ZYGMUNT WASYLEWSKI, ANDRZEJ JANIK and MARTA PASENKIEWICZ*

PROTEIN-CATIONIC DETERGENT INTERACTION

INTERACTION OF BOVINE SERUM ALBUMIN AND OTHER PROTEINS WITH ALKYLPYRIDINIUM BROMIDES STUDIED BY VISCOSITY, GEL FILTRATION AND SPIN-LABEL METHODS

Institute of Molecular Biology, Department of Animal Biochemistry and *Department of Biophysics, Jagiellonian University, ul. Grodzka 53; 31-001 Kraków, Poland

Viscosity, gel filtration and spin-labelling methods have been used to study the influence of alkylpyridinium bromides on the conformation of bovine serum albumin and other proteins. Cationic detergents cause partial unfolding of the native protein molecules. The magnitude of these changes increases with increasing length of the detergent hydrocarbon chain. When cationic detergents are added to reduced and carboxymethylated bovine serum albumin the observed changes are opposite to those found in native protein.

In the accompanying papers (Wasylewski, 1979a,b) we have tested by infrared and laser Raman spectroscopies the influence of dodecylpyridinium bromide on conformation of bovine serum albumin and a wide range of other globular proteins. Comparable hydrodynamic studies on interaction between cationic detergents and proteins are very few. In 1955 Few et al. observed an increase in viscosity of native albumin in dodecyltrimethylammonium bromide, and Nozaki et al. (1974) showed that tetradecyltrimethylammonium chloride induced changes similar to those induced by anionic detergents, i.e. the reduced, carboxymethylated lysozyme, immunoglobulin, ovalbumin, bovine serum albumin and conalbumin.

In the present work, hydrodynamic and spin-labelling methods were used for a more detailed investigation of the influence of alkylpyridinium bromides with different hydrocarbon chains on the conformation of bovine serum albumin and other proteins.
MATERIALS AND METHODS

Reagents. Catalase ex beef liver, cryst., was obtained from Koch-Light (Colnbrook, England). 2-Mercaptoethanol was obtained from Serva (Heidelberg, F.R.G.). Iodoacetamide was purchased from Fluka A.G. (Buchs S.G., Switzerland). Sephadex G-200 and Blue dextran were purchased from Pharmacia (Uppsala, Sweden). The paramagnetic spin label 2,2,6,6-tetramethyl-4-piperidone-1-oxyl was prepared as described previously (Wasylewski & Pasenkiewicz, 1977). Other reagents were the same as in the accompanying papers (Wasylewski, 1979a,b).

Viscosity measurements. Specific viscosity of bovine serum albumin was measured with Ostwald viscometers (outflow time of 145 s for 3 ml of water at 20±0.05°C). All measurements were made using 1% (w/v) solutions of the protein in phosphate buffer, pH 6.8, ionic strength 0.1. Solutions with the desired molar mixing ratios of alkylpyridinium bromides to protein were prepared and kept for 24 h at room temperature before ultracentrifugation (100,000 g, 30 min) and viscosity measurements. The outflow time of the solvent and protein solutions were measured separately four times.

The intrinsic viscosities of proteins were determined in Seide-Deckert semimicro viscometers with an outflow time of 270 s for 2 ml of water. The constant-temperature bath was thermostated to 20±0.05°C. To determine the reduced viscosity, samples of protein solution, initially 1% by weight, were diluted in the viscometer with phosphate buffer. After each dilution specific viscosity was measured. Intrinsic viscosity was calculated by extrapolation of the reduced viscosity to zero protein concentration.

Gel filtration. Stokes radii of proteins were measured at room temperature by gel filtration using a Sephadex G-200 column (1.2×100 cm) equilibrated with phosphate buffer, pH 6.8, ionic strength 0.1, containing 0.02% NaNO3. The column was calibrated using native proteins without detergent added; 0.5 ml samples of 1% protein solutions containing 2 mg of Blue dextran were layered on the top of the column and elution volume, Ve, and void volume, V0, were determined using the flow absorptiometer Uvicord (LKB, Sweden). After calibration of the column DPB was added to the buffer solution at a concentration of 10−2 M. Proteins (1% by weight) dissolved in phosphate buffer, pH 6.8, ionic strength 0.1, containing 10−2 M-DPB were kept for 24 h, then subjected to gel filtration using calibrated columns. Elution volumes for both native protein and protein-detergent associates were calculated as an average from 3 determinations.

Spin labelling. Spin labelling of native, reduced and carboxymethylated bovine serum albumin was performed with 2,2,6,6-tetramethyl-4-piperidone-1-oxyl as described previously (Wasylewski & Pasenkiewicz, 1977). Labelled proteins and cationic detergent were dissolved separately in phosphate buffer, pH 6.8, ionic strength 0.1. Solutions of the desired molar mixing ratio of alkylpyridinium bromides to bovine serum albumin were prepared by mixing 1 ml of protein solution with 1 ml of the detergent solution of various concentrations. The solutions were kept
for 24 h at room temperature. ESR spectra were recorded on the Varian E-3 spectrometer operated at 9.48 GHz using a modulation amplitude of 1G and a microwave power of 25 mW.

Reduction and carboxymethylation. Reduction of ribonuclease and lysozyme (concentration 1% by weight) was carried out in 0.01 M-Tris/HCl buffer, pH 8.6, containing 1% 2-mercaptoethanol and 6 M-guanidine hydrochloride. The mixtures were left for 2 h at 37°C. Sulphhydryl groups were then carboxymethylated by the addition of 5 moles of iodoacetamide per one mole of 2-mercaptoethanol. The solutions of modified proteins were kept for 24 h at room temperature, then dialysed exhaustively against phosphate buffer, pH 6.8, ionic strength 0.1, containing 10^{-2} M-DPB.

Critical micelle concentration. These values for alkylpyridinium bromides were determined at 20±0.05°C by the surface tension dropweight method using a stalgometer. The CMC\(^1\) values in citric acid - phosphate buffer, pH 3.4, ionic strength 0.1, of OPB, DePB, DPB and TPB were: 1.7×10^{-2}, 1.5×10^{-2}, 1.2×10^{-2} and 4.0×10^{-4} M, respectively. The corresponding CMC values of OPB, DePB, DPB and TPB in phosphate buffer, pH 6.8, ionic strength 0.1, were: 5.0×10^{-2}, 2.8×10^{-2}, 1.0×10^{-2} and 9.0×10^{-4} M, respectively.

RESULTS

Viscosity. A conventional plot of specific viscosity, \(\eta_{sp}\), of 1% bovine serum albumin in phosphate buffer, pH 6.8, ionic strength 0.1, as a function of the molar mixing ratio of alkylpyridinium bromides to bovine serum albumin is shown in Fig. 1. At low molar mixing ratio no significant changes in \(\eta_{sp}\) were observed. An increase of this ratio to about 7, 10 and 20 in the case of HPB, TPB and DPB, respectively, and 136 with DePB and OPB caused a significant increase of \(\eta_{sp}\). These changes indicate disorganization of the protein conformation and are more pronounced the longer is the hydrocarbon chain of the detergent.

The intrinsic viscosity, [\(\eta\)], of the proteins tested was measured both in DPB and TPB solutions at a given detergent/protein molar mixing ratio (Table 1). Figure 2 represents, by way of an example, a plot of \(\eta_{sp}/c\) versus protein concentration for myoglobin in DPB and TPB solutions. The ordinate intercepts of the obtained linear curves give the intrinsic viscosity values [\(\eta\)].

For unsolvated spheres of a molecule the intrinsic viscosity is independent of molecular weight or chain length. For extended polymers the relation between intrinsic viscosity, [\(\eta\)], and molecular weight can be described as follows (Reynolds & Tanford, 1970):

\[
[\eta] = k M^n = k M^n \\
(1)
\]

\(^1\) Abbreviations used: OPB, octylpyridinium bromide; DePB, decylpyridinium bromide; DPB, dodecylpyridinium bromide; TPB, tetradecylpyridinium bromide; HPB, hexadecylpyridinium bromide; CMC, critical micelle concentration; ESR, electron spin resonance.
where \( k \) and \( k' \) and \( a \) are constants, \( M \) is molecular weight and \( n \) is the number of aminoacid residues per polypeptide chain. The exponent, \( a \), in equation 1 varies between 0.5 and 0.8 for random coils, above 1.0 indicates rodlike particles of various flexibilities, and in the range 0.3 - 0.4 indicates partially unfolded globular molecules (Tanford, 1963, Cwietkow et al., 1968).

![Graph showing specific viscosity as a function of log (mole detergent/mole protein)](image)

**Fig. 1.** Specific viscosity of bovine serum albumin-cationic detergent associates as a function of the detergent/protein molar mixing ratio. HPB (△); TPB (●); DPB (○); DePB (△); OPB (□).

**Table 1**

<table>
<thead>
<tr>
<th>Protein-alkylpyridinium bromide associate with</th>
<th>Protein component</th>
<th>molar mixing ratio</th>
<th>([\eta])</th>
<th>molar mixing ratio</th>
<th>([\eta])</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPB</td>
<td>Ribonuclease</td>
<td>16</td>
<td>4.6</td>
<td>48</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>Myoglobin</td>
<td>17</td>
<td>4.7</td>
<td>50</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>α-Chymotrypsin</td>
<td>22</td>
<td>5.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Chymotrypsinogen</td>
<td>—</td>
<td>—</td>
<td>64</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>Bovine serum albumin</td>
<td>68</td>
<td>6.8</td>
<td>204</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>Haemoglobin subunit</td>
<td>24</td>
<td>6.4</td>
<td>77</td>
<td>6.3</td>
</tr>
</tbody>
</table>

The \([\eta]\) values of protein-DPB or protein-TPB associates were approximately linear functions of \( \log M \) (Fig. 3). The slopes of the lines for the two detergents were the same and equal to 0.34. This indicates a similar molecular shape and partially unfolded structure of the examined proteins in both detergents. A similar plot for the native proteins, included for comparison, shows the lack of dependence of intrinsic viscosities on \( M \) for globular proteins.
Fig. 2. Reduced viscosity of myoglobin-cationic detergent associates: with TPB (●) and DPB (○) at concentrations of 3 \times 10^{-2} \text{ and } 10^{-3} \text{ M, respectively. The molar mixing ratio of TPB and DPB to protein were 17 and 50, respectively.}

Fig. 3. Log [\eta] plotted versus log of molecular weight of proteins. Protein-TPB associates (▲); protein-DPB associates (○); native protein (●): 1, ribonuclease; 2, myoglobin; 3, β-lactoglobulin; 4, trypsin; 5, chymotrypsin; 6, chymotrypsinogen; 7, haemoglobin; 8, bovine serum albumin; 9, catalase.

**Gel filtration.** The data for globular proteins can be linearized if plotted according to the empirical equation of Laurent & Killander (1964):

\[
(− \log K_{av})^{1/2} = A + BR_v
\]

where \(K_{av}\) is the partition coefficient, \(A\) and \(B\) are constants for the gel, \(R_v\) is the effective Stokes radius of the protein. \(R_v\) is defined in terms of the frictional coefficient, \(f = 6\pi\eta R_v\) (where \(\eta\) is viscosity of the solvent).
The gel filtration data for native globular proteins are presented in Fig. 4B. As can be seen, there are no significant deviations from the linearity of the plot with the exception of lysozyme. This is due to the known fact that lysozyme is specifically bound by dextran (Siegel & Monty, 1966). For DPB-protein associates, a typical elution profile is exemplified in Fig. 4A. Since the elution volume of Blue dextran is not changed in the presence of DPB, one can exclude changes in gel properties under our experimental conditions. The values of the Stokes radii of the protein-DPB associates determined from the calibration curves based on the gel-filtration data, are presented in Table 2. Addition of cationic detergent to the native protein, with the exception of ribonuclease and lysozyme, increased Stokes radii. Reduction and carboxymethylation of disulphide bonds in lysozyme and ribonuclease increased their Stokes radii from 21.0 Å to 25.0 Å and from 18.5 Å to 30.5 Å, respectively. Changes in $R_s$ of haemoglobin and aldolase were apparently small. The relation between determined $K_w$ values of the protein-DPB associates and log $M$ plotted according to the method of Andrews (1965), indicates that these proteins are eluted in DPB solution at a volume corresponding to the dimeric form of haemoglobin and to subunits of aldolase.
Table 2

Stokes radii of the protein-alkylypyridinium bromide associates

<table>
<thead>
<tr>
<th>Protein component</th>
<th>Native protein*</th>
<th>Protein-DPB associates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mol. wt.</td>
<td>Stokes radius (Å)</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>17 000</td>
<td>18.5</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>16 900</td>
<td>18.8</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>14 400</td>
<td>20.6</td>
</tr>
<tr>
<td>Trypsin</td>
<td>24 000</td>
<td>19.4</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>25 700</td>
<td>22.5</td>
</tr>
<tr>
<td>β-Lactoglobulin</td>
<td>35 000</td>
<td>27.4</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>45 000</td>
<td>27.5</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>68 000</td>
<td>35.0</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>68 000</td>
<td>31.0</td>
</tr>
<tr>
<td>Haemoglobin subunit</td>
<td>34 000</td>
<td>—</td>
</tr>
<tr>
<td>Aldolase</td>
<td>149 000</td>
<td>46.0</td>
</tr>
<tr>
<td>Aldolase subunit</td>
<td>40 000</td>
<td>—</td>
</tr>
</tbody>
</table>

* Siegel & Monty (1966), Tanford (1963), Darnall & Klotz (1975).

For extended polymers the relation between $R_s$ and molecular weight of polymer is expressed by the equation (Tanford, 1963; Nozaki et al., 1974):

$$R_s = kM^b$$

where $k$ and $b$ are constants. The exponent, $b$, in equation 3 is equal to 0.5 for random coils, varies between 0.7-0.9 for rod-like particles and is about 0.33 for unhydrated globular spheres.

The relationship between log $b$ and log $M$ presented in Fig. 5 is linear for both native proteins and protein-detergent associates. The slopes of the curves give values of $b=0.44$ for native protein and $b=0.63$ for protein in DPB solution. This indicates partially unfolded structure of protein in DPB solution. For reduced and carboxymethylated proteins in DPB solution the slope of the curve is equal to 0.71 and indicates that these associates can be approximated as rod-like particles with some flexibility.

Spin labelling. As can be estimated from the ESR spectrum (Fig. 6) in the spin-labelled bovine serum albumin, approximately one molecule of label is attached per molecule of the protein. Addition of alkylpyridinium bromides to the labelled albumin caused changes proportional to the length of the hydrocarbon chain of the detergent. Narrowing of the line width and an increase of the maximum of spectral lines of labelled bovine serum albumin as a function of concentration of the cationic detergent is due to the increase in molecular tumbling of the spin label, caused by a change in the protein structure in the vicinity of the label. This can be quantitatively expressed by calculating the square root of the ratio of intensities of the lines $M$ to $H$ (Fig. 6; Berliner, 1972).
Fig. 5. Stokes radius as a function of molecular weight of proteins. Native proteins (○); protein-DPB associates (□); reduced, carboxymethylated protein-DPB associates (□). All proteins were dissolved in phosphate buffer; DPB concentration was $10^{-2}$ M. 1. Ribonuclease; 2. myoglobin; 3. trypsin; 4. chymotrypsin; 5. $\beta$-lactoglobulin; 6. haemoglobin; 7. ovalbumin; 8. aldolase; 9. bovine serum albumin; 10. lysozyme.

Fig. 6. ESR spectrum of spin-labelled bovine serum albumin in phosphate buffer, pH 6.8, ionic strength 0.1. Protein concentration $6.35 \times 10^{-3}$ M. $M$ is the middle field component, $H$ is the high field component.
The change in $(M/H)^{1/2}$ as a function of concentration of OPB, DePB, DPB, TPB and HPB in phosphate buffer, pH 6.8, ionic strength 0.1, is given in Fig. 7. When the detergent concentration increases to a detergent-protein molar mixing ratio equal to 3, 9, 18, 180 and 220 for HPB, TPB, DPB, DePB and OPB, respectively, $(M/H)^{1/2}$ slightly increases. The increase of $(M/H)^{1/2}$ indicates a small decrease in the average motion of spin-labelled amino groups of native bovine serum albumin, related to a slight folding of the protein moiety. Further increase in concentration of alkylpyridinium bromides up to concentrations approximately equalling their CMC values causes a large decrease in $(M/H)^{1/2}$ which indicates a considerable increase in the average motion of the spin label. This increase results from a significant unfolding of the protein molecule. Above CMC values, $(M/H)^{1/2}$ increases, particularly in the case of HPB and TPB, suggesting a small decrease in the average motion of the spin label. This decrease could be caused by interaction of the detergent micelles with protein or by association of protein chains.

![Graph](image)

Fig. 7. $(M/H)^{1/2}$ value of spin-labelled bovine serum albumin in phosphate buffer, pH 6.8, ionic strength 0.1, as a function of alkylpyridinium bromide concentrations: HPB (○); TPB (△); DPB (●); DePB (□); OPB (□). Protein concentration $2.2 \times 10^{-5}$ M.

As illustrated in Fig. 8, similar changes in the spin-labelled native bovine serum albumin were observed on addition of alkylpyridinium bromides in citrate buffer, pH 3.4, ionic strength 0.1. At this pH the net charge of the protein changes from negative to positive. If binding of the cationic detergent with protein were of an electrostatic character, the average motion of spin label should decrease remarkably at acidic pH. The results presented in Fig. 8 show that $(M/H)^{1/2}$ decreased when the molar mixing ratios of serum albumin and HPB, TPB, DPB and DePB increased from 1.3, 3.3, 12.0 and 104 to their respective CMC values. At pH 3.4 reorganization of the protein structure detected by the change in $(M/H)^{1/2}$ took place at a slightly lower detergent-protein molar mixing ratio than at pH 6.8 (Figs. 7 and 8).
Fig. 8. The \((M/H)^{1/2}\) value of spin-labelled, reduced and carboxymethylated bovine serum albumin in phosphate buffer, pH 6.8, ionic strength 0.1, as a function of alkylpyridinium bromide concentration: TBP (+); DPB (●); DePB (□). Protein concentration \(1 \times 10^{-4}\) M.

Fig. 9. The \((M/H)^{1/2}\) value of spin-labelled bovine serum albumin in phosphate-citrate buffer, pH 3.4, ionic strength 0.1, as a function of alkylpyridinium bromide concentration; HPB (○): TBP (+); DPB (●); DePB (□). Protein concentration \(3.37 \times 10^{-5}\) M.
When disulphide bonds of bovine serum albumin were reduced and carboxymethylated, addition of alkylpyridinium bromides caused folding of the protein molecule. As can be seen in Fig. 9, the increase of TPB concentration in the solutions of spin-labelled and reduced proteins from a molar mixing ratio of about 39 up to the CMC value increased the \((M/H)^{1/2}\) value. This change indicates a decrease in the average motion of spin label resulting from folding of the protein molecule. The increase in TPB concentration above CMC values caused a small increase in the average motion of the label. Similar changes in \((M/H)^{1/2}\) were observed in the presence of DBP and DePB, which induce significant folding of the protein molecule when the detergent-protein molar mixing ratios increase from 117 and 780, respectively, up to the CMC values.

DISCUSSION

The equilibrium dialysis study on binding of alkylpyridinium bromides to native serum albumin indicates 10, 13, 20 and 33 readily accessible binding sites for OPB, DePB, DBP and TPB, respectively, at low detergent concentration and at pH 6.8 and 25°C (Wasylewski, 1979a). The average number of detergent molecules bound to the protein was similar for OPB and DePB and increased with length of the hydrocarbon chain.

The changes in specific viscosity and in ESR spectra on addition of alkylpyridinium bromides to the solution of native albumin indicate a significant change in the protein conformation at the detergent concentrations exceeding those required for the binding to the readily accessible binding sites in the protein molecule. The extent of this change is similar for OPB and DePB and increases with the length of the alkyl chain of the detergent.

It should be noted that changes in conformation of the native albumin occur at higher concentration of cationic than of anionic detergents of the same hydrocarbon chain length (Reynolds et al., 1967; Wasylewski & Pasenkiewicz, 1977).

Intrinsic viscosity values of the proteins tested in TPB and DBP solution indicate that both detergent-associates have a similar shape. They also indicate, in agreement with the gel filtration data, partial unfolding of the globular protein molecules.

When alkylpyridinium bromides were added to the reduced and spin-labelled bovine serum albumin, changes in ESR spectra were opposite to those observed for native protein. The restriction of spin label motion can result from the folding of protein molecules.

Gel filtration data indicate that reduction of disulphide bonds in ribonuclease and lysozyme results in the increase of the Stokes radii of these proteins. The plot of log \(R_\theta\) versus log \(M\) for reduced ribonuclease and lysozyme, and also for aldolase and myoglobin (myoglobin and aldolase have no disulphide bonds, so the reduction processes were omitted) is essentially the same as for the reduced proteins in sodium
dodecyl sulphate and tetradecyltrimethylammonium chloride (Nozaki et al., 1974). These results indicate that conformation of these associates can be approximated as rod-like particles of some flexibility. This is in agreement with our observations on the influence of TPB on reduced and carboxymethylated proteins studied by Fourier transform infrared spectroscopy which showed that cationic detergent promotes formation of α-helix structure in the modified proteins (unpublished).

The authors wish to thank Prof Dr. Maria Sarnecka-Keller for helpful discussion.

REFERENCES


ODDZIAŁYWANIA BIAŁKA - DETERGENTY KATIONOWE

BADANIE ODDZIAŁYWAN BROMKÓW ALKILOPIRYDYNIOWYCH Z ALBUMINĄ SUROWICY WOŁU I INNYMI BIAŁKAMI PRZY POMOCY POMIARÓW LEPKOŚCI, FILTRACJI NA ŻELU ORAZ METODY ZNAKOWANIA SPINOWEGO

SREŚCZENIE

Wpływ bromków alkilopirydyniowych na konformację albuminy surowicy wołu i innych białek badano przy pomocy metody wiskozymetrycznej, filtracji na żelu oraz znakowania spinowego. Stwierdzono częściowe rozwinięcie cząsteczek natywnych białek pod wpływem kationowych detergentów. Wielkość tych zmian wzrasta ze wzrostem długości łańcucha węglowodorowego detergentu. Dodanie kationowych detergentów do roztworu albuminy z uprzednio zredukowanymi i karboksylowymi wiązaniami dwusiarzkowymi wywołuje przeciwwstawne zmiany aniżeli ma to miejsce w przypadku natywnego białka.

Received 10 October, 1978.