THE TERMINAL AMINO GROUPS OF CONALBUMIN, OVOMUCOID AND AVIDIN

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Recent advances in the elucidation of the structure of insulin[1] were facilitated by the prior recognition that this protein was composed of relatively short peptide chains[2]. No other protein has yet been found to conform to a similar pattern. In the present study, the search has been extended to three of the biologically active proteins of egg white, using the fluorodinitrobenzene method[2]. Conalbumin and ovomucoid of molecular weights 87,000 and 28,000, respectively, were found to consist of one peptide chain only, while avidin (molecular weight about 60,000) appeared to have three chains per molecule. Alanine was the N-terminal amino acid in all three proteins. Ovomucoid and avidin presented special problems, probably because of the carbohydrate and nucleic acid present.

METHODS AND MATERIALS

Conalbumin and *ovomucoid* were the same preparations as used in previous studies[3,4]**.

Avidin was available in small quantities only, from a variety of preparations. These were in three forms:

a. the glycoprotein (Avidin A).
b. the glycoprotein in complex with nucleic acid (Avidin N.A.).
c. the glycoprotein in complex with a second glycoprotein (Avidin X.A.).

The different preparations varied from 50% to 100% in purity. The characteristics of the different forms of avidin have been described[5,6].

The technique used for the condensation of F–DNB*** with the proteins was that described earlier[2,7,8]. In some experiments the reaction mixture was hydrolysed directly, after removal of ethanol *in vacuo* and extraction of excess F–DNB with ether. In other cases the DNP-protein*** was isolated and samples of the preparation subsequently taken for hydrolysis.

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*** Fluorodinitrobenzene will henceforth be abbreviated as F–DNB, and dinitrophenyl as DNP.

References p. 562.

DNP-ovomucoid, like DNP-salmine[7], is readily soluble in water, while DNP-avidin is also partially soluble. Both are precipitated by the addition of excess ethanol. This ethanol precipitate was washed with more ethanol and ether, and then dialysed free of salts, freeze-dried, and the preparations allowed to equilibrate with air. The composition of these air-dried preparations, in terms of the original protein, was calculated from the moisture content and the number of DNP groups expected to be present if all the amino, phenolic and imidazole groups were reactive. The values found are close to 75% in agreement with the figures for other proteins[8]. Owing to the limited supply of the preparations, confirmation of these values by amide estimation[2] was not attempted, but the similarity of the results obtained on the basis of this figure and those obtained from direct hydrolysis, where the original weight of protein was known, lends support to their validity. Thus DNP-conalbumin contained 76% protein, DNP-ovalbumin 78%, DNP-avidin 82%.

The methods of hydrolysis used were those described by SANGER AND PORTER[2,7,8]. Some experiments on the stability of DNP-alanine and ε-DNP-lysine when hydrolysed in the presence of bovine serum albumin or ovomucoid are summarized in Table I.

TABLE I

DESTRUCTION OF DNP-AMINO ACIDS DURING HYDROLYSIS OF VARIOUS PROTEINS*

Conditions of Hydrolysis	*Recovery of DNP-alanine after hydrolysis in presence of*			*Recovery of DNP-lysine after hydrolysis in presence of*	
	Bovine Serum Albumin (%)	*Ovomucoid* (%)	*DNP-ovomucoid* (%)	*Bovine Serum Albumin* (%)	*Ovomucoid* (%)
Refluxed in 6 *N* HCl					
for 4 hours	—	79	80	—	—
for 16 hours	72	64	—	95	74
for 4, then 12 hours**	—	—	—	—	89
12 *N* HCl, 100°, sealed tube	54	48	—	85	63

* To about 20 mg of protein was added about 1 μ mole of DNP-alanine and about 10 μ mole of ε-DNP lysine. The unchanged DNP-amino acids were recovered chromatographically as usual. Thus the losses are those of both hydrolysis and isolation.

** After 4 hours of hydrolysis, the solutions were extracted with ether, then refluxing continued with constant-boiling HCl.

The rate of breakdown of DNP-alanine in boiling 6 *N* HCl in presence of serum albumin is very similar to that previously found with horse globin[7]. When ovomucoid or DNP-ovomucoid is added, the decomposition shows a definite increase. In the case of ε-DNP-lysine the loss caused during 16 hours of refluxing with HCl is uninfluenced by addition of serum albumin, and ovomucoid again causes increased loss, which appears to be prevented if the hydrolysate is extracted with ether after 4 hours of hydrolysis. Hydrolysis with 12 *N* HCl in sealed tubes at 100° was even more destructive to the DNP-amino acids used, in presence of ovomucoid. It is probable that the high carbohydrate content (25%) of ovomucoid is responsible for the greater destruction of the DNP-amino acids during hydrolysis. It has recently been shown that addition of tryptophan increases the loss of di-DNP-lysine under similar conditions[9]. However, the tryptophan content of horse globin, bovine serum albumin and ovomucoid, 1.7%, 0.2% and 0.3%,

References p. 562.

bears no relation to the rates of destruction during hydrolysis in the presence of these proteins. These results serve to emphasize the need to estimate rates of breakdown of the DNP-amino acid concerned under identical conditions to those used in the estimations.

The ether-soluble DNP-amino acids from the acid hydrolysates were fractionated on silica gel columns according to the scheme described earlier[2,7,8]. Paper chromatography[10] of the coloured fractions from the silica columns was extensively used to check the identity of the DNP-amino acids. If paper chromatography is applied directly to the acid hydrolysate, one artefact believed to be dinitrophenol moves at similar rates to DNP-alanine and prevents detection of the latter. Prior fractionation on silica columns removes this artefact and enables quantitative estimation to be made at the same time.

ε-DNP lysine was isolated from the water-soluble fraction of the hydrolysate by running on buffered silica gel columns[11]. This system would readily separate any DNP-arginine from the ε-DNP lysine.

RESULTS

Conalbumin

Table II sumarizes the results obtained with conalbumin. Only one slow moving yellow band was visible when the ether soluble fraction from an acid hydrolysate was run on a chloroform column. It remained a single band when tested on a variety of columns using different solvents as the moving phase, and in each case the rate of movement was the same as that of synthetic DNP-alanine. Similar results were obtained using paper chromatography and in no case could a mixture of the unknown and DNP-alanine be separated, while separation of mixtures of the unknown and other DNP-amino acids was achieved. Hence it was concluded that alanine is the only N terminal amino acid in conalbumin. It can be seen from Table II that there is one terminal group per molecule. This quantitative result is much lower than the value of 10 free α-amino groups calculated from analytical data[12]. Similar calculations had permitted an accurate prediction of the existence of only 1 chain in lysozyme. A possible explanation of the discrepancy in the case of conalbumin was that part of its terminal amino groups might be masked and unreactive, as was found the case for part of the ε-amino groups of β-lactoglobulin and serum γ-globulins[13]. However, denaturation by various means did not release any additional end groups in conalbumin (Table II). It thus appears probable that the older indirect method[12], based largely on small differences between big numbers, is not as reliable with proteins more complex, or of higher molecular weight, than lysozyme.

The yield of ε-DNP lysine obtained from the aqueous fraction of DNP-conalbumin hydrolysates was in good agreement with that expected from the microbiological assay data of LEWIS *et al.*[12], *i.e.* 60 groups per molecule of 87,000 M.W.

Ovomucoid

DNP-ovomucoid also gave DNP-alanine on hydrolysis, this being identified as previously. As shown in Table III, amounts found indicated rather less than one end group per molecule, even after applying the higher correction figures necessary to compensate for the loss of DNP-alanine during hydrolysis in the presence of DNP-ovomucoid. It is possible that the losses are in fact higher when the DNP-alanine is

References p. 562.

TABLE II

REACTION OF CONALBUMIN WITH F–DNB

Preparation Used	*Condition of Hydrolysis*	*Yield of DNP Amino Acids (mol/mol protein)*	
		Alanine	*Lysine**
Native Protein			
Standard condition	16 hr reflux	1.0, 1.0	65, 58
Standard condition	4 hr reflux	1.1	63
Repeated treatment with F–DNB	4 hr reflux	0.9	66
Denatured Protein			
Heat	4 hr reflux	1.3	57
Guanidine HCl	4 hr reflux	1.2	60
Urea	4 hr reflux	1.2	67

* All lysine analyses after a total of 16 hrs of hydrolysis (See **, Table I).

TABLE III

REACTION OF OVOMUCOID WITH F–DNB

Condition of Hydrolysis	*Yield of DNP Amino Acids*	
	Alanine	*Lysine**
16 hr reflux	0.7, 0.77, 0.77	14.6, 13.7, 14.5
4 hr reflux	0.94, 0.86, 0.81, 0.85 0.64, 0.75	11.9, 11.4, 11.6

* See footnote *, Table II.

bound to the protein. When larger amounts of DNP-ovomucoid were hydrolysed, a much fainter second yellow band became visible with an R value slightly lower than that of DNP-glycine. When examined on other columns and by paper chromatography, it could not be identified with any of the known amino acids. It may arise from the decomposition of DNP-alanine or possibly from DNP-glucosamine.

The ε-DNP lysine content was in good agreement with the value of LEWIS *et al.*[12], *i.e.* 13 groups per mol.

Avidin

The results obtained with avidin were less consistent than those obtained with the other two proteins. This is surely in part due to the great number of different avidin preparations, none of which was available in appreciable amounts for sufficient replication. Avidin was studied as the water-soluble glycoprotein, and its natural complexes (avidin NA and avidin XA), with samples of varying purity. A further difficulty arose from the fact that DNP-avidin was partly water soluble. It was noted that in experiments in which the end group result was lower than usual, the ε-DNP-lysine result was proportionately lower, suggesting contamination of the preparation with salt.

Qualitatively, alanine was again the only appreciable ether soluble DNP-amino acid, although it was accompanied by apparently the same as yet uncharacterised

References p. 562.

component which was encountered with ovomucoid, amounting again to 10–15% of the DNP-alanine found. Quantitatively, there appeared to be 2, or more probably 3 chains with terminal alanine (Table IV), and no differences which seem significant under the circumstances were noted between the 3 types of avidin, nor between preparations of different degrees of purity. The average ε-DNP-lysine content was again in excellent agreement with the microbiologically determined value[12]. Denaturation had no definite effect on the number of reactive amino groups.

TABLE IV

REACTION OF AVIDIN WITH F–DNB

Preparation Used	*Condition of Reaction*	*Time of hydrol. (hrs)*	*Yield of DNP Amino Acid*	
			Alanine	*Lysine**
Avidin A	Direct Analysis	16	1.8	—
		12	2.4	26
Avidin XA	DNP protein isolated	4.3	3.3	25
	Direct Analysis	16	3.1	28
Avidin NA	DNP protein isolated	4	3.3	33
	Direct Analysis	16	3.1	27
	Direct Analysis	4	1.7	23
Denatured Avidin NA	Repeated treatment with F–DNB	4	3.3	32

* See footnote *, Table II.

TABLE V

SUMMARY OF RESULTS

Protein	*Terminal Alanyl Residues (mol/mol)*	*Reactive Lysine ε-amino groups (mol/mol)*	*Lysine Content Microbiological Analysis[12] (mol/mol)*
Conalbumin			
Native	1.0	63	60
Denatured	1.2	61	—
Ovomucoid	0.81	12.4	12
Avidin A	2.1	26	26
Avidin NA	3.2	28	—
Avidin XA	2.7	28	27

Since all avidin preparations contain hexoses and glucosamine (about 8% for avidin A and NA, 10–12% for avidin XA), the usual correction factors for destruction of DNP-amino acids during hydrolysis are probably slightly too low. However, the scarcity of avidin prevented a detailed study of the rate of destruction of DNP-amino acids in avidin hydrolysates.

The amino groups of the nucleic acid of avidin NA were expected to react with F–DNB, but, to our surprise, no differences were detected in the chromatographic behaviour of hydrolysates of avidin NA, as compared to the nucleic acid free preparations of avidin. This might have been attributed to instability of DNP-purines and

References p. 562.

pyrimidines, or possibly to their being obscured on the columns by another DNP-compound, such as ε-DNP-lysine. However, treatment of thymus desoxypentosenucleic acid with F–DNB yielded a completely colourless reaction product. Since DNP-adenine is strongly coloured, it must be concluded that the amino groups of desoxypentose nucleic acids are not available for reaction with F–DNB.

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SUMMARY

1. Conalbumin was found to consist of a single peptide chain, the terminal amino group pertaining to alanine.

2. Ovomucoid also appeared to consist of a single peptide chain, terminating in alanine. Probably because of its high carbohydrate content, recovery of DNP-alanine was incomplete.

3. Avidin, whether in form of the glycoprotein or one of its complexes, seemed to be made up of three peptide chains, terminating in alanine. Neither the desoxypentose nucleic acid of avidin, nor that of thymus, appeared to yield yellow DNP-derivatives.

RÉSUMÉ

1. Nous avons trouvé que la conalbumine contient une seule chaîne peptidique dont le groupe amino terminal fait partie d'un reste d'alanine.

2. L'ovalbumine consiste également en une seule chaîne peptidique qui se termine en alanine. La récupération de DNP-alanine était incomplète dans ce cas, probablement à cause de la haute teneur en hydrate de carbone.

3. L'avidine (sous forme de glycoprotéine ou d'un de ces complexes) semble être constituée de trois chaînes peptidiques qui se terminent en alanine. Ni l'acide désoxypentosenucléique de l'avidine, ni celui du thymus ne donnèrent de dérivés de DNP jaunos.

ZUSAMMENFASSUNG

1. Es wurde festgestellt, dass Conalbumin aus einer einzigen Peptidkette besteht, und dass seine endständige Aminogruppe einem Alaninrest angehört.

2. Weiters hat sich gezeigt, dass Ovomucoid aus einer einzigen Peptidkette besteht, die mit Alanin endet. Wahrscheinlich auf Grund des hohen Kohlenhydratgehaltes, verlief die Zurückgewinnung von DNP-Alanin unvollständig.

3. Avidin (als Glycoprotein oder in Form eines seiner Komplexe) schien aus drei Peptidketten zu bestehen, die auf Alanin enden. Weder die Desoxypentosenukleinsäure des Avidins, noch die des Thymus gaben gelbe DNP-Derivate.

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