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Studies on Peptides from a Peptic Hydrolyzate of Cytochrome c from *Rhodospirillum rubrum*

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Cytochrome c (550 *Rhodospirillum rubrum*) was prepared by acid extraction, precipitation with $(NH_4)_2SO_4$ and TCA, and chromatography on DEAE and CM–W. It was studied by spectrophotometry and electrophoresis. The cytochrome c was digested with pepsin, after which two peptides were isolated by partition chromatography. They have been subjected to amino acid analysis and N-terminal end group determination. The peptides were also investigated by ultracentrifugation and spectrophotometry.

Ever since a cytochrome c * was first shown to be present in the facultative photoheterotrophic organism *Rhodospirillum rubrum* [1], a great interest has been focused on it and other heme proteins [2–4] in this type of bacteria. The remarkable similarity between the spectra of this cytochrome c and cytochrome c from mammalian sources, in spite of dissimilarities in oxidation potentials and other physio-chemical properties, has called for an elucidation of the structural similarities and differences. Paléus and Tuppy [5] were able to determine the structure of a hemopeptide containing 13 amino acids residues from a tryptic hydrolyzate of *R. rubrum* cytochrome c. They established the fact that the "core" of the peptide chain had the same structural features earlier encountered in all cytochrome c samples. The aim of the present investigation was to isolate a hemopeptide after digestion of the cytochrome c from *R. rubrum* with pepsin.

EXPERIMENTAL

Material

Rhodospirillum rubrum. The method of growing the bacteria on a large scale has been described in an earlier paper [6]. The total yield of a fermentation was 600 g of lyophilized bacteria. As was pointed out previously, a large portion of the bacteria was disrupted by

* The following abbreviations will be used: cyt. c and cytochrome c = cytochrome c (550 *Rhodospirillum rubrum*), unless otherwise stated. TCA = trichloroacetic acid, DNFB = 2,4-dinitrofluorobenzene, DNP– = dinitrophenyl-, CM–W = carboxymethyl cellulose, DEAE = diethylaminoethyl cellulose.

the harvesting procedure and by the lyophilization. The material had been stored in an evacuated desiccator at + 4° for 5 months before use.

Ion exchange material. Cellulose powder ion exchangers CM—W and DEAE types were used throughout the preparations of the cytochrome. They were prepared strictly according to the procedures recommended by Peterson and Sober [7], using Whatman cellulose powder and Solca floc as starting material.

Talc. Ordinary commercial talcum powder was washed 6 times with N HCl, allowed to settle each time, and the supernatant solution discarded. After the last sedimentation the supernatant solution was practically clear.

Silica. The commercial product "Celite analytical filter-aid" was thoroughly washed as described by Tuppy and Bodo [8], using 50 % ethanol containing 3 % ammonia, water, 2.4 N HCl and ethanol. The product was dried for 24 h at 110°C.

DNP-amino acids. For qualitative experiments, a DNP-amino acids kit purchased from Mann Research Laboratories was used. Quantitative measurements were performed on DNP-amino acids kindly supplied by Dr. Alfred Arens.

Pepsin. 3 times crystallized pepsin, lot No. P 109—060 from Sigma Chemical Co., was used.

DNFB. 2,4-Dinitro-fluorobenzene, Merck No. 2966 was used.

Paper chromatography. Whatman No. 1 papers were used throughout this investigation. The solvents for the "Toluene" mixture were purified from commercial products as recommended by Biserte *et al.*[9]

Other chemicals. Except for the chemicals used in the crude preparations, good commercial analytical grade substances were used. Ordinary distilled water was used throughout. All buffer concentrations refer to the sum of the concentrations of the acidic and basic forms of the buffer.

Methods

Electrophoresis. This was carried out in a Tiselius electrophoresis apparatus (Beckman/Spinco Model H Electrophoresis-Diffusion Instrument) at + 0.6°C. The micro (2 ml) cell was used. A tungsten lamp was used, with a red filter introduced for the exposures. The samples were equilibrated with the buffer by dialyses for 48 h at + 4°C.

The mobility was calculated by the formula $u = Sq\varkappa/ti$ where S is the apparent speed of the boundary, q is the cross-section of the electrophoresis cell, $\varkappa$ is the specific conductance of the solution, t is the time and i is the current.

Spectrophotometry. Measurements at single wavelengths were carried out in a Beckman Model DU Spectrophotometer using cuvettes of 1 cm optical light path, and with a volume of 3 ml. For smaller quantities, 0.5-ml cells were used. For complete spectra, the recording Beckman DK 2 was used with scanning set from 700 to 250 mμ. The heights of the sharper peaks were checked with the DU instrument. Measurements were usually performed in 0.066 M phosphate buffer of pH 6.8. As reducing agent, solid sodium dithionite was used.

Ultracentrifugation. This was performed in a Spinco Analytical Ultracentrifuge Model E by the method described by Yphantis and Waugh [10]. The hemopeptides were dissolved in a 0.2 M Na_2HPO_4 solution. In order to avoid polymerization, sufficient N-acetyl-L-histidine was added to form the hemochromogen. The concentrations of the hemochromogen, both in the top and bottom compartments of the cell, were determined spectrophotometrically at 320, 410 or 550 mμ. The molecular weight was calculated as described in the original method, except for the Q-values which were calculated according to the formula:

$$Q = \frac{c_t(b^2-a^2)}{c_t(r_p^2-a^2) + c_b(b^2-r_p^2)}$$

where c_t and c_b are the absorbancies of the solutions in the top and bottom compartments of the cell respectively, a is the distance from the rotor center to the meniscus, b is the distance from the rotor center to the bottom of the cell when the rotor is rotating at full speed, and r_p is the distance from the rotor center to the separating plate when the rotor is at rest.

DNP-amino acid analysis. This was carried out chiefly as described by Wallenfels and Arens [11]. The coupling with the DNFB was achieved in a small water-mantled vessel which contained a glass electrode, an agar-KCl bridge to a calomel electrode, and the outlet tube from an Agla micrometer syringe. A Radiometer pH meter (type PHM 22 p) was used. Water at 40°C was circulated through the mantle. The vessel was provided with a magnetic stirrer. 0.3 to 1 mg of material was used in each experiment. To the solution which was to be reacted was added KCl to make it 0.1 M. The pH was adjusted to 8.9 with 0.100 N NaOH from an Agla syringe, after which 0.02—0.1 ml of DNFB was added. The course of the reaction was followed by observing the amount of 0.1 N NaOH needed to keep the pH constant at 8.9. Readings were made every third minute and continued until the alkali consumption showed a linear course, which was after 90 to 120 min. The excess of DNFB was then extracted with ether.

In the case of the end-group analysis, the dinitrophenylated peptide was precipitated by acidifying the solution. The precipitate was centrifuged, and the supernatant solution discarded. The peptide was dissolved in alkaline water and reprecipitated. The precipitate was then washed with alcohol and with ether. The peptide was dried in a desiccator, and subjected to hydrolysis with 6 N HCl for 24 h at 110°C. The hydrolyzate was then evaporated to dryness in a vacuum desiccator at room temperature. The residue was dissolved in water and extracted 3 times with ether. The two fractions of DNP-amino acids were evaporated to dryness separately, and dissolved in acetone or ethyl acetate. Each of these solutions was then transferred to a separate sheet of 45 × 45 cm paper. The spots on each sheet were formed in one corner, 7 cm from each edge. The papers were subjected to descending chromatography with the solvents recommended by Levy [12]. First they were developed with the "toluene"-mixture for 7 h and, after drying in a forced-draft oven at 35°C for 6 h, developed in the 1.5 M phosphate buffer, pH 6, for 10 h. The spots were identified by comparison with runs made with known DNP-amino acids, and with other published charts [9,11,12]. In a confirmatory experiment, a mixture of 99 % formic acid:acetic anhydride:70 % perchloric acid (20:11:3 volume ratios), which is said to cause less destruction of DNP-glycine [13], was used as the hydrolyzing agent for the peptide. This hydrolysis was carried out at 100°C for 2 h. The hydrolysis was performed in a thick-walled, sealed glass tube which had to be opened very carefully to avoid explosions. The hydrolyzate was diluted 10 times and extracted 3 times with benzene that contained 10 % *tert*-amyl alcohol. The extract was then evaporated to dryness and chromatographed as described above.

In the case of the total amino acid analysis, the unreacted peptide was hydrolyzed with HCl as described above, and reacted with DNFB. The solution was then acidified, and the ether-soluble DNP-amino acids separated from the water-soluble derivatives by extraction. The solvents were evaporated, and the residues dissolved as quantitatively as possible in acetone or ethyl acetate, and transferred to spots on the papers. The chromatography was carried out as before. After identification of the spots, they were cut out, leaving 5 mm margins along the edges, and the DNP-amino acids were extracted in test tubes with 5 ml of 1 % $NaHCO_3$ solution at 60°C for 20 min. The relative concentrations were determined by reading the absorbancy at 360 mμ, and multiplying these values, after correcting them for the paper and cuvette blank values, by the factors given by Wallenfels and Arens [11] to give the corrected absorbancy readings. Since the extinction coefficients at 360 mμ of the DNP-derivatives of the common amino acids are about the same, the ratios of the absorbancy readings are approximately proportional to the molar ratios of amino acid residues in the original peptide. The DNP-aspartic acid and DNP-glutamic acid were determined together. They were separated by a second chromatogram in one dimension with 2.5 M phosphate buffer at pH 6. The mixed preliminary value was divided up in proportion to the content of each of the two.

Amino acid analysis. For this analysis, a sample of the peptide was hydrolyzed in an evacuated ampule with 6 N HCl at 110°C for 20 h. It was then dried, dissolved in water, dried again, and subjected to ion exchange chromatography as described by Moore *et al.*[14]

Preparations

Preparation of the cytochrome c. The preparation was performed at + 4°C except for the first centrifugations and the column chromatographies which were done at room tem-

perature. The extraction was performed by suspending 550 g of the lyophilized bacteria in 11 l water to which 22.9 ml concentrated H_2SO_4 had been added. The pH, after the addition of the bacteria, was 4. The suspension was stirred as vigorously as possible without causing excessive foaming. After standing for 2.5 h, the pH was adjusted to 7.0 by the addition of 4 N NH_4OH. After standing for 3 h, the suspension was centrifuged at 2 200 *g* for 1 h. To the turbid supernatant solution, 313 g of solid $(NH_4)_2SO_4$ per liter was added, and the solution allowed to stand overnight. After centrifugation for 1 h at 2 200 *g*, the supernatant solution was clear and had a reddish-brown colour.

Another 203 g of $(NH_4)_2SO_4$ was added to each liter of the resulting supernatant solution, after which it was allowed to stand overnight, and centrifuged as before. The supernatant solution was then saturated with $(NH_4)_2SO_4$, and left for 48 h with slow stirring. It was not possible to obtain a satisfactory sedimentation in the centrifuge at this stage. Therefore, 25 ml of 20 % TCA was added per liter of the suspension, after which it was immediately centrifuged for 0.5 h. The supernatant solution, which was now perfectly clear and free from any reddish colour, was discarded, and the precipitate washed with water that was saturated with $(NH_4)_2SO_4$. The brown residue was dissolved in 220 ml of 0.066 M phosphate buffer at pH 6.1. To this solution, which contained about 23 % $(NH_4)_2SO_4$ as determined by electrical resistance, was added 80.5 g solid $(NH_4)_2SO_4$ to give about 75 % saturation. The large amount of precipitate was sedimented by centrifuging at 3 500 *g*. The supernatant solution was strongly red coloured.

In order to diminish losses, all of the $(NH_4)_2SO_4$ precipitates were combined and subjected to repetition of the process, and the final red solution added to the main batch.

To the combined final red solutions was added 2.5 ml of 20 % TCA per 100 ml. The heavy gelatinous precipitate which appeared immediately was centrifuged, washed once with saturated $(NH_4)_2SO_4$ solution, and dissolved in 0.1 M phosphate buffer pH 7. A viscous, deeply red-coloured solution containing a large amount of 260-mμ-absorbing material was obtained. The solution was dialysed against water until free of salts, and mixed with half its volume of a settled suspension of DEAE which had been equilibrated with 0.01 M sodium acetate at pH 7. The suspension was transferred to a chromatography column and the solution allowed to pass through the ion exchanger twice. If not all of the red material was adsorbed by the ion exchanger, the column was washed with water once, and the solution allowed to pass through once again. The column was then carefully washed with water, and the cytochrome c eluted with 0.1 M ammonium acetate buffer of pH 5.4. The cyt. c could now, after dialysis against 0.01 M ammonium acetate buffer of pH 5.4, be adsorbed on a CM—W column which had been equilibrated with the same buffer. The column was washed with 4 volumes of buffer. When the buffer strength was increased to 0.033 M a red band moved slowly down the column. It contained the reduced cyt. c in a highly pure form. Finally, the oxidized cyt. c was eluted with 0.2 M buffer. The yield of cyt. c was about 100 mg. Recently Horio and Kamen [4] have introduced a new method of preparation, which gives a better yield of cytochrome c.

Preparation of the peptic peptide. Digestion: 51 mg of the cyt. c, prepared as described, was dissolved in 22 ml of water and adjusted to pH 1.55 with 0.3 N HCl. 5.9 mg of pepsin was dissolved in 0.01 N HCl and added to the cytochrome solution. The digestion was performed at 25°C for 22 h. After this, the pH was rapidly adjusted to 8.7 with 0.2 NaOH, and the solution was brought to 85 % saturation with solid $(NH_4)_2SO_4$ to precipitate the pigment. The solution was filtered to give a colourless filtrate. The precipitate was dissolved in a small quantity of ammoniacal water, and precipitated once more at about 80 % saturation of $(NH_4)_2SO_4$.

Desalting. A column of talc, 50 mm long and 15 mm in diameter, was prepared, and washed with 4 volumes of 0.05 M phosphate buffer pH 5.0. The pigment in ammoniacal water was adsorbed on the talc, and washed with 0.5 % acetic acid until no more sulphate ion could be demonstrated in the eluate on addition of $BaCl_2$. The pigment could then be completely eluted with a mixture of equal parts of acetone and 10 % ammonia solution. The coloured part of the eluate was evaporated in an evacuated desiccator over H_2SO_4 and NaOH.

Partition chromatography. 0.92 ml of the aqueous phase of a 20-hours-old mixture of butanol:acetic acid:water (4:1:5 volume ratios) was added to 2.50 g of washed and dried celite. This was suspended in the organic phase of the same mixture, and carefully packed in an 11 mm wide column. The pigment easily dissolved in 1 ml of the organic phase,

and was subsequently chromatographed on the column using the organic phase as the developing solvent. One main fraction of the coloured material travelled down the column as a sharp band, the R_F of which was calculated, as described by Martin and Synge [15], to be 0.7. This band was collected, and was found to contain 7.5 mg of material when dried in an evacuated desiccator over H_2SO_4 and NaOH.

RESULTS AND DISCUSSION

Analysis of the cytochrome c

Spectrophotometry. The absorption spectra were recorded on the reduced and oxidised fractions eluted from the CM—W column. They were found to be identical with the spectra published by Horio and Kamen [4], except for a small elevation of the 275 mμ maximum, probably caused by small amounts of protein contaminants. The differences in absorption in the range 250—290 mμ between the oxidised and reduced forms is considered to be of interest as it shows that the ratio $E_{550\ red}/E_{275\ ox}$, which was used as an index of the purity during the preparation, can only be used if the measured sample is fully oxidised.

Electrophoresis. The cyt. c preparation was concentrated on a CM—W column in a similar way as was described above. Solutions of 0.6 % concentration were dialyzed for 48 h against the buffers to be used. The electrophoresis were performed with acetate buffers at pH 5.02 and 5.32, and with phosphate buffers at pH 5.90 and 7.11. The ionic strength of the buffers was 0.1, and the pH was determined at 20°C. The cytochrome c was fully oxidised in all runs, as determined spectrophotometrically. The mobility was calculated after running times of between 2 and 3 h. A plot of the obtained mobilities indicates an isoelectric point close to pH 5.5 (Fig. 1). Horio and Kamen [4] have found the isoelectric point of the cyt. c to be 6.4. This might depend on dissimilarities of the protein configuration due to the different ways of preparation. The only certain difference in the experimental conditions is that they used buffers of ionic strength 0.2 instead of 0.1.

The electrophoresis showed that the preparations were not entirely homogeneous, but consisted of one main fraction and about 10 % impurities. The impurities were isolated, and upon examination showed an absorption maximum at 280 mμ and very little absorption in the Soret-band region. They were therefore considered to be uncoloured proteins.

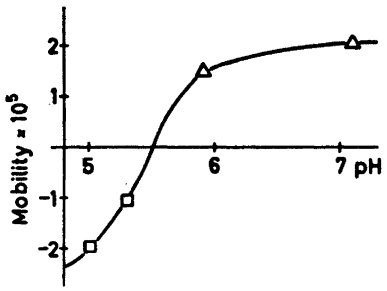

Fig. 1. Electrophoretic mobilities of oxidised cytochrome c from *R. rubrum*. △-phosphate buffers, □-acetate buffers. Ionic strength 0.1.

Analysis of the peptic hemopeptide

Spectrophotometry. The spectrum of the isolated peptide was recorded in 0.066 M phosphate buffer of pH 6.8. It showed similarities with the spectrum of the oxidised cyt. c, but there were several differences. The Soret-band had its maximum at 400 mμ whereas the 360 mμ band was seen only as a weak shoulder, obviously because of the widening of the Soret-band. Upon addition of an excess of N-acetyl-L-histidine there were several spectral changes which tended to bring the spectrum of the peptide closer to that of the cyt. c, but did not make it identical. The spectrum of the reduced N-acetyl-histidine hemochromogen was not quite identical with that of the reduced cyt. c, in contrast to what has been shown for the mammalian cyt. c peptic hemopeptide [16,17]. Another observation made in this experiment was that the molar extinction coefficient at 550 mμ was only about 50 % of that observed in other hemopeptides.

Ultracentrifugation. The Yphantis and Waugh [10] method was chosen because it required very small quantities of peptide. Different concentrations of the peptide were used. Several centrifugations were performed lasting from 1 to 5 h. Unfortunately, the peptide was not stable in these dilute solutions, but showed a decrease of extinction in the visible light region of about 10 % per 24 h upon storage at room temperature. This destruction was accelerated when the sample was introduced into the cell of the ultracentrifuge. By introducing the formula stated above, for calculating the Q value, this could be corrected for if it was postulated that the destruction took place uniformly throughout the solution being centrifuged, and that no compound of different molecular weight than the investigated peptide contributed to the extinction. The Q values thus obtained indicated a molecular weight of 2 600.

Amino acid composition. The results of the analyses are presented in Table 1. With the DNP-method, the histidine determination is very uncertain because of the formation of both α-amino-DNP-histidine and di-DNP-histidine. The same is true for the cysteine determination where only a part of it can be recov-

Table 1. Amino acid composition of the peptic hemopeptides of cytochrome c from *R. rubrum*. For details see text.

Amino acid residue	DNP-method	Stein and Moore	Number of residues
Alanine	2.06	2.10	2
Aspartic acid	0.55	0.55	0.5
Cysteine		1.28	2
Glutamic acid	0.98	0.98	1
Glycine	0.90	0.88	1
Histidine		0.94	1
Leucine	1.16	1.12	1
Lysine	3.84	2.81	3
Phenylalanine	1.09	0.97	1
Serine	1.04	1.05	1
Threonine	0.95	0.97	1
Valine	0.90	0.99	1

ered as DNP-cysteic acid and DNP-cysteine. These values are therefore omitted. The lysine determination is probably too high. This may be because the DNP-lysine spots have very low R_F values in the phosphate solvent system and therefore are probably contaminated with unhydrolyzed peptides. In the Stein and Moore analysis, the sum of the cysteine and cysteic acid gives the cysteine value and it is well known to be too low. The value of 2 residues is a minimal one and is deduced from the work of Paléus and Tuppy. Leucine cannot be distinguished from the *iso*leucine with the DNP-method but can be identified by the Stein and Moore method. Neither method makes it possible to distinguish between glutamine and glutamic acid or between asparagine and aspartic acid because they are all in the acid form after hydrolysis.

A comparison of the two methods of amino acid analysis show that they give comparable results. For the DNP-method 0.2 μmole of peptide was used whereas in the Stein and Moore analysis 0.4 μmole was required. The DNP-method may be considered easier to perform.

End group analysis. The end groups were identified as DNP-aspartic acid and di-DNP-lysine, each of which was present in only half of the amount predicted by the amount of peptide used in the experiment. The washing of the dinitrophenylated peptide made it improbable that one of them could have been present as a free impurity. It was thus concluded that the sample contained two peptides. At least one of them must be a hemopeptide, because the spectrum is that of a typical cytochrome c hemopeptide. High-voltage paper electrophoreses has failed to demonstrate the presence of any ninhydrin-positive spot other than the red-coloured hemopeptide spot. The most probable explanation therefore is that both of the peptides are hemopeptides, one having lysine as an end group, and the other having asparagine or aspartic acid as the end group, with lysine as the second residue. This is also in good accordance with the total amino acid analysis.

Paléus and Tuppy [5] established the sequence of the amino acids in the tryptic hemopeptide. The sequence CyS—Leu—Ala—CyS—His—Thr—Phe is probably included in the present hemopeptide because phenylalanine is very likely to be the C-terminal residue after digestion with pepsin. The above-mentioned authors also pointed out that due to the specific action of trypsin, the amino acid residue preceding the cysteic acid must be either lysine or arginine. As arginine was not found to be present in the peptic hemopeptides this residue must be lysine. With this in mind one can suggest the following amino acid sequence for the longer of the two hemopeptides that are assumed to be present: Asp—Lys(Ala, Glu, Gly, Lys, Ser) Lys—CyS—Leu—Ala—CyS——His—Thr—Phe. The molecular weight of this peptide, including the heme, is 2385, which is close to the value of 2 600 obtained by ultracentrifugation. The low molar extinction coefficient and the observations on the instability of the spectrum when the peptide was stored before the ultracentrifugations suggest that the hemin part of it is unusually unstable.

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