*ON THE MONOHEME NATURE OF CYTOCHROME C' (RHODOPSEUDOMONAS PALUSTRIS)**

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Various purple photosynthetic bacteria contain two predominant classes of soluble *c*-type cytochromes,[1] namely, cytochromes c_2 which resemble mammalian cytochromes *c* with regard to size and many other molecular properties, and cytochromes *cc'* which bear two covalently bound heme groups on a single polypeptide chain[2] roughly twice the size of cytochromes c_2 and which exhibit absorption spectra and chemical properties different from those of the mammalian *c*-type cytochromes.[3] Cytochromes *cc'* have previously been referred to as "RHP"-type cytochromes (cf. ref. 1). We now report the first example of a member of this class of heme proteins which contains only one heme group per molecule.

It was noted earlier[4] that the heme protein, designated as *Rps. palustris* (van Niel strain #2.1.37) cytochrome *cc'*, differed from other cytochromes of this class in that it exhibited a more positive oxidation-reduction potential ($E_{m,7} = +0.105$ v) than previously encountered in the cytochromes *cc'*, a relatively low molecular weight (<20,000), a basic isoelectric point (pH >9.0), and a blocked NH_2-terminal group. A recent improvement[5] in the purification procedure for this protein has made available highly purified samples suitable for a careful study of the amino acid composition of this protein. Table 1 shows its amino acid composition,[6] as compiled from experiments using acid hydrolyses with 6 *N* HCl at 105°C for 24 and 48 hours, as well as from complete enzymic hydrolyses of the protein with pronase followed by leucine aminopeptidase. The contents of cysteine + cystine and of methionine were determined as cysteic acid and methionine sulfone, respectively, from hydrolysates of performic acid–oxidized samples.[7] The absence of tryptophan in the enzymic hydrolysate was confirmed by spectrophotometric titration of protein samples with N-bromosuccinimide.[8]

The amino acid composition given in Table 1 is in fairly good agreement with data published previously from this laboratory[1] as well as with the data of Henderson and Nankiville,[9] the main improvement being a more accurate characterization and determination of the acid-labile amino acid residues of the protein. We find a higher lysine content (about four residues) than that reported by Henderson and Nankiville,[9] who comment on the difficulty of explaining the markedly basic isoelectric point of the protein on the basis of their amino acid composition. In consideration of the presence of at least eight amide groups, a comparison of the total basic (19 lysine and 2 arginine) and acidic (18 aspartic acid plus glutamic acid) amino acid residues indicates an excess of three basic residues, which is in good agreement with the strongly basic isoelectric point observed for this protein.[9] Similarly, our present analysis indicates a higher content in alanine than was observed by Henderson and Nankiville.[9] Unusually high alanine contents are a characteristic property of all cytochromes *cc'* so far investigated.[10]

The residue numbers in Table 1 are based on the occurrence of a single residue

each of histidine and tyrosine. The minimal molecular weight of the protein was calculated by adding the sum of the formula weights of the amino acid residues to the weight of a single protoheme IX group. The presence of a single heme group was established by quantitative comparison of the absorption values of the pyridine hemochrome of the protein and the micromoles of amino acids recovered from identical aliquots after hydrolysis, as listed in Table 2.

The spectral characteristics of this monoheme cytochrome c'[4] are almost indistinguishable from those reported for the various double-heme proteins[3] except for the complete lack of tryptophan absorption in the UV region of the spectrum (Fig. 1). Thus, in this monoheme protein there is a pronounced splitting of the Soret absorption band of the reduced form, as in the corresponding Soret bands of cytochromes cc' which are diheme proteins.

The minimal molecular weight based on the presence of 137 amino acid residues, as found by amino acid analysis, plus one protoheme IX is 14,820. A similar value

TABLE 1

AMINO ACID COMPOSITION OF *Rps. palustris* CYTOCHROME c'

	Acid Hydrolyses				
	1: Untreated protein, 24 hr	2: Untreated protein, 48 hr	3: Performic acid–oxidized protein, 24 hr	4: Enzymic hydrolysis	5: Integral values
Cysteic acid	—	—	1.8	—	2
Aspartic acid	15.3	14.9	15.1	5.5	15
Methionine sulfone	—	—	2.0	—	2
Threonine	6.9	6.5	6.8	6.4	7
Serine	6.2	5.8	6.4	6.9	7
Glutamic acid	11.1	10.9	10.7	4.6	11
Proline	5.0	5.2	4.8	1.2	5
Glycine	8.7	8.6	8.9	9.0	9
Alanine	29.0	28.5	28.8	23.4	29
Valine	2.6	3.6	2.8	3.9	4
Methionine	1.7	1.5	—	1.8	—
Isoleucine	6.1	6.8	6.3	6.8	7
Leucine	10.5	10.8	10.7	10.9	11
Tyrosine	0.7	0.5	0.3	1.0	1
Phenylalanine	5.0	5.1	5.3	4.9	5
Histidine	1.0	0.9	1.2	1.0	1
Lysine	19.1	19.0	19.2	14.5	19
Arginine	1.9	1.5	2.1	1.8	2
Tryptophan	—	—	—	0	0
Amides	—	—	—	7.8	8
Total number of residues:					137
Molecular weight of the protein, based on a single protoheme IX group					14,820

Column 1 refers to acid hydrolysis of the untreated protein with 6 *N* HCl at 105°C for 24 hr.
Column 2 refers to acid hydrolysis of the untreated protein with 6 *N* HCl at 105°C for 48 hr.
Column 3 refers to acid hydrolysis of the performic acid–oxidized protein with 6 *N* HCl at 105°C for 24 hr.
Column 4 refers to total enzymic hydrolysis using pronase followed by leucine aminopeptidase.
Column 5 shows the tentative amino acid composition based on the results in columns 1–4.

TABLE 2

COMPARISON OF PYRIDINE HEMOCHROME EXTINCTION AND AMINO ACID ANALYSIS

	Aliquots (ml)	Micromoles heme	Micromoles protein	Heme/protein (%)
Horse heart cytochrome *c*	0.2	0.0933	0.096	97
	0.3	0.1400	0.147	95
R. rubrum cytochrome cc'	0.2	0.0499	0.027	185
	0.4	0.0998	0.054	178
Rps. palustris cytochrome c'	0.2	0.0449	0.047	96
	0.4	0.0898	0.094	95

A molar extinction coefficient of 31.18[15] was assumed for the alpha band of the pyridine hemochromes of all three proteins. The micromoles of protein in each aliquot were estimated from the micromoles of histidine and tyrosine found after 24 hr of hydrolysis.

for the molecular weight was obtained by chromatography on thin layers of Sephadex G-50-F. In these experiments the *Rps. palustris* protein moved significantly faster than did mammalian cytochrome *c* and more slowly than did horse metmyoglobin.[11]

Ultracentrifugal studies indicate a molecular weight of 13,400,[5] obtained by use of the Ehrenberg modification[12] of the method of approach to sedimentation equilibrium. In these studies we used the measured partial specific volume, $\bar{v} = 0.723$. The two values for the molecular weight are in reasonable agreement within the range of experimental errors inherent in the two methods of measurement. These results exclude the possibility that the protein exists as a dimer.

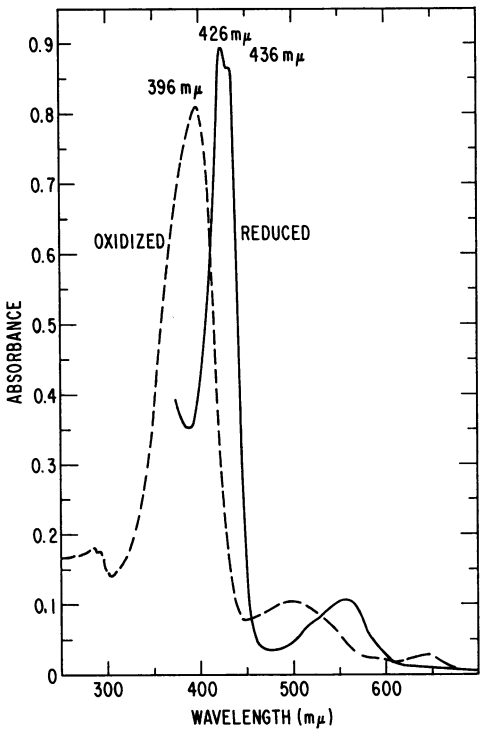

FIG. 1.—Absorption spectra of oxidized (———) and sodium dithionite-reduced (———) cytochrome *c'*. The protein concentration is 0.122 mg/ml in 0.05 *M* potassium phosphate buffer, pH 7.0.

Preliminary studies of a partial enzymic digest of *Rps. palustris* cytochrome *c'* led to the isolation and purification of a tryptic heme peptide which contained both cysteinyl residues and the single histidyl residue of the protein together with 21 other amino acid residues.[13] A heme-splitting experiment performed on this heme peptide using the $HgCl_2$-procedure in dilute acid solution[14] yielded a heme-free peptide and the free heme group in 80 per cent recoveries.[13] These data are taken as evidence that the heme group in *Rps. palustris* cytochrome *c'* is probably attached to the polypeptide chain of the protein by two thioether bonds as in cytochrome *c*.

Cytochrome *c'* of *Rps. palustris* is the first example of a protein with a single heme group which nevertheless belongs to the class of cytochromes *cc'*. It is appropriate to designate it as cytochrome *c'*, indicating its affinity to the previously

described group of double-heme proteins, in accordance with the recommendations of the International Union of Biochemistry Commission on Enzyme Nomenclature.[2]

The major finding in this research is the persistence of a pronounced splitting in the Soret absorption band of the reduced protein. In other cytochromes *cc'* this splitting could be interpreted as arising from separate contributions of each of the two heme groups present. However, the existence of a split Soret peak in the monoheme *Rps. palustris* protein invalidates this suggestion. It may be inferred that the heme group is bound to the protein in a very asymmetric environment, although other explanations are possible. The experimental elucidation of the mechanism for the nondegeneracy of the Soret transition may provide additional data for evaluation of current theories which deal with the problem of heme-protein interaction.

Summary.—The variant *c*-type cytochrome present in *Rhodopseudomonas palustris* (strain #2.1.37) has been characterized as a monoheme protein, differing from all other cytochromes *cc'* so far studied in electrochemical potential, molecular weight, isoelectric point, and in the presence of a blocked amino terminal residue. Nevertheless, it belongs to this class of variant cytochromes *c* on the basis that its spectroscopic properties and covalent binding of the prosthetic group are identical with those of other cytochromes *cc'*. The possession of a single heme by this cytochrome *c'* warrants its characterization as the first example of a cytochrome *c'*, as well as providing the first example of a monoheme protein in which a Soret band occurs with widely split components.

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