase activity was assayed by measuring spectrophotometrically the production of urocanate from L-histidine at 277 nm[7]. The pure histidine ammonia-lyase displayed a single homogeneous band, which had enzymic activity, on polyacrylamide gel electrophoresis as reported before[4].

Quantitative amino acid analysis. Histidine ammonia-lyase samples were hydrolyzed in 6 M-HCl before quantitative amino acid analysis. The protein solution (5 ml) was dialyzed extensively (24 h) at 0 °C against 5 mM-KH_2PO_4 buffer, pH 7.0 in double distilled water. Duplicate samples, containing 0.8–1.0 mg protein were placed in separate hydrolysis tubes and freeze dried. Aliquot, 2 ml of 6 M-HCl was added to each sample, and the tubes flushed with nitrogen gas, evacuated and sealed. The tubes were placed in an oven at 110 °C for 48 and 72 h respectively. This procedure ensures that all peptide bonds are broken and that the values obtained for amino acids partially destroyed during hydrolysis (serine and threonine) may be extrapolated to zero time. After hydrolysis, the tubes were cooled, opened and the HCl removed under vacuum; water was then added and the samples freeze-dried to ensure complete removal of acid.

This treatment causes the total destruction of tryptophan and may bring about partial oxidation of cysteine and methionine residues. To determine the accurate value for cysteine and cystine residues, a small sample (0.5 mg) was oxidized with performic acid before hydrolysis to convert cysteine and cystine residues to cysteic acid[8]. The solution of performic acid was prepared as described[9] and 0.15 ml of this solution was added to 1–2 mg protein dissolved in 0.15 ml of a solution of 0.87 ml 98% (w/v) formic acid and