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Short Communication

Disulphide Interchange Reactions involving Cyclocystine and their Relevance to Problems of α-Keratin Structure

By H. LINDLEY and T. HAYLETT*
Division of Protein Chemistry, Commonwealth Scientific and Industrial Research Organisation, Wool Research Laboratories, Parkville, Melbourne, Vic. 3052, *Australia*

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We have recently shown that CyS-CyS sequences occur very frequently in α-keratins (Lindley & Haylett, 1967). Until recently insulin was the only other protein known to contain this sequence, but it has since been recognized in lactoglobulins (Frank & Braunitzer, 1967). Of the possible structural consequences of this sequence for α-keratins the only one that seemed readily accessible to experimental test was the possibility of the occurrence of cyclocystinyl sequences, CyS-CyS. The parent compound, cyclocystine, has been described by Wade, Winitz & Greenstein (1956), but there has been little systematic study of its properties. Since α-keratins are quite resistant to proteolytic enzymes except in the presence of reducing agents, it follows that evidence on the occurrence of cyclocystine in keratins can only be sought in partial acid hydrolystates, and hence it further follows that the stability of cyclocystine in acid solutions is of major importance to any such investigation. We have accordingly investigated the problems of disulphide interchange and peptide bond stability in systems involving cyclocystine and CyS-CyS sequences and used this information to attempt to obtain evidence relating to the possibility of the occurrence of cyclocystine in wool.

Cyclocystine was prepared by the oxidation of a dilute solution of L-cysteinyl-L-cysteine with air as described by Wade *et al.* (1956); its properties agreed with their published data. We attempted to devise a quantitative procedure for determining cyclocystine by following the conventional amino acid analysis procedures on Dowex 50 columns, but cyclocystine seems to be completely destroyed under the usual conditions for amino acid analysis.

Model building studies with space-filling atomic models suggested that cyclocystine has a *cis*-amide group, and if this is so it could imply some instability of this bond to acid hydrolysis. An infrared spectrum of our sample of cyclocystine showed bands at 1470cm.$^{-1}$ and 1295cm.$^{-1}$, which are characteristic of *cis*-amide bonds (Blaha, Smolikova & Vitek, 1966) and thus reinforced this possibility.

Oxidation of cyclocystine produces cysteicyl-cysteic acid, which is readily characterized by its electrophoretic behaviour at pH 1·9. In the present work, proof of identity of an unknown material with cyclocystine was assumed if the unknown satisfied the following two criteria: (*a*) it had the same R_F as cyclocystine on paper chromatograms with pyridine–3-methylbutan-1-ol–water (7:7:6, by vol.); (*b*) it could be oxidized to give material with the same electrophoretic mobility as cysteicyl-cysteic acid at pH 1·9. It was convenient to use the two techniques consecutively in a two-dimensional fashion. Further, if the cysteicyl-cysteic acid was detected with the cadmium–ninhydrin reagent of Atfield & Morris (1961) then quantitative results can be obtained.

Oxidation of L-cysteinyl-L-cysteine with air produces in addition to cyclocystine a mixture of polymers made up of CyS-CyS dipeptide molecules linked together into cyclic structures through disulphide bonds. This material, freed from lower-molecular-weight derivatives by Sephadex filtration, was also isolated and used as a model compound for disulphide interchange studies. When this material was incubated with conc. HCl at 37° there was a rapid formation of cyclocystine, which reached a maximum of 50% of the theoretical yield after 5hr. and thereafter declined steadily. In confirmation of the findings of Ryle & Sanger (1955) it was found that the addition of catalytic amounts of thioglycollic acid prevented this rapid interchange, but studies over extended periods indicated that the effect of the thiol was merely to delay the onset of the reaction rather than function as a true inhibitor.

Similar studies on the stability of cyclocystine to conc. HCl in the presence of thioglycollate at 37° showed that it disappeared approximately exponentially with a half-life of about 1 day. Paper chromatograms of samples removed at various times showed a great complexity of disulphide

* Present address: National Chemical Research Laboratories, Council for Scientific and Industrial Research, Pretoria, South Africa.

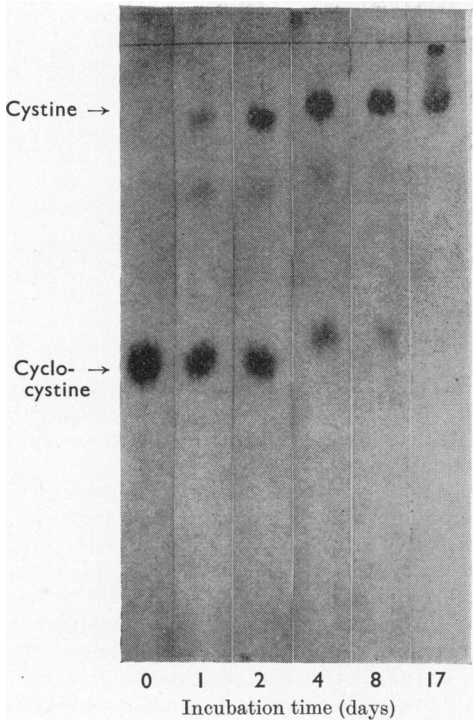


Fig. 1. Paper-strip chromatograms illustrating the almost complete absence of disulphide exchange reactions when cyclocystine is incubated with 10N-H_2SO_4 in 50% acetic acid containing thioglycollate at 37°. Experimental conditions were as follows. Cyclocystine (15mg.) was incubated at 37° with 10N-H_2SO_4 in 50% (v/v) acetic acid (6ml.) containing thioglycollic acid (2μl.). A sample (1ml.) was removed immediately and similar samples were removed after 1, 2, 4, 8 and 17 days. The samples were freed from H_2SO_4 by elution from a column of Dowex 2 (X8; acetate form; 200–400 mesh) with 20% (v/v) acetic acid (30ml.). The eluate was evaporated to dryness and dissolved in water (250μl.), and 25μl. samples were spotted on the Whatman 3MM paper for chromatography overnight with pyridine–3-methylbutan-1-ol–water (7:7:6, by vol.). The figure shows separate strips cut from chromatograms run on different occasions and hence there is some slight variability between the different chromatograms.

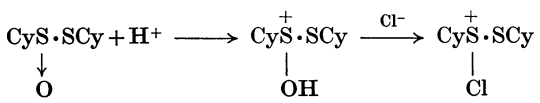
interchange products in addition to the gradual formation of cystine by peptide bond hydrolysis. All these results emphasize very strongly the finding that conc. HCl even in the presence of thiols is quite useless for the investigation of peptides containing a CyS-CyS sequence.

Ryle, Sanger, Smith & Kitai (1955) used 10N-H_2SO_4 in 50% (v/v) acetic acid plus thioglycollic acid in their work on the disulphide bonds of insulin and we have studied the behaviour of cyclocystine in this system at 37°. In this case also the overall disappearance of cyclocystine follows an approximately exponential course with a half-life of 3–4 days. Fig. 1 shows paper chromatograms of samples removed after various times of reaction, and it can be seen that in this case the reaction product is almost exclusively cystine with very little evidence of disulphide exchange reactions. It would seem therefore that the H_2SO_4–acetic acid–thioglycollic acid hydrolysis mixture is adequate for preventing disulphide interchange. Its use for partial hydrolysis studies of keratins is subject only to the proviso that the cyclocystine peptide bond seems somewhat labile, since Ryle *et al.* (1955) found it necessary to hydrolyse insulin for 17 days. However, in the absence of any alternative the procedure was used to investigate partial hydrolysates of wool as follows.

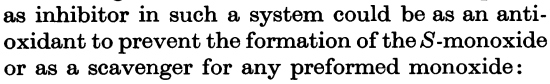
Wool (50mg.) was wetted out with 10N-H_2SO_4 in 50% acetic acid (4ml.) containing thioglycollic acid (2μl.) and incubated at 37° for 4 days. The hydrolysate was diluted and put on to a column (2cm. × 25cm.) of Dowex 2 (X8; acetate form; 200–400 mesh) and eluted with 100ml. of 20% (v/v) acetic acid. The eluate was concentrated *in vacuo* to dryness, and dissolved in 100μl. of water. Samples (10μl.) were then examined directly for cyclocystine by the two-dimensional technique without any further fractionation. With this technique it is possible to detect cyclocystine in HCl–thioglycollic acid partial hydrolysates of wool even after only 1–2 days' hydrolysis. On the other hand cyclocystine was never detected in a H_2SO_4–acetic acid–thioglycollic acid hydrolysate of wool or insulin. Various times of hydrolysis from 4 to 10 days were investigated and we also explored the possibility of using the alternative conditions employed by Ryle *et al.* (1955) for insulin (i.e. 45min. at 100°), but again we could find no evidence for the occurrence of cyclocystine in such partial hydrolysates.

Though this work cannot definitely exclude the possibility of cyclocystinyl sequences occurring in α-keratins it seems very improbable that they play a major role. However, the surprising ease which which cyclocystine can be formed from CyS-CyS sequences suggest they may conceivably occur as intermediates in for example the keratinization process in keratin biosynthesis and also in some technologically important reactions in wool processing such as permanent setting.

The mechanism of the disulphide exchange reaction seems of some interest. Benesch & Benesch (1958) have suggested a mechanism, but despite their claim to the contrary it seems to us that it offers no satisfactory explanation of the inhibition of the reaction by thiols. However, it has occurred to us that possibly the active intermediate in disulphide interchange might be the *S*-monoxide or the derived sulphonium ion:



Traces of free chlorine either pre-existent in the HCl or formed by photodecomposition could produce small amounts of the *S*-monoxide from the disulphide. For wool it is probable that small amounts of the *S*-monoxide occur naturally as a result of exposure of the wool to the atmosphere and radiation while on the sheep's back. The *S*-monoxides enter very readily into interchange reactions and these are known to be catalysed markedly by halide ion (Savige, Eager, Maclaren & Roxburgh, 1964). The role of a thiol compound as inhibitor in such a system could be as an antioxidant to prevent the formation of the *S*-monoxide or as a scavenger for any preformed monoxide:



Such a scheme not only explains why the interchange occurs more readily in HCl than H_2SO_4, but it also offers an explanation of our observation that added thiol tends only to delay the onset of disulphide interchange reactions, not to slow them down.

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