within individuals, and the extent of variation is much smaller than the differences we have found in callus culture[23].

As the existence of degrees of polyploidy higher than triploidy can be expected within the callus tissue grown in vitro, we have tried to determine the degree of polyploidy by estimating the content of heterochromatin per interphase nucleus by means of the PV-8 Image Analyser. The results [figure 2] show that there is no perceivable variation of heterochromatin contents in root tip cells of intact plants, the total area of chromocentres measuring 0.2–1.9%, whereas in callus culture there are considerable variations in the 9th and 11th passage, the corresponding figures being 2.2–11.4%, and 1.4–8.5% respectively. Considering that the root tips of the intact plant have diploid nuclei, and excluding the possibility of differential heterochromatin replication, in the callus tissue the ploidy levels of non-dividing nuclei are estimated to reach values as high as 12n. Detailed studies are in progress on the C-band karyotypes of different *Vicia faba* callus lines and their sources, to define the nature of the observed extra chromosome and of the karyotypic alterations.

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Reoxidation of reduced hen egg white lysozyme fragment 1–123

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Summary. The reactivation of reduced lysozyme, whose 6 COOH-terminal amino acid including cysteine 127 were cut off, was studied. The results show that the disulfide bridge I–VIII as well as the COOH-terminal hexapeptide do not play a decisive role in the acquisition of the native 3-dimensional structure of the enzyme.

It is generally accepted that the conformation adopted by a polypeptide chain is a direct function of its amino acid sequence. Numerous studies including those on insulin, chymotrypsin, ribonuclease and staphylococcal nuclease have suggested that the entire amino acid sequence of the protein is required for folding from a disordered state to a native-like conformation[2–9].

In the case of hen egg white lysozyme, however, the COOH-terminal dipeptide, Arg-Leu, was recently proved to be not essential for the acquisition of the native 3-dimensional structure of this enzyme[10]. Moreover, it was suggested that the formation of disulfide I–VIII, linking cysteinyl residues 6 and 127, one of the last events of the reoxidation process, is accompanied by only minor effects on the biological activity as well as on the 3-dimensional structure[11].

Checking such a role for cysteinyl residue 127 and for the COOH-terminal amino acid sequence of lysozyme, has been made possible by the discovery of carboxypeptidase Y from *Saccharomyces cerevisiae*. This enzyme conserves its proteolytic activity in denaturing media such as 6 M urea[12]. The study of the refolding of reduced lysozyme cut off between residues 122 and 123 is reported in this communication.

Material and methods. Dithiothreitol (lot 124C-0218), Tris base (lot 104C-5000), 2-mercaptoethanol (lot 32C-0170) and an aceton powder of *Micrococcus Luteus* (lot 128C-1980) were provided by Sigma. Hen egg white lysozyme was purchased from Boehringer and 5,5′-dithiobis(2-nitrobenzoic acid) from Fluka. Lysozyme was further purified by means of a chromatography on Biorex-70 according to Johnson et al.[10]. Carboxypeptidase Y was purified from *Saccharomyces cerevisiae* according to previously described procedures[12,13]. Urea and cuppric chloride were provided by Baker. Only freshly prepared solutions of deionized urea were used. All the other chemicals were of the best grade available. Reduction of lysozyme and regeneration of the reduced protein were performed according to Ristow and Wetlaufer[14].

The removal of the COOH-terminal hexapeptide from reduced lysozyme was performed by incubating the protein (2 mg/ml) in a 0.1 M acetate buffer containing 2 M urea, 10 mM 2-mercaptoethanol and 10 mM EDTA at pH 5.5 in the presence of carboxypeptidase Y (1% enzyme to substrate protein). Digestion proceeded for 5 h at room temperature and was stopped by adjusting the pH of the reaction mixture to 2 with 6 M HCl. Amino acid analysis revealed that the terminal hexapeptide COOH-Leu-Arg-Cys-Gly-Arg-Ile had been quantitatively removed from lysozyme. As a consequence, the new derivative was named lysozyme fragment 1–123. It was desalted on a Sephadex G-25 column and lyophilized.

Amino acid analysis was performed in a Beckman Unichrom analyzer according to Spackman et al.[15]. The lysis of *Micrococcus Luteus* cells by lysozyme at 25 °C in a 0.066 M sodium phosphate buffer containing 0.9% NaCl at pH 6.2 was determined by means of a standard assay similar to that used by Jolles[16].

The concentration of lysozyme and carboxypeptidase were determined spectrophotometrically assuming the extinction coefficient at 280 nm for a 1% solution of enzymes to be equal respectively to 26.3 and 15.0[12,17]. The free thiol content was estimated after reaction with Ellman's reagent according to the procedure described by Habeeb[18]. Absorbance measurements were made using either a Zeiss PMQ II or a Cary 15 M spectrophotometer. Fluorescence measurements were performed as indicated by Polastro et al.[19].

Results and discussion. Reduced lysozyme and reduced lysozyme fragment 1-123, completely devoid of tridimensional structure as well as of enzymatic activity, were submitted to the reoxidation using cuppric ions as the catalyst. A molar ration of 2 moles of cuppric ions per mole of each reduced proteins was used in all the experiments described here. This molar ratio was indeed found to lead to an optimal regain of enzymatic activity (measured 1 h after reoixdation had begun).

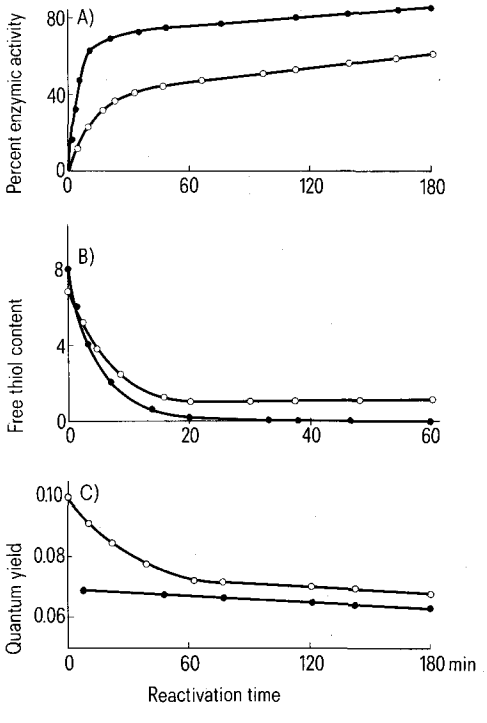
From reduced lysozyme, 84% of the enzymatic activity was recovered after 24 h, 65% being regenerated from reduced lysozyme fragment 1-123. Although lower than the value obtained with the entire polypeptide chain, the lytic activity recovered with reduced lysozyme fragment 1-123 remained exceptionally high (around 1% activity might be expected to regenereate if reoxidation had proceeded in a random way). It was therefore claimed in the abstract that the disulfide bridge I-VIII and the COOH-terminal hexapeptide of lysozyme did not play a decisive role in the acquisition of the native tridimentional structure of the protein.

From part A of the figure, it appears that the rate of regain of lysozyme activity for reduced fragment 1-123 was markedly lower than that for intact reduced lysozyme. To regenerate 50% of the final activity necessitated 3 min in the former case and 11 min in the latter. Despite this, the decrease of the free thiol content as a function of time (show in part B of the figure) was found to be very similar for the 2 derivatives.

The free thiol content reached a constant value within 15 min. At this stage, 75% of the final activity was already regenerated in the case of lysozyme, 38% only for lysozyme fragment 1-123. This observation strongly suggests that the number of initially mispaired disulfide bonds is greater in the latter than in the former case.

At this stage also, reoxidized lysozyme possessed fluorescence parameters (see part C of the figure) nearly identical to those of native lysozyme. The transition from a disordered state (with a quantum yield of 0.124) to a nearly native one, as observed by fluorescence, took place very rapidly (in less than 3 min). On the contrary, in the course of the reoxidation of reduced lysozyme fragment 1-123, the fluorescence quantum yield decreased only slowly, the value characteristic of native lysozyme being reached after 60 min in this case.

As previously outlined by Saxena and Wetlaufer, the transformation of reduced to reoxidized lysozyme, can be viewed as composed of 3 kinds of rate process: a) a rapid polypeptide backbone refolding, b) formation of intramolecular disulfides, either native or mismatched and c) the slow shuffling of mispaired to correct disulfide bonds[20]. Our results suggest the presence of the COOH-terminal hexapeptide to be required to allow the 1st step of the reoxidation to proceed rapidly.



Time course of the reoxidation of lysozyme (filled points) and of lysozyme fragment 1-123 (open points). *A* Reoxidation was followed with the use of lytic activity; *B* free thiol content and *C* fluorescence quantum yield.

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