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Interaction of allylisothiocyanate with bovine serum albumin

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Abstract. The interaction of allylisothiocyanate with bovine serum albumin was monitored by fluorescence titration. The interaction was weak with an apparent association constant of 2×10^2. The interaction was unaffected in the pH range of 5·0 to 8·3 and by NaCl. However, the addition of dioxane upto 4% increased the value of the association constant. N-Methyl bovine serum albumin and bovine serum albumin with sulphydryl groups blocked had the same affinity for allylisothiocyanate suggesting that amino and sulphydryl groups may not be involved in the interaction. Polyacrylamide gel electrophoresis and estimation of available lysine suggested that there were perhaps two types of groups involved in the interaction of allylisothiocyanate with bovine serum albumin.

Keywords. Serum albumin; allylisothiocyanate interaction.

Introduction

Glucosinolates (thioglucosides) occur in the seeds of *Brassica* species. On hydrolysis, they yield isothiocyanates, and nitriles which are toxic (Josefsson, 1972). Sinigrin (allylglucosinolate) occurring in the seeds of *Brassica juncea* (Indian mustard) yields, on hydrolysis, allylisothiocyanate.

In an effort to understand the nature of interaction of glucosinolates and isothiocyanates with proteins, the interaction of allylisothiocyanate (AIT) with bovine serum albumin (BSA) was studied. BSA was chosen for the following reasons : (1) it was difficult to prepare mustard protein fractions free from glucosinolates/isothiocyanates ; (2) considerable information is available on the chemical and physico-chemical properties of BSA and (3) BSA is known to interact with a variety of small molecules (Steinhardt and Reynolds, 1969).

Materials and methods

Materials

Bovine serum albumin, N-ethylmaleimide and tris-buffer were from Sigma Chemical Co., St. Louis, MO., USA and AIT from Fluka AG, Buchs, Switzerland

Abbreviations used : allylisothiocyanate, AIT; bovine serum albumin, BSA.

All the chemicals used were of the analytical reagent quality. AIT was distilled and the fraction distilling at 148° C was collected and used.

Protein concentration

This was determined by measuring the absorbance of the protein solution at 279 mm and using a value of 6 · 67 for $E_{1cm}^{1\%}$ (Foster and Sterman, 1956).

Allylisothiocyanate concentration

The concentration of a stock solution of AIT in ethanol was determined by measuring the absorbance at 240 mm and using a molar extinction coefficient of 770 (Schwimner, 1961).

Ainino group modification

Amino groups of the protein were modified by reductive methylation using aqueous formaldehyde and sodium borohydride (Means and Feeney, 1968). The modified protein was dialysed extensively against water and diluted with 0·01M phosphate buffer, pH 7·5, to a final concentration of 0·8 μM. The concentration of the dialysed solution was determined by absorbance measurement at 279 mm and using the same value of $E_{1cm}^{1\%}$ as for BSA.

Sulphydryl group modification

Sulphydryl group was blocked by the addition of N-ethylmaleimide (0·3 mM) to BSA solution (3 μM) in 0·01M phosphate buffer of pH 7·5 and keeping the solution for 2 h at room temperature before use.

Absorption spectra

The absorption spectrum of the protein with and without added AIT was recorded in the range 200-300 mm with a Perkin-Elmer double beam spectrophotometer (model 124). Measurements were made at pH 7·5.

Fluorescence measurement

The measurements were made in an air-conditioned room where the ambient temperature was ~21°C with Perkin-Elmer fluorescence spectrophotometer (model 203). To 3 ml of 0·8 μM BSA solution, varying amounts of AIT solution (in absolute ethanol) were added with an Agla micrometer syringe, stirred well and fluorescence measured. The maximum volume added was 0·1 ml and added AIT concentration varied from 0·5 mM to 8mM. The increase in volume was only 3% and no corrections were applied. The blanks were 3 ml of 0·8 μM BSA containing corresponding volume of absolute ethanol. The excitation wavelength was 280 nm and emission spectrum was recorded in the range 300-400 nm.

The measurements were made at different pH values in the range 5·0 to 8·3. The buffers used were acetic acid-sodium acetate, pH 5·0 ; sodium phosphate,

pH 6·0-7·5 and tris-glycine, pH.8·3. The concentration of the buffer was 0·01 M. The effect of NaCl (0-1 M) and dioxane (0-20%) was also studied.

Electrophoresis

Polyacrylamide gel electrophoresis using 7·5% gels was carried out in 0·01 M phosphate buffer, pH 7·5, at a constant current of 3 mA per tube for 90 min. The gels were stained for 30 min in 0·5% Amido Black solution in 7% acetic acid and were destained in 7% acetic acid solution.

Estimation of available lysine

This was estimated by a modification of Carpenter's procedure (Carpenter and Ellinger, 1955).

Results and discussion

The fluorescence quenching method has been used for following protein-small molecule interactions (Chen *et al.,* 1969 ; Steiner *et al.,* 1966 ; Kelly *et al.,* 1976 ; Reddy and Rao, 1977 : Evrain *et al.,* 1978). BSA gives an emission spectrum with a maximum at 335 mm. Addition of AIT quenches the fluorescence, the extent of quenching increasing with AIT concentration (figure 1). There was no

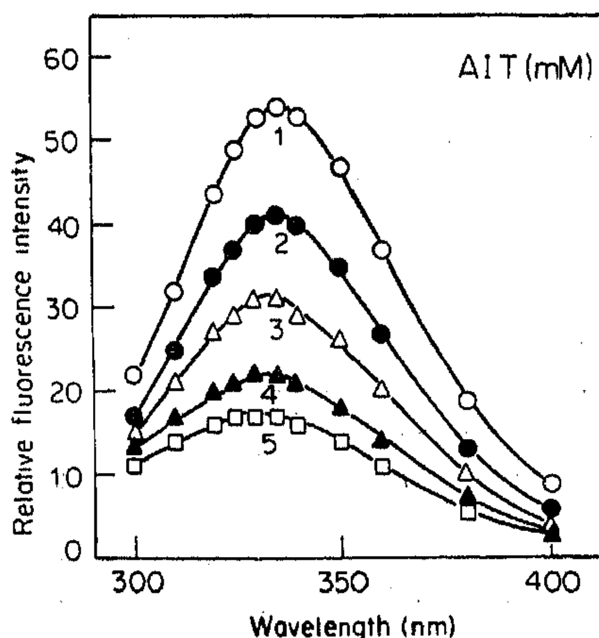

Figure 1. Fluorescence emission spectra of BSA. To a solution of BSA (0·8 μM) in 0·01 M phosphate buffer, pH 7·5)· 0, 1, 2, 3, 4 mM AIT was added and the fluorescence spectra corresponding to 1, 2, 3, 4 and 5 were recorded.

marked shift in the fluorescence maximum. The fluorescence quenching was found to be reversible (figure 1). These experiments were performed in the following way. A solution of BSA which contained 4 mM AIT was diluted with different volumes of the same concentration of protein solution and the spectra recorded. These were compared with the spectra of BSA containing the same concentration of AIT. There was good agreement between the direct and reversed spectra.

The binding curve, ΔF as a function of AIT concentration, $[A]$, is given in figure 2 A; here ΔF is the difference in fluorescence intensity. A Scatchard plot of $\Delta F/[A]$ vs ΔF gives a straight line (figure 2B, inset). From the slope, which gives the apparent association constant K_a and the intercept on the ordinate which corresponds to the product of association constant and the total number of binding sites, ΔF can be calculated as a function of $[A]$. The calculated curve is superimposable on the experimental curve in figure 2A.

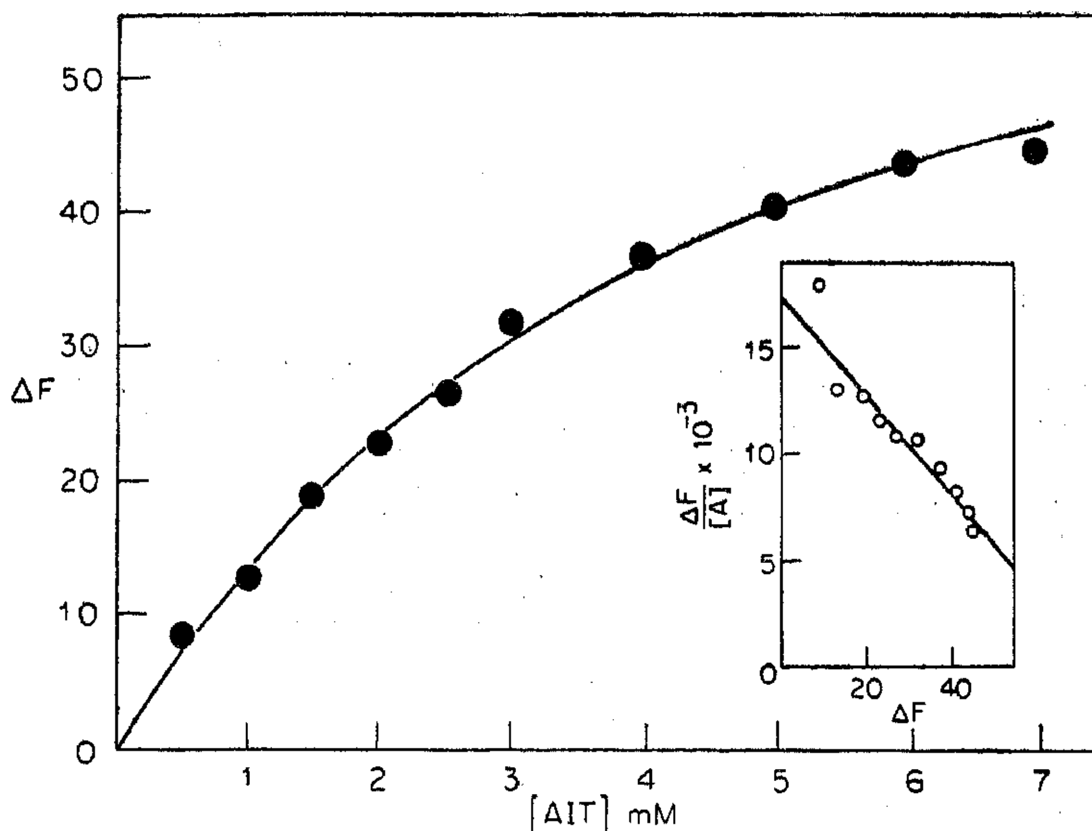

Figure 2A. Binding of AIT by BSA;
B. (Inset)—Scatchard plot of the binding data.

The apparent association constant has a value of 2×10^2. The interaction is obviously of a weak type. Scatchard *et al.* (1957) have reported that the association constant for the interaction of KCNS with BSA has a value of $1{\cdot}9\times10^3$ and also three classes of sites are involved. From the type of analysis described above, it is not possible to state, if interaction of BSA with AIT involves more than one type of site. Equilibrium dialysis experiments which can provide such information could not be performed because of the volatile nature of AIT.

Measurements at different pH values in the range 5 to 8·3 indicated that pH had no effect on binding as indicated by the values between $1{\cdot}9$-$2{\cdot}5\times10^2$. The apparent association constant was nearly the same indicating that the interaction was pH-independent. Also, addition of NaCl upto 1 M had no effect. Thus the interaction did not seem to be ionic in nature.

The effect of dioxane was also studied to determine if dielectric constant of the medium had any effect. Addition of dioxane upto 4% increased the association constant; above this concentration, the value decreased and at 20% dioxane it was the same as in the absence of dioxane (figure 3). This could be due to the effect of decrease in dielectric constant on the ionisation of amino groups and

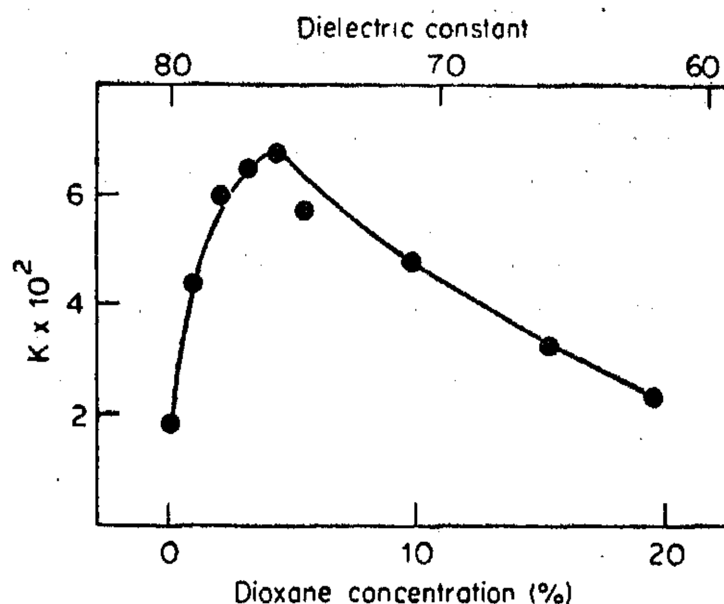

Figure 3. Variation of K_a with dioxane concentration. The values of dielectric constant of the dioxane-water mixture were taken from Akerlof and Short (1936).

on ion-pair formation (Singer, 1962). It is difficult to evaluate the interplay of these two factors. Decrease in dielectric constant may be expected to lead to an increase in pK of amino groups (Edsall and Wyman, 1958) and thus to a decrease in interaction. However, the observed increase in the association constant may be due to more favoured thiocarbamyl formation.

In an effort to determine the nature of the binding site on BSA molecule, experiments were also done with chemically modified BSA.

Drobnica and Gemeiner (1976) have reported that the affinity of phenylisothiocyanate for -SH groups is 400-4000 times greater than that for the amino groups at alkaline pH values. The -SH group of BSA was blocked by N-ethylmaleimide and the measurements were made at pH 7·5. Fluorescence quenching by different concentrations of AIT was the same as obtained with the unmodified protein. However, it cannot be concluded from this that -SH groups are not involved in the interaction. BSA contains only 0·6-0·7 -SH group per molecule (Benesch and Benesch, 1948) and because of the low content, possibly no measurable, difference in quenching could be observed.

Measurements were made with N-methylated BSA where the amino groups of lysine residues had been methylated to different extents. All the derivatives gave association constant values in the range 2·0 to 2·4 $\times 10^2$. Thus, the affinity of the amino group-modified protein for AIT was the same. This would suggest that the amino groups are perhaps not involved in the interaction.

Phenylisothiocyanate reacts with proteins to form phenylthiocarbamyl derivatives which in aqueous acid solutions give phenylthiohydantoins (Edman, 1970). Drobnica and Gemeiner (1976) have suggested that other organic iosthiocyanates may also form thiocarbamyl derivatives with proteins and these are fairly stable. The lysine groups available for interaction with fluorodinitrobenzene (FDNB), after interaction with AIT, were estimated. As the ratio of AIT to BSA increased, the number of groups available for interaction with FDNB decreased (table 1).

These results would suggest that AIT interacts with the amino groups of the lysine residues. This apparently contradicts the results of measurements with

N-methyl BSA; this derivative has the same affinity for AIT as the unmodified derivative. Since at pH 8·3 (and below) the ammo group of the mono- or dimethyl BSA may still carry a proton, thiocarbamyl formation would be possible and thus there may not be any difference in affinity for AIT.

Other measurements also suggest the possibility of thiocarbamyl formation This reaction is pH-dependent in the alkaline region (Edman, 1970). When the same ratio of AIT to BSA was used at pH 7·5 and pH 10·0, the available lysine content of the latter was much less (table 1). Polyacrylamide gel electrophoresis

Table 1. Reduction in available lysine of BSA-interacted with AIT.

Composition	pH	Available lysine (g/100 g protein)	Reduction (%)
BSA	7·5	11·0	..
BSA and AIT (1 : 0·2)	7·5	9·2	16
BSA and AIT (1 : 3)	7·5	5·8	47
BSA and AIT (1 : 6)	7·5	3·9	65
BSA and AIT (1 : 0·2)	10·0	5·6	49

The numbers in parentheses indicate the ratio of BSA to AIT (w/w).

of BSA treated with AIT showed greater (negative) mobility than BSA at pH 7·5 (figures 4 B and C). BSA has one major band and 3 minor bands. As judged by the intensity of the bands compared to those bands in BSA, AIT-treated BSA

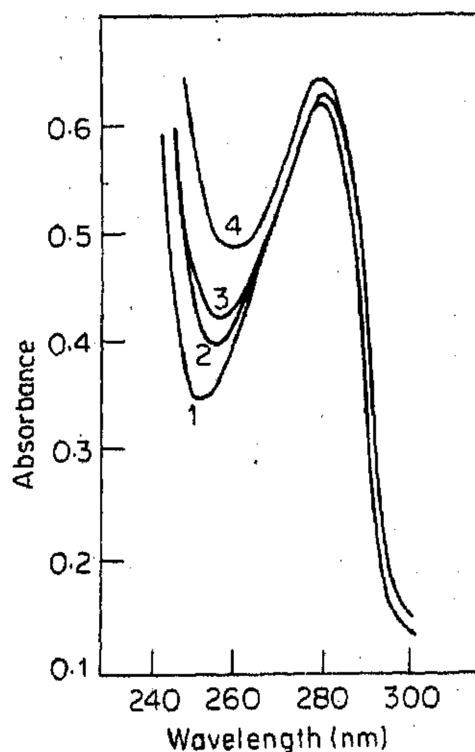

Figure 5. Absorption spectra of BSA.
The spectra were recorded in 0·01 M phosphate buffer, pH 7·5, at a BSA concentration of 13 μM. The concentrations of AIT indicated (1—0·0, 2—0·1; 3—0·2 and 4—0·4mM) were added and spectra taken.

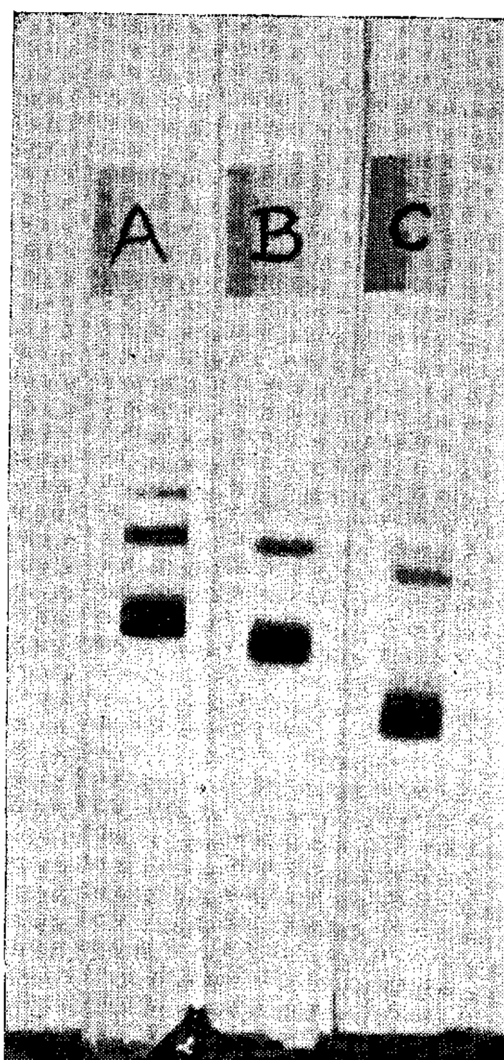

Figure 4. Polyacrylamide gel electrophoresis of BSA. The electrophoresis was performed in 0·01 M phosphate buffer, pH 7·5.

Amount of protein used = 120 μg.

A. BSA

B. AIT-treated BSA (reduction in available lysine—16%).

C. AIT-treated BSA (reduction in available lysine—49%).

has four bands in the same proportion. But all the bands showed higher mobility. This could be due to reduction in the positive charge which, at this pH, is contributed mostly by amino and guanidinyl groups. To our knowledge, there are no reports of interaction of AIT with guanidinyl groups. Thiocarbamyl formation results from the removal of proton from amino groups and thus could lead to greater negative charge.

The adducts of organic isothiocyanates with -SH groups give a characteristic absorption in the UV region at 252 and 277 nm (Drobnica and Gemeiner, 1976). The absorption spectrum of BSA in the presence of AIT did not reveal the characteristic peaks at 252 nm (figure 5) suggesting that the interaction did not involve -SH group. However, the absorption minimum of BSA at 250 nm showed a red shift.

Estimation of available lysine and Polyacrylamide gel electrophoresis expermerits suggests the involvement of amino groups in the interaction of AIT with BSA. The thiocarbamyl derivative appears to be fairly stable and is not decomposed even when it is dialysed extensively against distilled water. However, fluorescence quenching is reversed by dilution (figure 1). This may suggest that two different reactions are occurring, one thiocarbamyl formation with lysine and the other a reversible complex formation with some other group. Our results seem to suggest that lysine modification is not monitored by fluorescence. This is supported by the observation that N-methylated BSA, where lysine amino groups have been modified, gives the same fluorescence spectrum as BSA.

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