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Cyanocarbons as ligands for electron donor acceptor chromatography of human serum proteins

Patrick P. Berna[a,*], Jerker Porath[a,b]

[a]*Biochemical Separation Centre, Uppsala University, BMC, Box 577, S-751 23 Uppsala, Sweden*

[b]*ARL Division of Biotechnology and Department of Biochemistry, University of Arizona, P.O. Box 210088, Biosciences West 350, Tucson, AZ 85721-0088, USA*

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Abstract

The adsorption of human serum proteins to mono-, di- and tri-cyanocarbon ligands was studied in the presence of the water-structuring salt Na_2SO_4. All of the ligands adsorbed proteins to varying degrees when coupled to agarose via a divinylsulfone-derived spacer arm (DVS), whereas an insignificant or very low protein adsorption capacity occurred when the ligands were coupled through the bisoxirane-derived spacer arm. Studies of the DVS-coupled cyanocarbons showed that adsorption capacity increases with the number of cyano substituents carried by the ligand. The selectivity toward human serum proteins appeared to be similar to that of other electron acceptor ligands, but different from that of hydrophobic ligands. Tricyanoaminopropene–DVS–agarose was found to be the most potent protein adsorbent.

Keywords: Adsorbents; Tricyanoaminopropene ligand; Proteins; Cyanocarbons

1. Introduction

Electron donor acceptor chromatography (EDAC) has been primarily used for the separation of organic molecules through formation of labile complexes with electron donors or acceptors adsorbed or covalently coupled to a solid phase. Organic modifiers present in the mobile phase presumably allow for isocratic elution of analytes and for the resolution of complex mixtures. Two decades ago, the application of EDAC to protein fractionation in polar aqueous phase was successfully demonstrated by Porath and Caldwell [1], Porath and Larsson [2] and by Porath [3]. High concentrations of water-structuring salts were used in these studies to enhance protein adsorption, thus avoiding the use of deleterious organic solvents.

Among the different ligands tested, electron acceptor ligands composed of an aromatic ring substituted with electron-withdrawing groups, including nitro- and halogen groups, were most effective for protein adsorption [4,5]. The cyano group, CN, represents an effective electron-withdrawing substituent that can potentiate the electron acceptor properties of aromatic or unsaturated compounds. Nevertheless, the electron acceptor properties of cyanocarbons have rarely been applied in EDAC [6,7].

Here we have studied four alkanes or alkenes of short length (C2 to C6) substituted with mono-, di-

*Corresponding author. Present address: Institut de Recherche Jourcinal, Department of Biotechnology and Biochemistry, 3, 9 rue de la loge, F-94265 Fresnes, France.

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and tri-cyanocarbons as ligands for protein adsorption. Each of the studied ligands was coupled to bisoxirane- or divinylsulfone (DVS)-activated agarose to confirm the increase in protein adsorption capacity noted earlier [8] when electron acceptors were coupled to a DVS spacer arm and compared to the effect of coupling to the bisoxirane spacer arm. The experiments were carried out with human serum as a model sample containing proteins with a wide range of physico-chemical properties. Water-structuring salts were used to fulfill the EDAC requirements of protein adsorption in an aqueous polar chromatographic phase. Among the ligands tested, the most potent for protein adsorption, namely tricyanoaminopropene (TCP), was selected for further study of its protein specificity and binding mechanisms.

2. Experimental

Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). Normal human serum was obtained from the Blood Bank at the University Hospital (Uppsala, Sweden). Antisera against human serum proteins were kindly supplied by Prof. C.B. Laurell (Department of Clinical Chemistry, Malmö Hospital, Malmö, Sweden).

The ligands propylamine, allylamine, amino acetonitrile (AN), amino crotonitrile (ACN), diamino maleonitrile (DAMN) and tricyanoaminopropene (TCP) were coupled to Sepharose 6B using two different coupling methods, one based on bisoxirane (bisepoxy) and the other based on DVS. For coupling via bisoxirane, agarose was activated with 1,4-butanediol diglycidyl ether, as described by Winzerling et al. [9]. The ligands were coupled as follows: 50 g of bisoxirane-activated agarose were equilibrated with 0.2 *M* sodium carbonate (pH 11), suction-drained and added to 50 ml of a mixture of 0.2 *M* sodium carbonate (pH 11) and a 0.1 *M* amount of the desired ligand. The pH of the mixture was adjusted to pH 11. After shaking for 24 h at room temperature, the slurry was transferred to a glass filter and was washed with water, dimethyl sulfoxide and again with water. For coupling via DVS, the agarose was equilibrated with 0.2 *M* sodium carbonate (pH 11). A 50-g amount was suction-drained and added to 50 ml of a mixture of DVS (15%, v/v) [2.2% (v/v) for activation of the TCP gel] in 0.2 *M* sodium carbonate (pH 11). After shaking for 4 h at room temperature, the slurry was washed with deionized water on a sintered-glass filter until the pH of the filtrate was neutral. The coupling step was done as for the bisoxirane-activated gel but using 0.2 *M* sodium hydrogen carbonate (pH 10) throughout the procedure.

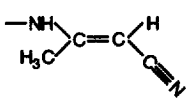
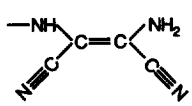
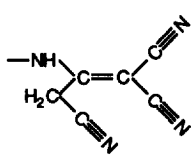
For chromatography studies, a 7.4×0.5 cm I.D. column was packed with the test gel. A program for equilibration, adsorption, desorption and regeneration of the adsorbent was executed by means of a programmable fast protein liquid chromatography (FPLC) system from Pharmacia. Solid sodium sulphate was added to the serum sample to a final concentration of 0.5 *M*. The gel was equilibrated with the adsorption buffer, 50 m*M* MOPS–acetate buffer (pH 7.6), containing 0.5 *M* Na_2SO_4, at a flow-rate of 60 ml/h. A 0.5-ml sample containing approximately 68 mg of protein/ml of centrifuged (5000 *g*, 5 min) human serum was applied to the column and the gel was washed for 40 min with the adsorption buffer. The adsorbed material was eluted by deletion of the salt from the adsorption buffer (50 m*M* MOPS–acetate buffer, pH 7.6). Finally, the gel was rinsed for 30 min with 50 m*M* MOPS–acetate buffer (pH 7.6) containing 6 *M* urea as a final cleaning step. The eluate from each step was recovered as a single fraction.

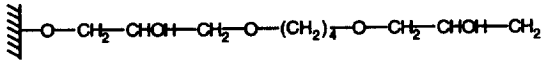
The protein content of the chromatographic fractions was measured using the Bradford assay [10]. Nitrogen and sulphur analyses of the coupled gels were performed by Microkemi (Uppsala, Sweden). Serum proteins were immunologically identified by the conventional Ouchterlony technique, using monospecific antibodies. Two-dimensional electrophoretic analysis of the adsorbed fraction from the TCP gel was accomplished using the Immobiline drystrip kit (pH 3.5–10 Linear, 11 cm) and an ExcelGel SDS gradient (8–18%) from Pharmacia, following the manufacturer's procedure. The electrophoresis gels were silver-stained and scanned on a Bio-Rad GS-700 Imaging Densitometer (Hercules, CA, USA) using the Molecular analyst 2-D PAGE software version 1.0 from Bio-Rad for image analysis.

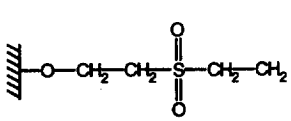
3. Results

The cyanocarbon ligands were coupled to DVS- or bisoxirane-activated agarose. Their salt-promoted adsorption capacity for human serum proteins was tested in the presence of 0.5 *M* sodium sulphate and the results are listed in Table 1. Although ligand densities were comparable for all gels, a clear difference in protein adsorption capacity was observed for ligands coupled to bisoxirane-activated compared to those coupled to DVS-activated gels. In all cases, the use of DVS as the spacer arm strongly enhanced the protein adsorption at the salt concentration tested. However, the reference gel, DVS–propylamine agarose, did not show any protein adsorption suggesting the inability of the DVS moiety to adsorb protein when not associated with an electron donor or acceptor substituent. A comparison of the protein adsorption capacities of the different cyanocarbon gels showed that protein adsorption is a

Table 1
Structure of the different ligands coupled to agarose gels via a bisoxirane or divinylsulfone (DVS) spacer arm. Degree of substitution and percentage of human serum proteins desorbed by salt deletion from the adsorption buffer for the different prepared gels

Ligand	Spacer arm	Degree of substitution (μmoles of ligand/g of dried gel)	% of protein desorbed by salt omission
—NH—CH_2—CH_2—CH_3	Bisoxirane[a]	320	0.0
Propylamine	DVS[b]	456	0.0
—NH—CH_2—CH=CH_2	Bisoxirane	383	0.0
Allylamine	DVS	305	1.3
—NH—CH_2—C≡N	Bisoxirane	356	0.1
Amino acetonitrile (AN)	DVS	420	1.4
	Bisoxirane	428	0.2
Amino crotonitrile (ACN)	DVS	303	2.0
	Bisoxirane	235	0.0
Diamino maleonitrile (DAMN)	DVS	312	6.8
	Bisoxirane	367	0.6
Tricyanoaminopropene (TCP)	DVS	453	14.0

[a] Butanediol diglycidyl ether: 

[b] Divinyl sulfone: 

function of ligand complexity; the more cyano substituents carried by the ligand, the more proteins are being adsorbed. The TCP gel was found to be the strongest adsorbent tested, with a protein capacity of 9 mg/ml of gel.

Characterization of the various kinds of proteins in the different chromatographic fractions by means of the Ouchterlony immunodiffusion technique showed that albumin, prealbumin, haptoglobulin, α-lipoprotein and β-lipoprotein passed through all of the cyanocarbon gels. The adsorbed proteins on the different gels are listed in Table 2. IgG and fibrinogen were consistently retained on all cyanocarbon gels, whereas IgA, IgM, α_2-macroglobulin, α_1-antichymotrypsin and the C3 and C4 complement proteins were not retained on the AN- and ACN-gels. Image analysis of the two-dimensional electrophoretogram of the proteins adsorbed on the TCP gel resulted in a more detailed picture and allowed for quantification of the relative percentages of each protein (Table 3). The major proteins adsorbed were IgG, IgA and IgM, representing 40%, 20% and 1% of the adsorbed proteins, respectively. Fibrinogen, transferrin, α_1-acid glycoprotein, ceruloplasmin, α_1-antitrypsin, α_2-macroglobulin, α_2HS-glycoprotein, α_1-antichymotrypsin, and the C3 and C4 complement proteins were minor components representing (in total) 14% of the adsorbed proteins. A portion (25%) of the proteins in this fraction remained unidentified but was composed of proteins, each of which represented less than 0.5% of the total protein content.

Table 3
Relative percentages of the different human serum proteins present in the desorbed fraction of the tricyanoaminopropene–DVS–agarose (TCP)

Protein	Relative percentage (%)
IgG	40
Unknown	25
IgA	20
Fibrinogen	5
Transferrin	2
α_1-Acid glycoprotein	1.4
Ceruloplasmin	1
α_1-Antitrypsin	1
α_2-Macroglobulin	1
IgM	1
C3 complement	1
α_2-HS-glycoprotein	0.7
α_1-Antichymotrypsin	0.6
C4 complement	0.5

Quantification by image analysis of a two-dimensional electrophoretogram.

In all chromatographic analyses, the total protein recovery exceeded 95%. The TCP gels gave reproducible results for the different coupling batches. Protein adsorption capacity decreased by less than 5% after fifteen chromatographic cycles, which demonstrates the reusability of the gel under normal

Table 2
Human serum components in the desorbed fraction eluted by salt omission from different derivatized agarose gels

Protein	AN	ACN	DAMN	TCP	Mercaptopyridine	Thiophilic	Octyl
IgG	+	+	+	+	+	+	
Fibrinogen	+	+	+	+	+	nd	nd
C3 complement			+	+	+	nd	
C4 complement			+	+	+	nd	
IgA			+	+	+	+	
IgM				+	+	+	
α_2-Macroglobulin				+	+	+	--
α_1-Antichymotrypsin				+	+	nd	nd
Albumin							--
α-Lipoprotein							--
β-Lipoprotein							--

+ = protein detected in the eluate, blank = protein not detected in the eluate, nd = not determined.
Protein determined using the conventional Ouchterlony technique, using monospecific antibodies.
The pooled fractions were concentrated to have an A_{280} value of 1.0.

conditions. No changes in protein retention specificity were observed.

4. Discussion

The cyano group, CN, is an effective electron-withdrawing substituent that potentiates electron acceptor properties of saturated and especially of unsaturated compounds. We observed adsorption of serum proteins on all of the different cyanocarbon gels coupled via the DVS spacer arm. The protein adsorption was weak on mono-cyano-substituted alkanes or alkenes like AN (1.4%) or ACN (2%) and was, in fact, equivalent to protein binding on an unsubstituted alkene, the allylamine gel (1.3%) (Table 1). This suggests poor electron acceptor properties for these two ligands. In contrast, the di- and tri-cyanocarbon-substituted gels (DAMN and TCP) showed a significant increase in protein adsorption with 6.8 and 14% of proteins adsorbed, respectively. This increase in protein adsorption may be attributed to either an enhancement of the electron acceptor properties of the ligands combined with the degree of cyano substitution or to an increase in the hydrophobic character of the ligands in parallel with an increase in the number of carbons. However, a comparison of the protein adsorption for ligands with a constant cyano substitution but an increased number of carbons [AN (1.4%) and ACN (2%)] with those ligands having a constant number of carbons but an increased degree of cyano substitution [ACN (2%) and DAMN (6.8%)], clearly shows that the degree of cyano substitution has a distinct impact on the protein adsorption capacity, whereas hydrophobicity seems to be of secondary importance at the ligand density tested.

In this study, we also demonstrated that no protein was adsorbed on the gels if bisoxirane was used in place of DVS as the spacer arm. A similar fact had been noted for the T-gel, where association of DVS with 2-mercaptoethanol led to the discovery of thiophilic adsorption. The mechanism of interaction was interpreted as a synergistic two-point attachment between the unsaturated ligand and the DVS residue (7). Although this mechanism is applicable to our results, we suggest an alternative hypothesis where the DVS residue is not directly involved in the binding but rather its presence is necessary to modify the physico-chemical microenvironment of the matrix. In this scenario, the DVS moiety is considered to be an immobilized chemical structure that indirectly augments the interaction resulting in protein binding at a lower concentration of water-structuring salt.

With respect to the mechanism of interaction, the use of water-structuring salts to promote protein adsorption clearly eliminates the possibility of an ionic type of interaction and further supports the existence of short-range, weak-type interactions. However, hydrophobic interaction certainly is not the primary cause of the mechanism, as was shown by the difference in the protein specificity between cyanocarbons and hydrophobic ligands (Table 2) and by the ability of cyanocarbon gels to adsorb protein in the presence of non-ionic detergents (Tween 80, Brij 35 or Triton X-100) (results not shown). Therefore, electron donor/acceptor interactions and hydrogen bonding are the most plausible candidates among the short-range weak interactions to rationalize the binding mechanism of proteins on cyanocarbon gels. For complexation to depend on partial electron transfer occurring, the proteins must have available counterparts to the ligands. In our view, the only reasonable candidates are the aromatic side-chains. These amino acid side-chains, present to varying extents on the surface of different proteins, would then act as electron donors. Finally, the similitude of protein specificity among the cyanocarbon gels and other gels such as the T-gel or the mercaptopyridine gel [11] (Table 2) strongly suggests common sites of interaction for these different ligands.

It is also worth mentioning that these types of salt-promoted chromatographic effects can be of importance to the field of halophilic protein purification, where proteins need to be stabilized by high salt concentrations.

Clearly TCP–agarose can be used for efficient group fractionation of serum proteins due to its opposed protein selectivity when compared to hydrophobic adsorbents. Whether more refined fractionations of individual proteins from a complex such as a serum mixture are possible or not can only be answered after a more detailed study of displacement effects and attempts to find selective eluents. The stability of the adsorbent and the reproducibility of

the chromatograms indicate that such studies may be rewarding.

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