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USE OF THIOL–DISULPHIDE INTERCHANGE REACTIONS IN PREPARATIVE WORK ON PLASMA PROTEINS

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CONTENTS

1. INTRODUCTION

Thiol–disulphide interchange reactions with solid-phase (agarose gel beads)-linked thiol compounds (homocysteine, glutathione)[1,2] have been proposed as aids for immobilizing and stabilizing enzymes with labile SH groups and for fractionating proteins and peptides[3]. These SH–SS interchange reactions have been based on the mixed disulphides of the solid-phase-linked thiol after activation with 2,2′-dithiopyridine. This mixed disulphide renders exchange reactions with thiol compounds possible even below pH 7[4].

Our interest in SH–SS interchange reactions arose from clinical observations. On electrophoretic analysis of a myeloma serum, we observed an atypical occurrence of small protein bands in the inter-α-zone. They could not be explained by any myeloma proteins or by any free light chains. The atypical bands were identified as SS-linked complexes between κ-chains and three plasma proteins: prealbumin, albumin and α_1-antitrypsin (α_1AT)[5]. κ-Chains are known to have a reactive terminal cysteine. The albumin and α_1AT had formed complexes in the highest and similar concentrations. Prealbumin had given a series of complexes. This relation was unexpected as albumin was considered to be the dominating plasma protein with a reactive thiol group whereas each prealbumin subunit had been claimed to have a very unreactive thiol[6] and no cysteine had at that time been found in α_1AT. These statements on the content of reactive thiols in the proteins and their formation of SS-linked complexes *in vivo* were contradictory and prompted further studies.

2. EXPERIMENTAL AND RESULTS

The occurrence of complexes between κ-chains and albumin and α_1AT was explored in a few hundred filed myeloma sera with immunochemical methods. The

results suggested the conclusion that secretion of increased amounts of free κ-chains into blood regularly caused the formation of disulphide-linked complexes with α_1AT and albumin in similar proportions. The content of complexes in plasma showed a significant correlation ($r = 0.66$) to the amount of κ-chains secreted as estimated from the amount of κ-chains excreted with the urine[7]. The complexes could be formed *in vitro* on mixing of plasma and κ-chains. The yield of complexes was much enhanced if the κ-chains were added after their cysteine had formed a mixed disulphide with Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoate) (Nbs_2^{2-}). The complex formation was most rapid at about pH 8[8]. This suggested that the complex-forming plasma proteins had reactive thiol groups with p*K* values above 7.5. Of the other major plasma proteins, subfractions of IgA and β-lipoproteins also gave κ-chain complexes. The tendency for complex formation at biological pH was found to be 10–20 times greater for α_1AT and prealbumin than for albumin. This was a new property of α_1AT, which was shown[9] to depend on a single reactive cysteine in α_1AT.

One of the tasks of our research group was to develop methods for the isolation of native α_1AT without changing its electrophoretic microheterogeneity. Such α_1AT was available only in small amounts but was requested in large amounts for biological and chemical studies. The charge heterogeneity would not be altered in purification steps utilizing SH–SS interchange reactions. Therefore, we coupled monomeric κ-chains with Nbs-blocked terminal cysteine to cyanogen bromide-activated Sepharose. On slow passage of plasma (2 ml/ml of κ-Sepharose per minute) through such columns, about 50% of the retained proteins at pH 8.1 was α_1AT. Proteins bound through SS bridges could be stripped off efficiently by reduction with 0.02 *M* β-mercaptoethanol and the κ-chain column was ready for a new interchange cycle after reactivation of the κ-thiol with Nbs_2[10].

The SS bridges between κ-chains and the various plasma proteins varied slightly in their sensitivity to reduction. Fractionated elution was achieved by reversing the reaction used for the linkage (1) through application of a pH 8 buffer with a molar ratio of Nbs to Nbs_2 of 2:1. The free SH group of uncoupled α_1AT molecules immediately reacted with Nbs_2 (reaction 2):

$$\alpha_1AT(s^-) + Nb(ss)\kappa \rightleftharpoons \alpha_1AT(ss)\kappa + Nbs^- \quad (1)$$

$$\alpha_1AT(s^-) + Nbs_2 \rightleftharpoons \alpha_1AT(ss)Nb + Nbs^- \quad (2)$$

SH–SS interchange chromatography has been combined with antibody affinity chromatography to eliminate remaining impurities[10]. This isolation procedure has been automated by utilizing a series of pumps and valves. The flow system is governed by a punch strip and an electronic unit with a time constant of 10 min. When starting with the κ-chain in the Nbs_2 derivatized form, the sequence of the various steps of the fractionation cycle are as follows:

I	Washing off excess Nbs_2 from the κ-chain column with Tris buffer, pH 8.
II	Sample application, pH 8.
III	Washing off plasma with Tris buffer, pH 8.
IV	Washing off plasma with phosphate buffer, pH 5.5.

V Washing off plasma with Tris buffer, pH 8.
VI Uncoupling of SS-linked proteins with Nbs/Nbs_2 in Tris buffer, pH 8.
VII Reduction of the κ-chains with β-mercaptoethanol in Tris buffer, pH 8.
VIII Washing with Tris buffer, pH 8.
IX Reactivation of the κ-chains with Nbs_2 in Tris buffer, pH 8.

The eluate obtained during step VI is transfered to a column with Sepharose-linked antibodies to absorb the undesired proteins, while α_1AT passes. The antibody column is reactivated by passage of 3.5 *M* ammonium thiocyanate solution, which dissociates the antigen–antibody complexes. The production capacity of an automated system using 200-ml κ-chain and antibody columns is about 250 mg/day of α_1AT (more than 95% pure) from plasma in a yield of about 40%. So far the column capacity has remained roughly unaltered after about 50 cycles.

The SH–SS interchange reactions can be used as an efficient step for enrichment of some other plasma proteins with reactive thiols, *e.g.*, prealbumin[11].

Nbs_2-activated λ-chains showed much slower interchange reactions with plasma proteins than activated κ-chains with COOH-terminal cysteine. λ-Chains have their reactive cysteinyl in a penultimate position with a COOH-terminal serine, similar to Sepharose-linked glutathione with glycine as the COOH-terminal. However, glutathione bound more albumin in relation to α_1AT from plasma than the κ-chains. Spacer-linked cysteine was tested as a κ-chain substitute. Its efficiency in linking thiol proteins was high but its interchange pattern with various plasma protein resembled glutathione more than κ-chains. It is therefore apparent that the microchemical surrounding of the thiol group of the protein has a great influence on the interchange reaction[12].

It was less surprising that Nbs_2-activated β,β-dimethylcysteine (penicillamine) was relatively inactive to thiol proteins because of probable steric hindrance through the bulky methyl groups adjacent to the SS bridge. The protein thiol interchange reactions of Nbs-cysteine methyl ester showed a selectivity in protein linkage similar to κ-chains. The selectivity of the SH–SS reactions of Nbs-cysteinyl is thus little influenced by the presence of a carboxyl adjacent to the thiol group.

The highest recoveries from the interchange reactions were obtained when the plasma proteins had been freshly reduced before their application on the columns and the Sepharose-coupled thiol compound had been "activated" to a labile mixed disulphide with Nbs_2 or similar compounds[12]. Part of the plasma protein thiols probably occur as reduction sensitive mixed disulphides in plasma. One problem with thiol chromatography is that freshly reduced proteins give the highest protein retention but any remaining reducing substance in the loading solution immediately inactivates the κ-chains by cleaving their mixed disulphides. Excessive reduction of the proteins of the sample may create difficulties by cleaving internal SS bridges of some proteins that normally have no reactive thiol. This will cause contamination with undesired proteins. Irregular yields in thiol chromatography are usually secondary to varying degrees of reduction of the thiols of the protein in the samples.

The Nbs_2-activated forms of κ-chains and of other simpler thiol compounds are efficient in forming SS bridges with albumin, α_1AT and prealbumin, but these plasma proteins never form mutual complexes through SS linkage or dimers, with the exception of albumin. Affinity chromatography on solid-phase-linked thiol com-

TABLE I

AMOUNT (mg) OF MAJOR PLASMA PROTEINS BOUND TO κ-CHAIN-, GLUTATHIONE- AND CYSTEINE-SEPHAROSE (50 ml) ON LOADING WITH 100 ml OF PLASMA

The SH–SS interchange reactions were performed at pH 8.1 with mildly reduced plasma and the matrix-bound thiol compounds in the form of mixed disulphides with 4,4′-dithiopyridine, 6,6′-dithiodinicotinic acid or 5,5′-dithiobis-(2-nitrobenzoate).

Protein	*4,4′-Dithiopyridine*			*6,6′-Dithiodinicotinic acid*			*5,5′-Dithiobis(2-nitrobenzoate)*		
	κ-Chain	*Glutathione*	*Cysteine*	*κ-Chain*	*Glutathione*	*Cysteine*	*κ-Chain*	*Glutathione*	*Cysteine*
Prealbumin	2	4	9	11	13	18	5	10	11
Albumin	2	10	58	14	121	160	5	23	68
α_1-Antitrypsin	56	48	71	63	55	62	65	58	59
IgA	5	19	6	30	23	12	6.8	20	8

pounds will be more or less efficient depending on the varying reactivity of the thiols of the proteins and of the interchanging mixed disulphide.

Nbs_2-activated thiols have been regularly used in a comparative study of the efficiency and selectivity of various Sepharose-linked compounds in thiol chromatography of plasma proteins[12]. Brocklehurst and Little[4] compared the reactions of some aromatic disulphides with papain and found differences in their influence on the SH–SS interchange reactions. Therefore, some commercially available, related aromatic disulphides were compared as "activators" in plasma fractionation experiments, namely 2,2′-dithiopyridine, 4,4′-dithiopyridine, 2,2′-dithio-5-nitropyridine, 6,6′-dithiodinicotinic acid and 5,5′-dithiobis-(2-nitrobenzoate). Three types of thiol columns, κ-chain-, cysteine- and glutathione-Sepharose, were used for each activator. In each experiment the three columns were simultaneously loaded from the same batch of plasma protein to avoid any effect of varying degree of reduction in the samples. Both the efficiency and the selectivity of the interchange reaction were found to vary. The extreme results were obtained with 6,6′-dithiodinicotinic acid and 2,2′-dithiopyridine, like 4,4′-dithiopyridine. The former gave a higher yield and a lower selectivity than the latter. The yields of four major plasma proteins using three matrix-bound thiol compounds in combination with three "activators" are given in Table 1. The selectivity pattern of each column was characteristic, like its relative efficiency, when using the same activator. The absolute amounts of protein obtained varied slightly in replicate experiments because a reproducible reduction of plasma proteins before

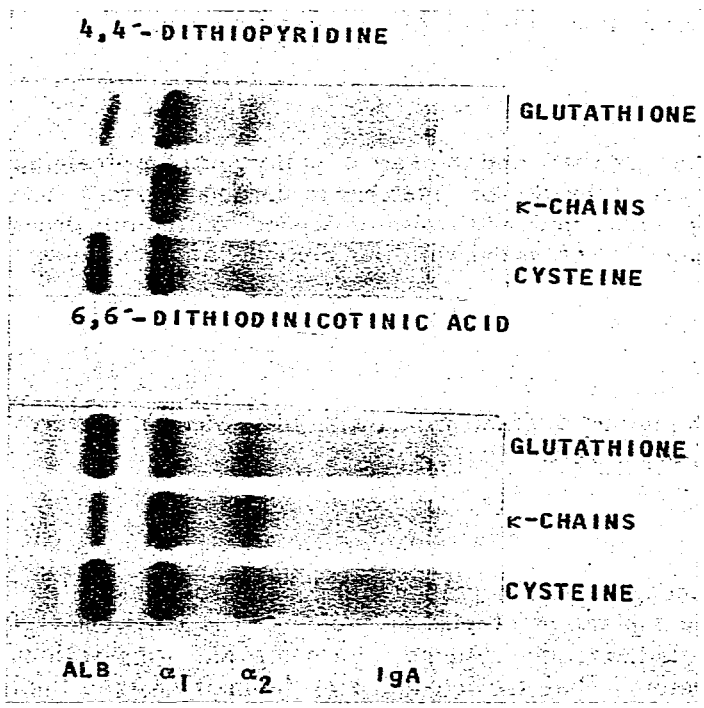

Fig. 1. Electrophoretic comparison of plasma proteins bound to glutathione-, κ-chain- and cysteine-Sepharose after activation with 4,4′-dithiopyridine and 6,6′-dithiodinicotinic acid.

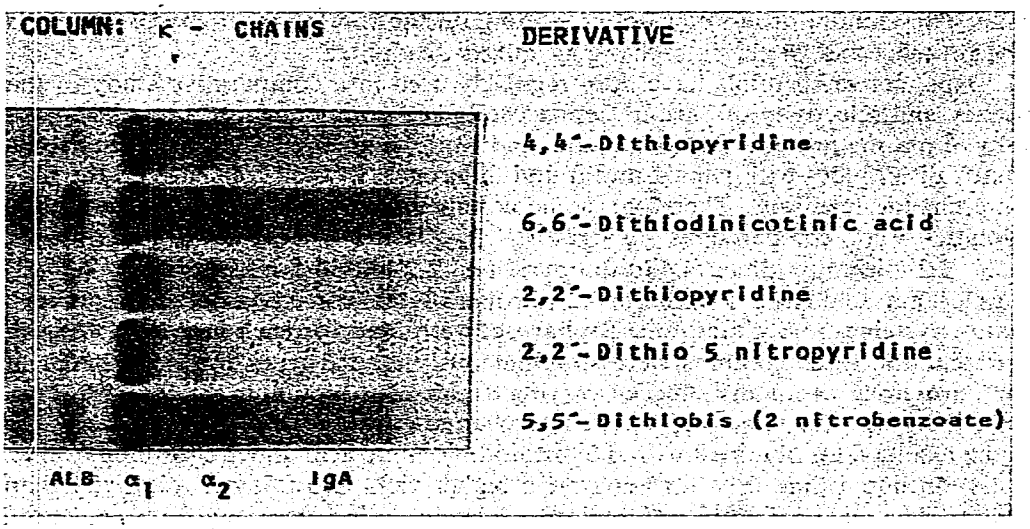

Fig. 2. Electrophoretic comparison of plasma proteins bound to κ-chain-Sepharose after activation with various aromatic thiol compounds.

loading the columns has not yet been developed. The qualitative difference (selectivity) in the SH–SS interchange reaction of plasma proteins is also apparent from Figs. 1 and 2, demonstrating the electrophoretic pattern of the proteins released from the three types of columns after elution with 0.02 *M* β-mercaptoethanol.

3. CONCLUSIONS

Both the activator and the matrix-bound thiol are of importance for obtaining maximal efficiency on fractionation by SH–SS interchange chromatography. The optimal combination of matrix-bound thiol and activator depends on the properties of the desired protein and cannot be predicted with our present knowledge of the interchange reactions.

4. ACKNOWLEDGEMENT

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5. SUMMARY

Thiol–disulphide interchange chromatography using Sepharose-linked thiol compounds is effective in the separation of plasma proteins. The efficiencies of Sepharose-linked κ-chains, glutathione and cysteine were compared using various aromatic disulphides as "activators". Both the activator and the matrix-bound thiol influence the efficiency and the selectivity of the fractionation of plasma proteins with SH–SS interchange chromatography.

REFERENCES

1 P. Cuatrecasas, *J. Biol. Chem.*, 3059 (1970) 295.
2 K. Brocklehurst, J. Carlsson, M. P. Y. Kierstan and E. M. Crook, *Biochem. J.*, 133 (1973) 573.
3 T. A. Egorov, A. Svenson, L. Rydén and J. Carlsson, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 3029.
4 K. Brocklehurst and G. Little, *Biochem. J.*, 133 (1973) 67.
5 C.-B. Laurell, *Immunochemistry*, 7 (1970) 461.
6 L. Rask, P. A. Peterson and S. Nilsson, *J. Biol. Chem.*, 246 (1971) 6087.
7 C.-B. Laurell and E. Thulin, *Immunochemistry*, 11 (1974) 703.
8 C.-B. Laurell and E. Thulin, *J. Exp. Med.*, 141 (1975) 453.
9 J.-O. Jeppsson and C.-B. Laurell, *Eur. J. Biochem.*, 82 (1978) in press.
10 C.-B. Laurell, J. A. Pierce, U. Persson and E. Thulin, *Eur. J. Biochem.*, 57 (1975) 107.
11 G. Fex, C.-B. Laurell and E. Thulin, *Eur. J. Biochem.*, 75 (1977) 181.
12 C.-B. Laurell and E. Thulin, *Anal. Biochem.*, 88 (1977) 30.