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Interaction of acrylamide with proteins in the concentration range used for fluorescence quenching studies

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Abstract

^{14}C labelled acrylamide was synthesized and used in equilibrium dialysis measurements to study the binding of acrylamide to the proteins: human serum albumin (HSA), ovalbumin and cod parvalbumin III. Our intent was to determine whether binding takes place in the concentration range that is used for the study of fluorescence quenching by acrylamide. In contrast to previously published reports, we found that all the proteins investigated did bind acrylamide. The affinity of this interaction, when interpreted in terms of multiple independent binding equilibria, was very low, $K_{ass} \approx 0.5\text{–}2\ M^{-1}$; however, due to multiple binding sites, a considerable amount of acrylamide is to be found in the protein phase at concentrations used for quenching experiments. The number of binding sites seems to vary with the protein. A pH 7.0 the binding to the 69 kD HSA corresponds to 14 ± 8 sites, the binding to the 45 kD ovalbumin corresponds to 40 ± 25 sites, whereas for the 11 kD cod parvalbumin the binding corresponds to only a few sites. The binding is very sensitive to pH and to the presence of cosolvents such as glycerol. At pH 5.2, close to the isoionic point, the number of binding sites for acrylamide on HSA increases to > 100. The very weak binding justifies an alternative description of the phenomena as a distribution equilibrium between two phases. In such a model we see that the formal concentration of acrylamide in the protein volume is in some cases higher than in solution ($K_{eq} = 1.2$ for HSA at pH 5.2). These findings suggest that any model describing the quenching of fluorescence in proteins by acrylamide has to account for the presence of two pools of acrylamide and consequently for the presence of multiple modes of quenching.

Keywords: Human serum albumin; Ovalbumin; Parvalbumin; Acrylamide binding; Equilibrium dialysis

1. Introduction

Quenching of the native fluorescence of proteins by acrylamide is widely used as a tool in the study of structural fluctuations of proteins or as a measure of the accessibility of indoles in the protein matrix to solvent [1]. Acrylamide quenching of the fluorescence of small indole compounds is probably not entirely collisional but involves the formation of a charge transfer complex [1–3]. The processes leading from encounter pair to charge transfer complex are, however,

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very fast and the reaction is shown to be viscosity limited [4].

In order to explain the observed quenching of buried tryptophan residues in proteins by acrylamide we have to postulate the existence of a mechanism providing access to acrylamide molecules, because encounter pairs and charge transfer complexes involve physical contact between the fluorophore in the excited state and the quencher molecule. A simple and attractive model is represented by a gating mechanism, where structural movements of the protein transiently expose the indole residue to quencher trajectories in the bulk solvent. Such a mechanism has been suggested [5] and has received support from findings that no or very little correlation can be found between the size and efficiency of different quencher molecules [6,7]. The alternative explanation, quencher penetration into the protein matrix, modeled after the mechanism of oxygen quenching and penetration [2,8], has received support from the observed absence of viscosity effects predicted for a gating process [9,10].

It follows from the basic tenets of a penetration mechanism that some concentration of acrylamide, however small, has to be present in the protein phase. The experimental evidence pertinent to this point is, unfortunately, contradictory. Reports of considerable binding of acrylamide to HSA, visualized by equilibrium dialysis experiments [11], could not be reproduced [12] and the latter authors concluded that no binding of acrylamide to HSA takes place. Our interest in this problem stems from our study of protein fluctuations by isotope exchange at the indole residue quenched by acrylamide [13]. Reconsidering the problem of acrylamide binding we were reminded that the static quenching of tryptophan fluorescence in HSA by acrylamide when interpreted in terms of a binding equilibrium [14,15] gives us a binding constant of 3–30 M^{-1}. Consequently, in order to observe excess acrylamide in the protein phase after dialysis equilibrium has been reached, we have to use very high concentrations of protein and acrylamide, radioactively labelled. Experiments of this type have not been carried out previously. We used a microdialysis technique suitable for high protein concentrations, and synthesized ^{14}C labelled acrylamide for a detailed study of acrylamide binding to HSA and ovalbumin. We also carried out some experiments with cod III parvalbumin. We show that, indeed, a weak binding of acrylamide takes place in all of these proteins and, due to the high capacity for acrylamide, respectable amounts are found in the protein phase at acrylamide concentrations corresponding to those used in quenching studies. We also show that the presence of high concentrations of glycerol can increase the amount of acrylamide in the protein phase for some proteins. If the excess of acrylamide found in the protein phase is modeled as bound to definite sites we can calculate affinity constants and numbers of binding sites; however, the binding data show such a wide distribution of affinities that the data for HSA are best interpreted in terms of a distribution of acrylamide between two phases, the protein and the solution phases.

2. Materials and methods

2.1. Materials

Human serum albumin (fatty acid free), Dextran T70 and T40, glycerol and acrylamide (electrophoresis grade) were all purchased from Sigma Inc., St. Louis, MO. Ovalbumin (two times crystallized) was obtained from Worthington Inc. Glycine-UL-^{14}C was also from Sigma Inc. ^{14}C labelled acrylamide was prepared from acrylonitrile-1-^{14}C (Sigma Inc.) by reaction with sulfuric acid monohydrate, followed by neutralization with solid sodium carbonate and extraction with methanol [16]. Acrylamide-1-^{14}C was then recovered by evaporation at reduced pressure and recrystallization (2 ×) from acetone. Melting point 83–84°C. After mixing with "cold" acrylamide it was recrystallized once more. Specific activity of the preparation was 1.37 $\mu Ci/mM$. All buffer reagents were of analytical grade and the water was glass distilled.

2.2. Methods

2.2.1. Equilibrium dialysis

For the equilibrium dialysis studies we chose a micromethod where we equilibrate 400 μl of protein solution with 200 μl of ligand solution [17].

Such a micro dialysis cell represents an equilibrium between three compartments, the two solution sides and the volume of the membrane acting as a third compartment. Consequently, the method for determining acrylamide concentration has to be able to measure acrylamide on both sides, especially in the presence of protein. We synthesized radioactively labelled acrylamide which allows the determination of acrylamide concentration with an error of $< 1\%$.

When calculating the binding, another problem presents itself in the form of a change of the free volume available to acrylamide due to the presence of large amounts of macromolecules. This excluded volume represents, in the case of 10% HSA, about 7% of the total volume. It is the concentration of acrylamide in the free volume that is, at equilibrium, equal to the acrylamide concentration in the protein free compartment. This volume exclusion leads, if no acrylamide is bound to the protein, to a situation where at equilibrium the apparent concentration of acrylamide is predicted to be lower on the protein side. For a correction accounting for the excluded volume, we have to know the specific volume of the protein and the effect of glycerol on the specific volume. We used published specific volume measurements for HSA and ovalbumin [18]. For the glycerol effect we used data for BSA [19] and for ovalbumin we determined the effect in this laboratory. The different values used are compiled in Table 1. We have two additional corrections to consider. First let us address the problem of Donnan equilibrium and effects of ionic strength. In view of the known binding of ions by HSA, the possibility of producing high ionic strength solutions without binding some of the buffer ions is difficult to realize. We minimized this problem by carrying out our measurements in phosphate buffer, known to show little binding [20] and in the presence of added glycine. We determined the binding of glycine to HSA by a separate experiment using radioactively labelled glycine.

Table 1

The specific volume (V, in ml/g) at 25°C for HSA, BSA and ovalbumin

Solution	HSA, pH 7.0	BSA, pH 7.0	Ovalbumin, pH 7.0
Buffer	0.733 [a]	0.734 [a]	0.748, 0.748 [a]
Buffer + 30% glycerol		0.729 [b]	
Buffer + 40% glycerol		0.727 [b]	
Buffer + 50% glycerol			0.736

[a] From Kuntz and Kauzmann [18].
[b] From Gekko and Morikawa [19].

The second correction is due to osmotic pressure effects. The relative volumes on both sides of the membrane are not the same at equilibrium as they were at the beginning of the experiment. This changes the final concentration of the protein and influences the calculation of the number of moles bound per protein molecule. We can compensate for that by a determination of the protein concentration in the beginning and at the end of a separate experiment carried out in the absence of acrylamide. We have corrected all of our values in this fashion. In order to check our volume corrections, we also carried out a series of binding experiments under identical conditions as before, but in the presence of Dextran T-70 for HSA and T-40 for ovalbumin in the protein free compartment. We adjusted the Dextran concentration as to be equiosmolar to the protein containing solution. In such experiments we found no change in protein concentration at the beginning of the experiment or at equilibrium. We have to realize of course that the introduction of a macromolecule to the other side introduces an excluded volume effect on that side. The excluded volume of Dextran is somewhat different from the volume of the protein studied [21], 0.6 ml/g as compared to 0.73 for HSA, thus we expect a small but visible shift in the binding curves due to the presence of Dextran.

Taking into consideration the problems discussed above we carried out the dialysis experiments as follows. The cells were made from commercially available 1.5 ml Eppendorf centrifuge

tubes. The membrane was Spectrapore 2 from Fischer. Before use it was soaked, first in distilled water, then in the appropriate buffer. For the experiment 200 μl of the acrylamide solution was introduced into the inverted cap of the tube, covered with the membrane (1.5 × 1.5 cm) and then closed with the tube itself. The bottom of the tube had a hole in it through which we now introduced 400μl of the protein solution. We closed the hole with parafilm and placed the tube with others on an Orbitron rotator. The solution was then equilibrated at room temperature, 22 ± 1°C up to 4 days. The time course of equilibration was determined in experiments using HSA, and 0.3 *M* acrylamide. The time to reach equilibrium in systems containing buffer only was 24 hours whereas, as seen in Fig. 1, in 50% glycerol the necessary time had stretched to 80 hours. The concentration of acrylamide on both sides of the equilibration vessel was determined by withdrawing 100 μl of the solution. The volume of the sample delivered was also controlled by weighing. The sample was transferred to a scintillation vial, mixed with 10 ml toluene based scintillation liquid and then counted in a Beckman LS-7500 scintillation counter with the appropriate quenching corrections applied. Every point on our graphs is the average of 5–10 independent experiments. The specific activity of the acrylamide was checked for each experimental series. The protein concentration in these experiments was 1.8 $\times 10^{-3}$ *M* equal to 120–130 mg/ml for HSA and 80 mg/ml for ovalbumin. The proteins are easily soluble at these concentrations and, even in 50% glycerol, show no apparent increase in light scattering. Still, the high protein concentration presents a problem with regard to possible dimerization. Thus the moles of acrylamide bound per mole protein strictly represents the preferentially interacting acrylamide as moles per 69000 g of HSA and 45000 g of ovalbumin. In our case we cannot determine the possible effect of dimerization by the customary way of carrying out experiments at a range of protein concentrations, because the dialysis method is not sensitive enough outside the concentration window used.

2.2.2. *Calculations*

At equilibrium the following relations hold: if we added volume A of labelled acrylamide to either side of the dialysis cell and the factor converting CPM (counts per minute) to concentration is F, we can write a material balance

$$AF(\mathrm{CPM}_0) = BF(\mathrm{CPM}_1) + CF(\mathrm{CPM}_2) + DF(\mathrm{CPM}_3) \quad (1)$$

CPM_0 is the count in the acrylamide solution added: B, C and D stand for the volumes of the compartments of the dialysis cell at equilibrium. The compartment B contains protein, C is without protein and D represents the membrane compartment. CPM_1, CPM_2 and CPM_3 represent the

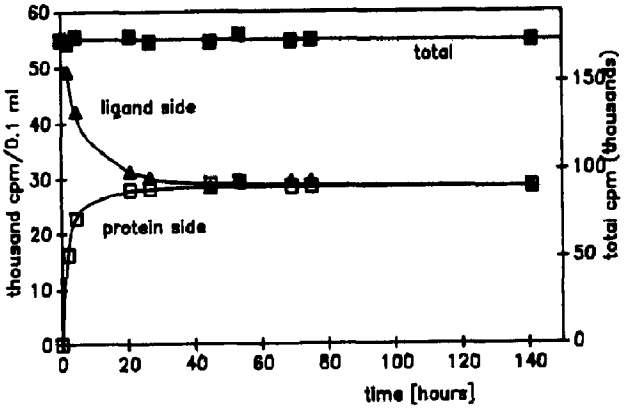

Fig. 1. The time course of the equilibrium of HSA–acrylamide binding determined with 0.3 *M* acrylamide, 13% HSA in 50% (w/w) glycerol, 0.10 *M* glycine containing phosphate buffer, pH 7.0 at 25°C. The error bars are smaller than the size of the symbols.

counts in the respective compartments. By comparing the sum $B(\mathrm{CPM}_1) + C(\mathrm{CPM}_2)$ to $A(\mathrm{CPM}_0)$, as seen in Fig. 1, we can rapidly eliminate the contribution, if any, of membrane binding.

We also have to remember that the volumes B and C are the corrected volumes, corrected for transfer of solvent due to osmotic differences. We found in experiments with 12% HSA but without Dextran a difference of $5.7 \pm 1\%$ in the volumes B and C at equilibrium. No difference was found in the presence of Dextran. The equilibrium is defined by equal activity of the acrylamide in compartments B and C. In compartment C in the absence of Dextran we can set the activity of acrylamide equal to concentration. For compartment B we have to consider one additional effect. The free acrylamide molecules are excluded from the volume occupied by the protein, V_{ex}. The true activity of acrylamide in compartment B is thus CPM_1'

$$BF(\mathrm{CPM}_1) = (B - V_{ex})F(\mathrm{CPM}_1') \qquad (2)$$

It is this CPM_1' that at equilibrium is equal to CPM_2, provided no binding to protein occurs. If, as in our experiments, CPM_3 is negligible, the excess of counts on the protein side can be determined by two different ways of subtraction. First, we can express it as a difference between the counts in compartments B and C. We can alternatively determine the difference between the measured counts in compartment B and the expected value for that compartment derived from CPM_0. The latter method has the advantage that CPM_0 can be determined as an average of multiple samples, whereas compartment C usually yields only one sample. In both cases we measure the difference between two independently determined activities. The use of CPM_0 in our case resulted in a somewhat reduced scatter of data.

We can eliminate F on both sides and write

$$(\mathrm{CPM}_1') = B/(B - V_{ex})(\mathrm{CPM}_1) \qquad (3)$$

$$(\mathrm{CPM}_1') - (\mathrm{CPM}_2) = 0$$

if no acrylamide binds to protein. (4)

$$(\mathrm{CPM}_1') - (\mathrm{CPM}_2) = \Delta\mathrm{CPM}$$

if acrylamide binds to protein. (5)

ΔCPM is the excess count representing acrylamide concentration due to binding. If we now assume that this excess is due to acrylamide binding to a number of defined sites we can write

$$\nu = (\Delta CPM)FB/P \qquad (6)$$

where P is protein concentration in grams or moles per unit volume and ν is the number of molecules bound per gram or mole.

2.2.3. Specific volume determinations

To correct for the excluded volume effect in calculating CPM_1' in eq. (3), we need the specific volume data for the proteins used. In addition, changes in protein volume affect the chemical activity of acrylamide residing there, thus influencing the value of CPM. Consequently, we also need to know the magnitude of the changes in specific volume due to the presence of cosolvents. We have available data of high precision for BSA in up to 40% glycerol [19], and we use these values for HSA. For ovalbumin we carried out separate determinations of its specific volume from density measurements. We used a SODEV 03-D densimeter and measurements techniques described in the literature [22], to determine the density of ovalbumin solutions in buffer and in 50% glycerol. We summarized the specific volume values utilized in Table 1. There is no measurable change in apparent specific volume at higher protein concentrations.

3. Results

In concentrated polymer solutions the nominal solute concentrations used in calculating binding isotherms are, as a rule, not identical with solute activities. Equilibrium dialysis, however, leads at equilibrium to equal solute activities in the two compartments. If the experiments are carried out at moderate to high ionic strength, the major effect of polymers on the activity coefficients of solutes is due to the excluded volume. We accounted in the previous section for the steps we took to correct our data for excluded volume and osmotic pressure differences.

There are two possible ways to check for the adequacy of our corrections and for the presence of additional effects.

First, we can carry out experiments with an inert polymer at equiosmolar concentration in the protein free chamber of the dialysis cell. We compensate in this manner for both osmotic and excluded volume effects. The second and the more sensitive way is to measure the distribution of a solute known not to bind to the protein, glycine in this case. Experiments with Dextran as the inert polymer are included in all of our data sets. The agreement of the results with those from Dextran free experiments is, after the corrections are applied, gratifying.

The second procedure, measurement of glycine binding, is described in the following section.

3.1. Binding studies with ^{14}C-glycine

We chose to examine glycine, a strong dipole without net charge. We have found no literature that indicated glycine binding to HSA. The experiments designed to study glycine binding were identical with the experiments used for measurements of acrylamide binding. The results are summarized in Table 2. The ratio of counts, B/C, in the table characterizing the differences between the two sides of the dialysis cell, were calculated after the application of all the corrections we used in our studies of acrylamide binding. We can at this point say with confidence that glycine is a good model for representing a lack of binding and will in this work represent the level of what we call no binding. The results also tell us that our corrections, calculated as described, seem to work satisfactorily.

Table 2

Binding of glycine to HSA. Equilibrium dialysis experiments utilizing ^{14}C-glycine in 0.05 M phosphate buffer, pH 7.0

Content of the dialysis cells		Ratio of DPM B/C after 24 h	Glycine [M]
B side	C side		
HSA in buffer	Buffer, acrylamide, ^{14}C-glycine	0.9920 0.9938, 0.9723	0.1
HSA in buffer	Buffer, ^{14}C-glycine	0.9831	0.1
HSA in buffer, ^{14}C-glycine	Buffer, ^{14}C-glycine acrylamide	0.9935	0.1
HSA in buffer ^{14}C-glycine	Buffer, ^{14}C-glycine	0.9912 0.9921	0.1
HSA in buffer ^{14}C-glycine	Buffer	1.0187	0.1
Average:		0.9921 ± 0.0130	

The concentration of HSA was 13% on all occasions. Acrylamide concentration, if present, was 0.628 M. Glycine contained 0.25 μC/l ^{14}C-labelled molecules.

3.2. Analysis of covalently bound acrylamide

In order to account for the possible covalent attachment of acrylamide to the reactive groups of the protein, SH especially, we carried out the following reaction. A solution 10^{-3} M in ovalbumin and 0.137 M in ^{14}C-labelled acrylamide was stored in 0.05 M phosphate buffer pH 7.0 for 30 h at room temperature. The reaction mixture was then loaded onto a Sephadex-G25 column (1 × 4 cm) and eluted with buffer. We collected 1 ml fractions and measured the absorbance at 280 nm. We also removed 0.1 ml from each fraction and counted them after mixing with 10 ml toluene based scintillation liquid in a beta counter. The protein peak is free from acrylamide, and all the radioactivity travels with the peak due to free acrylamide. These results clearly show that ovalbumin, in spite of its four free sulfhydryl groups [23], does not react with acrylamide at room temperature and neutral pH.

3.3. Acrylamide binding

The basic data for acrylamide binding to HSA at pH 7.0 are shown in Fig. 2 in the form of a binding isotherm where ν, representing the excess acrylamide in protein phase, is expressed in moles per 69 000 g of albumin. It is important to state that we cannot distinguish between concepts such as binding to assigned sites, or excess acrylamide dissolved in protein phase. The localization of acrylamide molecules is not determined by our experiments, however, the form of the binding isotherm allows us to choose between different models as more or less useful. We will return to this later in the discussion section.

The experiments were carried out at four different conditions. In the first series of experi-

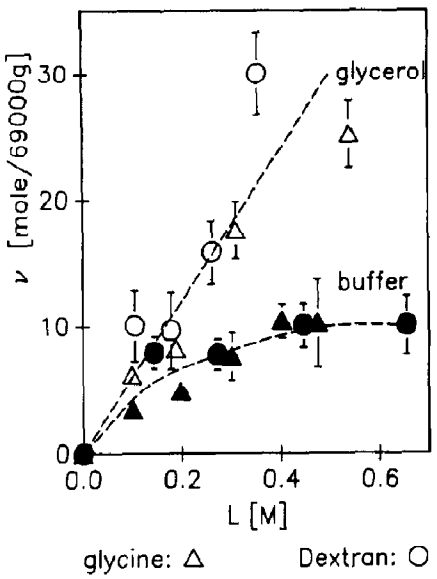

Fig. 2. The binding of acrylamide to human serum albumin at pH 7.0 and 0.05 *M* phosphate buffer and 50% (w/w) glycerol containing buffer, measured in the presence of 0.10 *M* glycine (△), and alternatively with equiosmolar Dextran T-70 in the protein free compartment (○). See Table 3 for details.

ments the protein was equilibrated with acrylamide in buffer containing glycine to minimize the Donnan effect. In this series the osmotic pressure difference results in volume changes that have to be corrected. In the second series, the protein free compartment contained besides buffer and acrylamide Dextran in amounts equimolar to the protein content. Both series were then repeated in the presence of 50% glycerol. In the absence of glycerol the differences between solutions with and without Dextran T-70 were small and within the error of our measurements (see Figs. 2a,b). The difference in the presence of glycerol is somewhat larger.

The addition of 50% glycerol nearly triples the amount of acrylamide bound to HSA. However, it becomes experimentally difficult under these circumstances to determine whether saturation has been reached or not. We succeeded in the case of HSA at pH 7.0 without glycerol to reach acrylamide values in excess of 0.6 *M*, in which case the binding curve clearly shows saturation (see Fig.

Table 3

Summary of the results of acrylamide binding studies with HSA at 22°C. All the data have been determined from plots according to eq. (7), where n represents the number of binding sites, and K is the affinity constant

Glycerol content	Side		pH	n	K (M^{-1})
	B	C			
–	Buffer (1) Protein	Buffer (1) Acrylamide	7.0	16±11	2±2
–	Buffer (2) Protein	Buffer (2) Acrylamide Dextran	7.0	11±2	18±12
50%	Buffer (1) Protein	Buffer (1) Acrylamide	7.0	≥ 90	0.7±2
50%	Buffer (2) Protein	Buffer (2) Acrylamide Dextran	7.0	> 140	≈ 2
–	Buffer (3) Protein	Buffer (3) Acrylamide Dextran	5.2	170±170	≈ 0.4
50%	Buffer Protein	Buffer (3) Acrylamide Dextran	5.2	> 100	

Buffer (1) = 0.05 *M* phosphate buffer, containing 0.10 *M* glycine.
Buffer (2) = 0.05 *M* phosphate buffer.
Buffer (3) = 0.10 *M* acetate buffer.

2). We first investigate the nature of acrylamide binding for all the experiments by assuming a possibility of saturating n sites, sites with a narrow distribution of affinity constants, a distribution that can to a first approximation be represented by a binding constant K. With such an approximation the data can be plotted in terms of the familiar reciprocal form of the mass action law,

$$\frac{1}{\nu} = \frac{1}{n} + \frac{1}{nK} \times \frac{1}{L} \qquad (7)$$

where ν represents the amount acrylamide bound, n the number of binding sites, L the free acrylamide concentration (in our case, within error, equal to the total acrylamide concentration) and K the binding constant. The number of sites and the average binding constants calculated for all our measurements of acrylamide binding to HSA are compiled in Table 3. Whereas the degree of acrylamide binding, expressed as n, clearly changes with pH and with the presence of 50% glycerol, the average affinity, represented by K, seems to be less affected.

The data for ovalbumin showed less scatter. We feel this is the result of considerably lower protein concentration used. Figure 3 shows again the number of acrylamide molecules bound per 45000 g protein (ν) plotted against the concentration of acrylamide. It also shows the results of cod parvalbumin III binding measured only at one concentration of acrylamide in buffer, and in 50% (w/w) glycerol.

Figure 4 shows the corresponding reciprocal plot of the ovalbumin binding. In this case we obtain $n = 39 \pm 25$ and $K = 1 \pm 0.3\ M^{-1}$ both for the buffer and in the presence of 50% glycerol. We have however to realize that this and the other n values are large and intercept of the reciprocal plot is perilously close to the origin. A curve passing through the origin stands for an infinite number of binding sites, same as in the case of HSA (data not shown). We are dealing maybe with a simple absorption isotherm of some type. In these circumstances we can approach the problem from a different angle. We will consider the apparent binding resulting in excess acrylamide in the protein phase as a distribution equi-

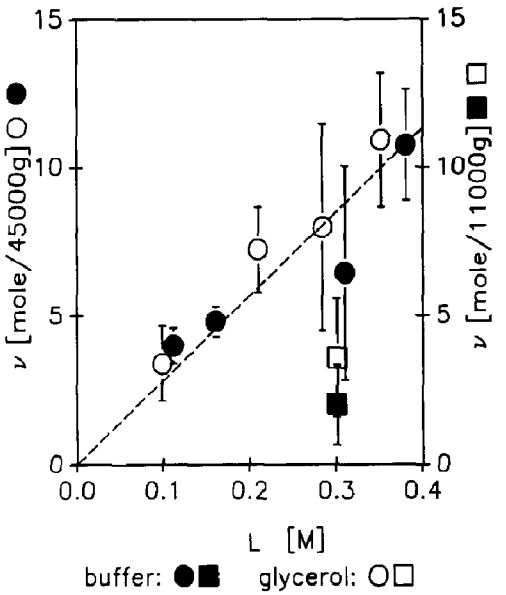

Fig. 3. The binding of acrylamide to ovalbumin and to cod parvalbumin III at pH 7.0 in 0.05 M phosphate buffer and in 50% (w/w) glycerol containing buffer. (Cod parvalbumin III was measured at one acrylamide concentration.) In these experiments we used Dextran T-10 and Spectrapore 3 membrane. Ovalbumin concentration was 8% and parvalbumin 5% (w/v).

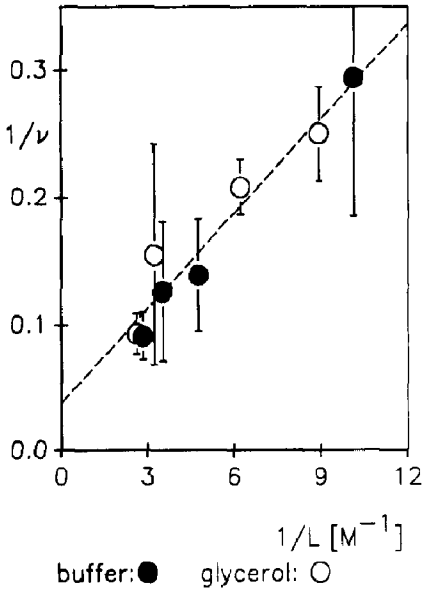

Fig. 4. A reciprocal plot of acrylamide binding to ovalbumin at pH 7.0 in 0.05 M phosphate buffer and in 50% (w/w) glycerol containing buffer. The ovalbumin concentration was 8% (w/v).

Table 4

The binding results interpreted as an equilibrium distribution of acrylamide between the bulk solution and the protein phase; K_p is the distribution constant

Solution conditions	K_p	[L] range (M)
pH 5.2, HSA in buffer	1.20±0.10	0.16–0.49
Same conditions but 50% glycerol added	1.75±0.39	0.18±0.28
pH 7.0, HSA in buffer	0.52±0.09	0.10–0.40
Same conditions but 50% glycerol added	1.47±0.38	0.10–0.36
pH 7.0, ovalbumin in buffer	0.95±0.09	0.10–0.35
Same conditions but 50% glycerol added	0.86±0.38	0.10–0.38

librium where the concentration in the bulk solution c_{out} relates to the concentration of acrylamide in the protein phase c_{in} as $K_p = c_{in}/c_{out}$. We can, from the values of acrylamide considered as bound and the specific volume of the protein phase, calculate both the concentration in the protein phase and the distribution constant K_p.

We have carried out such calculations for HSA at pH 7.0 and pH 5.2 and for ovalbumin at pH 7.0. The resulting K_p values and their standard deviations are shown in Table 4.

3.4. Error propagation

The primary data used in plotting our graphs, ν, excess acrylamide found in the protein phase is a function of several *independent* variables, y_i. If we write $\nu = \mathrm{CPM} \times F \mathrm{x} B/P$ as in eq. (6), the expected standard deviation, σ_ν, can be written as

$$\sigma_\nu = \left\{ \sum_i \left(\frac{\mathrm{d}\nu}{\mathrm{d}y_i} \right)^2 \sigma_i^2 \right\}^{1/2} \qquad (8)$$

where σ_i represents the standard deviation of each independent variable. The variables CPM, B and P are in turn functions of the primary data, ΔCPM, volume and protein concentration. The σ for each variable can thus be calculated by using eq. (5) and the standard deviation of each measurement. If measurements utilizing acrylamide labelled in a separate procedure are to be combined, the error in F propagates in a similar fashion to the error in, for example, B.

The counting error for CPM is 0.1%; however, the lowest error in sample volume is between 0.5–1.0%. This leads to a final error in CPM of 10 to 15%. The variation in volume occupied by the protein is less than 1%. The calibration error is 1–2%, and the error in protein concentration is at least 2%. If these errors are propagated they lead to a final error of 15 to 20% in the number of moles of acrylamide bound or present in excess. The error in the averages of multiple measurements plotted in Figs. 2 and 3 are indicated by error bars or the size of the symbols. We have another way of estimating the error in individual data points. Looking at glycine binding data, we expect the scatter around the ratio 1.000 of acrylamide activities in the compartments B and C to represent the expected error. The ratio varies to ±0.01 (Table 2). Comparing this with ratios found in binding experiments, we predict an error of 20% for the individual determinations.

The points in Figs. 2, 3 and 4 have been fitted by weighted least squares methods to straight lines represented by eq. (7). We considered the customary weight with $1/\nu^4$ not satisfactory because the error on individual determination varied. The actual error was transferred graphically to the reciprocal plot. The fitting used the length of the error bar as weight. The error in fitting parameters was transformed, resulting in the error shown in Table 3. The data set for HSA in buffer in Fig. 2 was fitted to

$$PL = nK[\mathrm{L}]/(1 + K[\mathrm{L}]) \qquad (9)$$

where PL represents the amount acrylamide bound, n the number of binding sites, [L] the concentration of acrylamide and K the equilibrium constant. The errors for the slope and intercept are shown in Tables 3 and 4.

4. Discussion

The question of a possible interaction between acrylamide and proteins and the relationship of such an interaction to the observed quenching of

fluorescence from tryptophan residues in proteins has received less attention than quenching studies per se. The experimental findings are not concordant and have been interpreted in a contradictory manner. The focus of the early work was on possible covalent bond formation between acrylamide and the thiol and ϵ-amino groups of the protein. It has been established that acrylamide reacts with amino groups at basic pH values [24,25] and with thiol groups under reducing conditions [26]. Indirect measurement, by a rather difficult method, of acrylamide binding to bovine serum albumin (BSA) was reported [27] to show that an incubation for 3 h at 37°C at pH 5 producing binding corresponding to an affinity constant of 38 M^{-1}. This was interpreted as due to covalent bond formation. Our measurements of covalent binding using a radioactive label clearly established that between pH 5–7 and at room temperature such interactions do not take place. Thus if an interaction between the protein and acrylamide is seen, it is of a reversible equilibrium type [28].

Equilibrium dialysis studies of acrylamide binding to HSA and ovalbumin, using acrylamide quenching of eosin fluorescence as a measure of acrylamide concentration, carried out at pH 2.2 have been interpreted to show the binding of a very large number of molecules, 200–300, with a distribution of affinity constants, 120–1000 M^{-1} [11]. These authors then suggested that the Scatchard plots of their data were best interpreted as representing a distribution of acrylamide between the protein and the bulk phases, with a distribution constant K_p of 25–300 at pH 7.0. More recent studies, however, could not confirm these findings. Equilibrium studies of acrylamide binding to HSA at pH 7.0 were reported to show no binding [12]. The methods used in the determination of acrylamide concentration in the latter case, UV at 240 nm, or quenching of eosin fluorescence in the first case, are not sensitive enough, especially because the analysis can be carried out only on one side, the protein free side, of the dialysis vessel. We carried out experiments with both of these methods and concluded from calculations of error propagation and from experimentally determined variances that the results obtained by these methods are not reliable in the affinity range encountered in acrylamide binding. Consequently we believe that the disagreement between the different laboratories is not real and all the previously published results fall within the very wide limits of error, thus allowing no conclusions about acrylamide interactions to be drawn.

In these circumstances it was imperative that we carry out our investigations in great detail and present the calculations, plots and error ranges. The application of the different corrections to the raw measurements due to excluded volume, osmotic inequality, effects of glycerol and the propagation of measurement errors through the calculations have all been discussed in the results section. We also discuss there the use of Dextran as a control for volume corrections. The data for HSA, seen in Fig. 2 and compiled in Tables 3 and 4, show good agreement between the data sets with and without the use of Dextran.

It is also important to note that we were fortunate enough to be able to establish a no-binding control using radioactive glycine. In view of the well known tendency of HSA to bind even the simplest ions [29] it is difficult to predict the structure for a small molecule that does not bind to HSA. The data for glycine in Table 2 clearly shows that, under the conditions we use and in the affinity range observed for acrylamide binding, glycine does not bind to HSA. In other words, it is excluded from the protein volume.

What do our studies of acrylamide binding to HSA and ovalbumin show? We can first say that although the affinity of acrylamide for HSA and ovalbumin is low, ~ 5–15 M^{-1}, a surprisingly large number of acrylamide molecules are found bound to a protein molecule under conditions similar to those used for fluorescence quenching studies.

The reciprocal plots all extrapolate close to the origin indicating that we are dealing with a very large number of sites and no saturation seems to be possible.

In view of these observations, we decided to turn to the two phase distribution model where we assume that the acrylamide molecules distributed between the protein and bulk phases

obey a simple distribution law. The protein molecules represent a defined volume and, as we know the specific volume of the proteins and the formal number of excess molecules of acrylamide in the protein phase, the concentrations in equilibrium and the distribution constant K_p can be calculated for all of our experimental series. The data in Table 4 shows that a stable K_p can be obtained over the range of acrylamide concentrations used in quenching studies. We can say that the two-phase distribution model is the model of choice because it corresponds to a very wide distribution of weak binding sites where n, the number of sites, is high. The model also allows rationalization of the observed increase in acrylamide content with glycerol, although the affinity, when determined from a definite site model, does not seem to change too much (see Table 3 and the reciprocal plot on Fig. 4). The effect of glycerol on the acrylamide distribution between the two phases is partially due to the change in the activity of acrylamide in the presence of glycerol. Another part of the effect could be due to changes in protein activity. The protein in most cases interacts preferably with water [19,30]. Possible alteration of protein structure produced by changes in water activity could modify the specific volume of the protein and thus influence the distribution constant. Direct determinations of the specific volume of our proteins in the presence of glycerol argues against this possibility. Consequently we believe the effects we see are dominated by changes in acrylamide activity in the presence of glycerol.

We have no stress that this does not mean that acrylamide is distributed randomly in the protein space. The probability of finding acrylamide varies from site to site. The protein matrix is not a homogeneous solution, and the effect of glycerol will, we predict, vary from protein to protein depending on the sensitivity of the free volume accessible to acrylamide to changes in bulk solvent. It is interesting to note that although their equilibrium dialysis data are difficult to reproduce [11], the model suggested earlier [10] is a useful one.

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