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PURIFICATION AND PROPERTIES OF CYTOCHROME c_{555} FROM A PROTOZOAN, *CRITHIDIA FASCICULATA*

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SUMMARY

Cytochrome c_{555} was isolated and purified from a protozoan, *Crithidia fasciculata*. This cytochrome exhibits a unique spectrum. In the reduced form, the maxima appear to have shifted 5 nm towards the red region in comparison to the mammalian mitochondrial cytochrome c_{550}. In spite of this spectral difference, cytochrome c_{555} is similar to the cytochromes c_{550} in many respects. Both are cationic proteins and can be isolated and purified by essentially the same methods even though the former has an isoionic point at pH 8.8, while the latter at pH 10.0. Both have molecular weights around 13000 and have similar amino acid compositions except that cytochrome c_{555} contains 1 to 2 residues of ε-*N*-trimethyllysine per molecule of protein. Although the protozoan's protein reacts with mammalian cytochrome oxidase, it reacts at a reduced rate compared with that exhibited by the cytochromes c_{550}.

INTRODUCTION

Functionally there are two types of cytochromes *c*[1]: those from the mitochondria of eukaryotic organisms, which react with cytochrome oxidases from mammalian sources, and those which react with oxidases that are derived from bacterial sources. The former group consists of proteins derived from both plants and animals[2]. Spectra of this group of proteins are nearly identical; the α-peak is at 550 nm in the reduced state. The isoelectric point is close to pH 10.0, and all are reactive with mammalian cytochrome oxidases and effectively substitute one for another in the terminal oxidation chain of mitochondria. On the other hand, proteins in the latter group have somewhat varied spectra[3]. Thus, in the reduced form, the α-peak of cytochromes from various bacteria may be at 550, 551, and 555 nm, etc. Furthermore, the isoionic point may also vary considerably from acidic, to neutral, to basic. These proteins react with oxidases from bacterial sources and react very slowly, if at all, with mammalian cytochrome oxidase[3]. The primary structures among the cytochrome *c* of the first group are very similar to one another, and it has been firmly established that these proteins are in fact ancestral homologues[2]. Because the amino acid sequence of cytochromes *c* of the second group is grossly different from that of the first group, the question of whether there is a homology between them becomes a subject of discus-

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sion. With the use of a computer program, CANTOR AND JUKES[4] were able to show a certain degree of homology between these two groups of proteins.

DUS *et al.*[5] and SLETTEN *et al.*[6], after establishing the primary structure of cytochrome *c* of *Rhodospirillum rubrum*, added strength to the hypothesis that sequence homology exists among these heme-proteins. Thus, these recent data raise the possibility that the two groups of cytochromes *c* may be derived from a common evolutionary origin even though these two types of cytochrome have distinctly different enzymic properties. If this hypothesis is correct, it seems reasonable to assume that there exist in nature groups of *c* type cytochromes whose structure and enzymic properties may establish a link between those two groups of proteins previously mentioned. It is expected that the primary structures, the physicochemical properties as well as the enzymic properties of this special group of cytochromes *c*, should be between those of the two diverse groups of proteins discussed above. The purpose of this report is to describe the isolation and purification of such a cytochrome and identify those properties which may be considered to have fulfilled these requirements as the missing link. This is the cytochrome c_{555} isolated from *Crithidia fasciculata*, a unicellular insect trypanosome.

Based on spectral evidence, KUSEL AND WEBER[7] and HILL AND WHITE[8] presented clear evidence for the presence of cytochrome *b* and $a + a_3$ in *C. fasciculata*. In addition, the latter authors succeeded in demonstrating the existence of cytochrome c_{555} in this protozoan by concentrating extracts derived from the organism with ion-exchange resins. The latter observation was confirmed subsequently by KUSEL *et al*[9]., who had prepared a purified sample of the cytochrome and investigated some of its physical and chemical properties. The data obtained in our present investigation are generally in agreement with those obtained by these authors, but there are some significant differences. In addition, we have extended our investigation to the enzymic properties of the cytochrome.

MATERIALS AND METHODS

Culture of the organism and the preparation of cells

C. fasciculata were grown in a medium as described by HILL AND WHITE[8]. The culture was grown in the dark at room temperature in carboys (capacity 20 l) with ample aeration. The cells were harvested at late log phase by centrifuging at 500 × *g* for 10 min and stored at −70°.

Extraction and purification of cytochrome c

The method described by MARGOLIASH AND WALASEK[10] was followed. Concentrating and desalting of cytochrome solution were carried out as described by these authors. Their procedures were applied whenever necessary throughout the purification steps. Cells of *C. fasciculata* were sonicated for 4 min at 10° with a Branson sonifier in a solution containing 0.3 % $Al_2(SO_4)_3 \cdot 17H_2O$. Extraction at pH 4.5 was carried out at room temperature for 1 h. The solution was centrifuged at 500 × *g* for 10 min and the supernatant solution was adjusted to pH 8.0 with concentrated ammonia. After filtration, the cytochrome in the clear solution was concentrated and then brought to 50 % saturation with solid $(NH_4)_2SO_4$. The precipitates were discarded, and the cytochrome in the supernatant solution, after appropriate desalting

steps, was further purified by chromatography on Amberlite IRC-50 resins. The ion-exchange resins were equilibrated in sodium phosphate buffer solution, 0.02 M, pH 8.0 and eluted with a linear NaCl gradient from 0 to 0.5 M in the same phosphate buffer solution. The Na^+ content in the fraction was estimated with a Radiometer conductivity meter, calibrated against NaCl standards.

Analytical procedures

Spectra were determined with a Cary model 14 recording spectrophotometer. The spectra of derived pyridine hemochromogens were determined by a procedure described by FALK[11]. Molecular weight determinations were carried out by the use of a Spinco model E ultracentrifuge and by gel filtration through Sephadex G-75. Molecular weight obtained from data with the ultracentrifuge was calculated as described by MARGOLIASH AND LUSTGARTEN[12]. Iron content in the cytochrome was estimated according to ADLER AND GEORGE[13] and CAMERON[14]. Amino acid composition was calculated from data obtained by quantitative analyses of amino acids in protein samples after hydrolysis[15] using the Technicon autoanalyzer[16]. Samples of the proteins were hydrolyzed under reduced pressure (5 mm Hg) in 3 times glass-distilled 6 M HCl for 24 and 40 h. Duplicate analyses were performed on each hydrolyzate. An authenic sample of ε-*N*-trimethyllysine was obtained from Dr. W. K. Paik. The identification of this unusual amino acid in the hydrolysates of cytochrome c_{555} was made by comparison with an authenic sample of ε-*N*-trimethyllysine on the Technicon autoanalyzer[16]. Isoionic point of the cytochrome was determined by electrophoresis as described by BARLOW AND MARGOLIASH[17]. Reactivity of the protein to mammalian cytochrome oxidase was determined by the spectrophotometric assay described by SMITH AND CONRAD[18]. The oxidase was a Keilin–Hartree type of preparation, isolated from beef heart and had been treated with detergents[19]. The reaction was run in a Tris–malate buffer, 0.1–0.05 M (pH 7.0) at 25°. Samples of cytochromes *c* from the protozoan and from beef heart were dialyzed extensively against a Tris–malate buffer, 0.01–0.005 M (pH 7.0), then reduced by adding a minimal quantity of sodium borohydride to give maximal absorption at the α-absorption peak. The concentration of the pigment was calculated by assuming that the protozoan pigment has the same extinction coefficient at the α-absorption peak as the beef cytochrome *c*

RESULTS

Isolation and purification of cytochrome c_{555} from C fasciculata

A total of 50 mg of cytochrome was isolated from 2.5 kg (wet wt.) of cells which contained 200 g of cellular proteins. This represents extraction of approximately 50 % of the heme *c* present in the intact cells. The chromatographic profile of the protein on Amberlite IRC-50 is shown in Fig. 1. For reference, cytochrome c_{550} isolated from chicken heart[20] was eluted at 0.23 M NaCl solution. The major fraction of the protozoan's cytochrome was eluted at 0.16 M NaCl solution and was used in the subsequent investigations. There are three minor cytochrome fractions representing about 10 % of the total which were eluted earlier in the gradient. These fractions were not studied further.

Absorption spectra

The spectra of the reduced cytochrome c_{555} of the protozoan and cytochrome

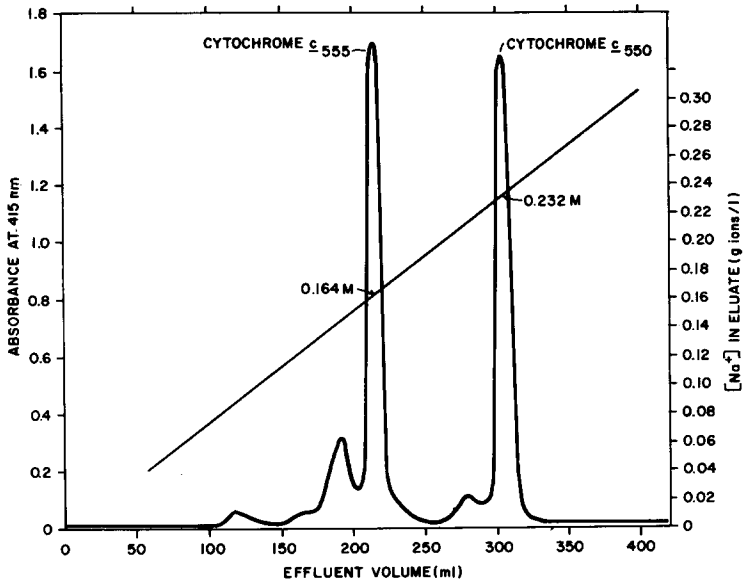

Fig. 1. The chromatographic profiles of chicken cytochrome c_{550} and c_{555} on cationic exchange resin Amberlite IRC-50.

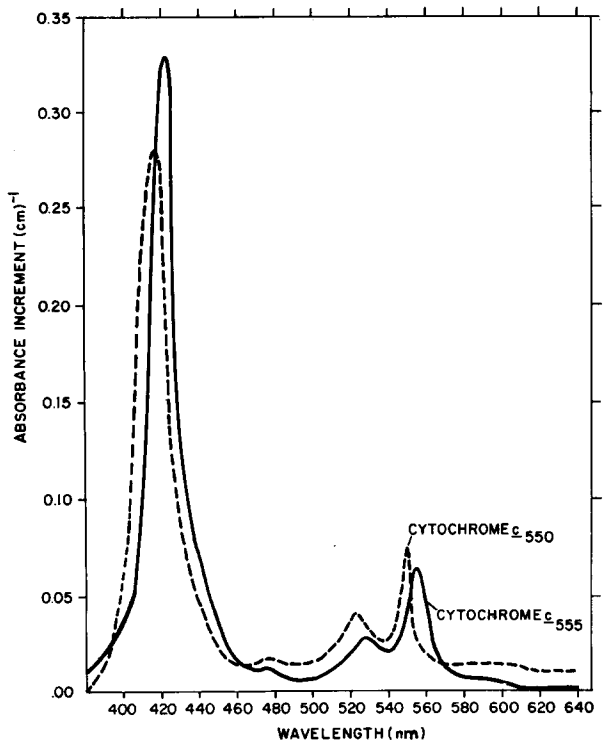

Fig. 2. Absorption spectra of chicken cytochrome c_{550} and c_{555} in their reduced state.

c_{550} of chicken heart are depicted in Fig. 2. The maxima of the protozoan's cytochrome are at 555, 525 and 421 nm, respectively. In comparison to the chicken cytochrome, the maxima appear to have shifted 5 nm towards the red region of the spectrum. Also, the α-peak of cytochrome c_{555} in pyridine is at 553 nm while that of cytochrome c_{550} is at 550 nm.

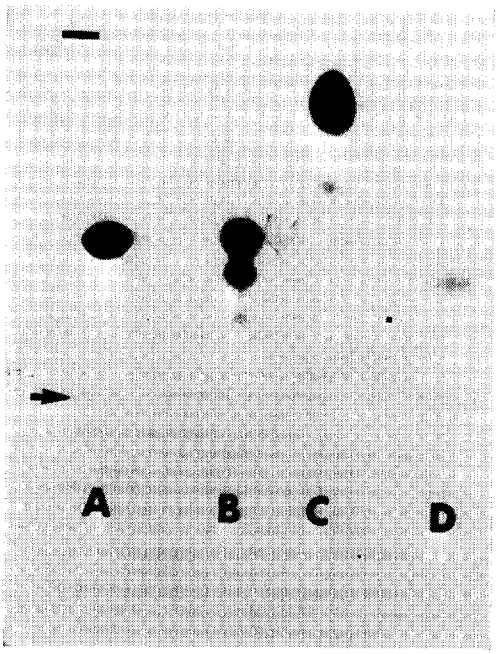

Fig. 3. Electrophoresis of cytochrome c_{550} and cytochrome c_{555} on cellulose acetate strips. Samples were applied at the origin as indicated by →. The cathode was marked by the sign −. 0.05 M phosphate buffer, pH 7.0, was used in this experiment. A was the major fraction of cytochrome c_{555} after Amberlite IRC-50 column chromatography as shown in Fig. 1. B was cytochrome c_{555} before chromatography. C was the major fraction of cytochrome c_{550} after chromatography. D was the minor fraction of cytochrome c_{555}, eluted prior to the major fraction as shown in Fig. 1.

Electrophoretic pattern and isoionic pH

The electrophoretic patterns of the two cytochromes from the protozoan and from chicken heart were compared in Fig. 3. At pH 7.0, both proteins migrated toward the cathode; the chicken cytochrome ran considerably faster than the protozoan's protein, an indication that at this pH the two proteins bear different net positive charges. When the isoionic points of these proteins were determined and compared, it was shown that the chicken's cytochrome c[17] along with those of the "mammalian type" has an isoionic pH of about 10.0, while that of the protozoan's cytochrome is at pH 8.8.

Molecular weight and amino acid composition

The sedimentation coefficient of the protozoan's cytochrome c_{555} was determined to be 1.65 and the corresponding molecular weight was calculated to be about 13000. These figures are in agreement with the figure obtained from the data of amino acid composition (see below). However, the molecular weight as determined by gel

filtration on Sephadex G-75 is considerably higher. As is shown in Fig. 4, the protozoan's cytochrome is clearly separated from cytochrome c_{550} [20]of the chicken heart. By comparison with several proteins of known molecular weights, the molecular weight of the protozoan's cytochrome c_{555} was estimated to be 16000.

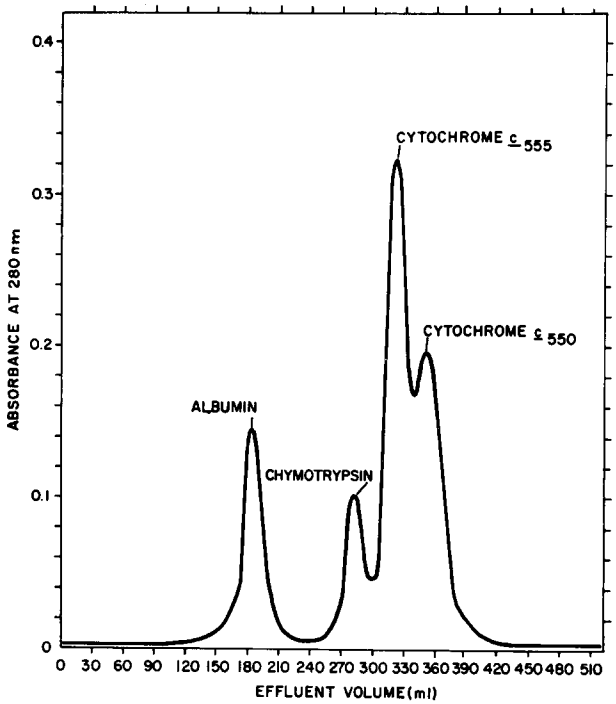

Fig. 4. Elution profiles of cytochromes c_{550} and c_{555} on Sephadex G-75. Albumin and diisopropylphosphofluoridate-chymotrypsin were used as known markers.

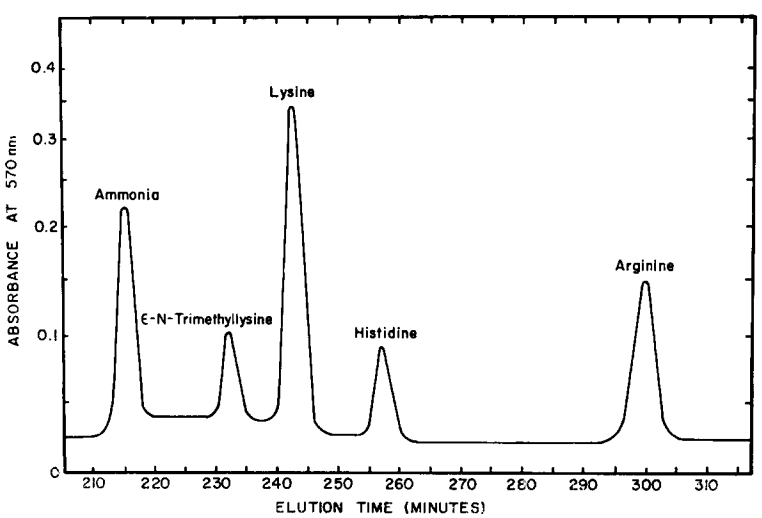

Fig. 5. Composite elution profiles of ε-*N*-trimethyllysine along with ammonia, lysine, histidine and arginine on the chromatographic column of the Technicon amino acid autoanalyzer.

The amino acid composition of the protozoan's cytochrome is reported in Table I. The two cysteine and one tryptophan amino acid residues shown in the table are assumed values and are not derived experimentally. Based on the data shown in Table I there are 117 amino acid residues per heme group. Assuming one heme group per protein molecule, the protein contains 117 amino acid residues. The amino acid composition data are similar to those reported by KUSEL *et al.*[9]. However, it should be pointed out that an unusual amino acid, ε-*N*-trimethyllysine, in an amount equivalent to 1–2 residues per mole of protein was shown to be present in the protozoan's cytochrome. A composite chromatographic profile of an authenic sample of this unusual amino acid along with three other basic amino acids and ammonia is shown in Fig. 5. The amino acid ε-*N*-trimethyllysine was eluted from the chromatographic column in a position between ammonia and lysine. The unusual amino acid present in the hydrolysates of the protozoan's cytochrome was eluted at the same position as the authenic sample of ε-*N*-trimethyllysine.

Reactivity toward mammalian cytochrome oxidase

The reactivities of cytochrome c_{555} and cytochrome c_{550} of beef heart toward mammalian cytochrome oxidase were determined. The oxidation of the ferro cytochrome from both sources was first order with respect to cytochrome concentrations. In each assay an amount of beef oxidase was added containing 0.08 mg of protein to 3.0 ml containing the buffer and cytochrome *c* (2.7 μM final concentration). The K (sec^{-1}) for the protozoan's cytochrome is 0.141, 0.162, while that for the mammalian's cytochrome is 0.794, 0.764. The rate for the former is only one-sixth of that for the latter.

TABLE I

AMINO ACID COMPOSITION OF CYTOCHROME c_{555} FROM *Crithidia fasciculata*

Amino acid	*μmoles of amino acid per μmole of Fe*	*Amino acid residues per molecule of protein*
Lysine	9.6	10
ε-*N*-Trimethyllysine	1.5	1–2
Histidine	2.2	2
Arginine	5.0	5
Aspartic acid	9.9	10
Threonine	5.2	5
Serine	5.3	5
Glutamic acid	10.0	10
Proline	8.3	8
Glycine	15.6	16
Alanine	14.2	14
Half-cystine	—	2*
Valine	6.9	7
Methionine	1.3	2
Isoleucine	2.2	2
Leucine	8.5	9
Tyrosine	3.8	4
Phenylalanine	3.2	3
Tryptophan	—	1*
Total number of residues		117

* Assumed values.

DISCUSSION

Isolation

Since cytochrome c_{555} of the protozoan and cytochrome c_{550} are both cationic proteins the isolation and purification of the protozoan's protein were made simple and straight-forward by following the method described by MARGOLIASH AND WALASEK[10]. It was reported that cytochrome *c* from vertebrate sources was not precipitated in 80–90 % $(NH_4)_2SO_4$ saturated solution while the homologous proteins from non-vertebrate sources such as the insects[15] were precipitated in 70 % $(NH_4)_2SO_4$ saturated solution. Cytochrome c_{555} of the protozoan, in this respect, is like the proteins of the non-vertebrates. At $(NH_4)_2SO_4$ saturation above 50 %, some cytochromes were observed to precipitate. For this reason, the protozoan's cytochrome was not subjected to salt fraction above the 50 % saturation level.

Physical, chemical and biological activities of cytochrome c_{555}

The absorption spectrum of the protein was shown to have shifted 5 nm towards the red region in comparison with that of the mammalian cytochrome c_{550}. The α peak of the pyridine ferrohemochrome maximum in cytochrome c_{555} is at 553 nm, while the cytochrome c_{550} from mammalian sources as well as cytochrome c_{553} from *Tetrahymena pyriformis* is at 550 nm[21]. Cytochrome c_{555} is a cationic protein but its rate of electrophoretic migration towards the cathode is considerably slower than that of the mammalian cytochrome c_{550}. The isoionic point of cytochrome c_{555} was determined to be at pH 8.8, distinctly different from the cytochrome c_{550} series[17] where all have isoionic points around pH 10.0. In contrast, KUSEL *et al.*[9] have determined the isoelectric points of cytochrome c_{555} and cytochrome c_{550} by pH gradient electrophoresis and reported both of them were close to pH 10.0. It is possible that determination of isoelectric points by pH gradient electrophoresis on electro-focuser column is not always accurate especially when very cationic proteins such as cytochrome *c* are studied, since some irreversible binding of the protein with the column support materials may occur.

The molecular weight of cytochrome c_{555} was determined to be around 13000 by KUSEL *et al.*[9] Our data based on amino acid composition as well as on ultracentrifuge analysis confirm these authors' findings. However, the molecular weight as determined by gel filtration on Sephadex is considerably higher. It was observed that the protozoan's cytochrome was running clearly ahead of the chicken's cytochrome on Sephadex; the molecular weight for the latter has been established to be around 12000. Since there is no significant difference in the relative amounts of aromatic amino acids present in the two cytochromes, it is unlikely that the slower migration rate of the protozoan's cytochrome on Sephadex is due to the binding between the protein and the Sephadex. It is possible that there exists a rapid equilibrium of interconversion between the monomer and dimer of cytochrome c_{555} and thus, on the Sephadex column, the cytochrome may emerge in a position between the monomer and dimer. However, it should be pointed out that no such monomer–dimer equilibrium was observed in the ultracentrifuge. This is probably due to the fact that the protein was in a solution of higher ionic strength when the experiment was performed in the ultracentrifuge. In any case, it appears more reasonable at the present time to consider cytochrome c_{555} to have a molecular weight of 13000.

The amino acid composition of cytochrome c_{555} is similar to that reported by KUSEL *et al.*[9] and to a large extent also similar to those reported for cytochrome c_{550} of the mammalian type[2]. One unusual feature is the presence of ε-*N*-trimethyllysine in the protein molecule of cytochrome c_{555}. This was not reported by KUSEL *et al.*[9] presumably because of failure to resolve this amino acid from lysine in the analytical procedures these authors employed. The presence of 1–2 residues of ε-*N*-trimethyllysine per molecule of protein is similar to that reported[22,23] for cytochrome *c* isolated from wheat germ, while the cytochrome *c* of the fungi contains a single residue of ε-*N*-trimethyllysine. On the other hand, DELANGE *et al.*[24], in a comprehensive survey, failed to detect any of the methylated lysine derivatives in cytochrome *c* obtained from various classes of vertebrates and from an insect. Based on these data, these authors suggested that there appears to have been an evolutionary differentiation in the development of specific methylation enzymes in Ascomycetes and higher plants. We may now add the protozoa to the picture. It is reasonable to assume that the evolutionary development of any enzyme system is for the benefit of the evolving organism. It would be of interest to determine in what functional respects the methylated cytochromes *c* differ from the non-methylated cytochromes *c*.

It has been shown[1,2] that various cytochromes c_{550} for which homologous primary structures had been established all have an isoionic point around pH 10.0 and all those tested react with mammalian cytochrome oxidase at an identical rate. This is true even among those cytochromes whose amino acid sequences differ as much as 50 %. On the other hand the bacterial cytochromes c^1, including the photosynthetic bacteria, *Rhodospirillum rubrum*, react very slowly, if at all, with the mammalian enzyme even through the structures of these cytochromes have been shown to have a homologous relationship with the mitochondrial cytochrome c_{550} series. In our present investigation, it was shown that the protozoan's cytochrome c_{555} does react with the mammalian oxidase but at a reduced rate when compared to cytochromes c_{550}. This is in contrast to cytochrome c_{553} present in another protozoan, *Tetrahymena pyriformis*. This latter cytochrome had been reported[21] to react slowly with Pseudomonas cytochrome oxidase but not with beef cytochrome oxidase. In addition, the isoionic point of cytochrome c_{555} was shown to be different from that exhibited by the cytochrome c_{550} series. All these considerations argue that the protozoan's cytochrome *c* may well be an evolutionary link one expects between the cytochromes c_{550} and the bacterial cytochromes *c*. If this is the case, homology of primary structures among these cytochromes should be easily recognized. Based on the data of amino acid composition, there is no doubt that the protozoan's cytochrome *c* is very similar structurally to the cytochromes c_{550}. Confirmation of this hypothesis, of course, awaits the elucidation of the complete amino acid sequence of the protozoan's cytochrome *c*.

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REFERENCES

1 T. YAMANAKA AND K. OKUNUKI, in K. OKUNUKI, M. D. DAMIN AND E. SEKUZU, *Structure and Function of Cytochromes*, University Park Press, Baltimore, Md., 1968, p. 390.
2 C. NOLAN AND E. MARGOLIASH, *Annu. Rev. Biochem.*, 37 (1968) 727.
3 R. G. BARTSCH, *Annu. Rev. Microbiol.*, 22 (1968) 181.
4 C. CANTOR AND T. JUKES, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 177.
5 K. DUS, K. SLETTEN AND M. D. KAMEN, *J. Biol. Chem.*, 243 (1968) 5507.
6 K. SLETTEN, K. DUS, H. DE KLERK AND M. D. KAMEN, *J. Biol. Chem.*, 293 (1968) 5492.
7 J. P. KUSEL AND M. M. M. WEBER, *Bacteriol. Proc.*, 68 (1968) 139.
8 G. C. HILL AND D. C. WHITE, *J. Bacteriol.*, 95 (1968) 2151.
9 J. P. KUSEL, J. R. SURIANO AND M. M. WEBER, *Arch. Biochem. Biophys.*, 133 (1969) 293.
10 E. MARGOLIASH AND O. E. WALASEK, *Methods Enzymol.*, 10 (1967) 339.
11 J. E. FALK, *Porphyrins and Metalloporphyrins*, Elsevier, Amsterdam, 1964, p. 182.
12 E. MARGOLIASH AND J. LUSTGARTEN, *J. Biol. Chem.*, 237 (1962) 3397.
13 A. D. ADLER AND P. GEORGE, *Anal. Biochem.*, 11 (1965) 159.
14 B. F. CAMERON, *Anal. Biochem.*, 11 (1965) 164.
15 S. K. CHAN AND E. MARGOLIASH, *J. Biol. Chem.*, 241 (1966) 335.
16 Technicon AutoAnalyzer, *Res. Bull.*, 10 (1966).
17 G. H. BARLOW AND E. MARGOLIASH, *J. Biol. Chem.*, 241 (1966) 1473.
18 L. SMITH AND H. CONRAD, *Arch. Biochem. Biophys.*, 63 (1956) 403.
19 L. SMITH AND P. W. CAMERINO, *Biochemistry*, 2 (1963) 1432.
20 S. K. CHAN AND E. MARGOLIASH, *J. Biol. Chem.*, 241 (1966) 507.
21 T. YAMANAKA, Y. NAGATA AND K. OKUNUKI, *J. Biochem. Tokyo*, 63 (1968) 753.
22 F. STEVENS, A. N. GLAZER AND E. L. SMITH, *J. Biol. Chem.*, 242 (1967) 2764.
23 R. J. DELANGE, A. N. GLAZER AND E. L. SMITH, *J. Biol. Chem.*, 244 (1969) 1385.
24 R. J. DELANGE, A. N. GLAZER AND E. L. SMITH, *J. Biol. Chem.*, 245 (1970) 3325.