FORMATION OF PERSULFIDE GROUPS IN ALKALINE TREATED INSULIN

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1. Introduction

Alkaline treatment of disulfide-containing proteins is known to produce a number of unusual compounds related with the degradation of the cystinyl residues. Lanthionine [1] and lysinoalanine [2] are among the products identified with certainly, while dehydroalanine [2], thiocysteine [3,4], cysteine sulfenic acid [5] and other products are supposed, by indirect evidence, to rise in the meantime. Although for experimental purposes the effect of alkali on proteins is usually studied under heavy alkaline conditions, sensitivity of proteins to alkali is sufficiently high to represent a possible nuisance to proteins under the mild alkaline conditions employed in many biochemical studies [6]. The mechanism of degradation of the cystinyl sulfur by alkali is still controversial and it is not yet clear whether the main reaction is hydrolysis of disulfide bond or β-elimination of the cystinyl sulfur with the formation of persulfide groups (R-SSH). We have recently detected persulfide groups by spectral analysis of proteins in the presence of sodium sulfide [7]. The results of that study encouraged us to extend the investigation to the formation of persulfide groups during the alkaline cleavage of proteins. In the present note we report the results obtained by using insulin as a representative disulfide-containing protein.

2. Experimental

Crystalline zinc-insulin was a gift of Wellcome Italia; it had a content of 0.38 percent Zn, 6 percent of humidity and an activity of 24 units per mg. Other products were of the best available analytical grade. Persulfide groups were detected by the absorbance at 335 nm in alkaline solution [7]. Quantitative analysis of persulfides by cyanolysis was performed as reported earlier [7]. Spectrophotometric curves were obtained with the Beckman DK2 spectrophotometer and spectral changes at fixed wavelengths were followed with a Beckman DU2 spectrophotometer. The temperature ranged from 22° to 26°. A silica cell converted into a Thunberg tube was used for the anaerobic experiments. The Thunberg tube was evacuated by a water pump and refilled three times with extra pure nitrogen. The conventional Warburg apparatus was used for the determination of O_2 uptake.

3. Results

As reported earlier [7] the absorbance at 335 nm of insulin dissolved in 0.01 N NaOH did not change appreciably during the time of our experiment; when the NaOH concentration was raised to 0.025, or over, the absorbance increased with time (fig. 1). The spectral curves shown in fig. 2 indicate a time dependent production of a chromophore absorbing in the range of 300 to 400 nm with characteristics similar to those found by interacting insulin with Na_2S [7]. The curves relating absorbance at 335 nm with time are complex and do not allow a simple kinetic treatment, but show an initial lag phase, a two-step increase and, after having reached a maximum, a decay phase.

Correlation of the absorbance at 335 nm with the formation of persulfide groups by cleavage of cystinyl sulfur is shown by tests reported in fig. 3. The addition of 0.01 M cyanide, when the absorbance has reached the maximum, abolished the absorbance. This test was based on the ability of cyanide to cyanolize persulfides producing thiocyanate [7]. The addi-

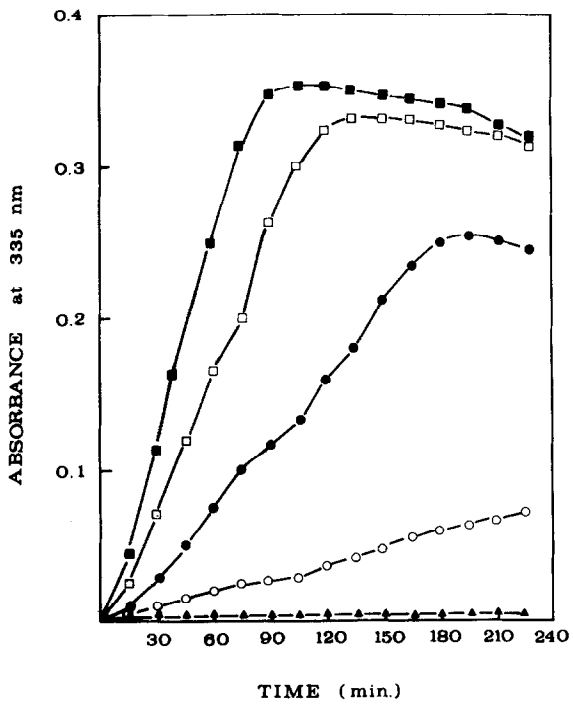

Fig. 1. Change of absorbance at 335 nm of 3.33 mg insulin per ml of NaOH solution at room temperature. Concentration of NaOH solution was: ▲—▲ 0.01 N; ○—○ 0.025 N; ●—● 0.05 N; □—□ 0.075 N; ■—■ 0.1 N.

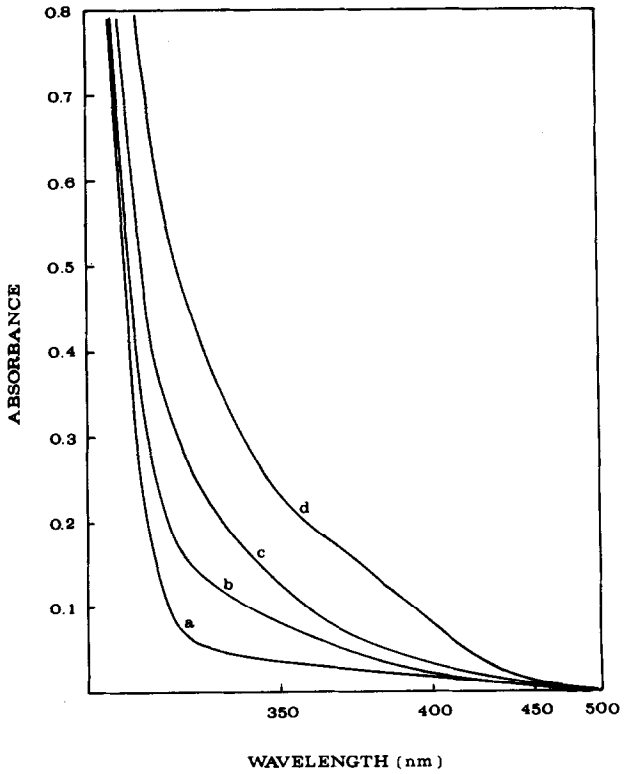

Fig. 2. Spectral curves of 3.33 mg insulin per ml 0.05 N NaOH at room temperature. a) as soon as dissolved; b) after 60 min; c) after 120 min; d) after 180 min.

tion of equimolar amounts of hypotaurine or sodium phenyl sulfinate (Fluka) at maximum absorbance also abolished the absorbance. The disappearance of the 335 nm absorbance upon the addition of sulfinates is known to be due to the transulfuration of sulfinates by persulfides, yielding thiosulfonates [7–9].

Further proof for the correlation of the 335 nm absorbance with formation of persulfide groups was obtained by the addition of sodium monoiodacetate. Reaction of persulfides with iodacetate to produce mixed disulfides has been established with the persulfides produced by the cleavage of cystine by cystathionase [10] and with the persulfides produced by the cleavage of cystamine by diamine oxidase [11]. The addition of 0.01 M sodium monoiodacetate under the conditions described for the addition of cyanide or sulfinates promtly abolished the absorbance at 335 nm. However the absorbance reappeared again after a certain time indicating that the new disulfide groups produced were cleaved a second time. This finding will be carefully investigated in separate experiments.

The lag phase observed in the curves reported in fig. 1 suggests that a preliminary denaturation of the protein is necessary before the attack of the disulfide groups by the hydroxyl ions. To test for this possibility, the change at 335 nm was followed with insulin denatured in 6 M urea. As reported in fig. 3, denaturation abolishes the lag phase and speeds up the production of persulfide groups.

The decay phase observed after the absorbance at 335 nm has reached a maximum is due to the instability of protein persulfide groups. This has been verified for persulfides produced by addition of Na_2S [7]. However, because persulfides are sensitive to oxygen [12], we have tested the effect of air on the decay phase of the curve. Alkaline treatment of insulin in a Thunberg tube with N_2 as the gas phase instead of air showed a faster rate of production of persulfides and a final higher absorbance at 335 nm (fig. 3). This re-

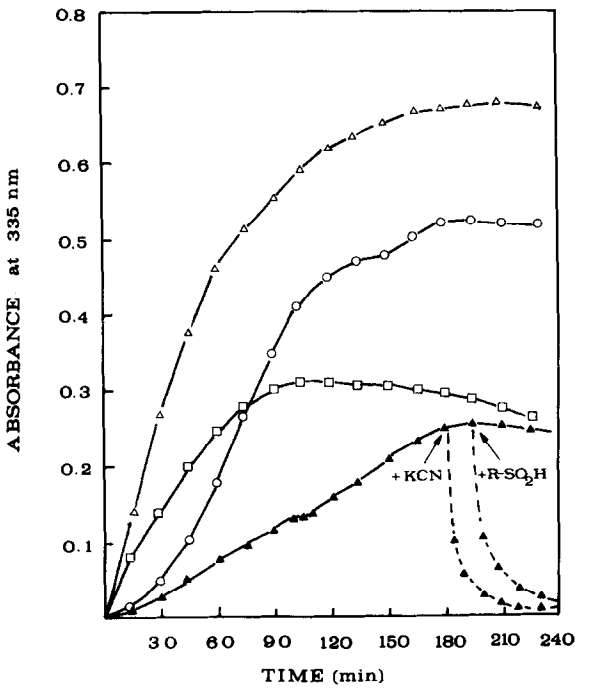

Fig. 3. Change of absorbance at 335 nm of 3.33 mg insulin per ml of 0.05 N NaOH at room temperature. ▲—▲, in air (in other experiments 0.01 M KCN or 0.01 M hypotaurine has been added at the arrows); □—□ in air and 6 M urea; ○—○ in N_2; △—△ in N_2 and 6 M urea.

Table 1

Number of persulfide groups produced by treatment of 3.33 mg insulin per ml 0.05 N NaOH at room temperature. Persulfide determination was performed by cyanolysis except in experiment 2 where the extinction coefficient was used [7]. Values reported are the number of persulfide groups per mole of insulin.

Exp. no.	Hr of solution in 0.05 N NaOH	Conditions	Number of -SSH detected
1	3	–	1.0
2	3	zinc-free insulin	1.0
3	1.5	6 M urea	1.35
4	3	under N_2	2.3
5	3	under N_2 and 6 M urea	3.0

sult is in accord with the prevention, in the presence of N_2, of degradation of persulfides by an oxidative reaction. The occurrence of an oxidative side reaction has been verified by performing the alkaline cleavage of insulin in a Warburg flask. An uptake of 10 μl of O_2 per hr was obtained for 10 mg insulin in 0.05 N NaOH.

A control has been performed using zinc-free insulin. The behaviour of zinc-free insulin, prepared as described by Lens and Neutelings [13], towards 0.05 N NaOH was essentially the same as that observed for the natural product, indicating no appreciable effect due to the zinc ion.

The number of persulfide groups produced by treatment of insulin in 0.05 N NaOH under various conditions has been established by cyanolysis (table 1). Under optimal conditions, the conversion of disulfide groups into persulfide groups seems to be quantitative.

4. Discussion

The appearance of the absorbance between 300 to 400 nm when insulin is dissolved in NaOH solutions higher than 0.01 N is attributed to the production of persulfide groups. No other chemical change is known to produce this absorbance in an unconjugated protein, except the oxidation of tyrosine by oxidizing agents [14]. However, the fact that the absorbance shown here also appears in the absence of oxygen and disappears when persulfide breaking agents – cyanide, sulfinates, and iodacetate – are added rules out the involvement of tyrosine.

The detection of persulfide groups in proteins by spectral analysis has two advantages: it permits a continuous assay of persulfides in proteins in the course of their formation; it is possible to establish the contribution of the cystinyl β-elimination compared with the hydrolysis of disulfide bonds during the alkaline degradation of proteins.

The quantitative conversion of disulfide bonds into persulfide groups obtained in the present work under optimal conditions indicates that the cleavage of the cystine residues by the β-elimination is the main, if not the only, path operating in the degradation of insulin by alkali.

Acknowledgments

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References

[1] M.J. Horn, D.B. Hones and S.J. Ringel, J. Biol. Chem. 138 (1940) 141.
[2] Z. Bohak, J. Biol. Chem. 239 (1964) 2878.
[3] N. Catsimpoolas and J.L. Wood, J. Biol. Chem. 239 (1964) 4132.
[4] J.F. Schneider and J. Westley, J. Biol. Chem. 244 (1969) 5735.
[5] J.W. Donovan, Biochem. Biophys. Res. Commun. 29 (1967) 734.
[6] L.O. Anderson, Biochim. Biophys. Acta 200 (1970) 363.
[7] D. Cavallini, G. Federici and E. Barboni, European J. Biochem. 14 (1970) 169.
[8] D. Cavallini, C. De Marco, B. Mondovì and R. Mori, Enzymologia 22 (1960) 161.
[9] C. De Marco, M. Coletta and D. Cavallini, Arch. Biochem. Biophys. 93 (1961) 179.
[10] M. Flavin, J. Biol. Chem. 237 (1962) 768.
[11] C. De Marco, G. Bombardieri, F. Riva, S. Duprè and D. Cavallini, Biochim. Biophys. Acta 100 (1965) 89.
[12] C. De Marco, M. Coletta and D. Cavallini, Arch. Biochem. Biophys. 100 (1963) 51.
[13] J. Lens and J. Neutelings, Biochim. Biophys. Acta 4 (1950) 501.
[14] K.T. Yasunobu, E.W. Peterson and H.S. Mason, J. Biol. Chem. 234 (1959) 3291.