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The Reactivity of the Iminazole Ring in Proteins

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(Received 22 September 1949)

The sulphhydryl, disulphide (cf. Anson, 1945), phenolic (Crammer & Neuberger, 1943) and amino groups (Porter, 1948) of proteins show varying degrees of reactivity in the native molecule. On denaturation of the protein by any means, these groups, with very few exceptions, become capable of reacting completely with the several reagents examined. This has been interpreted as suggesting the existence, in the native protein, of labile bonds or steric factors which are altered on denaturation. It has now been found, using the reagent 1:2:4-fluorodinitrobenzene (FDNB), that the iminazole rings of the histidine residues of β -lactoglobulin are less reactive in the native than in the denatured protein. Other proteins examined did not show this difference in reactivity. In insulin the iminazole group was reactive in the native molecule, but on heat precipitation, or fibrous insulin formation, almost half the residues became unreactive. Fibrous insulin, superficially resembling denatured protein, is believed to be a long-chain polymer of the insulin molecule (Waugh, 1948), and it would appear that in this case intermolecular association, rather than intramolecular rearrangement is responsible for the change in reactivity of the histidine residues.

MATERIALS

Haemoglobins. These were prepared as described by Porter & Sanger (1948).

Bovine serum albumin. A sample prepared by cold ethanol

fractionation was obtained from Armour Ltd., Chicago, U.S.A.

β -Lactoglobulin. This was a crystalline sample given by Prof. Linderström-Lang.

Ovalbumin. This was a five-times recrystallized sample obtained by Prof. A. C. Chibnall from Prof. R. K. Cannan.

Insulin. Crystalline ox insulin from Boots Pure Drug Co. Ltd. was used. Heat-precipitated insulin was prepared by keeping a 3% solution of insulin in 0.1N-H₂SO₄ at 95° for 30 min. (du Vigneaud, Siffert & Sealock, 1933). The precipitate was centrifuged and washed with distilled water. Fibrous insulin was prepared as an opalescent gel according to Waugh (1948).

METHODS

The conditions of denaturation and of condensation of FDNB with proteins have been described by Porter (1948).

Estimation of unreactive histidine residues in proteins

It was suggested by Sanger (1945) that FDNB did not form a stable compound with the iminazole ring of histidine, but it has since been found that a hydrolysate of dinitrophenyl-(DNP-) globin contains no free histidine. Dr Sanger has re-examined the reaction of FDNB with *N*'-acetyl-histidine and found that a stable crystalline compound, m.p. 221° (decomp.), is in fact formed. After removal of the acetyl group the colourless crystalline DNP derivative, m.p. 280° (decomp.), has a nitrogen content corresponding to mono-DNP-histidine and its probable structure is 1-(2':4'-dinitrophenyl)-4(5')-iminazole- α -aminopropionic acid (im-DNP-histidine). On acid hydrolysis it did not give rise to histidine (Sanger, unpublished).

The condensation of FDNB with protein is carried out in aqueous ethanol at room temperature; the reaction is so rapid that it precedes and prevents ethanol denaturation of

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β -lactoglobulin and probably of other proteins (Porter, 1948). A comparison of the reactivity with FDNB of the iminazole ring in native and denatured proteins should give some indication of the condition of this group in the native molecule. As im-DNP-histidine is a colourless compound a simple method of fractionation and colorimetric estimation was not possible. The amount of histidine unable to react with FDNB was therefore estimated directly in the hydrolysate of the DNP-protein.

Experimental procedure. The procedure was as follows: (1) The protein was allowed to react with FDNB and the protein content of the air-dried DNP-protein calculated from amide estimations as described previously (Sanger, 1945). (2) A weighed amount of air-dried DNP-protein was hydrolysed for 24 hr. in boiling 5.7 N-HCl. Excess HCl was removed by repeated concentration *in vacuo*; the residue was neutralized to pH 4.5 with N-NaOH and made up to a known volume with 0.2 M-acetate buffer pH 4.5. Owing to the small amounts of free histidine remaining in DNP proteins it was necessary to work with concentrated solutions; thus, a hydrolysate from 200 mg. would be made up to 10 ml. and 2.5 ml. used per estimation. A precipitate frequently appeared on neutralization but did not interfere. (3) Histidine was estimated manometrically using the specific decarboxylase (Gale, 1945). The products of hydrolysis of DNP proteins did not affect the activity of the enzyme, but they would, of course, hinder colorimetric estimation. Satisfactory duplicates were obtained and all the results quoted are the mean of two or more assays.

RESULTS

In some of the proteins examined, FDNB condensed almost quantitatively with the iminazole ring and only a small blank value equivalent to 2 or 3 % of the original histidine remained. As indicated in

Table 1, native lactoglobulin was an exception; 50 % or two of the four histidine residues per molecule did not condense with FDNB. After denaturation by any of the three methods tried, all the histidine residues of lactoglobulin reacted with FDNB.

The result with human haemoglobin (Table 1) is of interest because it might be expected that, if haem is attached to the iminazole ring, four of the histidine residues (12.5 % of the total) would fail to react with the FDNB. As the linkage of haem to globin is very labile, the reaction was carried out at -3° for 30 hr., but again the histidine residues reacted almost quantitatively with FDNB. Though no haem was removed when the DNP-haemoglobin was washed with water, ethanol and ether, subsequent spectroscopic examination, kindly carried out by Miss J. Keilin, showed that a considerable amount of free haematin and haem were present together with haemochromogen. The haem-globin bond had been broken and no conclusion as to its nature could be drawn.

Insulin proved exceptional in that a small but significant fraction of the histidine did not react when crystalline insulin was used and a much larger proportion was unreactive in heat-precipitated and fibrous insulin (Table 2). Throughout the experiments with insulin, the sodium bicarbonate, FDNB and ethanol-water concentrations were the same, the only variable being the condition of the insulin. The sodium bicarbonate concentration used was 4 % not 10 % (w/v of aqueous protein solution) as in the other experiments, because the higher concentration throws insulin out of solution and is not necessary to keep the mixture alkaline throughout the reaction.

Table 1. *Reaction of histidine residues of proteins with FDNB*

Protein	Histidine content (N as % of total N)	Histidine content after condensation with FDNB. (N as % of total N of original protein)	Percentage of unreactive histidine residues
Bovine serum albumin	7.24 (a)	0.36	5.2
Native β -lactoglobulin	2.85 (b)	1.46	51.0
Heat-denatured β -lactoglobulin	2.85	0.04	1.4
Guanidine-denatured β -lactoglobulin	2.85	0.04	1.4
Ethanol-denatured β -lactoglobulin	2.85	0.07	2.0
Native ovalbumin	4.1 (b)	0.12	2.9
Heat-denatured ovalbumin	4.1	0.10	2.5
Horse globin	13.5 (b)	0.34	2.5
Human haemoglobin	13.1 (c)	0.5	3.9

References. (a) Brand (1946); (b) Macpherson (1946); (c) Roche (1936).

Table 2. *Reaction of insulin with FDNB*

Conditions of insulin in reaction with FDNB	Unreactive histidine (as % of total histidine)	No. of unreactive groups/submolecule of wt. 12,500
Solution of insulin (0.1 %)	7.5	0.30
Solution of insulin (1 %)	9.0	0.36
Solution of insulin (3 %)	10.1	0.42
Solution of insulin (5 %) in 6 M-urea	11.2	0.45
Heat-precipitated insulin in suspension	44.0	1.75
Fibrous insulin in suspension	45.0	1.80

DISCUSSION

 β -Lactoglobulin

Under the conditions of the reaction, the histidine residues of serum albumin, egg albumin and haemoglobin are able to condense with FDNB whether the proteins have been denatured previously or not, but in β -lactoglobulin only two of the four residues are available to the reagent, prior to denaturation. In view of the rapidity of the reaction of FDNB with β -lactoglobulin (Porter, 1948), and the failure of a considerably increased reaction period, in the presence of excess reagents, to affect the number of reacting iminazole groups, it is probable that there is a qualitative distinction between the reactive and unreactive groups. It is suggested that, in this native protein, histidine is an additional example of a residue whose reactivity is limited by the specific structure of the molecule.

Insulin

In native insulin there is a persistent content of unreactive histidine which appears to increase slightly with concentration (Table 2); the presence of 6M-urea has no effect and it has equally little effect on the biological activity or crystallizing power of the insulin. These values of unreacted histidine, about 10% of the total, are significantly higher than the blank values found for other proteins. As a number of different preparations of DNP-insulin were used, some of which had been made by allowing the reaction to continue for a considerably greater time than the usual 2 hr., it is most unlikely that the free histidine arose from a fortuitous failure of the reaction to go entirely to completion.

It has been postulated that the basic units of insulin have a molecular weight of about 12,000 (cf. Sanger, 1949), and it is believed that these units are associated in solution, reaching, under optimal conditions of pH and concentration, maximal aggregates of four units. At 3% concentration and pH 8.5, insulin would be such an aggregate of four units, but under these conditions only 0.4 residues of histidine are unreactive per unit, and it is therefore clear that there can be no connexion between this association and the lack of reactivity of the histidine residues. In contrast, the lack of reactivity of nearly two out of four of the iminazole groups in the submolecule of fibrous or heat-treated insulin is due, probably, to the association of the units by endwise attachment. The insolubility of heated insulin is unlikely to explain the altered reactivity of the protein as amino groups react completely in the same sample and experience with β -lactoglobulin and other proteins suggests that the reactivity of all groups increases rather than decreases when the soluble native material becomes denatured and insoluble. Waugh (1948) has postu-

lated that the formation of long fibres is due to the presence of two 'active centres' in each unit. If this is accepted, it could be assumed that two histidine residues become unreactive because they are part of such combining centres, or are sufficiently close to these centres for increased steric hindrance to prevent access of the FDNB molecule. The reactivity of the N¹ and N⁶ amino groups (six/submolecule) is unaffected by heat precipitation. This evidence supports the view that the combining centres are localized on the surface of the molecule.

In pointing out that aggregation occurred only at low pH, Waugh (1948) suggested that suppression of the ionization of the acidic groups might be essential to reduce electrostatic particle repulsion. By similar reasoning it seems unlikely that the two iminazole residues involved are situated on the two combining centres, since this would lead to the association of two similarly charged groups. It is more probable that both of the iminazole groups involved are in one combining centre, the second centre being of different composition. Such asymmetry of the molecule would be in keeping with the finding of Sanger (1949) that all the basic amino-acid residues are in the two peptide chains which have terminal phenylalanyl residues, the other two peptide chains with terminal glycyl residues containing no basic amino-acids.

If the blocking of the iminazole residues in heat-precipitated or fibrous insulin is due to the polymerization of submolecules, the small amount of unreactive material in crystalline insulin is perhaps explicable by the assumption of contamination of the protein by a little polymerized material. Seeding experiments carried out by Waugh showed that such contamination could not occur at acid reaction, but in neutral or alkaline solution it may not be impossible. This, or the presence of some other bound impurity, seems to be the only explanation of the results obtained with the crystalline material.

SUMMARY

1. 1:2:4-Fluorodinitrobenzene reacts with the iminazole ring of N¹-acetyl-histidine to give a compound which does not yield histidine on acid hydrolysis.

2. With the aid of this reaction it has been found that two of the four histidine residues in β -lactoglobulin fail to react with fluorodinitrobenzene unless the protein is first denatured. In ovalbumin, serum albumin, haemoglobin and globin the histidine residues react fully whether the proteins are denatured or native.

3. In crystalline zinc insulin 0.3-0.4 histidine residues of the four present per submolecule fail to react, and after heat precipitation or conversion to fibrous insulin, 1.8 residues become unreactive. The

significance of these findings in relation to fibre formation is discussed.

4. Unsuccessful attempts to use this reaction to establish the mode of linkage of haem to globin in native haemoglobin are described.

I wish to thank Prof. A. C. Chibnall, F.R.S., and Dr F. Sanger for their interest in this work. The receipt of a personal grant from the Medical Research Council is gratefully acknowledged.

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Studies on the Synthesis of Lactose by the Mammary Gland

I. PRECURSORS

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(Received 15 August 1949)

The main object of our work is to throw some light on the intermediary metabolism of the mammary gland, with particular reference to the mode of lactose synthesis. Although a number of workers, using a variety of experimental techniques, have made an approach to this problem, all studies hitherto reported have been primarily concerned with defining the initial blood precursors of the milk sugar, and there is little information on the detailed mechanism of this synthesis. *In vivo* experiments, in which the concentrations of possible precursors in simultaneously drawn samples of arterial and mammary venous blood were compared, have demonstrated a large uptake of glucose when blood passes through the active mammary gland (for references see Folley, 1940, 1949), and it is regarded as reasonably certain that this uptake is far in excess of the energy requirements of the tissue, and is, in fact, an indication that glucose is a precursor of lactose.

This conclusion has been confirmed and amplified by *in vitro* studies on surviving mammary tissues, notably by Grant (1935) and Knodt & Petersen (1945). Grant (1935) succeeded in demonstrating lactose synthesis from glucose by tissue slices from the lactating glands of guinea pigs killed 2-5 days after parturition, and was able to obtain a quantitative estimate of the synthesis by using a lactose-fermenting yeast, *Saccharomyces fragilis*. Knodt & Petersen (1945) claim to have shown lactose synthesis by tissue slices from the lactating udders of cows killed at slaughter, and in their work glucose, glycogen and maltose were found to be equally effective substrates. Lactic acid, on the other hand,

was ineffective, a result contrasting with earlier claims based on arterio-venous studies by Graham (1937). Knodt & Petersen (1945) used fermentation techniques (Scott & West, 1936) in determining lactose and, like Grant (1935), were dependent on changes in reduction values following fermentation for their estimation of lactose formed.

It seemed to us that a first step in our work should be a reinvestigation of these findings, using methods for the estimation and detection of lactose which could be regarded as more specific for this sugar than methods ultimately dependent on reduction measurements. This paper gives an account of this initial study.

EXPERIMENTAL

Reagents. Glycogen was prepared from rabbit livers by extraction with trichloroacetic acid and subsequent precipitation with 50% (v/v) ethanol, 80% (v/v) acetic acid and 50% (v/v) ethanol in that order (Good, Kramer & Somogyi, 1933; Bell & Young, 1934). *s*-Collidine was purified according to Partridge (1948).

Sources of tissues. The collection of cow udders from the local abattoir conformed as closely as possible with the procedure adopted by Knodt & Petersen (1945). Glands showing a high degree of milkiness on removal were selected and brought without special cooling direct to the laboratory. The time from slaughter to the start of an experiment was normally 1.0-1.5 hr.

Guinea pig glands were obtained from animals in our own colony. Parturient does were suckled normally by their litters and killed in active lactation on the sixth day post-partum. In these experiments the incubations of tissue started within 30 min. of killing by neck dislocation.