Proceedings of the National Academy of Sciences
Vol. 68, No. 3, pp. 629–631, March 1971

The Question of Histidine Content in *c*-Type Cytochromes

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Communicated December 31, 1970

ABSTRACT **Reports that histidine may not occur in heme peptides derived from *c*-type cytochromes isolated from chloroplasts of *Euglena gracilis* and *Porphyra* sp. have not been substantiated in the present investigation, in which the amino acid composition and a partial sequence were determined for a heme peptide derived from the *c*-type cytochromes of a strain of *Euglena* closely related to that used in the previous studies. It is concluded that no evidence exists to challenge the generalization that histidine is always present vicinal to the heme-binding site in *c*-type cytochromes.**

Histidine is invariably found adjacent to the second cysteine residue in the heme-binding site of *c*-type cytochromes. Thus, in all such cytochromes studied, i.e., mitochondrial cytochromes (1), *Pseudomonas fluorescens* cytochrome *c*-551 (2), *Desulfovibrio vulgaris* cytochrome c_3 (3), *Rhodospirillum rubrum* cytochrome c_2 (4), and *Chromatium*, strain D, cytochrome *c'* heme peptide (5), this generalization holds. It would be a finding of major importance, therefore, to observe cases of *c*-type cytochromes in which no histidine occurs vicinal to the binding site. The presence of a histidyl residue in this specific location in the structure of *c*-type cytochromes is a cornerstone of all present speculations on the relation of structure to function in these heme proteins (6).

However, there are reports of heme peptides isolated by peptic or tryptic digestion from *c*-type algal cytochromes in which histidine has not been demonstrated to be present. (Such peptides should, of course, contain the characteristic Cys-X-Y-Cys-His sequence invariably present at the heme-binding site.) Thus, Mitsui and Tsushima (7) have reported that acid hydrolysis of a tryptic heme peptide from cytochrome *c*-552 of a strain of *Euglena gracilis* (*var. bacillaris*) gave Asp, Glu, Gly, Ser, Ala, Thr, Leu, Ile, Lys, and Cys residues but no detectable histidine. No amino acid composition analyses for the whole protein were presented, however. Perini *et al.* (8), in earlier work on presumably the same organism, reported that *Euglena* cytochrome *c*-552 contained two histidines out of a total of some 120 amino acid residues, but could not state with certainty that in a peptic heme peptide containing the same residues reported by the Japanese workers there were any histidine residues. The latter group also stated (7) they found no histidine in a tryptic heme peptide obtained from *Porphyra tenera* sp. We have had available the "z" strain of *Euglena gracilis*, which is closely related if not identical to the strains used in the previous studies, and have isolated the corresponding cytochrome *c*-552, from which we have prepared a peptic heme peptide representing the heme-binding site of this protein. In this report, we present results on its amino acid composition and partial sequence analysis.

METHODS

Euglena gracilis strain z was grown in an autotrophic medium as described by Evans and San Pietro (9). Cells were harvested by centrifugation after growth for one week at 30°C.

Euglena cytochrome *c*-552 was prepared essentially as described by Perini *et al.* (8). We used DEAE-cellulose column chromatography until an apparently homogeneous protein was obtained, as judged by the elution profile. The purified *c*-552 had an absorbance ratio, 280 nm to 416 nm, of 0.200. Approximately 7 μmol of pure cytochrome was obtained from 1200 g wet weight of cells.

RESULTS AND DISCUSSION

Perini *et al.* (8) used three independent methods to estimate the molecular weight of *Euglena* cytochrome *c*-552. Use of iron and heme content yielded values between 13,000 and 14,000, assuming a millimolar extinction coefficient for the reduced α-peak of 28.1 $mM^{-1}\,cm^{-1}$. This value was consistent with the range of values found by amino acid composition analysis. However, sedimentation analyses showed values ranging up to 17,400. As an index of purity, the absorbance ratio (A_{280}/A_{416}) was given as 0.018. Perini *et al.* obtained approximately 21 μmol of cytochrome *c*-552 of this purity from the same quantity of cells we used to prepare approximately 7 μmol of the protein with a degree of purity equal to or perhaps slightly better. Mitsui and Tsushima reported absorbance data which indicate that the purity of their preparation was similar to ours. Unfortunately, it is unlikely that the degree of purity for any of these preparations can be stated to be identical. If one compares the amino acid compositions for the preparations of Perini *et al.* and of this study (Table 1)

TABLE 1. *Apparent molecular weight of Euglena cytochrome c-552 from gel filtration*

Protein	Mol wt	V/V_0
Horse heart cytochrome *c*	12,400	2.28
Sperm whale myoglobin	17,800	2.10
Chymotrypsinogen	25,000	1.88
Chromatium flavocytochrome *c*	72,000	1.30
Euglena cytochrome *c*-552	10,000 ±500	2.42

All proteins were chromatographed separately, with Blue Dextran 2000 as the void volume marker, on a 1.4 × 50 cm column of Sephadex G-75-F, equilibrated with 0.1 M Tris-HCl (pH 7.3)–0.5 M sodium chloride.

TABLE 2. *Amino acid composition of Euglena cytochrome c-552*

	Residues/heme	
	This work*	Perini *et al.* (8)
Asp	14	15
Thr	7	6
Ser	9	6
Glu	15	13
Pro	4	4
Gly	14	16
Ala	15	13
Cys/2	2	2
Val	9	11
Met	1	2
Ile	5	5
Leu	7	5
Tyr	5	5
Phe	3	3
His	2	2
Lys	8	7
Arg	3	2
Trp	1†	3‡
Total	124	120
Heme	1	1

Quantitative amino acid analyses were performed on a Beckman Spinco Model 120 B, modified as described by Dus *et al.* (17). Conditions for acid hydrolyses were 6 N HCl at 110°C in Pyrex tubes sealed under vacuum. Cysteine was determined as cysteic acid after performic acid oxidation as described by Hirs (18).

* Based on 24, 36, and 48 hr acid hydrolyses.

† Assumed number of residues.

‡ Estimated by the method of Bencze, W. L., and K. Schmid, *Anal. Chem.*, **29**, 1193 (1957).

there are seen to be only slight differences—which may, however, be real, and could arise from a species difference.

To obtain data relevant to this point we chose to study the molecular size of our protein sample by means of a Sephadex G-75 sizing column (10). In this procedure the molecular sieve material, as is well known, retains molecules roughly in an inverse relation to molecular size. By careful calibration with standard proteins similar in size and shape, as in homologous series, one may obtain relative sizes with considerable accuracy. By appropriate calibration, a direct proportionality between size and molecular weight can be demonstrated. It suffices to state that elution volumes for the *Euglena* cytochrome *c*-552 were obtained and compared with *Chromatium* flavocytochrome *c*-552, chymotrypsinogen, sperm whale myoglobin, and horse-heart cytochrome *c*. The "void" volume used as reference was obtained by using the high molecular weight dye, Blue Dextran 2000. Results are shown in Table 1.

It may be remarked, as extensively discussed in the literature (10, 11), that "size" and molecular weight as determined by this procedure are not necessarily identical. The sieving process depends on size and shape, rather than weight. Differences in degree of hydration as well as abnormalities in shape enter into the relation between size and weight. The required linear relation between the ratio of elution volume to void volume (V/Vo) and the logarithm of the molecular weight was found to obtain for the four standard proteins of known molecular weight, covering the desired ranges of weights. The *Euglena* sample fell nicely on this straight line at a value corresponding to a molecular weight which could be no less than 10,000 ± 500. That is to say, a molecule with "size" less than 10,000 (assuming no appreciable difference in shape and degree of hydration compared to the other *c*-type cytochromes used) could not have percolated through the column as rapidly as did the *Euglena* cytochrome *c*-552. It is also very unlikely from our extensive observations of the behavior of many different types of *c*-type cytochromes that any association took place at the high dilutions and salt concentrations employed in this gel chromatography. Our result is in accord with most molecular weights found for algal *c*-type cytochromes, as, for example, that (10,000) found by Sugimura *et al.* (12).

If 10,000 is accepted as the true molecular weight, then the results of amino acid composition analysis (Table 2) showing some 120 residues indicates that the samples in our studies and in those of the other workers may have been of degree of purity no better than 70% despite evidences of homogeneity obtained either by our chromatographic criteria, or by the ultracentrifugal studies (8).

The N-terminal sequence was determined by Edman degradation (13–15) and is compared with that obtained by Dus and Kamen (16):

Cytochrome *c*-552 (this work): H_2N-Gly-Gly-Ala-Asp...
Cytochrome *c*-552 (16): H_2N-Gly-Thr-Ala-Asp...

Cytochrome *c*-552 was denatured with ethanol (19) and then digested (17) with pepsin (Worthington Biochemical Corp.) in 5% formic acid, 1 μmol/ml for 7 hr at 37°C, pepsin to protein molar ratio, 1:10.

The digestion mixture was applied to a talc (Baker) column, 1 × 5 cm, which had been equilibrated with 0.01 N HCl. The column was washed with 0.01 N HCl and the heme peptide was eluted with 3% NH_4OH–ethanol 1:1. 90% of the heme applied was recovered (Table 3).

TABLE 3. *Amino acid composition of heme peptides from Euglena cytochrome c-552*

	Peptic peptide after talc	Final peptic peptide	Probable composition of final peptide	Peptic peptide Perini *et al.* (8)
Asp	4.0	3.9	4	1–2
Thr	0.9	1.1	1	1
Ser	1.3	1.3	1	1–2
Glu	0.5	0.6	1	1
Gly	2.5	2.1	2	3
Ala	1.4	1.5	2	1
Cys	...	1.7	2	1–2
Val	1.0	1.1	1	1
Met	0.5	0	0	?
Ile	0.3	0.3	0*	1
Leu	0.3	0.3	0*	1
Phe	0.4	0.3	0*	...
His	0.8	0.8	1	?
Lys	0.2	0.6	1	1
Arg	0.2	0	0	...
Heme	1.0			
Total			16	~13–16

* See discussion in text.

Heme was removed by performic acid oxidation (18). As a final purification step, the peptide was filtered through a Sephadex G-25 column, 1.5 × 40 cm, which had been equilibrated with 0.01 N NH_4OH. Approximately 90% of the applied peptide was recovered in one fraction. The purity was further tested by low-voltage electrophoresis at 15 V/cm on Whatman No. 1 paper in 2% formic acid–8% acetic acid (pH 2.2) for 1.5 hr. Only one ninhydrin-positive spot was observed. The final peptide (Table 3) was submitted to Edman degradation, as described by Doolittle (13), except that the conversion of the thiazolidones to phenylthiohydantoins was performed in 0.3 ml of 1 N HCl for 10 min at 80°C (14). The phenylthiohydantoin amino acids were identified by thin-layer chromatography (Eastman 6060 plates) in solvent systems D and E (15). The N-terminal sequence of this heme peptide was found to be H_2N-Ala-Asp-Asp. Although further Edman steps were attempted, no phenylthiohydantoin amino acids could be identified.

Three of four of the N-terminal amino acids of *Euglena gracilis* cytochrome *c*-552 used in this work were the same as those found by Dus and Kamen (16), which indicates that the two *c*-552 cytochromes studied were homologous, but not identical, in sequence. Although we purified our peptic peptide to a high degree, there were indications that all of the Phe, a small amount of the Asp, Gly, and Val residues, and perhaps the Leu and Ile residues, found in the amino acid composition analyses arose from contaminating peptides. These considerations were taken into account in deducing the probable composition of the peptic heme peptide shown (Table 3).

It is apparent that histidine is present in the peptic heme peptide isolated from our sample of *Euglena* cytochrome *c*-552, and in quantities which indicate clearly that the required single residue is present. The discrepancy between this result and those of the previous studies probably lies in varying degrees in the rate at which histidine may be released from oligopeptides. In any case, the data available clearly indicate that the results of previous studies cannot be interpreted as evidence for absence of histidine from the binding site of the heme in *c*-type cytochromes. Clearly, further studies on more highly purified samples of the *Euglena* cytochrome *c*-552 are needed. Further, more researches are indicated to define parameters which control release of histidine from peptides during serial degradation to establish sequence and to establish the location of the histidine residue found in the heme peptides isolated from algal *c*-type cytochromes.

These researches were supported by grants-in-aid from the National Institutes of Health (HD-01262) and the National Science Foundation (GB-7033X). T.M. was supported by a predoctoral training grant (5TI-GM-1045) from the National Institutes of Health.

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