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A CLASS OF BLUE QUINONE–PROTEIN COUPLING PRODUCTS: THE ALLAGOCHROMES?

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Abstract—Blue derivatives, with absorption bands near 680 nm were produced when enzymically generated chlorogenoquinone reacted in alkaline conditions with the following proteins: several strains of potato virus X, bovine serum albumin, human γ-globulin, and a protein fraction from spinach. Blue derivatives were also produced from insoluble lysine–Sepharose, but not from polylysine or tobacco mosaic virus. The amount of chlorogenoquinone bound to potato virus X and to lysine–Sepharose, suggested that two molecules of quinone react with each lysine ε-amino group to produce the chromophore. The blue colours turned blue-green above pH 10.5 and red below pH 5, and were reversibly bleached by reducing agents. These spectral properties, suggesting the presence of a hydroxylated quinone, resemble those of the pigment(s) allagochrome described by Habermann. As the original allagochrome may have been an isolation artifact, it is suggested that this name be extended to all chlorogenoquinone derivatives of amino-containing compounds with these spectral characters.

INTRODUCTION

Adducts are formed between proteins and the quinones produced during the enzymic oxidation of phenols; biological situations in which they occur are during the infection of plants with pathogens, during the decay of plant matter to humus-like material, and during the harvesting and treatment of vegetable products [1, 2]. We previously studied the adduct formed *in vitro* between the coat protein of potato virus X (PVX) and chlorogeno-quinone (CQ), the quinone derived from chlorogenic acid (CA), and were aided by the ease with which modified virus could be quickly and completely separated from polymerized phenols [3, 4]. At pHs near neutrality, one molecule of chlorogenoquinone, measured as its quinic acid moiety, was bound per molecule of coat protein, probably having reacted with the ε-amino group of one of the 12 lysine molecules in the protein [5]. At pHs near 8, however, the virus was converted to a blue derivative (λ_{max} 680 nm) in which two molecules of quinone were bound per subunit of protein. The nature of the blue chromophore was unknown, one possibility being that at pH 7.8, chlorogenoquinone had reacted with a protein moiety other than ε-NH_2. In this paper, however, we provide evidence that the blue chromophore is formed from the reaction of more than one molecule of chlorogenoquinone with each lysine ε-NH_2, and argue that the protein–phenol adducts have the same chromophore as the pigment(s) previously isolated from plant tissues rich in chlorogenic acid and termed 'allagochrome' by Habermann [6, 7].

RESULTS

Reaction of chlorogenoquinone with PVX-protein

The amount of CQ bound by four different strains of PVX, which had been exposed to an excess of chlorogenic acid being oxidized at pH 7.8, was almost exactly twice the amount bound at pH 7 (Table 1). This holds for a virus strain, X^4, which only binds one molecule of CQ per two molecules of protein at the lower pH; it also holds for a derivative of the X^N strain, X^N-PLP [5], in which the two most reactive ε-amino groups have been partly (70–80%) modified with pyridoxal phosphate (Table 1). It suggests that the blue chromophore formed at pH 7.8 is derived from the same group that binds 1 molecule of CQ at pH 7.0, by the binding of an extra molecule of CQ. The 2:1 relationship breaks down for preparations of strain T_8, and some preparations of X^{HB}, both of which contain proteolytically degraded forms of the virus protein (Table 1). This degradation, bought about by trypsin-like enzymes during extraction [8, 3] produced α-amino groups which are not present in undegraded virus protein and which may react with CQ [9]. If these, unlike the ε-amino groups, bind one molecule of CQ at pH 7.8 as at 7.0, the amounts of CQ bound at the two pHs by these strains (Table 1) can be accounted for satisfactorily.

Reaction of CQ with other proteins

Little or no CQ (0–0.04 mol/protein subunit) was bound to tobacco mosaic virus (TMV) after exposure at either pH 7 or 7.8, and no blue chromophore was formed at the higher pH. The coat protein of most strains of this virus contain two lysine residues per subunit (MW 17 500) but in the intact virus their ε-NH_2 groups are unreactive to a number of reagents [10]. Serum albumin and γ-globulin contain many available ε-amino groups [9, 11] and both apparently bind substantial amounts (>20 molecules/protein molecule) of CQ. There is some uncertainty in the quantities that are covalently bound because of the difficulty of completely separating these proteins from excess of polymerized phenol; however, reaction mixtures at pH 7.8 have distinct absorption peaks near 680 nm (Fig. 1); and some fractions of γ-globulin, separated by CC, are visibly blue. Chromato-graphic fractions of an $(NH_4)_2SO_4$ preparation of

Table 1. Amount of chlorogenogenoquinone bound by different strains and derivatives of PVX

Strain	CQ bound (molecules/protein subunit) pH 7	CQ bound (molecules/protein subunit) pH 7.8	Virus protein in degraded form (%)	Reference
X$^{\backslash}$	1.00	1.97	None	[3]
	0.95	1.95	None	[3]
CS 35	1.03	2.07	None	[4]
X^{HB}	1.12	2.06	Trace	This work
X^{4}	0.55	1.11	None	[4]
	0.48	0.93	None	[4]
	0.59	1.18	None	This work
X$^{\backslash}$-PLP	0.23	0.6	None	This work
T8	1.39	2.4	*ca* 40%	[4]
X^{HB}	1.80	2.7	More than 50% (2 degraded forms)	This work

Preparations made from different strains of PVX, and a pyridoxylated form of one strain (X$^{\backslash}$-PLP) were incubated with an excess of chlorogenic acid and polyphenoloxidase at either pH 7 or 7.8. They were separated from the reaction mixture, and the bound quinone estimated as the quinic acid that was liberated on hydrolysis. Samples of the virus preparations were examined by gel electrophoresis for protein degradation.

proteins from spinach leaves were also blue (λ_{max} 680–700 nm) after reacting with CQ at pH 7.8 (Fig. 1).

Reaction of CQ with lysine derivatives

When chlorogenic acid was oxidized in the presence of excess lysine, a small but distinct absorption peak near 700 nm was produced at pH 7.8 but not at 7. Attempts to separate a blue product were unsuccessful. There was no evidence for the formation of blue products from a number of preparations of polylysine at either pH.

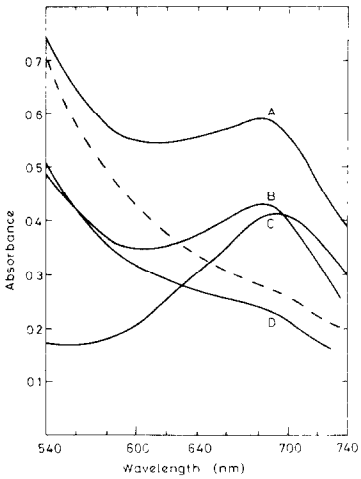


Fig. 1. Visible absorption spectra of proteins modified by chlorogenoquinone in alkaline conditions. Spectra were taken of the products of the reaction of serum albumin (A), γ-globulin (B), and spinach leaf protein (C) with chlorogenoquinone at pH 7.8. Spectra were also taken of the reaction of γ-globulin at pH 7 (D) and of the oxidation of chlorogenic acid without added protein at pH 8 (- - -). The product of the reaction of spinach protein was separated by chromatography and examined in cells of 4 cm light-path, whereas other spectra were on unfractionated reaction mixtures contained in 2-mm cells.

Commercial lysine–Sepharose 4B (Pharmacia), in which the α-amino groups of L-lysine are bound to beads of agarose gel (possibly via –NH CO O– or NH CNH–CO– linkages), reacts with excess of CQ to give a blue product at pH 7.8, a bluish brown product at pH 7, and a brown one at pH 6.5. The amounts of quinic acid bound in the blue and brown forms of lysine–Sepharose (LS) were more variable than that in PVX-derivatives; it approximated to two and one molecules per lysine molecule respectively in some experiments (Table 2), whereas in others it was more than two in both forms. Some of the variability was due to the absorption of completely polymerized CQ onto lysine–Sepharose (Expt 4, Table 2), probably by electrostatic attraction, which was not completely prevented by exhaustive washing. However, even after making corrections for this absorption, the ratios of quinic bound in the blue and brown forms was not as close to 2:1 as it was with most strains of PVX (Expt 5, Table 2).

When unsubstituted Sepharose (2B) was incubated with enzymically generated chlorogenoquinone its colour was not changed and little or no chlorogenoquinone bound; this argues that lysine is the quinone-reactive site of lysine–Sepharose. Consistent with this interpretation, less free lysine was recovered by acid hydrolysis of both brown and blue derivatives of LS than from unreacted LS (Table 3). Moreover, much of the lysine that was liberated from the quinone derivatives may not have been unmodified, but may have been regenerated from lysine–quinone adducts during hydrolysis [12, 13]: treating LS with fluorodinitrobenzene (FDNP) which reacts with free NH_2 groups [14] removed nearly all

Table 2. Products of reaction of CQ with lysine–Sepharose (LS)

Expt	Type of preparation	Lysine content (μmol)	Quinic acid bound (μmol)		Molecules quinic acid/ lysine residue	
			at pH 6.5	at pH 7.8	at pH 6.5	at pH 7.8
1	Quinoned-LS	0.63	0.64	1.11	1.0	1.75
2	Quinoned-LS	0.62	0.93	1.6	1.5	2.6
3	Quinone-LS	0.90	1.34	1.8	1.5	2.0
4	LS + pre-oxidized CA	0.90	0.4	0.39		
5	Quinoned-LS corrected for absorption of pre-oxidized CA	0.74	1.0	1.5	1.3	2.1

Lysine–Sepharose was incubated with chlorogenic acid and polyphenoloxidase at either pH 6.5 or 7.8. The products were exhaustively washed, freeze-dried, and their quinic-contents measured. In expt 4, the oxidation was allowed to proceed for 2 days, by which time the enzymically generated quinone had polymerized, before LS was added and the mixture incubated for 2 hr at 25°. In expt 5, some samples of LS were treated in the same way to estimate the amount of completely polymerized CQ that was absorbed.

Table 3. Recovery of lysine from derivatives of lysine–Sepharose

Preparation	Lysine recovered after hydrolysis (μmol)	(%)	Lysine recovered after reaction with FDNP and hydrolysis (μmol)
Lysine–Sepharose (LS)	0.675	100	0.014
Quinoned-LS (brown)	0.365	54	0.16
Quinoned-LS (blue)	0.263	39	0.06

Samples of lysine–Sepharose and the quinone derivatives formed at pHs 6.5 and 7.8, were hydrolysed in 6 M HCl and analysed for lysine, both before and after reaction with FDNB.

(98%) the lysine that could be recovered after acid hydrolysis, whereas the same reagent only partly decreased the lysine that could be recovered from brown, quinoned LS (Table 3).

Spectral properties of the blue chromophore

The spectral properties of PVX-Q_2, [15], of the protein stripped from it by acetic acid, of quinoned serum albumin and of the blue derivative of lysine–Sepharose both before and after solubilization with 1 M HCl, were similar. They were most conveniently studied with the extracted protein from PVX-Q_2 (Fig. 2); the SDS necessary to keep the protein in solution at low pHs had a hypsochromic effect on the spectrum and might have been responsible for the lower ε (*ca* 3600 at 660 nm; pH 7) than that apparent for intact PVX-Q_2 (ε *ca* 5300 at 680 nm; pH 7, ref. [3]). Below pH 5, the blue colours changed to red, the peak around 660–680 nm disappeared and absorption at 500 nm increased. Above pH 10.5, the colour changed to a blue-green and, in the case of intact PVX-Q_2, a distinct peak near 580 nm appeared [15]. Both changes are reversed by readjusting the pH. Reduction with $NaBH_4$ or ascorbate at pH 7, also discharged the 660–680 nm band and produced an orange colour (Fig. 2). Absorption band and blue colour returned as the solution was allowed to stand in air, and did so more quickly if the air were bubbled through it.

No novel ninhydrin-positive substance that could be identified with the chromophore was observed in the amino acid-analyser traces after hydrolysis of either PVX-Q_2 protein or blue lysine–Sepharose with 6 N HCl. The 'background' of the trace in these analyses were more irregular than those in analyses of unmodified compounds, presumably because of the degradation of quinone–amino acid adducts [13].

DISCUSSION

Blue phenol–protein derivatives are clearly not specific to the protein of PVX. They can be formed from other proteins and polymers containing reactive lysine-residues. They are probably formed as a consequence of the reaction of two molecules of quinone with each lysine ε-amino group, and not with the α-amino groups produced by the partial degradation of PVX-protein. This explains the stoichiometry of the reaction between CQ and PVX, and also the inability of Pierpoint *et al.* [3] to separate by electrophoresis, two quinic acid-containing peptides from tryptic digests of the protein of PVX-Q_2. The quinic content of the blue derivative of lysine–Sepharose conforms less exactly to this model, partly because of adsorbed polymerized CQ, and partly, perhaps, because of the formation of some cross-links between amino groups of the type lysine–(quinone)–lysine. Some linkages of this type must be formed in PVX-Q_1 and PVX-Q_2 to account for the linking of protein subunits [3]: if such links are formed in preference to the blue chromophore when lysine groups are close together, they would conveniently explain the surprising lack of chromophore production from polylysine.

The structure of the chromophore is not known. It does not involve the quinic acid moiety of the quinone because saponified PVX-Q_2 is still blue. The colour changes produced by acid and alkali, and produced reversibly by

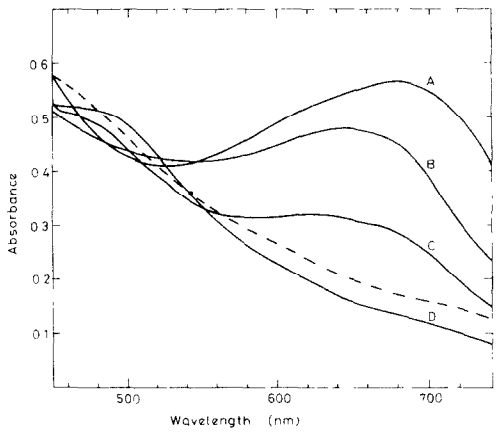
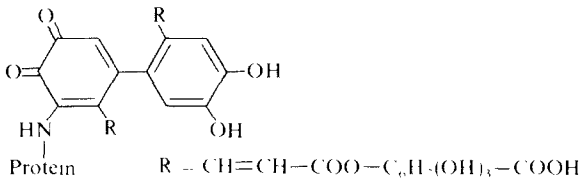
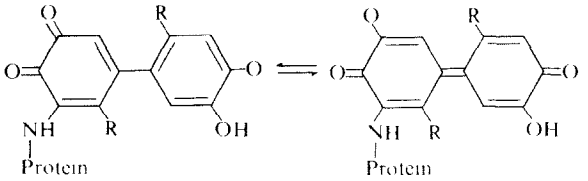


Fig. 2. Spectral properties of protein from PVX-Q_2. A sample (5 mg) of freeze-dried protein from PVX-Q_2, was dissolved in phosphate buffer (5 ml; 0.1 M containing 0.05% SDS) and its pH adjusted to 11.3 (A), 6.8 (B), 4.5 (C) and 2.2 (D) with 1 M NaOH or HCl. A sample was also reduced at pH 7.8 with sodium borohydride (– –). Spectra were measured in a cell of 4-cm light path.

reduction, suggest the presence of an ionizing group and of a quinone. One possibility, suggested by Prof. R. H. Thomson, is a structure of the type:



which may ionize and resonate between the forms:



Attempts to isolate the chromophore grouping have so far failed. Acid hydrolysis of protein from quinoned-PVX leads to its breakdown, as it does with other lysine–phenol adducts [13]. A soluble preparation of quinoned–lysine–Sepharose, which retains some of the characteristic colour properties can be obtained by mild acid-hydrolysis, but has not been sufficiently separated from hydrolysis products of the agarose to facilitate any chemical identifications. Attempts to detect an alkali-stabilized ESR signal characteristic of quinones [16] in the protein from PVX-Q_2 have not so far been successful.

In a series of papers, Habermann [6, 7, 17–19], following Kozlowski [20], described a blue-green pigment(s) which she extracted from a variety of vegetable sources and which had spectral properties which are qualitatively the same as those of the adducts described here. She called it allagochrome (Greek 'allago' = changing in form). It was extracted most effectively in alkaline, glycine-containing buffers, and often, although not invariably, from tissues rich in chlorogenic acid. With some species of plants, larger quantities were extracted when exogenous chlorogenic acid was present in the extraction medium. Its redox properties enabled the pigment to act as electron acceptors in some *in vitro* photosynthetic and respiratory systems, suggesting that if it existed *in vivo* it might be involved with these processes. Its purification however, proved difficult and very different estimates of its MW (50 000–1400) were obtained. More recent work, published in abstract [21, 22] suggests that at least one of the component pigments is probably a derivative of glycine and chlorogenoquinone, produced during the extraction of leaves by reactions similar to those studied here. It seems likely therefore, that the chromophore has the same general structure as that in the CQ-modified proteins, but involves α-, rather than ε-amino groups. In view of this similarity and the likelihood that these compounds are enzymic artefacts of extraction rather than metabolites, it may be worthwhile to extend the term allagochrome to include all chlorogenoquinone-modified amino-containing compounds that have the spectral properties summarized in Fig. 2.

Allagochromes derived from proteins, peptides or amino acids will be produced when vegetable tissue containing chlorogenic acid or its congeners is treated or extracted in an alkaline, aerobic condition. They may be responsible for some of the blue colouration that appears during the drying of *Mercurialis* roots [23]. They may be responsible for the blue component of ribulose bisphosphate carboxylase that was detected when parsley leaves were extracted at pH 8.3 in the absence of reducing agents [24]. The redox properties of the allagochrome-type chromophore may even have contributed to the apparently high ribulose bisphosphate oxygenase of the blue fractions, so suggesting the unexpected, and contested [25], separation of the oxygenase and carboxylase activities of this enzyme: it is relevant that an extract of spinach which was probably rich in the carboxylase [26] gave an allagochrome-colouration with chlorogenoquinone (Fig. 1). Finally, allagochromes almost certainly contribute to the greens and dark colours formed in proteins extracted by alkali from oil-extracted sunflower seed-meal [27], and which deleteriously affect its commercial potential and its nutritive value.

EXPERIMENTAL

Viruses and proteins. Strains of PVX were cultured in tobacco plants (*Nicotiana tabacum*, var. *Xanthi* nc), purified by centrifugation and estimated spectrophotometrically [3, 27]. The strain X^{HB} was handled as required by licence number HH 11797A/4 issued by the Ministry of Agriculture, Fisheries and Food under the Destructive Pests and Diseases of Plants Order, 1965. Virus protein was examined for proteolytic degradation using electrophoresis in SDS-containing polyacrylamide gels [3] by Dr. J. M. Carpenter. Protein was also stripped from virus and modified virus with acetic acid [5]. When required, PVX was modified with pyridoxal 5′-phosphate, dialysed and recovered by centrifugation at 50 000 g for 4 hr [5].

TMV was similarly cultured and prepared from tobacco plants.

Protein from spinach (*Spinacia oleracea*) was extracted and fractionated twice with $(NH_4)_2SO_4$ as described for the purification of ribulose diphosphate carboxylase [26]. Bovine serum albumin and γ-globulin were from commercial sources. Polyphenoloxidase (EC 1.10.3.1) was partially purified from *Xanthi* tobacco and estimated as previously described [28].

Lysine–Sepharose 4B (1–2 g) was swollen in 200 ml of Pi buffer (0.05 M; pH 7) containing 1 M NaCl as recommended by its manufacturers. It was allowed to settle, the buffer decanted, and it was washed with 4–5 more lots of buffered saline to remove dextran and azide preservatives. It was finally washed in Pi buffer, collected by centrifugation and suspended in 3× its packed volume of buffer. Samples were taken by pipette after thorough shaking.

Reaction of polymers with CQ. PVX, pyridoxylated-PVX and TMV were reacted with CQ and separated from the reaction mixture by chromatography on glass granules [3].

Lysine–Sepharose (0.5 ml suspension containing *ca* 0.9 μmol bound lysine) was diluted to 1.5 ml with Na-Pi buffer (0.05 M at pH 7; 0.1 M at pH 6.5 or 7.8) and incubated with chlorogenic acid (6 μmol in 0.1 ml) and polyphenoloxidase (0.03–0.05 ml) at 25° with frequent or continual shaking. Five or six more lots of CA and oxidase were added at *ca* 40 min intervals. The modified product was kept on ice overnight, and washed six times, usually twice with Pi buffer (0.05 M; pH 7), twice with buffer containing 1 M NaCl, and twice with H_2O and collected by centrifugation at 5000 *g* for 15 min. It was freeze-dried and stored at −15°.

Other proteins (10–15 mg) were incubated similarly, but usually with fewer (2–4) additions of CA and oxidase. Absorption spectra were taken on reaction mixtures after they had stood at 5° overnight. Serum albumin and γ-globulin were recovered from the reaction mixture by precipitation with saturated $(NH_4)_2SO_4$. Spinach-leaf protein was separated by chromatography on Sepharose 4 B (1.8 × 80 cm column) in Tris buffer, 0.05 M pH 7.5.

Reaction of polymers with fluorodinitrobenzene. The freeze-dried samples of lysine–Sepharose and its derivatives were reacted with FDNB in bicarbonate solution (8% w/v) at room temp. for 3 hr with constant shaking [14]. They were washed well with water and freeze-dried.

Polymer hydrolysis and lysine analysis. Derivatives of PVX-protein and of lysine–Sepharose were hydrolysed in 3 ml 6 M HCl *in vacuo* at 110° for 22 hr. Solutions were taken to dryness in a rotary evaporator, dissolved in 0.01 M HCl and examined on a Technicon TSM amino acid analyser by Mr. J. M. Hill or Mrs. S. Smith essentially as described [29].

Quinic acid estimations. Samples of modified polymers containing bound CQ were saponified with 1 M NaOH overnight at 5°. Solutions were neutralized with 2 M H_2SO_4, clarified on a bench centrifuge if necessary, and the liberated quinic acid estimated colorimetrically after treating aliquots with periodate and thiobarbiturate [3]. With some samples of thiobarbiturate it was necessary to prolong the period in the boiling water bath to 15–20 min to develop maximum colour. Chlorogenic acid (0.1–1.0 μmol) was used as a standard; its quinic acid moiety only reacted after it had been released by saponification.

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