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BOVINE SERUM ALBUMIN AS A CATALYST

VI. SPECIFICITY OF SEVERAL NUCLEOPHILIC GROUPS IN THE PROTEIN FOR *N*-DANSYLAZIRIDINE

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Summary

The reaction of *N*-dansylaziridine with serum albumin (both bovine and human) results in incorporation of about 3 mol of covalently bound dansyl label per mol protein. This indicates that a number of nucleophilic groups in these proteins (in addition to the free sulfhydryl group) will react with this reagent. The reaction has been studied in detail for bovine serum albumin and the results suggest that one of the sites labelled by the reagent may be at the unusual "catalytic site" responsible for the enzyme-like activity of bovine serum albumin recently described (Taylor, R.P., Chau, V., Bryner, C. and Berga, S. (1975) J. Am. Chem. Soc. 97, 1934—1942).

The reaction of *N*-dansylaziridine with a variety of other proteins indicates a pattern of labelling consistent with high specificity for the sulfhydryl group. The explanation for the unexpected excess reactivity of albumin with the "sulfhydryl specific" reagent *N*-dansylaziridine must be related to the three-dimensional structure in albumin which enables a number of specific residues to manifest unusually high degrees of nucleophilic reactivity.

Introduction

Recently Scouten et al. [1] reported that the reagent *N*-dansylaziridine could be used as a specific probe in a nucleophilic reaction for the fluorescent labelling of proteins at their free sulfhydryl groups. We have used this reagent in an attempt to label the lone sulfhydryl group in bovine serum albumin in order to prepare an immunogen in which a specific hapten is located in a well-defined chemical environment. However, our results indicate that a number of other functional groups on the protein are of sufficiently high nucleophilic reactivity that they also are labelled with this reagent.

Methods and Materials

N-dansylaziridine was obtained from Pierce Chemical Co. and its PMR spectrum was identical to that reported by Scouten et al. [1]. Bovine serum albumin was from Sigma or Armour, and iodoacetamide-blocked, monomeric defatted bovine serum albumin was prepared as described by Taylor et al. [2]. Human serum albumin was from Pentex and iodoacetamide-blocked, monomeric defatted human serum albumin was prepared in a manner analogous to that used for iodoacetamide-bovine serum albumin. Urea was Ultra-grade from Schwarz-Mann. Iodoacetic acid was from Aldrich and was resublimed before use. H^3-labelled iodoacetic acid was from Amersham and was diluted before use with cold iodoacetic acid and the mixture was then resublimed. Typically the specific activity of the material was approx. 0.1 mCi/mol.

The reaction of *N*-dansylaziridine with a variety of proteins was monitored by following the general procedure we now describe. Typically, a protein at a concentration of approx. 2—5 mg/ml was reacted at pH 7.5 in 0.1 M phosphate at room temperature with an approx. 10-fold molar excess of *N*-dansylaziridine. The reaction was stopped by acidification of the reaction mixture to pH 4 followed by prolonged (approx. 2—3 days) dialysis of the mixture against 0.1 M acetate buffer pH 4, and then against 0.1 M phosphate, pH 7.5. The degree of labelling of each protein was determined by measuring the absorbance of product at 279 nm (to determine protein concentration) and at 335 nm (to determine the dansyl label concentration). A molar extinction coefficient of 4440 was used for the dansyl chromophore. The contribution of the dansyl chromophore to the protein optical density was small, and corrections were made to determine the true protein concentration. Sulfhydryl titers of bovine serum albumin were measured by the method described by Ellman [3]. The sulfhydryl titer of iodoacetamide-bovine serum albumin was zero, as expected. Assays for the enzyme-like activity of bovine serum albumin and iodoacetamide-bovine serum albumin recently reported by Taylor et al. were performed exactly according to published procedures [2].

Reduction and alkylation studies

In a typical experiment 0.1—5 mg of dansylated or control protein was dissolved in 8 M urea containing 0.05 M phosphate, pH 8.5. A 5-fold excess of dithiothreitol (based on 17 disulfides per mol of albumin) was added and the sample was incubated for 1 h at 37°C. Then a 12-fold excess (over the dithiothreitol concentration) of iodoacetic acid (either radiolabelled, or cold, depending upon the experiment) was added and the sample was incubated for 3 h at 37°C. Subsequently the product was dialyzed exhaustively against 0.05 M phosphate, pH 8.5, and then the protein was assayed for covalently bound dansyl chromophore, and, where appropriate, the product was assayed for the degree of incorporation of radiolabel.

Results

The reaction of bovine serum albumin with *N*-dansylaziridine gives rise to a time-dependent increase in fluorescence intensity, as described by Scouten et

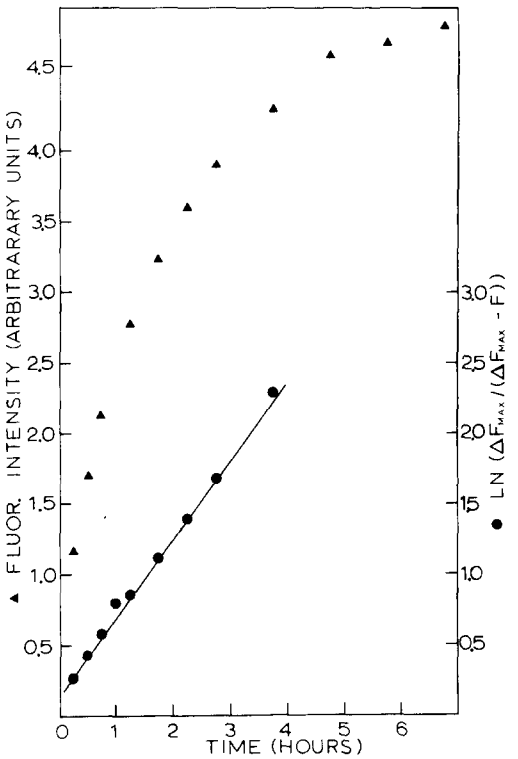

Fig. 1. Reaction of *N*-dansylaziridine with bovine serum albumin at pH 7.5 in phosphate buffer. The *N*-dansylaziridine was first adsorbed onto a cellulose matrix following the procedures of Scouten et al. [1], and the kinetics of the coupling reaction were monitored as described by the above authors. IA-BSA, iodoacetamide-bovine serum albumin.

al., and the reaction appears to be characterized by first order kinetics (Fig. 1). However, the results of the labelling reaction indicate that a number of groups are labelled with the dansyl chromophore. After exhaustive dialysis of the derivatized protein (see Methods and Materials) the absorption spectrum of the product indicated that there were approx. 2.8 mol dansyl label per mol protein. Preliminary studies with this derivatized protein indicated that it had less than 10% of the enzyme-like activity we recently reported for bovine serum albumin [2]. The degree of labelling was not decreased even after subjecting the protein to G-25 Sephadex gel chromatography in 1% dodecyl sulfate. Thus, we are confident that all the dansyl moiety is covalently bound to the protein. Further evidence that this is in fact the case is the observation that even after the protein was reduced and alkylated in 8 M urea the degree of labelling did not change, within experimental error.

We have also determined that iodoacetamide-bovine serum albumin reacts extensively with *N*-dansylaziridine. In fact, the rate at which the protein loses catalytic activity on reaction with the reagent is correlated with its rate of labelling (Fig. 2). The fact that incorporation of only two dansyl labels into the protein reduces its activity to approx. 10% of that of the control suggests that one of the dansyl labels may in fact bind covalently to the protein at the active

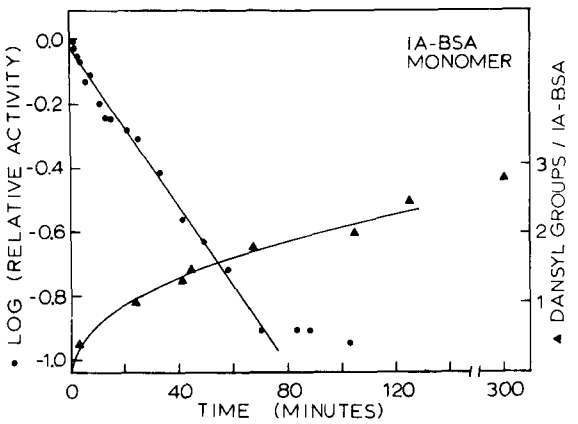

Fig. 2. Reaction of iodoacetamide-bovine serum albumin (approx. $7 \cdot 10^{-5}$ M/l) with *N*-dansylaziridine ($8 \cdot 10^{-4}$ M) at pH 7.5 in 0.1 M phosphate buffer. Aliquots of the reaction mixture were isolated at different times and assayed for both the degree of incorporation of the dansyl label and the residual "enzyme-like" activity of iodoacetamide-bovine serum albumin (see text for details). The *N*-dansylaziridine was introduced into solution via a stock solution in dimethylformamide. Control experiments indicated that dimethylformamide (approx. 1% v/v) was inert in these experiments.

site lysine (position 220 in Brown's sequence [4,5] or at least near this position. The enhanced reactivity of the protein toward *N*-dansylaziridine requires that the protein be folded in its native conformation. Iodoacetamide-bovine serum albumin which was unfolded in 8 M urea incorporated at the most 0.4 mol dansyl label per mole protein, and part of this appparent degree of labelling is probably due to the increase in light scattering of the unfolded protein. In addition, Scouten et al. [1] noted that in performate oxidized bovine serum albumin (which should be unfolded due to oxidation of all disulfide residues) there was no apparent reactivity with *N*-dansylaziridine.

We have confirmed that *N*-dansylaziridine does react with the free sulfhydryl group of bovine serum albumin (Fig. 3) but it is obvious that a number of other

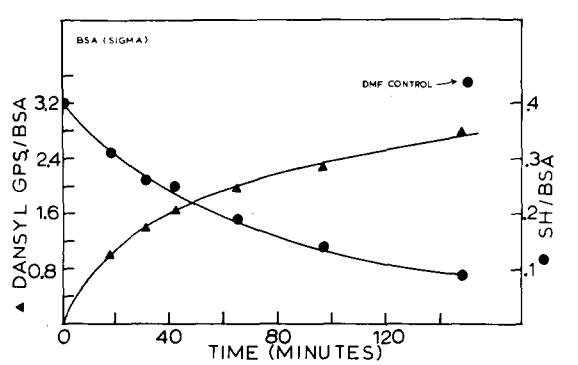

Fig. 3. Decrease in the sulfhydryl titer of bovine serum albumin upon reaction with *N*-dansylaziridine. Conditions were the same as in Fig. 2. It can be seen that considerably more dansyl label is incorporated on the protein than would be expected simply on the basis of the decrease in sulfhydryl titer. The fact that the sulfhydryl titer is initially less than 1 for bovine serum albumin is consistent with the known structure of the isolated protein [6]. SH/BSA, sulfhydryl bovine serum albumin titer.

TABLE I

Protein	Dansyl groups/molecule
Ribonuclease A	0.08
Lysozyme	0.08
Alpha-chymotrypsinogen	0.15
Beta-lactoglobulin	1.0 (per 18 000 subunit)

residues on the protein must react. This unusual reactivity is not only confined to bovine albumin. The reaction of human serum albumin and iodoacetamide-human serum albumin with *N*-dansyl aziridine under similar conditions gave a similar labelling pattern. In each case approx. 3 mol dansyl label were incorporated per mole protein, and the extent of labelling was not changed by a variety of treatments including extensive dialysis, or be reduction and alkylation of the unfolded protein.

The sites at which the *N*-dansylaziridine are bound to albumin (in addition to the sylfhydryl group) are still not known, but we can definitely state that the reagent does not attack disulfide bonds and then label the newly formed sulfhydryls. The total content of reactive sulfhydryl groups (i.e., cysteines) in the protein after reduction in 8 M urea with dithiothreitol followed by alkylation with radiolabelled iodoacetic acid was found to be identical (within 0.5%) for a "control" sample of iodoacetamide-bovine serum albumin and for a sample which had approx. 3 dansyl groups covalently attached. We have also verified that this procedure does not cause loss of covalently bound dansyl groups, so we are sure that the label must be located on groups other than cysteines.

Though the reaction of *N*-dansylaziridine with iodoacetamide-blocked albumin obviously involves groups other than the free sulfhydryl group of the protein, we have not yet been able to find similar groups of unusually high nucleophilic reactivity in other proteins. The degree of labelling of a variety of other proteins with *N*-dansylaziridine is completely consistent with complete specificity for the sulfhydryl group (Table I). Only β-lactoglobulin reacted significantly, incorporating one dansyl group per unit of molecular weight 18 000. As it is known that this protein contains one free sulfhydryl group per subunit [7], the degree of labelling is in excellent agreement with the predictions of Scouten et al.. The other proteins listed in Table I have no free sulfhydryl groups and the very low degree of labelling certainly attests to this fact.

Discussion

Our work is in basic agreement with that of Scouten et al. [1]. *N*-dansylaziridine is a reagent which shows high selectivity for free sulfhydryl groups in proteins. However, in the case of albumin other functional groups in the protein of apparently high nucleophilicity are capable of reacting with this reagent. Scouten et al. even noted that the selectivity of the reagent could be reduced if certain functional groups in a protein were "made unusually nucleophilic by virtue of their particular microenvironment" [1]. Clearly such a case obtains

for a number of groups in both bovine and human serum albumins. Our earlier findings [5] indicated that lysine 220 is at the "active site" of bovine serum albumin [2] and that it displays unusual nucleophilic reactivity toward such reagents as fluorodinitrobenzene and fluoresceinisothiocyanate. We have shown that this property of bovine serum albumin is intimately related to the structural organization in the protein [8] and so it appears likely that the microenvironment of lysine 220 confers unusual nucleophilic properties on this residue. This group is characterized by an unusually low pK_a, and it is tempting to speculate that the residue is buried in a hydrophobic environment which facilitates nucleophilic reactivity because dehydration of the substrate and the functional group is largely achieved in the binding step.

The situation is more complicated for albumin in general, because there is more than one non sulfhydryl group of high nucleophilic activity in both bovine and human serum albumin. In addition, we know that in human serum albumin, which has no catalytic activity [2], lysine 220 is replaced by arginine at its homologous position [9]. Thus we forced to conclude that there must be other highly nucleophilic groups in both proteins which are capable of reacting with *N*-dansylaziridine. This is not really surprising because it has been shown that certain functional groups (e.g., lysines and tyrosine) in both bovine and human serum albumin display high nucleophilic reactivity toward such diverse substrates as aspirin, halogenated polynitrophenyl compounds, and *p*-nitrophenylacetate [10—12]. There is evidence that some of these groups can be found on homologous positions in repeating structural domains in the protein [11], and this would be reasonable if the folding in different domains were repeated in such a way that these functional groups were situated in similar microscopic environments.

Whether these unusually reactive functional groups have any biological function or significance remains to be determined. It is possible that they may play some nonspecific role in detoxification of certain metabolites but it is clear that considerably more detailed studies would be needed to establish this point.

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