The Mechanism of Separation of Polythiolpeptides and Metallopolythiolpeptides by Covalent Affinity Chromatography

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Key Words

Column liquid chromatography
Affinity chromatography
Polythiolpeptides and metallopolythiolpeptides
Mechanism of separation

Summary

Covalent affinity chromatography with thiol-disulphide interchange (CAD-TDI) is a method for the separation of thiolproteins based on the interaction between disulphide bridges immobilised on a insoluble support and thiol groups in the protein undergoing separation. Mercury-thionein, cadmium-thionein and apothionein have been used as low molecular weight thiolproteins rich in cysteine residue. The proposed separation mechanism is more complex than those in the literature and can have anionic, cationic or a free radical character depending on the protein, the ligand and the conditions during separation.

Introduction

The terms "Covalent Chromatography" or "Covalent Affinity Chromatography" first appeared in the early 70 s [1–5]. Based on the mechanism of thiopyrydin metabolism in Ehrlich cancer cells [6, 9] and on the interaction of a low molecular weight thiols with the –SH groups in proteins [7–11], Brocklehurst [12] and Axen [13] proposed a method for the separation of proteins containing thiol groups by covalent affinity chromatography with thiol-disulphide interchange (CAC-CDI). The mechanism of separation is based on the exchange of sulphur-sulphur bridges between –SH groups in the proteins being separated and sulphur-sulphur bridges immobilised on the insoluble support [12–14] (Eqs. (1a)–(1c)):

$$/\!-\!\cdots\!-S\!-\!SX + P\!-\!SH \rightarrow /\!-\!\cdots\!-S\!-\!S\!-\!P + HS\!-\!X \quad (1a)$$

$$/\!-\!\cdots\!-S\!-\!S\!-\!P + R\!-\!SH \rightarrow /\!-\!\cdots\!-SH + P\!-\!SH + RSSR \quad (1b)$$

$$/\!-\!\cdots\!-SH + XS\!-\!SX \rightarrow /\!-\!\cdots\!-S\!-\!SX + X\!-\!SH \quad (1c)$$

where:

(1a) is the attachment of thiol proteins by disulphide bridges

(1b) is the specific elution of bound proteins with low MW thiols such as: cysteine, dithiotreitol, 2-mercaptoethanol, glutathione, etc.

(1c) is the regeneration of the support and re-formation of –S–S– bridges on the support.

This method was used mainly to for the separation of high MW proteins with a small number of –SH groups in the protein chain (Table I).

In the work described above a metallothioneine (MT) was used as the low MW polythiolprotein with a MW about 9–11,000 as determined by GPC and about 6,500 by amino acid analysis. The characteristic of MT is a very high cysteine residue representing about a third of the amino acid composition [27–29]. In native MTs the –SH groups bind with zinc and copper ions in metallothiolate clusters with different structures and with a variable affinity to the different metals [30, 31]. The clusters (A) and (B) are in two domains – α and – β which contain in their centers the metallothiolate clusters as cores around which polypeptide chain is wrapped in the form of two large helical toruses, probably in dumb-bell form [32]. The MT has a density of –SH groups greater than other high MW thiolproteins (Table I).

In the present paper the aim was try to determine the mechanism of separation of polythiolproteins and metallopolythiolproteins with high density of –SH groups in the peptide chain by covalent affinity chromatography with thiol-disulphide interchange.

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Table I The application of covalent affinity chromatography method for separation of thiolproteins with different numbers of thiol groups.

Protein	MW (g/mol)	Number of -SH groups (mol/mol)	Concentration of -SH groups (mol -SH/g protein $\times 10^{-5}$)	Ref.
Urease (EC 3.5.1.5)	483,000	44–49	9.11–10.14	[15–17]
Lipoamide oxireductase (EC 1.6.4.3)	110,000	10	9.09	[18–20]
Mercaptoalbumin	78,000	3	3.85	[14, 21, 22]
Ceruloplasmin (Ferroxidase EC 1.16.3.1)	134,000	15	11.19	[23–25]
Albumin	78,000	1	1.28	[21, 22]
Colagene α_1 (III)	95,000	2	2.10	[26]
Metallothionein	6,500	20	303.03	[27–29]

Table II Mean concentration of metallothionein's protein and metals in unbound and bound fractions with gel for covalent affinity chromatography.

Protein	Unbound fractions (A)					Bound fractions (B)				
	Protein**)		Metals		Concentration*)	Protein**)		Metals		Concentration*)
	mg	%	µg	%	moles metals/ /mol protein	mg	%	µg	%	moles metals/ /mol protein
Hg-Th	0.961	60.01	15.938	4.31	0.537	0.404	25.20	292.022	78.97	23.446
Cd-Th	1.547	64.33	127.655	71.93	0.911	0.466	22.13	27.185	17.38	3.571
Apo-Th	0.797	31.41	x	x	x	1.230	48.47	x	x	x

*) Initial concentration of metals:
-Hg-Th 7.482 moles Hg/mol protein
-Cd-Th 4.374 moles Cd/mol protein
4.281 moles Cd/mol protein
-Apo-Th 0.043 moles Cd/mol protein
**) protein determined by tannin method

Experimental

The experiments were carried out on female white rats (Wistar 180–220 g). Two groups of rats were exposed to $HgCl_2$ and $CdCl_2$ at a level of 1 mg of metal/kg body weight for 4 weeks in the case of mercury and 5 weeks for cadmium. The metals were administered subcutaneously in the form of physiological salt solutions three times per week. Homogenates of kidney tissue for mercury and liver tissue for cadmium were prepared. After initial dialysis and centrifugation the supernatants, were separated by GPC on Sephadex G-75 (Pharmacia-Uppsala) and fractions containing Hg-thionein (Hg-Th), Cd-thionein (Cd-Th) and apothionein (Apo-Th) were collected for subsequent experiments [33–35]

Sepharose-DTNB, {Sepharose-6-aminoheksylo-[5,5'-dithiobis-2(2-nitrobenzoate)]} was synthesised by a modified method of Lin and Foster [14] with Ellman reagent as the affinant with parameters similar to the commercial material (0.11–0.13 mmols DTNB/g Sepharose). Different elution regimes were used for the individual proteins as follows:

Hg-TH two step elution
Cd-Th two to four step elution
Apo-Th three and four step elution

The fractions of MTs obtained by covalent chromatography were rechromatographed by GPC on Sephadex G-75, K15/90 columns with 0,1 M ammonium-formate buffer, pH 8.0, ionic strength µ 0.1, and a flow rate of 10.0 ml/h. 5.0 ml fractions were collected and the MWs and homogenity of the fractions were obtained [42–45].

In the investigations the following determinations were carried out:

- protein concentration]36, 37],
- HS-groups concentration [38, 39],
- Hg by cold vapour AAS [40, 41],
- Cd by AAS using (Beckman AAS Model 1248 with a graphite Massman cuvette Model 1268 with a nonspecific correction by deuterium lamp Model CNA 1275,
- measuring absorbances at 220, 250, 270, 280 and 412 nm (UV/VIS Beckman Spectrophotometr Acta CIII).

Result and Discussion

In the process of MT separation by means of covalent affinity chromatography the yield of bound protein was low, ranging between 20 and 25 % for MT with blocked -SH groups (Hg-Th, Cd-Th). In the case of Apo-Th

Table IIIa Mean protein concentration and characterization of peaks after rechromatography on Sephadex G-75, unbound and bound fractions with the covalent affinity chromatography support.

Fractions		Mean protein concentration*)								
		Hg-Thionein			CdThionein			Apo-Thionein		
		MW	mg	%	MW	mg	%	MW	mg	%
Polyaggregate	A	> 70,000	0.422	27.61	> 70,000	0.232	30.24	> 70,000	0.093	9.57
	B	> 70,000	0.107	6.67	> 70,000	0.055	5.97	> 70,000	0.115	11.39
	Σ	x	0.549	34.28	x	0.287	36.21	x	0.208	20.96
Oligoaggregate	A	36,000	0.071	4.45	33–42,000	0.082	10.20	33,000	0.034	3.43
	B	36,000	0.044	2.75	33–42,000	0.037	3.61	33,000	0.070	6.99
	Σ	x	0.115	7.20	x	0.119	13.81	x	0.104	10.42
Monomer	A	11,000	0.228	14.27	9.8–16,000	0.135	16.61	8,400	0.136	14.10
	B	10,000	0.245	15.30	9.8–12,000	0.082	8.76	8,400	0.235	23.58
	Σ	x	0.473	29.57	x	0.217	25.37	x	0.371	37.68
Fraction	A	6,500	0.050	3.17	3,000	0.011	1.46	3,000	0.014	1.45
$MW < MW_{MT}$	B	4,000	0	0	3,000	0.010	1.02	3,000	0.020	1.99
	Σ	x	0.050	3.17	x	0.021	2.48	x	0.034	3.44

*) – proteins were determined by tannin method
A – unbound fractions
B – bound fractions
MW – molecular weight of protein fractions
MW_{MT} – molecular weight of native metallothioneins:
a/ Hg-Th MW = 11,000
b/ Cd-Th MW = 10,000
c/ Apo-Th MW = 9,600

Table IIIb Mean metals concentration and characterization of peaks after rechromatography on Sephadex G-75, unbound and bound fractions with the covalent affinity chromatography support.

Fractions		Means metals concentration							
		Hg-Thionein				Cd-Thionein			
		MW	μg	%	moles of metals/ /mol of protein	MW	μg	%	moles of metals/ /mol of protein
Polyaggregate	A	> 70,000	1.917	0.52	0.140	> 70,000	0.478	1.13	0.136
	B	> 70,000	21.376	5.78	6.479	> 70,000	1.982	2.47	2.041
	Σ	x	23.293	6.30	x	x	2.460	3.60	x
Oligoaggregate	A	36,000	1.065	0.29	0.484	33–42,000	0.362	0.85	0.436
	B	36,000	13.375	3.61	9.850	33–42,000	1.373	1.98	2.326
	Σ	x	14.440	3.90	x	x	1.735	2.83	x
Nonomer	A	11,000	1.756	0.47	0.249	9,8–16,000	1.856	4.34	1.443
	B	10,000	6.162	1.67	0.815	9,8–12,000	1.762	2.57	1.278
	Σ	x	7.918	2.14	x	x	3.618	6.91	x
Fraction	A	6,500	9.440	2.55	6.064	3,000	29.709	60.30	170.914
$MW < MW_{MT}$	B	4,000	232.041	62.72	u	3,000	5.452	7.94	40.293
	Σ	x	241.481	65.27	x	x	35.161	68.24	x

A – unbound fractions
B – bound fractions
u – undetermined
MW – molecular weight of protein fractions
MW_{MT} – molecular weight of native metallothioneins:
a/ Hg-Th MW = 11,00
b/ Cd-Th MW = 10,000
c/ Apo-Th MW = 9,600

containing free thiol groups the yield was about 50 % (Table II). Hg-Th bound to the support contained about 80 % of the added Hg and about 20 % Cd in the case of Cd-Th (Table II). These results are similar to those reported earlier [46–48]. Rechromatography of bound and unbound fractions (Table IIIa) demonstrated a lack of homogenity with the presence of three protein peaks with molecular masses approximate

Table IVa The yields of bond of the metallothionein protein depends on type of metals and their concentration, pH value and concentration of disulphide bridges on the chromatography support.

Protein	Parameters of support		The value pH of bonding	The characterization of bound metallothionein				
	Concentration of -SH groups(μM/g)	Content of -SH groups (μM) [C]		Content protein (μg)	Content protein (μM x 10-3) [Z]	Content of -SH (μM) [X]	Content of metals (μg)	Content of metals (μM) [Y]
Hg-Th	0.11	141.9	8.0	404.0	62.15	1.243	292.022	1.456
Cd-Th	0.13	92.3	9.0	408.0	62.69	1.254	22.288	0.198
Cd-Th	0.11	129.8	4.0	685.0	105.39	2.108	36.419	0.324
Cd-Th	0.11	129.8	8.0	314.0	48.31	0.966	25.307	0.225
Apo-Th	0.11	155.1	4.0	1,285.0	197.69	3.954	x	x
Apo-Th	0.11	155.1	8.0	1,175.0	180.77	3.615	x	x
Hg-HMWP	0.11	141.9	8.0	1,353.0	11.28	0.008	2.238	0.011

C - the total value of -SH groups content on covalent chromatography support (μM)
Z - the value of metallothionein protein bond with covalent chromatography support (μM)
X - the value of metallothionein's -SH groups bond with covalent chromatography support (μM)
Y - the value of metals in metallothionein's fractions bond with covalent chromatography support (μM)

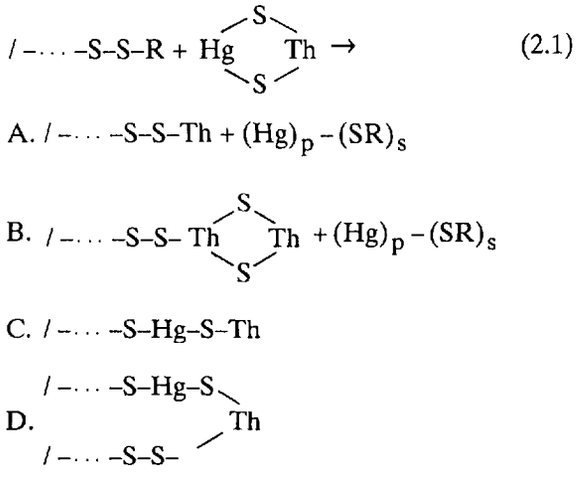
Table IVb The yields of metallothionein and the use of disulphide bridges from covalent affinity chromatography support.

Protein	The miscalculate of the effectivity of bonding for support unblocked -SH groups			The value of -SH unblocked with the metals [C'] (μM)	The miscalculate of the effectivity of bonding for support with blocked -SH groups		
	[C/Z] (μM -SH/ /μM protein)	[C/X] (μM -SH/ /μM -SH)	[C/Y] (μM -SH/ /μM metal)		[C'/Z] (μM -SH/ /μM protein)	[C'/X] (μM -SH/ /μM -SH)	[C'/Y] (μM -SH/ /μM metal)
Hg-Th	2,283.2	114,2	97,5	139,6	2,246.2	112.3	95.9
Cd-Th	1,472.3	73.6	446.2	92.0	1,467.5	73.4	464.5
Cd-Th	1,231.6	61.6	400.6	128.7	1,221.2	61.0	397.2
Cd-Th	2,686.8	134.4	576.9	128.3	2,655.8	132.8	570.2
Apo-Th	784.6	39.2	x	x	x	x	x
Apo-Th	858.0	42.9	x	x	x	x	
Hg-HMWP	88.6	17,737.5	12,900.0	141.8	88.6	17,725.0	12,890.9

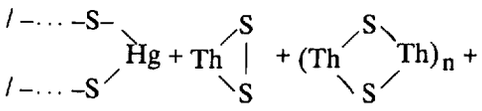
X - the value of metallothionein protein bond with covalent chromatography support (μM)
Z - the value of metallothionein 's -SH groups bond with covalent chromatography support (μM)
Y - the value of metals in metallothionein's fractions bond with covalent chromatography support (μM)
C - the total value of -SH groups content on covalent chromatography support (μM)
C' - the value of -SH groups of covalent chromatography support unblocked by metals and effectively bonding metallothionein protein (μM)

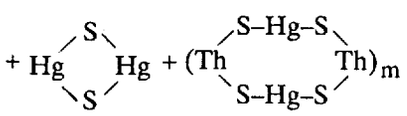
multiples of the mass of a single MT chain. Formation of poly- and oligoaggregates as well as tetra-, tri- and dimers of MT close to monomer fractions were observed (Table IIIa). These fractions showed a lower metal content than the starting MT (Table IIIb). Formation of low MW fractions with a high metal content and a high absorbance at 412 nm coming from TNB was also found [33, 34]. The concentration of -SH groups in the protein fractions obtained could not be measured by the Ellman method since this is specific to the reduced thiol groups. Table IVa shows the MT binding according to the type of protein and the pH used for binding, Table IVb shows the efficiency of -SH groups immobilised on the support under different conditions.

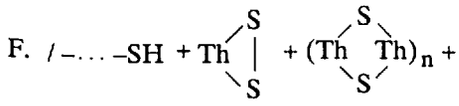
On the basis of the data obtained the following mechanism can be suggested (Eqs. (2), (3) and (4)):

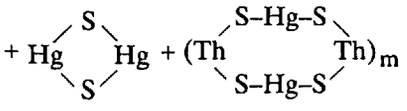


(2.1)

E. 



F. 

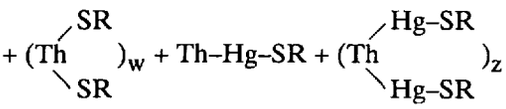


G. /–… –S–Hg–Hg–S–Th

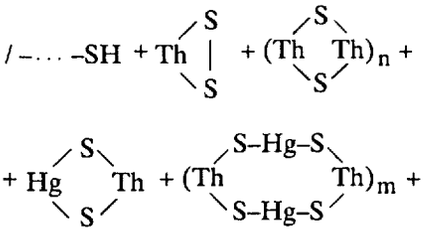
H. /–… –S–S–Th–SR

I. /–… –S–S–Th–Hg–SR

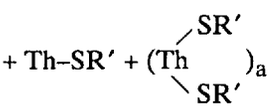
J. /–… –SH + $(Hg)_t$ – $(SR)_s$ + Th–SR +



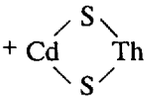
/–… –S–(…)-S–Th + R′–SH → (2.2)



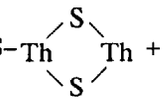
+ $(Hg)_p$ – $(SR)_s$ + $(Hg)_x$ – $(SR')_y$ +

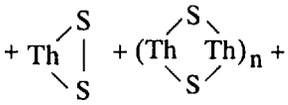


/–… –SH + RS–SR → /–… –S–S–R + R–SH (2.3)

/–… –S–S–R +  → (3.1)

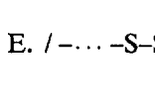
A. /–… –S–S–Th + $(Cd)_p$ – $(SR)_s$

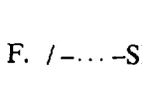
B. /–… –S–S–  + $(Cd)_p$ – $(SR)_s$

C. /–… –S–H + 

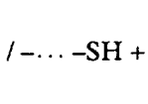
+ $(Cd)_p$ – $(SR)_s$

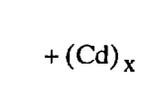
D. /–… –S–S–Th–SR

E. /–… –S–S– 

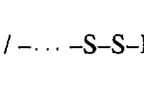
F. /–… –SH + Th–SR + 

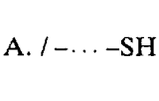
/–… –S–(…)–S–Th + R′–SH → (3.2)

/–… –SH +  + $(Cd)_p$ – $(SR)_s$ +

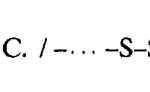
+ $(Cd)_x$ – $(SR')_y$ + Th–SR′ + 

/–… –SH + RS–SR → /–… –S–S–R + R–SH (3.3)

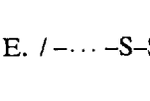
/–… –S–S–R + Th–SH +  → (4.1)

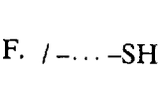
A. /–… –SH + 

B. /–… –S–S–Th + RS–SR

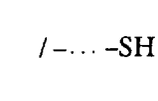
C. /–… –S–S–  + RS–SR

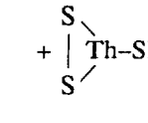
D. /–… –S–S–Th–SR

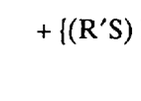
E. /–… –S–S– 

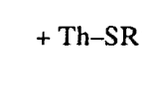
F. /–… –SH + Th–SR + 

/–… –S–(…)–S–Th + R′–SH (4.2)

/–… –SH + 

+ 

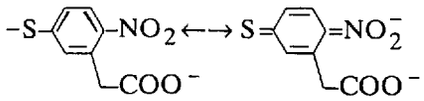
+ {(R′S) RS–  + (SR')}$_w$ +

+ Th–SR′ + 

/–… –SH + RS–SR → /–… –S–S–R + R–SH (4.3)

The appearance of high MW fractions (> 70,000) with intermediate MW material (M 33–42,000) is probably due to the creation of poly- and oligoaggregates composed of n > 7 and n = 3–4 protein molecules by MT molecules. Webb and Etienne [49] and other authors [50–52, 69–72, 115–117] have reported similar results during the separation of pure MT. Random aggregation through disulphide bridges also takes place during Cd-Th preparation [53]. Formation of disulphide bridges was also observed in the case of Hg-Th [54] and Zn-Th [55] where Zn(II) ion underwent dissociation from mercaptide bonds. Weser et al. [56–61] claimed uncontrolled anaerobic oxidation of –SH groups to disulphide bridges take place during the isolation of Cu-Th as evidenced by the appearance of high MW fractions and shifts in EPR and CD spectra. The oxidation of –SH groups results in intra- or intermolecular bridging in the presence of free metal ions such as Zn(II) [55]. A similar process takes place in the case of Cu-Th [58, 62] and the unblocked –SH groups undergo oxidation under neutral pH conditions [57]. According to Weser et al., –SH groups stable at pH 1.4 create disulphide bridges responsible for polymerisation and intramolecular bridging already at pH 6.6 [63]. These data conform the experimental results that the binding from the creation of disulphide bridges is lowest at pH 4.0 (Tables IVa and IVb). Other papers in the literature point out the exceptional predisposition of –SH groups to participate in Cu-Th and Zn-Th oxidation reactions [55, 57, 60, 61]. Hg-Th, Cd-Th and Apo-Th also have a similar predispositions [33–35]. The smaller tendency to oxidisation of the –SH groups in Apo-Th is probably due to the absence of metal ions in the reaction medium while the basic cause of the oxidation to disulphide bridges is the ability of –SH groups undergo autocatalysis [35, 74]. Intramolecular oxidation of the –SH groups appears in the form of an apparent loss of mass determined by GPC and changes in the protein spectra. The emerging monomers are more compact in structure and more densely packed with the hydrodynamic radius of the protein ball decreased. The structure of protein probably changed from a dumb bell form [32] into the single packed protein ball, that does not contain any atom of metal or it contains only single atoms that do not form metallothiolate clusters. Such packing, created as a result of intramolecular oxidation of –SH groups, is also suggested by earlier workers [28, 29, 64]. A different result was obtained by Philips [65] where a smaller radius occurs in the case of full MT saturation with Zn atoms caused by the coiling of the protein around the metal ions which are arranged individually and not in clusters. Another possibly but less likely explanation is that the reaction medium does not oxidise –SH groups so that the structure is not made denser and the size of the spherical protein ball is not decreased.

It is also stressed in the literature that, during the reaction of MT thiol groups, changes in the properties of metal-sulphur chromophores are observed [49, 53, 54, 56, 63, 66–68]. The loss of Cu during covalent affinite chromatography has been noted by Ryden and Deutsch [52]. The lose of metal content in proteins is due to the breakage of the mercaptide bonds in clusters and the formation of covalent bonds from TNB [52, 73–76]. A soft base such as thionitrobenzoic groups is better stabilised by resonance (Eq. (5)):



(5)

This results in the greater ability of metals (soft acids) to create bonds [77–79]. The tendency to form more durable complexes with a decrease of ligand mass (e.g. of polypeptide) has been noted previously [80–85] and the data in this paper are in full accord with this. The competetive character of a micromolecular thiol compound depend on its structure [86]. The fixity of the Hg–S– bond is then conditioned by such factors as concentration and location of –SH groups and the presence of an environment assisting the –SH groups and responsible for scattering charge [87, 88].

The bonding reaction, i.e. the exchange of disulphide bridges, should not take place at acidic pHs in which –SH groups are stable [63, 90] but in the present experiments Cd-Th and Apo-Th underwent bonding at pH 4.0. In this case oxidation and exchange of disulphide bridges catalysed by H_3O+ (H+) hydronium ions took place with the process of initiation, exchange and inhibition [91–93] (Eqs. (6a)–(6c)):

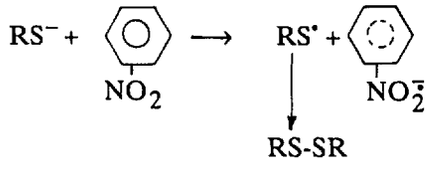
$$RSSR + H^+ \rightleftharpoons RS^+ + RSH \quad (6a)$$

$$RS^+ + R'SSR' \rightleftharpoons RSSR' + R'S^+ \quad (6b)$$

$$RS^+ + R'SH \rightleftharpoons RSSR' + H^+ \quad (6c)$$

These reactions should not proceed spontaneously in the pH range 1–6 [94, 95] but even a small addition of –SH groups is sufficient to start the reactions. This may be due to –SH and –S–S– groups bound to the support or MT itself may have strong autocatalytic properties [8].

Thiolate groups can also be oxidised in the presence of nitrobenzene [118] or other good electron transfer agents [119] which explains well enough the mechanism of oxidation and exchange in the presence of DTNB (Eq. (6d)):



(6d)

Oxidation of –SH groups also proceeds as a result of catalysis by the products of the reaction itself [136, 137]. Even the presence of –SH groups on the chromatographic support does not prevent the effects of oxidation as pointed out by Jellum [138].

Another factor which can starts the oxidation reaction of the thiolate groups and exchange of disulphide bridges in an aqueous medium is UV radiation [94, 95] (Eqs. (7a)–(7d)):

$$RSSR \overset{h\nu}{\rightleftharpoons} RS^{\circ} \overset{H_2O}{\rightleftharpoons} RSH + {}^{\circ}OH \rightleftharpoons RS^- + H_2O \quad (7a)$$

$$R'SSR' + RS^{\circ} \overset{h\nu}{\rightleftharpoons} R'SSR + R'S^{\circ} \quad (7b)$$

$$RSSR + R'S^{\circ} \rightleftharpoons R'SSR + RS^{\circ} \quad (7c)$$

$$RS^{\circ} + R'S^{\circ} \rightleftharpoons RSSR' \quad (7d)$$

An alternative mechanism has been described by Benesh [92] (Eqs. (8a) and (8b)):

$$RS^- \longrightarrow RS^{\circ} + \bar{e} \quad (8a)$$

$$2\,RS^{\circ} \longrightarrow RS\text{–}SR \quad (8b)$$

In this type of initiation the nitrobenzene groups in DTNB play an important part [118]. At low pH the speed of the reaction is limited by the speed of the first step. At sufficiently high concentration on RS^- ions the efficiency of the creation of disulphide bonds depends on the constant speed of reaction which probably does take place in the separations carried out.

In inactive or alkaline media at a pH of 8–9 the formation of disulphide bridges shows an anionic character [96] (Eqs. (9a)–(9c)):

$$R'\text{–}SH \overset{OH^-}{\longrightarrow} R'S^- + H^+ \quad (9a)$$

$$RSSR + R'S^- \longrightarrow RSSR' + RS^- \quad (9b)$$

$$RS^- + R'\text{–}SH \longrightarrow R\text{–}SH + R'S^- \quad (9c)$$

Thus the mechanism can be anionic, cationic or free radical in nature depending on the pH in which the covalent affinity chromatography is conducted.

The reactivity of –SH and –S–S– groups itself depends on the composition of the medium and the structure, conformation and geometry of the particular protein [87, 97, 123–125]. In the case of MTs the reactivity is quaranteed by the close proximity of the thiolate groups in the I and III order structures. Also the presence of serine coupled with cysteine containing –OH groups permits an appropriate medium and explains the behaviour during separation [98]. The existence of metal ions in solution acting as catalysts also facilitates the oxidation of the SH-groups and exchange of disulphide bridges [120–122].

A similar protein aggregation phenomenon due to –SH groups has been observed for alcohol dehydrogenase [99], vasopresin [100], cysteine peptides [101], fibrin and fibrinogen [102–104], zymogen [126, 127] and many others [105–111, 128, 139–145].

Protein aggregation using –S–Hg–S– mercaptide bridges has also been reported [112–114, 129–134] (Eqs. (10a) and (10b)):

$$\text{(P)}\text{–SH} + HgCl_2 \longrightarrow \text{(P)}\text{–S–HgCl} \quad (10a)$$

$$\text{(P)}\text{–S–HgCl} + \text{(P)}\text{–SH} \longrightarrow \text{(P)}\text{–S–Hg–S–(P)} \quad (10b)$$

$M_s(TNB)_z$ micromolecular complexes probably exist in different forms. In the case of mercury they create different forms of mercury-thiolate complexes; with Cd(II) ions they probably make complexes with lower molecular mass which do not take the form of clusters.

It is clear that the mechanism of polythiolate protein and polythiolate metalloprotein separations by means of covalent affinity chromatography using the exchange of disulphide bridges is much more complex than previously believed. Apart from binding to the support, an aggregation of polythiolate proteins through disulphide and mercaptide bridges takes place. In the case of monomers, intramolecular disulphide and mercaptide bonds make the structure denser and reduce the radius of the protein molecule. This is shown as an apparent loss of mass and a change in rheological properties. Due to the breaking of mercaptide bonds polythiolate metalloproteins also lose part of the metallic content. For all these reasons the proteins lose their original structure and become denaturated during the chromatographic process in addition to losing their physicochemical homogenity. The investigations carried out show that there is no possibility of reconstruction of the proteins after separation.

The separation yields are reduced to about 20–30 % where metal is present in the separating medium and about 50 % in the case of polythiolate proteins without metals. The results emphasise the care needed in quality control of large-scale protein separation by affinity chromatography in general and covalent affinity chromatography in particular when the products are required for pharmaceutical purposes.

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