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Journal of Chromatography A, 668 (1994) 101–106

JOURNAL OF CHROMATOGRAPHY A

Use of hydrophobic affinity partitioning as a method for studying various conformational states of the human α-macroglobulins

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Abstract

The serum proteins α_2-macroglobulin and pregnancy zone protein undergo major conformational changes when complexed with proteinases. It is shown that the changes in $\Delta\log K_{max}$ determined by hydrophobic affinity partitioning is a measure of the extent of changes in the conformation of these α-macroglobulins. We introduce a new term for the changes of surface hydrophobicity in a protein as $\Delta\log K_{acc}$. This defines the difference of $\Delta\log K_{max}$ between a modified and an unmodified conformational state of a specific protein and can be useful as a parameter to describe the apparent conformational changes in the protein.

1. Introduction

Many proteins exhibit their functions at certain conformational states, which can be reversibly or non-reversibly changed by various factors. Some proteins change conformation by binding ligands or ions and some by proteolytic cleavage. The changes in conformational states are caused by changes in the protein structure, which involve changes in the exposure of protein regions to the surroundings. Some of these regions can be hydrophilic or hydrophobic and these characteristics play dominating roles in the function and stability of the protein. Changes in exposure of hydrophobic regions to the surroundings have been detected in many proteins upon regulation of functions. Strong hydrophobic regions are often exposed for direct functional reasons, which, in the case of serum proteins, may involve binding and transport of specific ligands. In general hydrophobic regions are concealed and hydrophilic regions exposed in proteins [1]. Hydrophobic affinity partitioning has been documented to be a sensitive method for studying changes in hydrophobicity of the surface of proteins [2]. It has been demonstrated that calmodulin and α-lactalbumin undergo a considerable change in surface hydrophobicity upon Ca^{2+} binding [3–5]. These changes in hydrophobicity have been correlated with changes in conformation of these proteins [4,5].

Human α_2-macroglobulin (α_2M) and the related pregnancy zone protein (PZP) are two serum proteins which undergo large conformational changes during inhibition of proteinases. The two proteins have many characteristics in common and have 71% identically positioned amino acids [6]. The conformational changes in α_2M have been studied extensively by various methods such as polyacrylamide gel electropho-

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0021-9673/93/$06.00
SSDI 0021-9673(93)E1154-R

resis (PAGE) [7,8], hydrodynamic methods, absorption, fluorescence and circular dichroism spectra [9] and by electron microscopy [10]. α_2M is a homotetrameric protein with a molecular mass of 720 000; 1 mol is capable of inhibiting 2 mol of a proteinase [11]. Native PZP is a homodimeric protein with a molecular mass of 360 000, but can generate a 720 000 species after inhibition of a proteinase [12–14]. The conformational changes in the two proteins are complex, but the importance of three specific regions for the conformational changes is known, which are the bait region, the γ-glutamyl-β-cysteinyl thiol ester and the receptor recognition site [15]. The bait region is a region sensitive to proteolytic cleavage by a large number of proteinases. Cleavage of α_2M or PZP in this region leads to conformational changes by which thiol esters become susceptible to nucleophilic attacks. The γ-glutamyl residues of the thiol esters can generate covalent bonds with lysine residues of the proteinase and the sulfhydryl groups are liberated. The conformational changes due to cleavage of the bait region(s) and thiol ester(s) lead to "trapping" of proteinases by compaction of α_2M whereby the proteinases become sterically inhibited. In PZP the conformational changes also lead to steric hindrance of the proteinase by tetramerization of two PZP molecules, but no "trapping" occurs as in α_2M [13,14]. In both α_2M and PZP previously concealed receptor recognition sites are exposed by the conformational changes [16], whereby the inhibitor–proteinase complexes can be specifically removed from the circulation.

Previous studies have indicated that changes in hydrophobicity occur on the surface of α_2M, *i.e.* two conformationally different forms can be separated by high-performance hydrophobic chromatography [17]. Further Birkenmeier *et al.* [18] have documented differences in hydrophobic properties for α_2M and PZP by affinity phase partitioning. We have studied α_2M and PZP by hydrophobic affinity partitioning to obtain further information on the differences in conformation of these two unique proteinase inhibitors. This technique is especially advantageous in the study of different states of the same protein since most of the factors that can influence the partitioning, such as size, charge and polarity, are eliminated and only hydrophobic interactions are determined.

2. Experimental

2.1. Chemicals and enzymes

α-Chymotrypsin (EC 3.4.21.1), phenylmethylsulphonyl fluoride, sodium thiocyanate, methyl methanethiosulphonate and methylamine were purchased from Sigma (MO, USA). Poly(ethylene glycol) (PEG) 8000 was from Union Carbide (NY, USA, and Dextran T70 was from Pharmacia (Uppsala, Sweden). PEG-palmitate was synthesized as described [19] and had a degree of substitution of 0.82 mol of palmitate per mol PEG. All other chemicals were of analytical-reagent grade.

2.2. Proteins

α_2M was purified from fresh human plasma as described by Imber and Pizzo [20], and PZP was purified as described by Sand *et al.* [12]. The proteins were iodinated by the use of Iodobeads (Pierce, IL, USA) with $Na^{125}I$ from Amersham, UK. The specific activity (300–600 cpm/ng) was determined in a Unigamma γ-counter (LKB, Stockholm, Sweden). All derivatives of α_2M and PZP treated with methylamine and chymotrypsin were prepared according to Jensen and Stigbrand [14]. Modification of thiol groups was performed by incubation of α_2M with methylamine at a concentration of 0.4 *M* in the presence of methyl methanethiosulphonate at a concentration of 100 μM for 1 h at room temperature. Dimeric α_2M was prepared by incubation of α_2M with 1.2 *M* NaSCN in 0.1 *M* sodium phosphate, pH 8.0 for 4 h at room temperature. Excesses of reagents were removed by gel filtration on a NAP-5 column (Pharmacia) equilibrated with 0.1 *M* sodium phosphate buffer, pH 8.0, before analysis.

2.3. Hydrophobic affinity partitioning

Aqueous two-phase systems contained 7% Dextran T70, 4.5% PEG 8000, including PEG-palmitate, 20 m*M* potassium phosphate and 75 m*M* sodium chloride, pH 7.4. The systems were quickly mixed with protein (1–2.5 μg) at room temperature by approx. 60 inversions and phase separation was speeded up by centrifugation at 2000 *g* for 5 min. The concentration of PEG-palmitate expressed as percent of total PEG in the system was varied from 0.02 to 0.72%. K_0 and K_{max} are partition coefficients of the protein in the absence and presence of 0.7% PEG-palmitate, respectively. $\Delta\log K$ is defined as $\log K_{aff} - \log K_0$, where K_{aff} is the partition coefficients of the protein in the presence of PEG-palmitate in the system [2]. The values are averages from two experiments.

3. Results and discussion

Since human α_2M is a tetrameric protein consisting of two non-covalently associated dimeric molecules and PZP is dimeric in the native state, it is of interest to study the conformational changes occurring in these two homologous proteins in connection with inhibition of proteinases, since both can generate covalent tetrameric complexes with proteinases. The study of dimeric α_2M is included for comparison of differences in oligomerization.

The K_0 values for the native molecules as well as for the derivatives of α_2M and PZP are low. This reflects low affinity of these proteins and their derivatives for the upper PEG phase. However, the values demonstrate that there are differences in the overall conformation of PZP, tetrameric and dimeric α_2M, but the differences are too small to justify any specific conclusions.

The curves of $\Delta\log K$ values in Fig. 1 demonstrate that these proteins have high affinity for PEG-palmitate. The maximum affinity, $\Delta\log K_{max}$ of dimeric α_2M is more than twice that of native α_2M. This may indicate that the stabilization of the tetrameric form of native α_2M involves hydrophobic interactions and/or that the

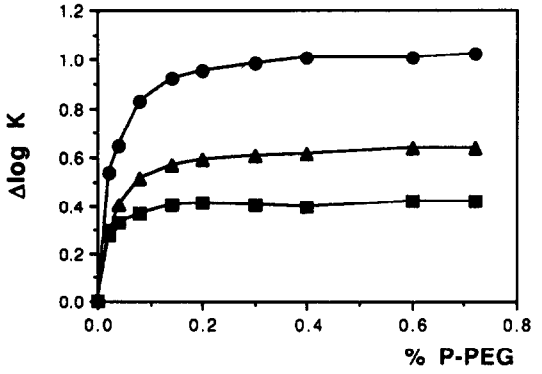

Fig. 1. $\Delta\log K$ plotted as a function of PEG-palmitate (P-PEG) for (▲) native PZP, (■) native α_2M and (●) dimeric α_2M. The partitioning coefficient, *K*, defined as the ratio of concentrations in the upper and the lower phases was taken as the ratio of radioactivities in equal aliquots of the upper and lower phases of the system. $\Delta\log K$ is defined in the Experimental section.

dimeric α_2M has changed conformation during the dissociation. Studies by use of dodecyl sulphate, urea or low pH to dissociate α_2M into dimeric forms have demonstrated drastic changes in conformation of the protein [21–23]. It has been proposed that hydrophobicity is involved in the non-covalent interactions between the dimers in α_2M [24]. The native dimeric PZP has a higher $\Delta\log K_{max}$ value than that of tetrameric α_2M, which may suggest that hydrophobic forces holding α_2M in a tetrameric form are at least partly exposed in the dimeric PZP, but if present, they may not be strong enough to retain PZP in a tetrameric form.

Treatment with methylamine cleaves the thiol esters by amidation of the γ-glutamyl residues leaving the bait regions intact.

By treatment of tetrameric α_2M with methylamine the $\Delta\log K_{max}$ value is drastically increased as compared to that of native tetrameric α_2M (Table 1). The increase suggests major conformational changes. These results agree with previous conclusions that methylamine treatment of α_2M generates large conformational changes [25]. In the case of dimeric α_2M and PZP, no significant changes occur upon cleavage of the thiol esters by methylamine, indicating that no major conformational changes occur when the thiol esters alone are cleaved in the dimeric

Table 1
K_0, K_{max} and Δlog K_{max} values for α_2M, dimeric α_2M and PZP (a); after treatment with methylamine (b); α_2M simultaneously treated with methylamine and methyl methanethiosulphonate (c)

	Protein	K_0	K_{max}	Δlog K_{max}
a	Tetrameric α_2M	0.14	0.36	0.41[a]
	Dimeric α_2M	0.23	2.35	1.01
	PZP	0.20	0.87	0.64[a]
b	Tetrameric α_2M	0.18	2.99	1.22[a]
	Dimeric α_2M	0.29	2.90	1.00
	PZP	0.20	0.94	0.67[a]
c	Tetrameric α_2M	0.08	0.21	0.41

[a] Results obtain from Jensen *et al.* [29].

forms as has been previously suggested for PZP [13,14].

Simultaneous treatment with methylamine and the thiol modifying reagent methyl methanethiosulphonate (MMTS), which binds to the liberated thiol groups of the thiol esters, generates a derivative of α_2M with similar conformation as native α_2M, *i.e.* with an open "trap" and a slow electrophoretic form (data not shown). This derivative of α_2M gives a Δlog K_{max} value similar to that of native α_2M in support of the similarity with native α_2M (Table 1). A derivative generated by treatment with methylamine and dinitrophenyl thiocyanate, which has been shown to be similar in conformation to native α_2M [14,26–28] also had a similar Δlog K_{max} [29].

Chymotrypsin treatment of the α_2M derivative modified by methylamine and methyl methanethiosulphonate restores the surface hydrophobicity close to that of methylamine-treated tetrameric α_2M (Table 2). This result suggests that the cleavage of the bait regions in the derivative generates a conformational state of the derivative, which is close to that of the methylamine-treated state. PAGE has demonstrated this form to be the electrophoretic "fast" form similar to the methylamine as well as the proteinase-treated forms of tetrameric α_2M (data not shown).

Table 2
K_0, K_{max} and Δlog K_{max} values of dimeric α_2M and PZP after treatment with methylamine and then chymotrypsin, and tetrameric α_2M treated with methylamine and methyl methanethiosulphonate before treatment with chymotrypsin

Protein	K_0	K_{max}	Δlog K_{max}
Tetrameric α_2M	0.18	2.32	1.11
Dimeric α_2M	0.35	2.37	0.83
PZP	0.52	1.31	0.40

The derivatives were generated as described in Experimental.

As seen in Table 2 both PZP and the dimeric α_2M treated with methylamine and then with chymotrypsin generate changes in Δlog K_{max} values as compared to those treated with methylamine alone. For the PZP derivative the Δlog K_{max} is the same as that of proteinase-treated PZP (Table 3). PAGE of the derivatives has demonstrated that the bait regions become cleaved in methylamine-treated dimeric α_2M even though this does not occur in methylamine-treated tetrameric α_2M (not shown), which supports the interpretation of the surface hydrophobicity changes by methylamine. The change in exposed hydrophobicity only occurs when the bait region is cleaved in PZP and cleavage of the thiol ester has no effect in accordance with ref. 14 as studied by use of monoclonal antibodies, PAGE and *in vivo* clearance studies.

The Δlog K_{max} values for the derivatives treated with chymotrypsin are different (Table 3). For the tetrameric α_2M the value is approxi-

Table 3
K_0, K_{max} and Δlog K_{max} values of PZP and dimeric and tetrameric α_2M after treatment with chymotrypsin at different molar ratios

Protein	K_0	K_{max}	Δlog K_{max}
Tetrameric α_2M (1:1)	0.23	1.32	0.76[a]
Tetrameric α_2M (1:4)	0.29	2.20	0.88[a]
Dimeric α_2M (1:1)	0.29	2.36	0.91
Dimeric α_2M (1:2)	0.34	2.15	0.80
PZP (1:1)	0.36	1.04	0.46[a]
PZP (1:2)	0.47	1.18	0.40[a]

The derivatives were generated as described in Experimental.
[a] Results obtained from Jensen *et al.* [29].

mately twice that for the value of the native molecule, which demonstrates large changes in the hydrophobic surface of α_2M. This can be correlated with the major conformational changes occurring in α_2M after bait region cleavage and inhibition of the proteinase, which have been documented using several methods [25]. The conformational state is similar to that of α_2M treated with methylamine as judged by various methods [8,9,30,31]. The surface hydrophobicity increases with an increase in molar ratio of proteinase to α_2M that is when more bait regions are cleaved. These results suggest that the extent of "trapping" in α_2M is correlated with the value of $\Delta\log K_{max}$. The $\Delta\log K_{max}$ values of the proteinase-treated dimeric form of α_2M indicate differences between the dimeric and tetrameric forms of α_2M. The value for the dimeric α_2M decrease at a molar ratio of 1:1 proteinase to dimeric α_2M and decrease further at a molar ratio of 2:1. For PZP, which, in contrast to dimeric α_2M, can tetramerize on proteinase binding, but also exists as a proteinase-complexed dimeric form, the $\Delta\log K_{max}$ values also decrease at a molar ratio of 1:1 proteinase to PZP and decrease further at a molar ratio of 2:1. It is of interest that the hydrophobic affinity of the dimeric α_2M decreases as it does for PZP.

In native α_2M the changes in surface hydrophobicity are sensitive to bait region cleavage as well as thiol ester modification, while the dimeric forms of PZP and α_2M seem only to be sensitive to changes upon proteinase treatment. The generation of dimeric α_2M might introduce a high degree of disorganisation, and cause the large differences in surface hydrophobicity observed between native α_2M and dimeric α_2M. This is supported by the fact that dissociation to dimeric α_2M caused by various agents (dodecyl sulphate, urea, thiocyanate, low pH) is irreversible [21–23].

For lucidity, we introduce a new term $\Delta\log K_{acc}$ to define the difference in $\Delta\log K_{max}$ between the modified and the unmodified forms of the same protein. This means that $\Delta\log K_{acc}$ can be written as:

$$\Delta\log K_{acc} = \Delta\log K_{max\ (modified)} - \Delta\log K_{max\ (unmodified)}$$

The index "acc" is the abbreviation of "apparent conformational change". Thus, $\Delta\log K_{acc}$ is correlated with conformational changes which result in changes in surface hydrophobicity. The values of this parameter are recorded in Table 4.

This table clearly demonstrates the difference between the tetrameric and dimeric forms, *i.e.* the dimeric forms present negative values (decreased surface hydrophobicity) while the tetrameric α_2M present positive values (increased surface hydrophobicity). The table further reveals the similarity in changes between dimeric α_2M and PZP in contrast to the changes in tetrameric α_2M, which may suggest that the differences in oligomerization between α_2M and PZP may to a high degree influence the differences observed in conformational states.

Table 4
$\Delta\log K_{acc}$ values of the apparent conformational changes in PZP, α_2M and dimeric α_2M

Δ log K_{acc}	α_2M	Dimeric α_2M	PZP
Methylamine	0.81	−0.02	0.03
Chymotrypsin (1:1)	0.35	−0.11	−0.18
Chymotrypsin (1:2)		−0.22	−0.24
Chymotrypsin (1:4)	0.47		
Methylamine, chymotrypsin (1:1)		−0.18	−0.24
Methylamine, MMTS	0.00		
Methylamine, MMTS, chymotrypsin (1:1)	0.70		

The numbers following chymotrypsin denotes the molar ratio of derivative to proteinase.

4. Conclusions

This study demonstrates that hydrophobic partitioning with PEG-bound palmitate is a sensitive method for monitoring changes in surface hydrophobicity of α-macroglobulins. All results obtained on tetrameric α_2M and PZP by the affinity partitioning can be correlated to conformational changes, since they are in line with results of conformational studies by several other methods. The method in addition reveals differences in the changes of structures, which have not been detected by other methods.

5. Acknowledgements

Mrs. Eva-Maj Hägglöf is thanked for skillful technical assistance and Luis F. Arbelaez for kindly providing PZP. This investigation was supported by grants from the Swedish Medical Research (MFR) and the Swedish Natural Sciences Research Council (NFR).

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