Air Reoxidation of SH Groups of Reduced α-Lactalbumin Fragments

As is well known, fully reduced proteins are reoxidized, in the absence of denaturing agents, to molecular species which have recovered most of the original three-dimensional structure[1]. In some instances a similar recovery of the native structure is obtained simply by mixing 2 fragments of a protein obtained by enzymatic or chemical cleavage of covalent amide bonds[2-4]. It is clear that the two phenomena are distinct in origin, but they also present some analogies owing to the reformation in both cases of the non-covalent interactions determining the native structures. Previous studies in our laboratory[5] have shown that fully reduced bovine α-lactalbumin can be reoxidized in the presence of Cu^{++} ions with significant recovery of the physico-chemical properties of the native protein. It seemed worthwhile to investigate whether a correct coupling of the cysteinyl residues could be accomplished even by association of 2 polypeptide fragments, comprising, respectively, the sequence 1–90 (CB–1) and 91–123 (CB–2), obtained by cyanogen bromide attack on the single methionyl residue of α-lactalbumin followed by disulphide bonds reduction with 2-mercaptoethanol.

Materials and methods. Commercial bovine α-lactalbumin (Pentex Inc, Kankakee, Ill.) was purified as elsewhere reported[5]. Attack by cyanogen bromide on α-lactalbumin was carried out according to Brew and Hill[6]. The isolated product (CB-Lac) showed amino acid analysis in good agreement with the expected values. Reduction was effected, either in presence or absence of iodoacetic acid, by 2-mercaptoethanol in 6 *M* guanidinium chloride, pH 8.6, under the same conditions as used by Brew et al.[7] for α-lactalbumin. The resulting peptides, either carboxymethylated or unprotected, were isolated on Sephadex G-100 equilibrated with 50% acetic acid[5,6]. Reoxidation of reduced α-lactalbumin was carried out according to Tamburro et al.[5]. The reoxidation of the equimolar mixture of unprotected CB-1 and CB-2 was effected by exposure to air of 3.2 mg of CB-a and 1.3 mg of CB-2 dissolved in 0.025 *M* *Tris*-Cl buffer (5 ml) containing 5×10^{-5}, or alternatively, 5×10^{-4} *M* $CuSO_4$. The process was followed by monitoring the disappearance of sulphydryl groups according to Ellman's procedure[8] (Figure 1). Amino acid analysis[9] gave values in agreement with those calculated from the sequences. Circular dichroism data were obtained wtih a Cary Model 60 recording spectropolarimeter equipped with a 6002 dichroism accessory.

Results and discussion. Figure 2 contains the circular dichroism (CD) spectra in the near UV of unprotected CB-1 plus CB-2 at various stages of the reoxidation process in the presence of 5×10^{-4} *M* $CuSO_4$. The spectra of reduced-reoxidized α-lactalbumin, CB-Lac, S-carboxymethylated CB-1 plus CB-2, are also shown for comparison. It is evident that, under these experimental conditions, the reoxidation of CB-1 plus CB-2 does not correspond to a renaturation in terms of recovery of the original structure present in the intact

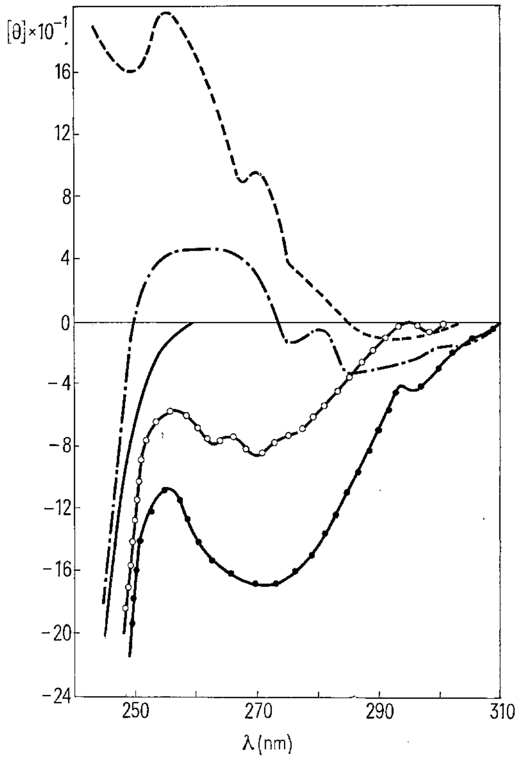


Fig. 2. Near-UV-CD-spectra of the equimolar mixture CB-1 + CB-2 in 0.025 *M* *Tris*-Cl buffer, pH 8.0, containing 5×10^{-4} *M* $CuSO_4$: (-·-·-·), after 23 h of reoxidation; (----), after 26 h. (-●-●-), reduced-reoxidized α-lactalbumin; (-○-○-), cyanogen bromide-cleaved α-lactalbumin; (——), equimolar mixture of caboxymethylated CB-1 + CB-2.

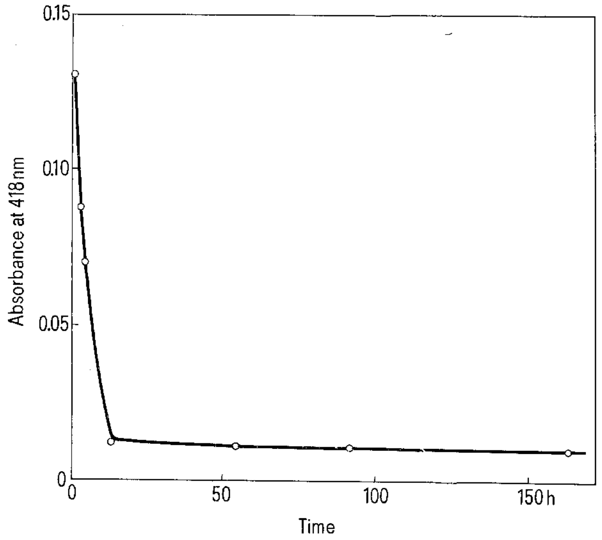


Fig. 1. Time course of reoxidation of the equimolar mixture CB-1 + CB-2 as monitored by Ellman's procedure[8]. Solvent: 0,035 *M* *Tris*-Cl buffer, pH 8.0, containing 5×10^{-5} *M* $CuSO_4$.

---

[1] C. B. Anfinsen, *New Perspectives in Biology* (Ed. M. Sela; Elsevier, Amsterdam 1964), p. 42.

[2] F. M. Richards, Proc. natn. Acad. Sci., USA *44*, 162 (1958).

[3] H. Taniuchi and C. B. Anfinsen, J. biol. Chem. *244*, 3864 (1969).

[4] G. Gorradin and H. A. Harbury, Proc. natn. Acad. Sci., USA *68*, 3036 (1971).

[5] A. M. Tamburro, G. Jori, G. Vidali, A. Scatturin and G. Saccomani, Biochim. biophys. Acta *263*, 704 (1972).

[6] K. Brew and R. L. Hill, J. biol. Chem. *245*, 4559 (1970).

[7] K. Brew, F. J. Castellino, T. C. Vanaman and R. L. Hill, J. biol. Chem. *245*, 4570 (1970).

[8] G. L. Ellman, Archs Biochem. Biophys. *82*, 70 (1959).

[9] D. H. Spackman, W. H. Stein and S. Moore, Analyt. Chem. *30*, 1190 (1968).

protein. The spectral features, characterized by large positive ellipticities, are by no means reminiscent of those of CB-Lac or even reduced-reoxydized α-lactalbumin. Most probably the coupling of the cysteine side chains is not the correct one but proceeds randomly yielding different molecular species. When the reoxidation of the mixture was carried out at lower concentration of the catalyst (5×10^{-5} *M* $CuSO_4$), quite different CD spectra were obtained (Figure 3). Although the spectra at various stages of the reoxidation process are still distinct from those of CB-Lac and even more from those of reduced-reoxidized α-lactalbumin, nevertheless they show a tendency towards features clearly reminiscent of the protein containing the correct disulphide bonds. Apparently, judging from the CD standpoint, some of CB-1 and CB-2 molecules interact together by restoring, through a correct coupling of sulphydryl groups, at least part of the non-covalent interactions determining the original structure. It is to be noted that an equimolar mixture of carboxymethylated CB-1 and CB-2 (in which disulphide formation is prevented) give a CD spectrum comparable to the sum of the curves of the individual peptides. The role played by the catalyst, Cu^{++} ions, deserves further comment. It is reasonably anticipated that the correct refolding of the polypeptide chain is initiated by the regeneration of a part of original disulphide bonds. Of course various kinds of mismatched disulphide bonds are possible: however, they could be corrected, at least partially, via disulphide interchange. In the presence of excess copper ion, the rate of reoxidation may be too high to allow preferential formation of the disulphide bond(s) which initiates the correct refolding. In this case disulphide bonds may be formed incorrectly, thus producing stable molecular species completely different from the native protein. A similar influence of the catalyst concentration has been observed in the reoxidation of reduced Taka-amylase A.[10].

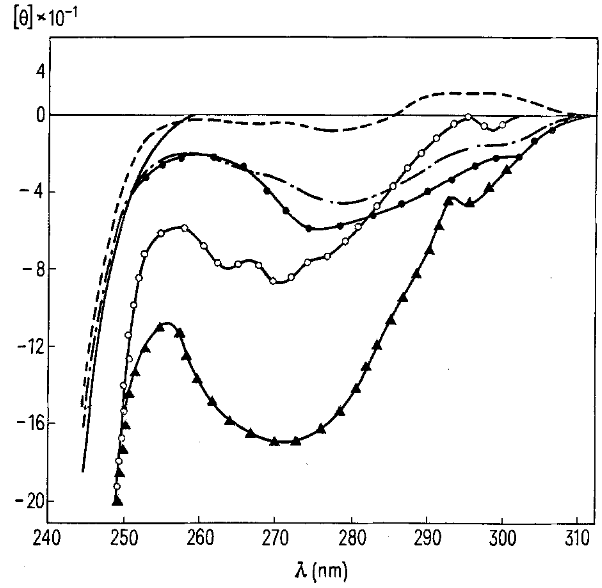


Fig. 3. Near-UV-CD-spectra of the equimolar mixture CB-1 + CB-2 in 0.025*M* *Tris*-Cl buffer, pH 8.0, containing 5×10^{-5}*M* $CuSO_4$: (---), after 1 h 30 min of reoxidation; (-·---), after 18 h 20 min; (-●-●-), after 168 h 20 min. (-▲-▲-), reduced-reoxidized α-lactalbumin. Other symbols as in Figure 2.

Riassunto. Si é studiato, mediante dicroismo circolare, il processo di riossidazione-ricombinazione di due frammenti ottenuti per attacco con Br-CN e successiva riduzione dell-α-lattalbumina. Si é trovato che la concentrazione del catalizzatore $CuSO_4$ svolge un ruolo importante nel dirigere il riaccoppiamento dei gruppi SH.

G. Chiaranda, G. Saccomani and A. M. Tamburro[11]

Istituto di Chimica Organica, Università di Padova,
Via Marzolo 1, I–35100, Padova (Italy), 19 June 1972.

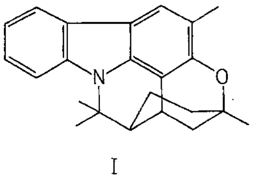
---

[10] T. Takagi and T. Isemura, J. Biochem. *56*, 344 (1964).

[11] To whom reprint requests should be addressed.

The Crystal Structure of Murrayazoline (Mahanimbidine, Curryangin)

In connection with an investigation on carbazoles of the Rutaceae, there was isolated a new alkaloid, murrayazoline, $C_{23}H_{25}NO$ (M^+ 331), mp 260–262°, from an alcoholic extract of the stem-bark of *Murraya koenigii* Spreng[1]. The UV-spectrum was suggestive of the 2-methoxy carbazole chromophore [λ_{max} 245 nm and 307 nm (log ε 4.69 and 4.16)][2,3]. Further studies revealed that like the known mahanimbine[4], murrayazoline was derived from a 2-hydroxy-3-methyl carbazole to which a monoterpene (C_{10}-unit) fragment was fused at the 2-position through an ether linkage.



I

This alkaloid was also obtained from the leaves and the stem bark of the plant by two other groups independently, and renamed mahanimbidine[5] and curryangin[6,7]. On the basis of additional chemical and spectral data, the structure I (9a, 10, 11, 12, 13, 13, 13a-hexahydro-2, 9, 9, 12-tetramethyl-1, 12-epoxy-9*H*-indolo [3, 2, 1-*de*]phenanthridine) was assigned to the compound. It was felt that an independent proof of the hexacyclic system would be of value, especially in view of the biological activity reported for various carbazoles[8–10].

Suitable, colorless crystals of I were obtained by a slow evaporation of an acetone solution. One Å intensity data (maximum sin $\theta/\lambda = 0.5$) was collected with copper radiation on a Syntex P1 diffractometer equipped with a graphite monochromator. The intensity data was processed and all subsequent crystallographic calculations were performed using subprograms of the CRYM system[11]. Phasing was accomplished by a routine application of symbolic addition[12]. A trial structure was obtained from