The Specificity of the Carotenoid-Protein Linkage in Crustacyanin

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Few carotenoproteins have been obtained in a sufficiently homogeneous state to merit chemical investigation. Of these, overubin, the green glycolipoprotein of lobster eggs (Kuhn & Sørensen, 1938; Ceccaldi, Cheesman & Zagalsky, 1966), overubin, the red glycoprotein of *Pomacea canaliculata* (Cheesman, 1958), and crustacyanin, the blue lobster-carapace pigment (Wald, Nathanson, Jencks & Tarr, 1948; Cheesman, Zagalsky & Ceccaldi, 1966), all have astaxanthin as prosthetic group. (The prosthetic group of overubin, originally described as an astaxanthin derivative, has since been identified as astaxanthin; D. F. Cheesman & P. F. Zagalsky, unpublished work.)

The apoproteins of overubin and crustacyanin have been prepared and the carotenoproteins reconstituted. Only with overubin has any attempt been made to study the specificity of recombination, astaxanthin dipalmitate not combining with the protein under conditions where astaxanthin did so with ease (Cheesman, 1958).

Apocrustacyanin (mean mol.wt. about 20000) shows up to eight components in starch-gel electrophoresis; two of these are always predominant. On recombination with astaxanthin, α' -crustacyanin (mol.wt. about 36000) is formed. This associates in the presence of salt to form α -crustacyanin, the naturally occurring pigment (mol.wt. 380000). On prolonged dialysis, α -crustacyanin irreversibly forms β -crustacyanin (mean mol.wt. about 36000), which shows four purple bands on electrophoresis (Cheesman *et al.* 1966; Gammack & Raper, 1966).

Since astaxanthin exerts a marked stabilizing effect on the tertiary and quaternary structures of crustacyanin (Cheesman *et al.* 1966), we thought that studies of the specificity of this interaction would be of interest.

Materials and methods. Apocrustacyanin was prepared by twice-repeated precipitation of crustacyanin (10mg. in 2ml. of 0.04M-KSCN) with acetone made 0.04M with respect to KSCN (30ml.). The protein, dissolved in 5–10ml. of 0.1M-KH₂PO₄-Na₂HPO₄ buffer, pH7, was dialysed against the same buffer overnight. Unchanged α -crustacyanin was removed on a short DEAE-cellulose column

(Whatman DE-32) equilibrated with the buffer. The effluent apoprotein was dialysed and concentrated against 0.04M-KSCN by ultrafiltration.

Zeaxanthin, canthaxanthin and echinenone were kindly supplied by F. Hoffmann-La Roche, Basle, Switzerland. 4-Hydroxy-4'-oxo- β -carotene and 4,4'-dihydroxy- β -carotene (isoeaxanthin) were obtained by partial and complete borohydride reduction of canthaxanthin respectively. 4-Hydroxy- β -carotene was formed by the reduction of echinenone. Lutein, astaxanthin dipalmitate and β -carotene were isolated from *Idothea granulosa*, *Homarus vulgaris* and maize respectively. Astaxanthin diacetate was prepared from astaxanthin by acetylation with acetic anhydride, and astacene by oxidation of astaxanthin in ethanolic KOH (5%, w/v). These carotenoids were purified on alumina columns and checked for purity by thin-layer chromatography (cf. Lee, 1966a).

The following method of recombination was found the most satisfactory. To 10mg. of apoprotein in 0.5ml. of 0.04M-KSCN was added 5ml. of carotenoid solution in acetone. The precipitated apoprotein was redissolved by gradual addition, with stirring, of 0.04M-KSCN until the acetone concentration was 10–15% (v/v). The solution was dialysed against 0.1M-phosphate buffer, pH7, passed through Celite to remove excess of carotenoid and concentrated by ultrafiltration. The solution was then dialysed again against water, and the chromoproteins were adsorbed on DEAE-cellulose and eluted stepwise with increasing concentrations of phosphate buffer, pH7. All operations were performed at 0–4°. A large excess of carotenoid was needed for significant recombination. Part of each apoprotein preparation was combined with astaxanthin under identical conditions, a good yield of α -crustacyanin indicating a satisfactory preparation.

Reconstituted material was split with 2vol. of acetone, and the carotenoid was transferred to light petroleum (b.p. 40–60°) and checked by thin-layer chromatography against samples of the carotenoid originally used.

Where the polarity of the carotenoids was considered to be insufficient to allow recombination by these methods, pyridine or ethanol was substituted for acetone, or detergents were used as solubilizers

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(0.5% Triton X-100 or Tween 20). This detergent concentration was without evident effect on α -crustacyanin.

The molecular size of the reconstituted chromoproteins was estimated by gel filtration on columns of Sephadex G-75 (medium grade). These were standardized with cytochrome c, β -crustacyanin and serum albumin (cf. Andrews, 1964). Molecular weights were assessed from a log (mol.wt.)-effluent volume plot.

The chromoproteins were compared electrophoretically with the original apoprotein and β -crustacyanin in starch gels at pH 8.35 (cf. Cheesman *et al.* 1966).

Results. Only canthaxanthin, astacene and 4-hydroxy-4'-oxo- β -carotene formed chromoproteins. Yields were about 3-10%.

Canthaxanthin formed a complex that separated into three purple fractions on DEAE-cellulose during stepwise elution with 0.02M- and 0.05M-phosphate buffer, pH 7. Their absorption spectra appeared to be identical, consisting of a smooth curve with a maximum at about 580m μ , slightly below that of β -crustacyanin (585m μ). The components corresponded, both in molecular size (about

36 000) and in electrophoretic mobility, to β -crustacyanin (Fig. 1).

Electrophoresis of the purple astacene-protein complex revealed four purple proteins (Fig. 1), again corresponding to those of β -crustacyanin. A small blue component, identical in mobility with α -crustacyanin, was also present. The blue and purple fractions were separated on Sephadex G-75. The purple material (mol.wt. about 39 000) had a smooth curve with a maximum at about 605m μ . The blue component, about 5% of the chromoprotein, moving in the void volume of the gel and evidently of large molecular size, had an absorption maximum at approx. 630m μ , similar to that of α -crustacyanin. Dialysis against water gave an almost completely irreversible conversion into the lower-molecular-weight purple material. Partial separation of the chromoproteins was obtained by DEAE-cellulose chromatography. The absorption spectra of the two main fractions, eluted with 0.02M- and 0.05M-phosphate, pH 7, had maxima at 610 and 590m μ respectively.

The violet product obtained with 4-hydroxy-4'-oxo- β -carotene had an absorption maximum at 560m μ . Electrophoresis revealed that carotenoid was bound to all the apoprotein units (Fig. 1). Gel filtration confirmed that association of apoprotein units had not occurred. Thus keto groups on both β -ionone rings would seem essential for the association of two apoprotein units. This suggests that echinenone (4-oxo- β -carotene) might bind to a single unit. However, no evidence for such combination was obtained. Perhaps another group such as a hydroxy substituent at the 4'-position is essential for additional anchorage to the protein.

A hydroxy group in the 4-position alone (isocryptoxanthin), in the 4- and 4'-positions (isozeaxanthin) or the 3- and 3'-positions (lutein and zeaxanthin) did not induce recombination. Failure to bind to protein was also encountered when the 3- and 3'-hydroxy groups of astaxanthin were esterified (diacetate and dipalmitate). This is noteworthy in view of the isolation of a carotenoprotein with an astaxanthin ester as prosthetic group (Cheesman & Prebble, 1966).

Discussion. The results suggest that the structural modifications of astaxanthin that still allow the formation of the complex quaternary structure of α -crustacyanin are rather limited. On present evidence keto groups at both the 4- and 4'-positions, as well as keto or hydroxy groups on the 3- and 3'-positions, are requirements for this structure, although with the 3,3',4,4'-tetraoxo compound (astacene) the β -crustacyanin structure is preferred. Dimerization of apoprotein units is possible with keto groups at the 4- and 4'-positions. If one of these is replaced by a hydroxy group, the carotenoid can bind to apoprotein but no dimerization is evi-

Fig. 1. Electrophoresis on starch gel of (a) apocrustacyanin, (b) β -crustacyanin, (c) astacene recombination product, (d) canthaxanthin recombination product and (e) α -crustacyanin. The solid bands represent major fractions, vertical lines represent bands of medium intensity and diagonal lines of faint intensity. Trace components are not represented.

dent. Keto groups are clearly essential in the binding of carotenoid to apocrustacyanin.

Kuhn & Lederer (1933) and Ball (1944) suggest that the blue and green colours of astaxanthin proteins result from enolization of the carotenoid and resultant salt formation between basic protein groups and the tetravalent carotenoid anion. Platt (1959) has stressed that polarization of carotenoids can cause large bathochromic shifts in their absorption spectra. The formation of purple chromoproteins from apocrustacyanin and astacene, which cannot enolize in the 4- and 4'-positions, and from canthaxanthin, which has no 3- or 3'-substituents, suggests that the observed bathochromic shifts result simply from polarization due to interaction with a specific site on the protein. Isolation of astacene from natural sources has been attributed to oxidation of astaxanthin during isolation procedures (cf. Goodwin, 1954). On the basis of the present study, a more careful examination of these cases may be warranted.

Naturally occurring canthaxanthin-proteins (Lee, 1966a,b) have absorption maxima further to the red than has α -crustacyanin; it is not known how far the quaternary structure of these depends on the structure of the prosthetic group.

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Mechanism for the Stimulation *in vivo* of Hepatic Gluconeogenesis by Glucagon

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Glucagon has a well-established glycogenolytic action on liver. This effect is mediated by 3',5'-(cyclic)-AMP, which is formed by the interaction of glucagon with the membrane-bound adenylate-cyclase system (Sutherland, Øye & Butcher, 1965). Glucagon not only stimulates glucose formation from glycogen, but also increases the rate of glucose synthesis from C₃ precursors (Struck, Ashmore & Wieland, 1965; Garcia, Williamson & Cahill, 1966; Exton & Park, 1966). However, the mechanism for the gluconeogenic effect of glucagon has not yet been clearly defined.

In addition to activating the phosphorylase system, glucagon and 3',5'-(cyclic)-AMP have been

shown to activate a triglyceride lipase of adipose tissue (Rizack, 1964) and of liver (Bewsher & Ashmore, 1966). Since fatty acids can stimulate gluconeogenesis when added *in vitro* to the perfused rat liver (Struck *et al.* 1965; Williamson, Kreisberg & Felts, 1966b) or to kidney-cortex slices (Krebs, Speake & Hems, 1965), the possibility arises that the gluconeogenic effect of glucagon is mediated by an enhanced availability of fatty acids.

To test this hypothesis, and some of the theories on the control of gluconeogenesis that have been advanced recently (Krebs, 1964; Walter, Paetkau & Lardy, 1966; Newsholme & Underwood, 1966), glucagon was administered to fed rats *in vivo*. The