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A Hemopeptide from a Tryptic Hydrolysate of *Rhodospirillum rubrum* Cytochrome C*

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Rhodospirillum rubrum cytochrome c purified by chromatography on carboxymethyl cellulose was subjected to hydrolysis with trypsin. A hemopeptide isolated from the digest has been found to consist of thirteen amino-acid residues arranged in a single polypeptide chain, the heme and polypeptide moieties being held together by two thioether bridges. In spite of marked dissimilarities, the sequence of amino-acids in this hemopeptide has certain characteristic features in common with the sequences previously worked out in hemopeptides prepared from other c-type cytochromes.

For the past few years the structure of cytochrome c has been studied with the aim of relating the catalytic activity of this hemoprotein to certain characteristic features of its chemical architecture. Much attention has been given to that portion of the protein moiety which adjoins the prosthetic group and which is supposed to contribute to a catalytically active site. In previous investigations [1,2] the sequence of twelve amino-acid residues including two heme-bound cysteine residues has been elucidated and found to be identical in the cytochromes of three different mammalian species (beef, horse and pig). When, however, the homologous amino-acid sequences were also determined in cytochrome c from fish (salmon)[2], bird (chicken)[2], insect (silk worm)[3] and from a fungus (yeast)[4], they appeared to be dissimilar though resembling each other more or less closely. The present communication will extend this comparative structural investigation to the c-type cytochrome produced by the photosynthetic bacterium *Rhodospirillum rubrum*, first described and subsequently studied in more detail by Kamen *et al.*[5–7]. There is a twofold interest in comparative structural work of this kind. In the first place, one may suppose that only those features which turn up invariably in all various cytochromes c, are likely to be essential to the specific catalytic function, whereas structural differences will indicate points not directly concerned with catalytic

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activity. Secondly, the species similarities and dissimilarities as revealed by comparative investigations of amino-acid sequences may give a clue to the genetic relation between the organisms from which the proteins studied have been obtained.

MATERIAL AND METHODS

Preparation of Rhodospirillum rubrum cytochrome c. Lyophilized bacterial cells were extracted with H_2SO_4 at pH 4. From the extract, the cytochrome c was obtained by fractionation with ammonium sulphate [8], dialysis and lyophilization. A sample thus prepared was kindly put at our disposal by Professor M. D. Kamen. It was further purified by chromatography on a column of CM-W cellulose (1.6 × 10 cm) prepared according to Peterson and Sober [9] and buffered with 0.015 M ammonium acetate of pH 6.5. The preparation of cytochrome c (470 mg) was dissolved in 2 ml of this buffer, reduced by the addition of solid $Na_2S_2O_4$ and transferred to the column. A dark brown, fast moving band separated from the red cytochrome band which moved slowly, if at all. When the buffer of pH 6.5 was replaced by 0.05 M ammonium acetate of pH 7.5, the red material started moving down the column and divided into three bands. The main fraction (R = 0.4, E_{270}/E_{551} = 0.96) yielded 71.5 mg of dry, salt-free cytochrome c which was used for all subsequent experiments.

Preparation of the hemopeptide and removal of the heme. After hydrolysis of the bacterial cytochrome c with trypsin, the hemopeptide formed could be precipitated from the digest with ammonium sulphate and purified by adsorption on talc and by column partition chromatography as described previously for other hemopeptides [1,2]. The hemin part of the hemopeptide was split from the peptide moiety using silver sulphate in acetic acid solution according to the method of Paul [10]. Performic acid was used to oxidize the thiol groups of cysteine residues to sulphonic acid residues. The hemin-free oxidized peptide obtained was essentially homogeneous as determined by electrophoresis on paper in a formic acid-acetic acid buffer of pH 2 [11].

Enzymic hydrolyses. The crystalline chymotrypsin used was purchased from the Worthington Biochemical Corporation. The subtilisin was a gift from Ing. M. Ottesen, Carlsberg Laboratory, Copenhagen. Digestions were carried out at pH 7.5 to 7.8 for 20 h at 25°C (with chymotrypsin) or for 24 h at 37°C (with subtilisin), the ratio between enzyme and substrate (w/w) being about 1:15.

End group determinations. For the characterization of N-terminal amino-acid residues the DNP method of Sanger [12,13] was employed. C-Terminal residues were determined using the hydrazinolysis procedure of Akabori *et al.*[14]

RESULTS

In the hydrolysate of the hemin-free oxidized polypeptide (prepared from the hemopeptide as described in the foregoing section) ten different amino-acids were identified by paper chromatography: alanine, aspartic acid, cysteic acid, glutamic acid, glycine, histidine, leucine, lysine, phenylalanine, and threonine. The lysine present in the polypeptide may be expected to be the C-terminal residue of the amino-acid sequence, since the polypeptide has been formed by the action of trypsin which is known to split bonds involving the carboxyl groups of lysine and arginine residues. The N-terminal residue was shown to be cysteic acid. After dinitrophenylation of the polypeptide and subsequent hydrolysis, however, the hydrolysate was found to contain, besides DNP-cysteic acid, free cysteic acid as well. This finding is accounted for by the presence in the polypeptide of two cysteic acid residues, one of which is terminal and the other is not.

Table 1. Determination of the amino-acid sequence in a polypeptide obtained from *Rhodospirillum rubrum* cytochrome c by tryptic hydrolysis, removal of the heme moiety, and oxidation with performic acid.

N-terminal residue (DNP method)		$CySO_3H$—. . .
C-terminal residue (specificity of trypsin)		. . .—Lys
Peptides obtained by *chymotryptic* digestion		$CySO_3H$—[Leu, Ala, $CySO_3H$, His] Thr—Phe Asp—Glu—[Gly, Ala, Asp]—Lys
Peptides found after digestion with *subtilisin*	Main split products	$CySO_3H$—Leu—Ala Thr—Phe NH_2 Asp —Lys $CySO_3H$—His [Asp, Glu, Gly, Ala]
	Minor split products	Ala—[$CySO_3H$, His] [Asp, Glu, Gly] [Thr, Phe, Asp, Glu, Gly] $CySO_3H$—[His, Thr] [Gly, Ala]
Sequence		$CySO_3H$—Leu—Ala—$CySO_3H$—His—Thr—Phe—Asp—Glu—Gly—Ala—Asp(NH_2)—Lys

Chymotryptic hydrolysis of the polypeptide (Table 1) gave rise to three main split products which were separated from each other by high-voltage electrophoresis at pH 2 and by paper chromatography in butanol-acetic acid: (I) $CySO_3H$-[Leu, Ala, $CySO_3H$, His], (II) Thr-Phe, and (III) Asp-Glu-[Gly, Ala, Asp]-Lys. Split product (I), with N-terminal cysteic acid, must be derived from the N-terminal side of the polypeptide chain and the lysine-containing peptide (III) from the C-terminal side. Thr-Phe, then, must be located between these two. After dinitrophenylation of peptide III and partial hydrolysis (with conc. HCl at 37°C, for 8 days) two ether-soluble products were separated and characterized, DNP-Asp and DNP-Asp-Glu, which demonstrates that glutamic acid must adjoin the N-terminal aspartic acid residue.

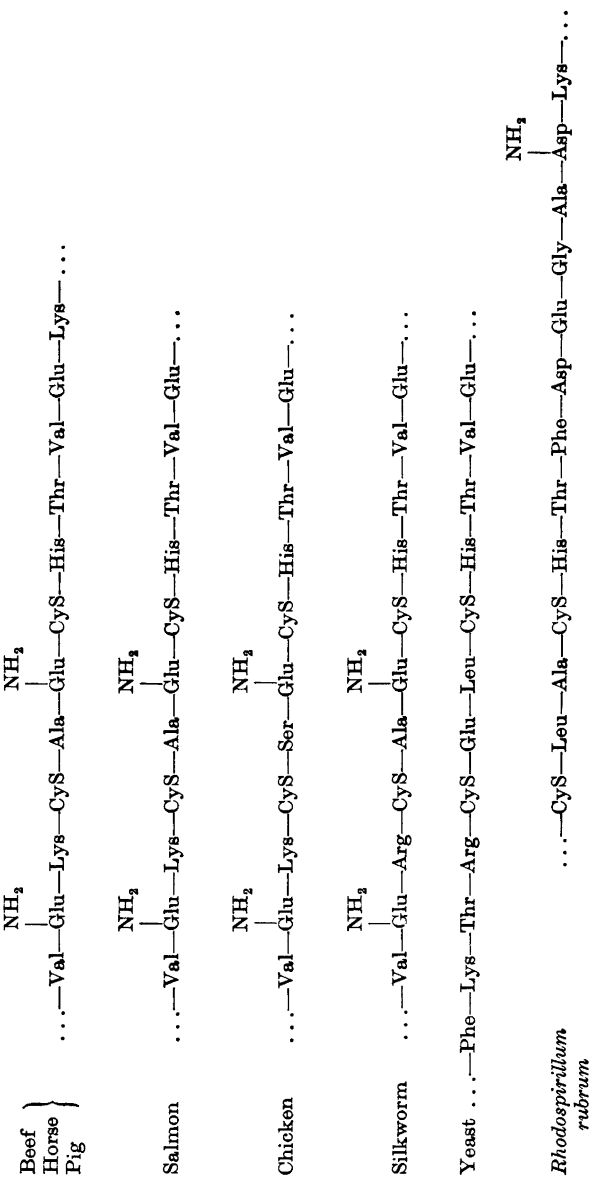
In the subtilisin hydrolysate of the polypeptide (Table 1) five products were present in relatively high concentration: $CySO_3H$-Leu-Ala, $CySO_3H$-His, Thr-Phe, [Asp, Glu, Gly, Ala], and Asp(NH_2)-Lys. Among the split products obtained in small amounts there were two tripeptides containing histidine: Ala-[$CySO_3H$,His] and $CySO_3H$-[His, Thr]. Other minor components of the hydrolysate were shown to be [Gly, Ala], [Asp, Glu, Gly], [Thr, Phe, Asp, Glu, Gly], and free alanine.

From the findings presented above it may be concluded that the amino-acid sequence in the hemin-free oxidized polypeptide is

1 2 3 4 5 6 7 8 9 10 11 12 13

$CySO_3H$-Leu-Ala-$CySO_3H$-His-Thr-Phe-Asp-Glu-Gly-Ala-Asp (NH_2)-Lys.

Table 2. Comparison of the sequences of amino-acids in the vicinity of the prosthetic group in c-type cytochromes of different organisms.



The results obtained leave it undecided whether residues 8 and 9 are free aspartic acid and free glutamic acid or whether these amino-acids are in the amide form, as asparagine and glutamine residues.

The two cysteic acid residues contained in the sequence have arisen from and taken the place of two heme-bound cysteine residues present in the original

Rhodospirillum rubrum cytochrome c and in the hemopeptide prepared from it by tryptic hydrolysis.

The specificity of trypsin already mentioned suggests that the amino-acid residue preceding the above sequence must be either lysine or arginine.

The cleavage by chymotrypsin of a bond between histidine and threonine (residues 5 and 6) may appear unexpected in view of the general belief that chymotrypsin will split bonds involving the carboxyl groups of the aromatic residues phenylalanine and tyrosine. The finding is in agreement with recent observations of Davis [15], however, who has reported that bonds involving the carboxyl groups of histidine residues are somewhat susceptible to chymotryptic hydrolysis.

A comparison of the amino-acid sequences that have been shown to occur in the vicinity of the heme moiety in different cytochromes demonstrates that they have various structural features in common (Table 2). The presence of two cysteine residues linked to side chains 2 and 4 of the porphyrin of the prosthetic group, first indicated by Theorell [16] in 1939 for beef cytochrome c, has been confirmed and found to be characteristic of all c-type cytochromes. The distance between the two cysteine residues along the polypeptide chain is always the same, two other amino-acids being situated in between. Invariably a histidine residue has been found to adjoin one of the heme-bound cysteine residues. Stereochemical considerations of Ehrenberg and Theorell [17] have indicated that the chain of amino-acids in which the two heme-bound cysteine residues and the histidine residue are included is likely to be in the form of an α-helix. This coiling will bring the histidine residue into a steric position favourable for a covalent attachment of an imidazole nitrogen to the iron of the heme disc.

There are still other structural features invariably encountered in all cytochrome c samples examined so far, such, *e.g.*, as the presence of a basic residue (lysine or arginine) close to cysteine and of a threonine residue close to histidine. The significance of these similarities is presently not known.

It is remarkable, anyhow, that in so many structural respects the cytochrome c of *Rhodospirillum rubrum* appears to resemble the c-type cytochromes of other organisms, in spite of an overall amino-acid composition, oxidation-reduction potential, and a specificity of action quite different from those of vertebrate and yeast cytochromes [18].

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