THE UNREACTIVE AMINO GROUPS OF PROTEINS

by

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INTRODUCTION

The varying reactivity of different groupings in the protein molecule has been the subject of a recent review by ANSON[1]. Most of the work discussed concerns SH and S-S groups, which have been extensively studied by many workers and which are indeed, with one exception, the only groups whose reactivity has been shown to alter when the protein has been denatured. It has been found that the number of SH groups, for example, which can be estimated in egg albumin increases after denaturation, the value found in the native protein depending on the reagent used for the estimation. The third group which appears to behave similarly is the phenolic hydroxyl of tyrosine. ANSON[1] quotes unpublished work by HERRIOTT as showing that FOLIN's Phenol Reagent oxidises more tyrosine in denatured pepsinogen and egg albumin than in the native form, and clear proof that the condition of this group can be altered was obtained by CRAMMER and NEUBERGER[2] who found, using ultraviolet absorption, that the p_H at which this group ionises in egg albumin was altered significantly by denaturation.

The significance of this type of work lies in that, if sufficiently extended, it should give us a clearer picture of the complex folding of the polypeptide chains of protein molecules, on which so many of their highly specific biological properties appear to depend. We have found that in several serum globulins and β-lactoglobulin, the reactivity of the lysine ε-amino groups depends on the condition of the protein, i.e., native or denatured, and on the reagent used in the condensation. This is in contrast to our findings with insulin[3] and a number of haemoglobins[4], where all the lysine ε-amino groups react quantitatively with the reagents tested. That such a variation in the reactivity could occur in certain proteins might have been anticipated from PAPPENHEIMER's observations[5] that ketene and formaldehyde react readily with only 40 % of the total NH_2 groups present in diphtheria toxin. BRAND[6] also noted a difference in the rates of deamination of proteins by nitrous acid, but did not name the proteins examined or give any data.

EXPERIMENTAL RESULTS

Throughout this paper 1:2:4 fluorodinitrobenzene has been abbreviated to F.D.N.B. and 2.4 dinitrophenyl to D.N.P.

PROTEIN PREPARATIONS USED

β-Lactoglobulin. Crystalline preparations obtained from cow's milk both by PALMER's[7] and SØRENSEN's[8] method were kindly provided by Professor LINDERSTRØM-LANG. Both preparations behaved similarly with the reagents used.

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Serum Globulins. Most of the work was carried out on horse serum pseudoglobulin prepared by sodium sulphate fractionation. Its approximate electrophoretic composition was 25% α, 50% β, 25% γ. Small samples of cow and pig β- and γ-globulin and cow albumin were also used; these had been prepared in the Armour Laboratory by cold ethanol fractionation.

METHODS OF DENATURATION

Heat denaturation. The protein solution, approximately 5%, was heated at neutral p_H to 90° for 30 minutes, cooled, and the coagulated protein washed several times with saline and water to remove any undernatured or reversibly denatured protein.

Ethanol denaturation. Two volumes of ethanol were added to one volume of 5% protein solution and the mixture allowed to stand 24 hours at room temperature, after which the protein was washed as before.

Acid denaturation. HCl was added, with stirring, to the protein solution until the p_H fell to 1, when the mixture was allowed to stand 24 hours at room temperature. The precipitated protein was centrifuged and washed as before.

Denaturation in guanidine solution. Solid guanidine hydrochloride was dissolved in the protein solution to give a final concentration of 6-molar. This solution, whose p_H did not fall below 6, was allowed to stand 24 hours at room temperature. In the case of β-lactoglobulin the solution was dialysed free of guanidine and the precipitated protein washed. With the serum globulin the solution of the denatured protein was allowed to react with F.D.N.B. in the presence of guanidine. The D.N.P. protein remained in solution but was precipitated on dialysis. We had previously established that although guanidine reacts readily with F.D.N.B. in N-NaOH, the reaction proceeds only very slowly in bicarbonate solution.

METHODS OF ESTIMATION

The estimation of the free α- and ε-NH_2 groups of the proteins using F.D.N.B. was carried out as described by SANGER[4] and PORTER and SANGER[5].

Acetylation was effected by treating the protein solution at 0°–5° with ketene, using an acetate buffer to maintain the p_H above 5. Acetylation of pseudoglobulin was also carried out with acetic anhydride by the method of HUGHES, as described briefly by OLCOTT and FRAENKEL-CONRAT[9].

Lysine was determined by the specific decarboxylase method of GALE[10] and, after electrodialysis of the hydrolysate, by the difference between the total base nitrogen and the arginine and histidine content[11]. The nitrous acid method of VAN SLYKE was used to estimate the free amino groups of the proteins, an eleven minute reaction time being allowed. For most of these amino N determinations, I wish to thank Mr. G. F. WILTSHIRE of this laboratory.

Results

Table I gives details of the protein preparations used. The number of NH_2 groups per molecule is calculated from the lysine content and the assumed molecular weight.

TABLE I

Protein	Lysine N as % total protein N	Assumed mol. wt	No. of NH_2 groups per mol
β-Lactoglobulin	13.9 (6)	40 000	31
Horse serum pseudoglobulin	8.6 (decarboxylase)	165 000	81
	8.4 (Nitrogen difference)		79
Cow serum albumin	14.6 (10)	70 000	59

As analysis suggests that the horse pseudoglobulin possesses 80 ε-NH_2 groups, and human γ-globulin 92 groups per molecule, respectively, it is believed that cow and pig globulins will have a value of the same order, and an arbitrary figure of 85 in these latter proteins has been assumed for comparison with the number of groups which have been found to react with F.D.N.B.

Table II lists the number of ε-NH_2 groups founds to react with F.D.N.B. when the

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proteins were either native or after treatment under various conditions. The number of groups which failed to react with F.D.N.B. was obtained by difference.

TABLE II

NUMBER OF LYSINE ε-NH_2 GROUPS REACTING WITH F.D.N.B.

Protein	Condition	No. of reacting groups per mol	No. of non-reacting groups per mol
β-lactoglobulin	Native	19	12
	Acid denatured	32	0
	Alcohol denatured	32	0
	Heat denatured	29	2
	Guanidine denatured	31	0
Horse serum pseudoglobulin	Native	57	23
	Acid denatured	76	4
	Alcohol denatured	77	3
	Heat denatured	75	5
	Guanidine denatured	78	2
	Acetylated native	23	0
Cow β-globulin	Native	40	45
Cow γ-globulin	Native	47	38
Pig β-globulin	Native	40	45
Pig γ-globulin	Native	41	44
Cow albumin	Native	57	2

The results quoted for β-lactoglobulin and horse pseudoglobulin are the mean of two or more estimations, the variation in no case exceeding 5 %. Only single determinations could be carried out on the small quantities of cow and pig proteins which were available.

The number of ε-NH_2 groups acetylated by ketene or acetic anhydride was deduced from the total amino N before and after acetylation. The results obtained are given in Table III.

TABLE III

THE NUMBER OF ε-NH_2 GROUPS ACETYLATED BY KETENE OR ACETIC ANHYDRIDE

Protein	Condition	NH_2 groups before acetylation	NH_2 groups after acetylation	No. of reacting groups
β-Lactoglobulin	Native	36	0	36
Horse serum pseudoglobulin	Native	79	24	55
	Heat denatured	80	4	76
	D.N.P. native	24	1	23

It can be seen that all the amino groups of β-lactoglobulin react with ketene, but in native horse pseudoglobulin 23 of the amino groups fail to react either with acetic anhydride or ketene, or to combine with F.D.N.B.

DISCUSSION

The results are summarised in Table IV. For the purposes of discussion it is considered that very small numbers of unreactive amino groups fall within the experimental error and are neglected.

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TABLE IV

SUMMARY OF RESULTS

Protein	Condition	Total no. of NH_2 groups per mol	No. of NH_2 groups failing to react with F.D.N.B.	No. of NH_2 groups failing to react w. ketene or Ac. An.
β-Lactoglobulin	Native	31	12	0
	Denatured	31	0	0
Horse serum pseudoglobulin	Native	80	24	23
	Denatured	80	2–3	2–3
Cow β-globulin	Native	85	37	—
Cow γ-globulin	Native	85	45	—
Pig β-globulin	Native	85	45	—
Pig γ-globulin	Native	85	44	—
Cow albumin	Native	59	2	—

Ethanol denaturation

The condensation of F.D.N.B. with proteins is carried out under the following conditions[3]. A 5 % to 10 % solution or suspension of protein in 10 % $NaHCO_3$ solution is added to twice its volume of a 10 % solution of F.D.N.B. in ethanol. This mixture is shaken for 2 hours at room temperature and the D.N.P. protein washed free from the excess reagents. Some denaturation would be expected to occur during the reaction, and it was therefore important to examine the effect of ethanol denaturation more closely in order that the significance of the results could be evaluated.

β-Lactoglobulin which had stood 24 hours at room temperature in 66 % ethanol had no unreactive amino groups, and it seemed possible that the 12 unreactive amino groups found for the native protein might represent an intermediary figure of partially denatured or mixed native and denatured protein. Samples of β-lactoglobulin were therefore allowed to stand in 66 % aqueous ethanol for 4 hours, 2 hours, and 2 minutes, respectively. In each case it was found that virtually the whole of the material was rendered completely insoluble in saline and that almost all the amino groups now reacted with F.D.N.B. To emulate the conditions of the reaction more closely, the protein was dissolved in 10 % $NaHCO_3$ solution and 2 volumes of ethanol added, the precipitated protein immediately centrifuged down and extracted with saline. Approximately 25 % was still soluble and thus 2 fractions could be obtained. Treatment with F.D.N.B. gave the following results.

TABLE V

THE DENATURATION OF β-LACTOGLOBULIN BY ETHANOL

Conditions of denaturation	Fraction	No. of ε-NH_2 groups reacting with F.D.N.B.
Sol. in saline + 2 vols. ethanol for 2 minutes	Insol. in saline	28
Sol. in 10% $NaHCO_3$ + 2 vols ethanol for 2 minutes	Insol. in saline	27
	Sol. in saline	18

It would appear that although the presence of $NaHCO_3$ slowed the denaturation, it was still rapid. The soluble fraction had the same number of unreactive amino groups

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as the native protein, while the insoluble fraction was similar to the fully denatured protein, the slight difference being probably due to incomplete extraction with saline. The sharp differentiation between these two fractions makes it unlikely that we are estimating the free amino groups of a partially denatured protein and suggests that the values obtained for the native proteins are correct. The reaction of the F.D.N.B. with the amino, sulphydryl, phenolic hydroxyl, and imidazole groups must fix the structure of the protein so that the alcohol is unable to disorient the configuration of the peptide chains and expose all the amino groups as occurs when F.D.N.B. is not present. This is perhaps comparable to the increased stability of diphtheria toxin, as measured by its ability to combine with antitoxin, after conversion to toxoid by reaction with formaldehyde or ketene.

BAILEY[13], when measuring the reactivity of SH groups in the edestin-edestan reaction, found that the number of free SH groups was proportional to the amount of edestan in the mixture. As in the ethanol denaturation of β-lactoglobulin, it would seem unlikely that partially denatured protein is present, and both are probably cases of all or none denaturation.

α-Amino groups. The α-amino groups of these proteins were also estimated, and no protein has yet been found in which the number of α-amino groups reacting with F.D.N.B. has been changed by denaturation. β-Lactoglobulin was foun dto have three terminal leucyl residues per molecule. The serum globulins, like the haemoglobins[4], showed a marked species variation, the horse pseudoglobulin presenting a complex picture of some ten different terminal residues, yet together they were hardly sufficient to account for one free α-amino group per molecule. It seems probable that we were dealing here with a mixture of different molecules each consisting of one long chain. This was in marked contrast to pig γ-globulin, in which a single estimation suggested about 6 free α-amino groups per molecule.

The detailed structure of the serum globulins is of great interest when considering the chemical identity of antibodies with each other and with 'normal' serum globulin. It may be remembered that CHOW and BACON[14] found that an immune horse serum globulin fraction, of which 80% was precipitated by pneumococcus polysaccharide, reacted with ketene so that all its amino groups were acetylated. The substituted globulin remained soluble and apparently none of the phenolic hydroxyls reacted. This is in contrast to the present results with normal horse serum pseudoglobulin, where the acetylation does not go to completion unless the protein is first denatured, or the p_H is allowed to fall during the reaction below 4, when a completely acetylated, insoluble protein results.

β-Lactoglobulin

CANNAN *et al.*[15] have shown that in this protein the whole of the α- and ε-amino groups can be titrated in aqueous solution. All these groups, moreover, react with ketene under conditions which would not be expected to cause denaturation. Both these results are in contrast to that obtained on condensation with F.D.N.B., when all the α-amino groups, but only 19 out of a total of 31 ε-amino groups, react. It is suggested that the configuration of the polypeptide chains prevent a sufficiently close approach of the F.D.N.B. molecule, though not of the small ketene molecule or the hydrogen ions. This steric hindrance could be local, i.e., due to the proximity of the side chains of neighbouring amino acid residues to the ε-amino group of the lysine; or distant, i.e., 12 of

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the lysine side chains could be directed to the interior of the molecule and the entry of F.D.N.B. prevented by the steric pattern of the surface of the molecule. It is impossible to decide which phenomena we are dealing with in the present case, but in steric hindrance of any type non-polar side chains would be expected to play an important part. They could thus influence the reactivity of polar groups and presumably therefore the biological behaviour of proteins. This emphasises the point recently made by EDSALL[16] that in considering the reactions of proteins, the numbers, types and positions of the non-polar residues cannot be neglected as has often been the tendency.

Serum globulins

The distinction between serum albumin and serum globulins is again stressed by the presence of unreactive amino groups in the latter and their absence in the cow serum albumin. The β- and γ-globulins of the species examined appear to be alike in that one half to one third of the lysine ε-amino groups of the native protein fail to react with F.D.N.B.

In horse serum pseudoglobulin a similar number fails also to react with ketene or acetic anhydride, suggesting a more complex state than exists in β-lactoglobulin. It was thought that the same groups were in each case unable to react with these reagents and that therefore they were protected by tighter steric hindrance, or possibly bound by a labile link to a neighbouring polar group. To test this, the acetylated native globulin, which was soluble and had 23 'free' amino groups per molecule, was allowed to react with F.D.N.B. Unexpectedly, as shown in Table II, the groups unable to react with acetic anhydride now reacted with F.D.N.B. Conversely, the unreactive amino groups of D.N.P. native pseudgolobulin reacted with acetic anhydride. This could be taken as evidence that the identity of the number of unreactive amino groups in each case was coincidental and that the presence of three classes of amino groups had been established. These classes would be: (1) of amino groups able to react with acetic anhydride and F.D.N.B.; (2) of amino groups unable to react with D.N.F.B. but reacting with acetic anhydride; (3) of amino groups able to react with D.N.F.B. but not with acetic anhydride. It is, however, possible that the acetylation of some of the amino and other groups prevents the F.D.N.B. from reacting and protecting against subsequent ethanol denaturation during the reaction in the manner discussed earlier. The D.N.P. globulin, which is insoluble, may be sufficiently altered to expose the unreactive groups to acetylation though not to condensation with F.D.N.B. The evidence is considered to be inconclusive and the question of classes (2) and (3) of the amino groups being distinct or the same remains open.

Effect of different types of denaturation

Table II shows that each type of denaturation examined has exposed all the amino groups. This result is similar to the findings with the tyrosine groups of egg albumin (2) and with the sulphydryl groups of various proteins.

It is interesting to note the contrast in the effect of different conditions of denaturation on the antigenic specificity of proteins. ERICKSON and NEURATH[17] found urea and guanidine denaturation did not influence the specificity of serum albumin, while MACPHERSON and HEIDELBERGER[18] found that heat, alkali and acid denaturation caused a marked alteration of the specificity of egg albumin, though all the denatured proteins reacted fully with antisera produced against the differently denatured proteins. If these

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results could be confirmed using the same protein, and correlated with the chemical reactivity, an indication might be obtained of the factors determining the antigenic specificity of native proteins.

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SUMMARY

1. Twelve of the thirty one lysine ε-NH_2 groups per molecule of β-lactoglobulin will not react with F.D.N.B. (1:2:4 fluorodinitrobenzene) unless the protein is first denatured. All the groups can be acetylated in the native protein.

2. One half to one third of the lysine ε-NH_2 groups of several native serum globulins will not react with F.D.N.B. In horse serum pseudoglobulin a similar number fails to react with ketene or acetic anhydride. All the groups become reactive to all the reagents after denaturation.

3. The mechanism of the ethanol denaturation of β-lactoglobulin has been studied by estimating the amino groups liberated.

4. The significance of these results in relation to the structure of the proteins is discussed.

RÉSUMÉ

1. Douze des trente et un lysines-ε-NH_2 groupes contenus dans une molécule de β-lactoglobuline ne réagissent pas avec F.D.N.B. (fluoro–1 dinitro–2:4 benzène), si la protéine n'a pas d'abord été dénaturée. Tous ces groupes peuvent être acétylés à l'état natif.

2. La moitié jusqu'au tiers des lysines-ε-NH_2 groupes de plusieurs globulines de sérum natives ne réagissant pas avec F.D.N.B. à la pseudoglobuline de sérum de cheval, un nombre égal manque dans la réaction avec le kétène ou l'acide acétique anhydrique. Après dénaturation de ces globulines, tous les groupes sont actifs avec tous les réactifs appliqués.

3. Le mécanisme de dénaturation de la β-lactoglobuline par l'éthanol a été étudié au moyen des groupes qui ont été réactifs additionellement.

4. La signification de ces résultats est discutée en relation de la structure des protéines.

ZUSAMMENFASSUNG

1. Von den 31 in einem Molekül β-Lactoglobulin vorhandenen Lysin-ε-Aminogruppen reagieren zwölf nicht mit F.D.N.B. (1:2:4 Fluordinitrobenzol), wenn das Eiweiss nicht vorher denaturiert wurde. Alle Gruppen können jedoch im nativen Eiweiss azetyliert werden.

2. Bei mehreren nativen Serumglobulinen reagiert die Hälfte bis ein Drittel der Lysin-ε-Aminogruppen nicht mit F.D.N.B. Bei Pseudoglobulin aus Pferdeserum bleibt bei einer ähnlichen Anzahl die Reaktion mit Keten oder Essigsäureanhydrid aus. All diese Gruppen werden nach Denaturierung für alle genannten Reagentia zugänglich.

3. Der Mechanismus der Aethanoldenaturierung von β-Lactoglobulin wurde durch Bestimmung der freigesetzten Aminogruppen untersucht.

4. Die Bedeutung dieser Resultate im Zusammenhang mit der Struktur der Eiweisskörper wird erörtert.

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