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On the Monoheme Character of Cytochromes *c′*

(monoheme peptide/heme/subunit structure/cytochrome nomenclature)

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ABSTRACT **Interpretations of data bearing on structures of cytochromes *cc′*—a class of variant *c*-type heme proteins from bacteria—in support of a diheme-bearing single chain as a basic structural unit, appear to be invalid in the light of recent studies. These reveal that nearly all members of this class exist as dimers that can be dissociated into, if they do not already exist as, monoheme-bearing monomers. The particular case of the *Chromatium* protein, held to be the source of a peptic-"core" peptide containing two covalently-bonded heme groups, has been re-examined by preparation of tryptic and chymotryptic peptides derived from the heme-bearing region of the protein, as well as by repetition of experiments on peptic digestion, with more rigorous purification of the resultant peptides than was previously done. It is shown that this protein can also be dissociated into identical subunits, bearing a single heme prosthetic group, and accounting essentially for all its heme content. Thus, the previous terminology—cytochromes *cc′*—based on supposition of heme group heterogeneity, is inconsistent with these findings and should be replaced by *cytochromes c′*.**

The class of variant bacterial *c*-type cytochromes, presently known as cytochromes *cc′*, is defined by the presence of heme prosthetic groups (usually 2 per mol) bound covalently as in cytochromes *c*, but with at least one heme group in a ligand field of high-spin character ("non-hemochrome"‡ (1–3) under normal physiologic conditions. Early studies (5–7) provided data that suggested that these proteins were diheme in character, and consisted of a monomer chain to which were attached both heme groups. Results of recent investigations in our laboratory (8–11) and elsewhere (ref. 12; Ambler, R. P., in preparation; Meyer, T. E., Ambler, R. P. & Kamen, M. D., in preparation) are at variance with this conclusion, and support rather the existence in this group of cytochromes of monoheme monomers which, with the single known exception of the protein from *Rhodopseudomonas palustris*, are isolated dimerized to produce a diheme form. Data that support this conclusion are presented in this report.

It is proposed that the proper terminology for this class of proteins, consistent with recommendations of the International Commission on Enzyme Nomenclature (4), is *cytochromes c′*. This terminology will be used in the text which follows.

MATERIALS AND METHODS

Cell Cultures and Protein Preparation. Chromatium vinosum, strain D,—the bacterium used in previous studies for production of *Chromatium* heme proteins—was grown as described (13). Cytochrome *c′* was isolated and purified by a procedure modified slightly from that reported (13). Washed cells were suspended in 3 volumes of 0.1 M Tris·HCl buffer (pH 7.3) and broken in a Ribi Cell Fractionator (20,000 lb/in.^{2}, temperature <25°). The resultant cell extract was clarified first by centrifugation (30,000 × *g*) in a Servall SS1 rotor for 10 min, followed by centrifugation of the supernatant fluid in a Spinco 42 rotor for 2 hr (200,000 × *g*). The soluble protein fraction so obtained was desalted by passage through Sephadex G-25-C with change of buffer to 0.02 M Tris·HCl (pH 7.3) adsorption on DEAE-cellulose (Brown Co., Type 20), and subsequent salt-gradient elution of the brown cytochrome *c′* band at about 0.10–0.14 M NaCl in 0.02 M Tris·HCl (pH 7.3). Further purification was accomplished by precipitation with ammonium sulfate (60–80% satn.), followed by chromatography with Sephadex G-100 and a repetition of the DEAE-cellulose treatment. The purity index (ratio of absorbance for oxidized protein at 280 nm to that for oxidized protein at the Soret maximum, 400 nm) was 0.31 for the best fractions obtained and used in subsequent studies (also see ref. 14), and corresponded to a spectrochemical purity of >95%—based on purity of N-terminal alanine, determined by amino acid analysis of the hydrolyzed phenyl thiohydantoin derivative prepared by the Edman procedure on the whole protein (see below).

Physico-chemical Determinations. Molecular weights were based in part on electrophoretic migration rates [sodium dodecyl sulfate–10% acrylamide gels in 0.1 M sodium phosphate (pH 7.2)] according to the method of Weber and Osborn (15), and in part on Sephadex chromatography. Amino acid compositions were determined as described by Dus *et al.* (16). N-terminal residues were identified by Edman degradation, as modified by Doolittle (17). The phenyl thiohydantoin derivatives of amino acids were hydrolyzed in evacuated tubes at 150° for 24 hr. Proteins were hydrolyzed in 6 N HCl for 24, 48, or 72 hr (110°). Labile amino acids, such as threonine and serine, were assayed by extrapolation of values observed to initial time. Cysteine in peptic peptides was determined as cysteic acid after performic acid oxidation (18). Control experiments with the peptic heme peptide from

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‡ Hemochromes—chelates invariably associated with low-spin ligand field character—are defined as compounds of any tetrapyrrolic chelate of iron in which the ligand field in extraplanar coordination is provided by basic nitrogen atoms (4). Official action to extend this definition to include sulfur atoms capable of electron-pair donation to the central iron atom, as well as other basic ligand atoms, is pending.

horse-heart cytochrome *c* showed that this procedure resulted in satisfactory recovery of cysteine.

Sequence analyses were based on dansylation procedures described by Ambler (19) and Gray (20); in the case of tryptic and chymotryptic peptides, analyses were obtained after removal of heme from the whole protein by the method of Ambler *et al.* (21). Performic acid oxidation of protein followed the procedure of Hirs (18). Peptic digestion and purification of the resultant peptide mixture was performed as described by Dus *et al.* (6). In addition, further treatment of these peptides to obtain higher degrees of purification required thin-layer chromatography on 3-mm preparative Silica Gel F_{254} plates (E. Merck Co., Darmstadt) developed 12 hr with the freshly prepared organic phase of the solvent mixture, butanol–acetic acid–H_2O 4:1:5. Bands were scraped off the dried plate and eluted with 0.1 M NH_3. High-voltage paper electrophoresis at pH 6.5 was used to monitor homogeneity; the purified peptides so examined were then submitted to composition analysis.

Heme content was determined spectrophotometrically by derivatization as the pyridine hemochrome (25% pyridine–0.2 M NaOH), except that the value of 31.18 for the millimolar absorbance at 550 nm was adopted (Flatmark, T., unpublished).

RESULTS AND DISCUSSION

As a preamble, a short history of the viscissitudes attending the progression of researches into the structural chemistry of cytochromes c' is indicated. The development of knowledge about these proteins has followed an erratic course—as mirrored in the various appellations given them, e.g., "pseudohemoglobin" (22), "yellow pigment" (23), "RHP" (24), "cytochromoid" (4, 25), "cryptocytochrome *c*" (26), and "cytochrome cc'" (4). The circumstances leading to their supposed diheme character have been described elsewhere (1). Briefly, the bases for this notion reached in the early 1960s were 3-fold: (*a*) the existence of a split Soret band in the reduced form, (*b*) electrochemical differential titration data that seemed consistent with differences in spectral characteristics of two hemes possessing apparently identical oxidation–reduction potentials (7), and (*c*) isolation and sequence determination of a diheme peptide from *Chromatium* cytochrome c' that possessed three cysteine residues adequate for binding of one heme as in cytochrome *c* (two thioether linkages to two cysteine residues), and another heme residue by a single thioether linkage (5–7).

Association of the split Soret maximum of the reduced protein with the presence of two heme groups was disproved as a valid basis for diheme character by isolation of a monoheme cytochrome c' from *Rhodopseudomonas palustris* in 1965 by DeKlerk *et al.* (9, 10). It became evident that its typical Soret splitting could be the result of asymmetric ligand fields around a single heme abolishing the usual degeneracy of transitions responsible for the Soret band in heme proteins. The second type of observation did not permit unambiguous interpretations.

Thus, the major basis remaining for belief in diheme cytochrome cc' was the existence of the diheme peptide. Doubts as to the validity of this observation arose as sophistication in heme protein characterization, coupled with rapid development of sequence methodology, increased in the latter half of the 1960s. Preliminary sequence studies in our laboratory (11) on cytochrome c'—isolated from a nitrate-reducing bacterium (tentatively identified as "*Pseudomonas denitrificans*")§ and described by Suzuki and Iwasaki as a "cryptocytochrome *c*" (26)—indicated that only a single heme subunit of molecular weight about 14,000 could be isolated from the protein of molecular weight about 28,000. Cusanovich (12) provided additional data on molecular weights of numerous cytochromes c' incubated in various denaturing solvents that strongly supported the contention that even the *Chromatium* and *R. rubrum* preparations, as well as other apparent diheme proteins, could be dissociated into smaller monoheme subunits. Most recently, sequence studies of several cytochromes c', including those of *R. rubrum* [recently completed (Meyer, T. E., Ambler, R. P. & Kamen, M. D., in preparation)] and of *Chromatium*, together with the complete determination of the amino-acid sequence of the protein from the nitrate-reducing bacterium (Ambler, R. P., in preparation), appear definitely to dispose of the existence of the diheme monomer as a plausible primary structure.

It was clear that the studies that provided the basis for the postulation of the existence of a diheme peptide from the *Chromatium* protein required reevaluation. The results obtained are described briefly in this section.

In Na dodecyl SO_4–gel electrophoresis, the protein, purified to a purity index of 0.31, migrated as a single homogeneous band. Its molecular weight was determined as 14,000 [estimated from its migration rate, as read from a linear calibration plot of migration rate against log of molecular weight, defined by five standard protein preparations (horse-heart cytochrome *c*, myoglobin, ferritin, ovalbumin, and bovine serum albumin) ranging in weight from 12,400 to 68,000]. Values (12, 13) determined by ultracentrifugation under nondenaturing conditions led to an estimate of about 28,000, i.e., twice the minimal size of about 14,000. Sephadex G-75 chromatography at pH 7 gave a molecular size of about 37,000 indicating nonideality in shape and/or hydration. The amino-acid composition of the protein is given in Table 1. Values for cysteine (determined as cysteic acid after performic acid oxidation of intact protein) were always less than about 60% of the theoretical value, and are not shown. This result was also true for recovery of cysteine from hydrolyzed heme proteins without prior performic acid oxidation.

The N-terminal sequence was Ala-Gly-Leu-Ser . . ., in agreement with that obtained from earlier studies (7). Spectral analysis (27) of the PTH-alanine produced by Edman degradation from aliquots of protein of known heme content and purified by paper chromatography gave 0.65 alanine per heme. Acid hydrolysis and amino-acid determinations of the N-terminal PTH amino acid resulted in a yield of 0.59 alanine per heme. Control experiments with a sample of pure spermwhale myoglobin gave assays of 0.60 N-terminal valine per heme. These data, indicating one end group per heme, were inconsistent with those of previous studies—based on derivatization with dinitrofluorobenzene—that had led to the conclusion that one N-terminal residue was associated with two hemes (7). The finding of a one-to-one correspondence between the end-group and the heme content accorded with the

§ Later examination of this bacterium by Dr. M. Doudoroff (personal communication) has revealed it possesses peritrichous flagellation—a feature inconsistent with a major criterion for classification as an aerobic pseudomonad—polar flagellation (see ref. 32). Prof. Doudoroff and his associates propose it be assigned tentatively to the genus *Alcaligenes*.

TABLE 1. *Amino-acid composition of Chromatium cytochrome c′*

Residue	*(1)* (a)*	*(2)* (b)*	*(3)* (c)*	*(4)* Integral composition/heme This work	*(5)* Ref. 30
Asp	12.63	12.65	12.55	13	15
Thr	5.89	5.52	5.11	6	(6)
Ser	2.42	2.30	2.10	3	(2)
Glu	19.95	19.60	19.78	20	22.5
Pro	3.33	3.30	3.15	3	4
Gly	15.82	15.52	16.12	16	19
Ala	25.50	26.75	25.00	26	29.5
Cys	—	—	—	(2)	(2)
Val	10.05	9.96	10.03	10	12.5
Met	4.05	4.25	4.08	4	5
Ile	4.74	4.76	4.86	5	6.5
Leu	4.29	4.25	4.20	4	5
Tyr	3.84	3.83	3.90	4	(3.5)
Phe	5.25	5.18	5.43	5	6
His	1.05	1.02	1.08	1	1
Lys	10.41	10.50	10.38	10	12
Arg	4.28	4.31	4.31	4	4.5
Try	—	—	—	—	—
Total	—	—	—	136	156

Averages of at least four analyses each.

* *(a) (b) (c)*: Hydrolysis times = 24, 48, and 72 hr, respectively.

Na dodecyl SO_4-gel-sizing experiment, which showed the dissociation of the protein into two identical monoheme subunits.

The unusual resistance of the protein to enzymic digestion was originally remarked upon in our laboratory by Dus *et al.* (6) and, indeed, dictated a limitation of attempts to define the heme-binding region to peptic digestion to produce heme-bearing peptides, rather than an application of the more-specific enzyme digestion procedures that use trypsin and chymotrypsin. On the other hand, peptic digestion resulted in peptide mixtures difficult to fractionate into homogeneous components (see below). Hence, in addition to re-examination of the original findings by peptic digestion, we attempted to characterize the heme-binding portion of the protein by searching for peptides containing cysteine and histidine, after treatment of the (heme-free) apoprotein with trypsin or chymotrypsin. Two such tryptic peptides were isolated (Table 2). The first tryptic peptide (column *A*)—the only one isolated containing histidine—exhibited the sequence Ser-Cys-His-Glx-Lys. The composition data available are shown. While these preliminary findings are in need of improvement, they are consistent with the more-reliable sequence data. The second tryptic peptide (column *B*) showed the sequence Thr-Ala-Phe-Gly-Asp-Val-Gly-Ala-Ala-(Cys, Lys), in satisfactory agreement with the composition data. The chymotryptic peptide isolated (column *C*) was not sequenced, but its composition, taken together with the sequence data for the tryptic peptides and comparisons with homologous regions in other cytochrome *c′* sequences (Meyer, T. E., Ambler, R. P. & Kamen, M. D., to be published), suggests a preliminary assignment for the sequence of the heme-binding region in *Chromatium* cytochrome *c′* as: Thr-Ala-Phe-Gly-Asp-Val-Gly-Ala-Ala-Cys-Lys-Ser-Cys-His-Glu (or Gln)-Lys-Tyr.

As no remotely comparable sequence is seen in the proposed diheme peptide of the earlier studies, peptic digestion was performed following strictly the procedure described therein. The result, including the Celite column chromatography used previously, yielded a peptide mixture that upon paper electrophoretic screening (a method unavailable in the earlier studies) was found to contain not the original two major bands reported (6), but various components that required further fractionation by thin-layer chromatography to obtain two pure heme peptides that appeared homogeneous by high-voltage electrophoretic examination. The compositions of these two peptides are shown in columns *D* and *E*, Table 2.

More than 80% of the total heme in the pepsin digest was contained in these two peptides. Peptide II differed from peptide I in amino-acid composition in that it contained two alanines, which occurred in the order Ala-Ala as N-terminal residues, as determined by Edman degradation. The compositions found were consistent with the sequence determined for the tryptic and chymotryptic peptides, as well as with the known specific hydrolytic action of pepsin.

Examination of Table 1, in which the composition of pure cytochrome *c′* (column *4*) is shown as well as that of the earlier preparation (column *5*), reveals that the earlier preparation contained some contaminating proteins or peptides, a conclusion also forced upon us by the differences in purity indices (0.31 for the pure preparation and 0.42 for the earlier preparation). However, it is unlikely that this amount of contaminant could have interfered significantly with sequence determinations of the peptic peptide derived, as only minor quantities of a few residues from contaminants appear to have been present. It is most reasonable to assume that the two major peptic peptides in the early studies were inhomogeneous. Sequence studies of such mixtures could well have led to the results that appeared to establish existence of the diheme peptide.

TABLE 2. *Derivative peptide compositions of Chromatium cytochrome c′*

Residue	*(A)* Tryptic peptides I	*(B)* Tryptic peptides II	*(C)* Chymotryptic peptide	*(D)* Peptic peptides I	*(E)* Peptic peptides II
Asp	—	1.18 (1)	1.08 (1)	—	—
Thr	—	0.78 (1)	—	—	—
Ser	0.63 (1)	—	0.91 (1)	0.93 (1)	1.07 (1)
Glu	0.86 (1)	—	1.10 (1)	1.22 (1)	1.15 (1)
Gly	—	2.03 (2)	1.79 (2)	—	—
Ala	—	2.96 (3)	2.19 (2)	—	1.70 (2)
Cys	1.22 (1)	0.78 (1)	1.88 (2)	1.24 (2)*	1.28 (2)*
Val	—	1.22 (1)	1.10 (1)	—	—
Tyr	—	—	0.89 (1)	—	—
Phe	—	0.95 (1)	—	—	—
His	1.14 (1)	—	0.96 (1)	0.94 (1)	0.96 (1)
Lys	1.17 (1)	1.10 (1)	2.08 (2)	2.14 (2)	2.00 (2)
Total	5	11	14	7	9

Values in parentheses denote assumed numbers of residues, based on a single analysis in the case of each peptide.

* Determined as cysteic acid (see text).

The present failure to obtain this peptide in the peptic digest of *Chromatium* cytochrome *c′* of highest purity and with application of the rigorous chemical procedures developed in the last decade removes the last basis for the suggestion that—at least in *Chromatium*—cytochrome *c′* is a single chain covalently binding two hemes. In addition, the anomalies associated with results of early analytical studies, as discussed earlier (7)—e.g., the abnormal cysteine to heme ratio of 3:2¶, rather than of 2:1 seen in other cytochromes *c′*, and some of the spectral features of the peptic peptides compared to the whole protein—no longer require rationalization.

The status of characterization of the heme prosthetic groups in cytochromes *c′* is still unclear. Early attempts (31) to achieve quantitative cleavage of the heme groups by treatment with silver or mercury salts in acid—demonstrated in control experiments to be effective in the case of cytochromes *c*—were unsuccessful, leading to the suggestion that binding of at least one heme group might be quantitatively different from that in cytochromes *c*. However, later experiments using such cleavage procedures, modified in certain particulars, have been observed by K. Dus and M. Morrison (private communication) to produce good yields (about 85–90%) of a product very similar to hematoheme with characteristics closely resembling those of the products obtained from horse-heart cytochrome *c*. These workers note that there may be slight differences between the spectra obtained for pyridine hemochromes of the prosthetic hemes from cytochromes *c′* and those seen with the product of cleavage of horse-heart cytochrome *c*. In view of the known lability of heme moieties, such spectral discrepancies provide no unambiguous basis for the conclusion that the prosthetic heme groups in cytochromes *c′* differ from those of cytochromes *c*.

The further study of these heme proteins can now proceed on the basis that the structural unit is a monoheme single chain of about 125 amino acid residues, most frequently isolated as dimers, in which the prosthetic heme groups are bound in identical fashion and in identical ligand fields in any given protein. Further, they may be considered as members of a homologous set of cytochromes not related to other *c*-type cytochromes in primary structure, except in the common mode of covalent attachment of the heme prosthetic group. The use of the term "cytochromes *cc′*" assumes existence of hemes in two different types of ligand-field—one low-spin ("hemochrome") and one high-spin ("non-hemochrome"). In view of the conclusion that only high-spin binding appears to exist under normal physiological conditions, the proper term is "cytochromes *c′*."

¶ It is of interest that such an abnormal situation does seem to exist in the case of a mitochondrial-type cytochrome *c* from the protozoan insect parasite, *Crithidia oncopelti* (see ref. 28) and, possibly, in the variant cytochrome *c* derived from mitochondria of the green alga, *Euglena gracilis* (29).

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