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On Bacterial "Cytochromoids"*

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A new type of haem protein, extractable as a soluble di-haem protein conjugate, is found in purple photosynthetic bacteria. The physical and chemical properties of two representative samples, obtained from the facultative photoheterotrophe, *Rhodospirillum rubrum*, and the obligate photoanaerobe, *Chromatium*, are reviewed briefly and discussed in relation to the recommendation for establishment of a new class of haem proteins to be called "cytochromoids".

There has never been a dearth of phenomena for the biochemist to ponder when he has had occasion to consider problems of structure and function presented by the haem proteins. Now there come more in the wake of studies on variant haem proteins which occur distributed widely throughout the group of purple photosynthetic bacteria, and which have been known since 1953 when the first specimen was observed in cell-free extracts prepared from the facultative photoheterotrophe, *Rhodospirillum rubrum*[1,2]. Subsequent researches have established a complex of properties[3] for these proteins which have prompted the Commission on Enzymes of the International Union of Biochemistry to propose[4] a new class of haem proteins to be termed "cytochromoids", defined as "Haemoproteins with a haemoglobin-like spectrum and a reactivity with ligands which do not react with cytochrome *c*".

In this article, this nomenclature will be adopted, in place of the previous "RHP-type". The properties of cytochromoids will be surveyed and present knowledge of structure and function discussed briefly, as based on studies of two representative proteins – the first, labeled "I", from *R. rubrum* and the second, labeled "II", from the strict photoanaerobe, *Chromatium* (strain D). Space available prohibits detailed presentations of data; for these the reader is referred to the bibliography.

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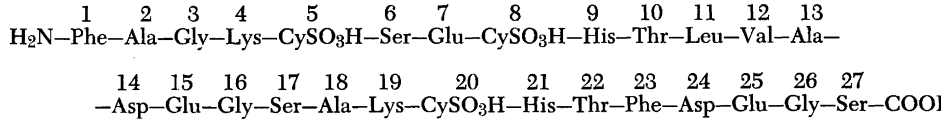
The prosthetic groups

Most studies have been limited by the minute quantities of pure protein available. At present, the nature of the prosthetic haems must be inferred more or less indirectly from spectrochemical studies[3]. It is certain that cytochromoids I and II are di-haem proteins. This conclusion follows from measurements[5] of molar absorbancy indices at the characteristic maxima of absorption exhibited by the reduced (ferro-) forms both in the native state, and as derivative haemochromogens formed by partial denaturation in alkali alone or by ligand interactions with pyridine and cyanide after denaturation in alkali. It is also certain that the prosthetic haems are bound covalently, because fission does not occur by the usual treatment with acid-acetone. Attempts to obtain the free haems by fission in the presence of dilute acid and heavy-metal salts (Ag^+ or Hg^{++}), a treatment effective to the extent of approximately 80 % or better for cytochrome *c* and myoglobin types of haem protein, have been disappointing[6]. Yields no greater than approximately 20 % have been obtained with a mixture of products which, after conversion to porphyrins, include a major fraction as a porphyrin very similar to haematoporphyrin and two others as yet unidentified. However, recent experiments, which employ a modification of the original conditions of the Paul procedure[7], have resulted in much improved yields (up to 70 % of total haem) with only a single product, haematohemin, as judged by chromatography and spectroscopic criteria.

From the position of the alpha maxima (550 mμ) of the pyridine haemochromogens, it appears certain that the majority of the substituent groups on the peripheries of the pyrrole moieties are not electron-withdrawing (unsaturated). However, it cannot be stated unequivocally that there is not a single such group (vinyl, or partially shielded unsaturated chain residue) present.

A promising approach to the eventual characterization of the haem groups is based on the partial enzymic hydrolysis of the cytochromoids after preparatory extensive denaturation by boiling or treatment with organic solvents. The cytochromoids are resistant to enzymic attack in the native state. The only procedure which is effective without prior denaturation is that with pepsin, which of course involves incubation in acid solvent (pH $\approx$ 1.9). Even such treatment leaves a large core peptide of some 27 residues which still contains both haem groups[8]. Further directed degradation to obtain the amino acid sequence requires first that the haem groups be split off (by performic acid oxidation). The resultant peptide reveals the following sequence:



Small but significant quantities of a monohaem peptide which is not resistant to enzymic attack, and which is made up of the sequence including residues 2–10, occur. This, together with the remarkable resemblance of this portion of the long peptide to sequences encountered invariably in *c*-type cytochromes[9,10] renders very plausible the possibility that one haem is attached, as in cytochrome *c*,

through thioether linkages to the cysteinyl residues 5 and 8. The placement of the other haem is still unknown. A possibility that it is linked by a single saturated bond either to serine 17 or cysteine 20, or to both by two saturated bonds, is obvious. No positive clue can be obtained from the absorption spectrum of the di-haem peptide which is that of the usual *c*-type haemochromogen, except for an anomalously high ratio of absorbancy of the oxidized form (haemichrome) at the 530 mμ maximum to that of the reduced alpha peak at 550 mμ[8].

Determinations of the paramagnetic susceptibility and electron spin resonance measurements on the native forms of I and II show[11] that both haems contain iron in the high spin form when the cytochromoids are reduced, but that there is an equilibrium mixture of high and low spin forms in the oxidized cytochromoids. These results are reminiscent of the behavior of alkali haematin[12]. No measurements have been made as yet on the peptic di-haem peptide.

When sufficient quantities of peptide are available, efforts will be made to obtain the two monohaem peptides expected from the above sequence studies. Such experiments, coupled with the improved cleavage procedures and application of the recently-developed proton spin resonance analysis[13], should clarify the nature of the two haem groups.

Attempts to resolve the prosthetic haems as attached in the native proteins have been only partially successful. Thus, low temperature absorption spectra[14] of reduced cytochromoid I show clearly resolution of the Soret absorption band into two components with maxima at 422 mμ and 433 mμ. However, no comparable resolution of two weakly differentiated maxima at 549 mμ and 565 mμ in the visible alpha region is affected. Differential reduction[15], in the presence or absence of CO, also fails to give evidence that two haem groups differ appreciably either in reactivity with CO, or in midpoint potentials at neutral pH ($E_{m,7}$ = = −8 to −5 mV).

Ligands interactions

As observed repeatedly[3], the responses of cytochromoids to all of the usual ligand reagents are negative, except for certain limited interactions with CO and NO, both small, uncharged molecules. The only other molecules, or solvent species, which may have access to the reactive sites, are molecular oxygen — as evidenced by the ready autoxidizability of I and II, hydronium ion — as seen[5] in the complex spectroscopic changes which occur when the oxidized cytochromoids are subjected to gradual changes in pH from 6 to 10, and possibly molecular hydrogen — because catalytic reduction by hydrogen is observed in the presence of palladium.

The changes noted with change in pH do not require that protons from the solvent medium actually penetrate to the active center, however. It is also possible that these effects are the results of configurational changes transmitted through the protein structure by alterations at the surface exposed to the solvent.

The oxidized cytochromoids act as though they are completely inaccessible to all charged ligand reagents. Thus, unlike other haem proteins with typical "open" spectra (myoglobins, peroxidases, *etc.*), no spectroscopic changes are noted in the presence of a long list of such reagents, *e. g.*, N_3^-, CN^-, HS^-, F^-, *etc.*[3]

The reduced cytochromoids appear even more inaccessible inasmuch as they

fail to respond spectrally to deprotonation[5], as do the oxidized forms. Large uncharged ligands, *e. g.*, nitrosobenzene[16], 4-methylimidazole[3], *etc.*, are also unreactive.

Even in the reactions which do take place, as with CO and NO, many anomalies occur[16]. Thus, while CO forms a typical haemochromogen with ferrocytochromoids, its affinity is much lower than for other haem compounds; thus, pressures for half saturation of I are $\approx$ 0.015 atmospheres, whereas they are only approximately $^1/_{10}$ this much for typical haemoglobin interactions. Even at atmospheric pressure, association is only approximately 90 % complete. Moreover, the photodissociation of the CO-complex is pH-sensitive, a phenomenon not noted with any other haem compound, as far as the writer is aware. At pH 4.8, the degree of association is twice that at pH 7.0.

The reactions with NO are complex and in many respects unlike any hitherto observed with other haem proteins[16]. The affinity of ferri- or ferro-cytochromoid I for NO is less than a tenth that displayed by the reduced form for CO – a relation just the reverse of that usually found in other haem compounds. The major interaction is one displayed by the ferri-form which appears to react with production of a pH-sensitive charge transfer adduct, *e. g.*, $Fe^{+++} + NO = Fe^{++} \cdot NO^+$, as in the case of ferricytochrome *c*[17]. The typical spectrum of a haemochromogen is produced. However, the residual ferro-form cannot be recovered by alkalination, as is found for the ferricytochrome *c*-NO adduct. Instead, the ferri-cytochromoid-NO adduct is already markedly unstable at pH 7, unlike the ferricytochrome *c* compound, and dissociates to yield the ferri-cytochromoid. As the pH is lowered progressively, the NO adduct is increasingly stabilized.

The ferrocytochromoid reacts weakly with NO to form a haemochromogen-like compound(s) of unknown structure which is not affected by changes in pH. At pH $>$ 11, irreversible changes which involve degradation of the haem moieties occur for both ferri- and ferro-forms.

In view of these limited ligand interactions, the wisdom of accentuating the *c*-type cytochrome character of these proteins by use of the term "cytochromoid" is evident. Taken with the results of the fission experiments, and sequence studies, the "cytochrome-like" character of the cytochromoids can be considered as outweighing the "peroxidase-like" character deduced from spectroscopic and magnetic properties.

Function

Every major function, other than oxygen transport or storage, has been suggested for the cytochromoids. These include roles as peroxidases, oxidases, and components of electron transport, with or without coupling to phosphorylation.

The peroxidase function has been eliminated as a reasonable possibility by the observations that peroxidase activity does not fractionate with cytochromoids I and II[1,3] and that peroxidation of ascorbate in the presence of cytochromoids I and II proceeds at rates much less than those observed for such pseudo-peroxidases as myoglobin and denatured cytochrome *c*[18].

Arguments for and against an oxidase function have appeared in the literature and need not be reviewed here[1, 3, 16, 19–21]. The most recent findings strongly

contraindicate an oxidase function. These are: (1) cytochromoid I is not present in significant amounts in non-photochemical aerobic cells[22,23]; (2) spheroplasts of *R. rubrum* with strong oxidase activity contain no cytochromoid I; (3) a cytochromoid found in *Rhodopseudomonas palustris,* is not auto-oxidizable; (4) immune sera specific for cytochromoid I react with extracts from light-grown cells but not with those from dark-grown cells[26,26].

The weight of evidence in favor of a strictly catalytic role in electron transport coupled to phosphorylation is considerable. Kinetic studies[14,27] show that cytochromoid I is a substrate for a flavin-mediated haem protein reductase which is a member of the electron-transport chain linked to photophosphorylation. Depleted chromatophores, with a low rate of photophosphorylation, can be activated by addition of either cytochrome c_2 or cytochromoid I, both endogenous haem proteins of *R. rubrum,* but the activity-concentration function shows the cytochromoid as more likely situated somewhere in the middle of the chain (as consistent with its midpoint potential, $E_{m,7} \approx 0$), rather than at the terminal end, as is the case with cytochrome c_2. (Many data implicate cytochrome c_2 as the terminal substrate for the photo-oxidase system but a discussion of these lies outside the scope of the present article). No data exist which run counter to a role for cytochromoids as intracellular electron carriers.

A definitive determination of the specific role of cytochromoid I in *R. rubrum* metabolism is difficult because of the very close coupling between oxidative metabolism and photoactivation processes[27]. Further progress will depend on development of techniques for resolution of the electron transport systems which are associated with dark and light metabolism.

General conclusions

On the basis of the evidence at hand, the creation of a new class of haem proteins to include cytochromoids appears well justified. Although the properties of the class must remain defined largely on the basis of those displayed by just two members, cytochromoids I and II, the data available are comparable with those which have been considered adequate for characterization of the more conventional classes of haem proteins. Thus, *c*-type cytochromes until recently were described almost entirely on the basis of the properties exhibited by horse-heart or yeast cytochrome *c*. A similar situation holds for the haemoglobins, chlorocruorins, myoglobins, peroxidases, catalases, *etc.,* in each of which relatively few specimens have been examined with a degree of thoroughness.

It is premature to attempt a more specific description than is implied in the present definition of cytochromoids. Already it is evident that there is a considerable spread in physicochemical properties of cytochromoids as a class. Thus, the amino acid compositions[28] and sedimentation and diffusion constants[3] are markedly different in cytochromoids I and II. More important, the *R. palustris* cytochromoid has a relatively high mid-point oxidation potential ($E_{m,7} \approx 250$ mV) and is not auto-oxidizable. However, such variations within a class of haem proteins are no more unusual than those which exist in the class of *c*-type cytochromes, for which similar greatly disparate degrees of autoxidizability and spreads in range of oxidation potential are found.

The apparent limitation in distribution of cytochromoids is also likely to vanish

as research continues. On comparative biochemical grounds, it can be anticipated that cytochromoids may exist in the groups of micro-organisms related to purple photosynthetic bacteria, such as the chemosynthetic facultative anaerobes which reduce nitrates, and the chemosynthetic anaerobes which reduce sulfates, as well as in the green photosynthetic bacteria. While attempts to reveal the presence of cytochromoids in such systems have been negative to the present, such results have been derived from studies on limited quantities of micro-organisms and prove only that the amounts of cytochromoids which may be present are much less than in the photosynthetic apparatus of the purple bacteria, in which the amounts of cytochromoids are very large. Recently, an apparently authentic example of a cytochromoid in a nonphotosynthetic facultative anaerobe, *Pseudomonas denitrificans*, has been described[29]. The possibility that cytochromoids may occur in conventional aerobic systems is not remote and has even been mentioned in one case, that of a new haem protein from liver microsomes[30].

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