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PURIFICATION AND PROPERTIES OF A PEPTIC HEME PEPTIDE FROM CYTOCHROME c_1

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SUMMARY

A novel purification procedure was devised to isolate a heme peptide from bovine cytochrome c_1. The procedure was based on unique properties of some heme peptides and was capable of processing small quantities ($\sim$ 100 n moles) of material with satisfactory yields. The dansyl reactions, two-dimensional thin layer chromatography, and electrophoresis demonstrated that the peptide was reasonably homogenous. The amino acid composition and spectral characteristics indicate a high degree of similarity with the peptic heme peptide of bovine cytochrom c.

Cytochrome c has been isolated from a wide variety of sources and its sequence, three-dimensional structure, and reactions with redox reagents have been well characterized. Other cytochromes of the mitochondrial respiratory chain are membrane bound and until recently little was known about their physical structure or their interaction with other components of the electron transfer chain. Knowledge about cytochrome c_1 is still fragmentary but with the introduction of several new purification procedures (1,2,3) there is now general agreement as to its molecular weight and other physical properties. Little, however, has been reported regarding its amino acid composition or sequence. Here we report on some of the properties of a peptic heme peptide isolated from cytochrome c_1. Some of the properties of a tryptic heme peptide have been previously described (4).

Methods:

Cytochrome c_1 was isolated from bovine heart by solubilization with Triton-X100, ammonium sulfate fractionation and DEAE chromatography (R. Holmquist, unpublished). The material was 50% cytochrome c_1 by weight (judged by amino acid, spectral, and iron analyses), had been freed of con-

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taminating cytochromes, and was identified as cytochrome $\underline{c}_1$ by a variety of physical techniques, including sodium dodecyl sulfate electrophoresis, isoelectric focusing, spectral and solubility characteristics. At this point it was subjected to peptic hydrolysis as follows. The solution was made 5% in trichloroacetic acid, centrifuged, and the precipitate dispersed in 5 mls of 0.02 N HCl with the aid of a sonicator. Digestion was for 20 hours at pH 2.0 at room temperature with a protein to enzyme ratio of 80:1. Shorter times gave incomplete digestion and the appearance of two heme peptides on two-dimensional thin layer chromatography and electrophoresis. Two-dimensional thin layer chromatography and electrophoresis, the dansyl reaction, and amino acid analysis were performed using standard methods (5,6). All chemicals were of standard reagent grade.

Results and Discussion:

The chromatography system used to purify the heme peptide was based on several unique properties that the heme moiety confers on heme peptides. Chromatography of the peptic digest on G-25 with 1 M NH_4OH took advantage of the well known (7) absorption of aromatic substances on dextran gels, a situation that is accentuated in strongly alkaline solutions. The peptic digest was lypholyzed and dissolved in 1 ml of 1 M NH_4OH. After centrifugation to remove insoluble material, the solution was applied to a 0.9 x 144 cm G-25 (fine) column and developed with 1 M NH_4OH at a flow rate of 15 ml/hr. The slow moving heme peptide band had an unusually high K_{av} ($K_{av} = \frac{V_e - V_o}{V_t \quad V_o}$, [8]) of 0.82. (Where K_{av} = the fraction of the gel phase available to the substance, V_e = the elution volume, V_t = the total volume of the gel bed, and V_o = the void volume--usually determined by chromatography with blue dextran.)

By rechromatographing the peptide band in another solvent it was possible to take advantage of the polymerization of heme peptides in non-liganding alkaline solutions (9). The heme band was collected, lypholyzed, dissolved in 0.1 M sodium tetraborate and chromatographed on a 0.9 x 144 cm G-50 (fine) column. Thus, on this G-50 eolumn eluted with borate, the cytochrome $\underline{c}_1$ heme peptide behaved as an aggregate of molecular weight 18,400 (see Figure 1). The peptide was desalted with 1 M NH_4OH on a 0.9 x 56 cm P-4 column. Total recovery was better than 60% (see Table 1). This procedure also successfully purified the peptic heme peptide of cytochrome $\underline{c}$ with

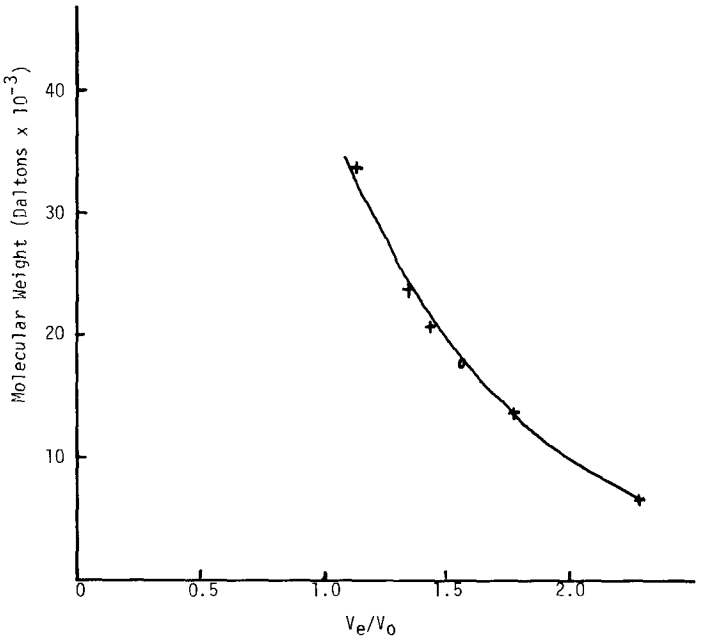

FIGURE 1. MOLECULAR WEIGHT VERSUS V_e/V_o FOR PEPTIC HEME PEPTIDE AND CALIBRATION PROTEINS

+ - proteins used for column calibration; Lipase (6,700), RNAase (13,700), Papain (20,700), Trypsin (23,800) and Myoglobin (Dimer = 33,750). 0 - peptic heme peptide. Standards and the peptide were chromatogramed on a 0.9 cm x 150 cm column of G-50 fine in 0.1 M sodium tetraborate at a flow rate of 15 mg/hr. The solid line represents a least square fit of the data.

TABLE 1

PURIFICATION OF CYTOCHROME $\underline{c}_1$ HEME PEPTIDE

	Volume(ml) Recovered	Concentration (nmoles/ml)	Total nanomoles	% Recovery
Before Digestion	19	10.4	198	100
G-25	7	20.6	144	73
G-50	14.5	8.5	124	62
P-4	2.8	42	118	59

better than 60% yields and thus has several advantages over the procedure devised by Tuppy and Paleus (10); among these are ease in processing smaller amounts of material and higher total recovery.

In general (11) it is usually possible, with good accuracy especially

in the lower molecular weight range, to calculate the probably stoke's radius of a polypeptide through the use of the equation $R_s = a_o + b_o \, erf^{-1} (1-K_{av})$, where R_s is Stoke's radius, a_o and b_o are constants dependent upon the pore size distribution of the gel, and K_{av} is the distribution coefficient [8]. K_{av}'s were experimentally determined for the standard proteins used (Lipase, RNAase, Papain, Trypsin and Myoglobin) and R_s's were calculated from the standard hydrodynamic formula, $f/f_o = \frac{KT/D}{6\pi\eta_o R_s}$ and sedimentation data in the literatures. This data gave a good straight line fit to the above equation. Calculation of the probable Stoke's radius of the heme peptide of cytochrome $\underline{c}_1$, using the K_{av} experimentally determined with G-50 chromatography, gave ~ 18 Å.

Two-dimensional thin layer chromatography and electrophoresis of the peptide gave a single spot whose R_f (0.56) in Butanol:Acetic Acid:Water (4:1:5) is the same as the R_f for peptic heme peptide of cytochrome $\underline{c}$. Reaction of the cytochrome $\underline{c}_1$ heme peptide with dansyl chloride gave a single spot, di-lysine, indicating that lysine is the N-terminal amino acid. As Table 2 shows, the amino acid composition of the peptic heme peptides of bovine cytochrome $\underline{c}$ and cytochrome $\underline{c}_1$ are very similar. Both heme peptides have eleven amino acids; of these seven are common to both peptides. In addition, the amino acids that are different (assuming that the Glx's are Glutamicacids as in cytochrome $\underline{c}$) are such that the total number of polar and non-polar amino acids are the same.

Figure 2 gives the spectrum of the ferro heme peptide in 5% pyridine. Absorption maxima and extinction coefficients are identical to those obtained with the unadecapeptide of cytochrome $\underline{c}$. It is apparent from the results of the gel filtration studies that the cytochrome $\underline{c}_1$ heme peptide undergoes reversible aggregation in a manner analagous to the previously studied heme peptides of cytochrome $\underline{c}$. Figure 3 shows that the aggregated state is favored by an increase in pH, with aggregation leading to hyperchromicity (an increase in extinction). Several pH dependent optical

TABLE 2

COMPARISON OF AMINO ACID COMPOSITION OF HEMOPEPTIDES OBTAINED FROM BEEF HEART CYTOCHROME c AND CYTOCHROME c_1

The results for the Cyt.c_1 peptic heme peptide are given as the average of triplicate 24 hour hydrolysis. In the case of the peptic heme peptide from Cyt.c the glutamic acid residues obtained upon acid hydrolysis are known to be glutamic acid in the primary structure. The numbers in parentheses represent the probable integer value for each residue.

	Cyt.c_1 Peptic Heme Peptide Analysis residues/heme		Cyt.c Peptic Heme Peptide[10] residues/heme	Cyt. c_1 Tryptic Peptide [4] residues/heme	Cyt.c_1 Chymotryptic Peptide [12] residues/heme
Lys	0.85	(1)	1	.13 (0)	1
His	0.85	(1)	1	.95 (1)	1
Arg	0.10	(0)	0	.44 (1)	0
Asx	1.15	1	0	1.01 (1)	2
Thr	0.21	0	1	0.06 (0)	1
Ser	2.33	2	0	2.06 (2)	2-3
Glx	1.73	2	3	0.97 (1)	2
Pro	0.29	(0)	0	0.73 (1)	1
Gly	0.42	0	0	0.16 (0)	2
Ala	0.37	0	1	1.25 (1)	1
Cys	n.d.	2	2	1.26 (2)	(2)
Val	1.17	1	2	1.88 (2)	1
Met	0.90	1	0	0.91 (1)	1
Ile	0.18	0	0	0.03 (0)	1
Leu	0.16	0	0	0.07 (0)	1
Tyr	0	0	0	1.19 (1)	0
Phe	0	0	0	0.07 (0)	0
Total		11	11	14	20

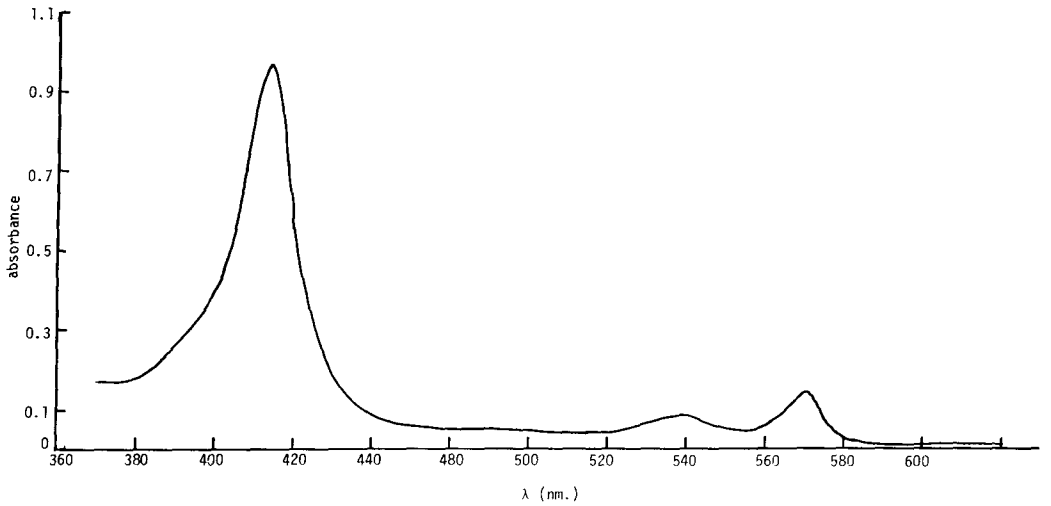

FIGURE 2. THE SPECTRUM OF THE REDUCED PYRIDINE HEMOCHROMOGEN OF THE HEME PEPTIDE

The spectrum (obtained with a Cary 14) is of 13.3 nmoles of the peptide dissolved in 1.11 ml of 0.01 M phosphate buffer (pH 7.5) that was 1.12 M in pyridine.

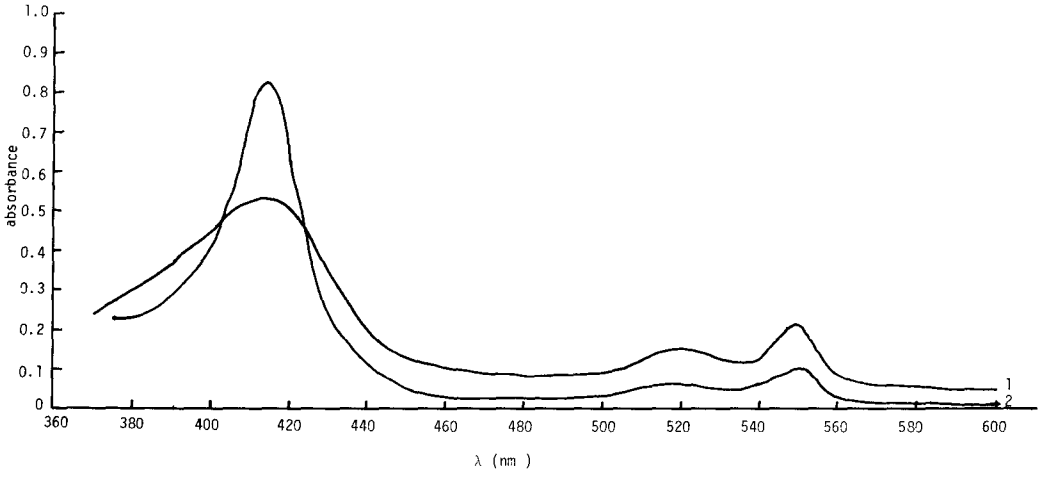

FIGURE 3. SPECTRA OF THE HEME PEPTIDE AT DIFFERENT pHs

The spectra were obtained with a Cary 14 spectrophotometer. In curve 1 the peptide (2.4×10^{-5} M) was in 0.01 M phosphate buffer, pH 5.45. In curve 2, the concentration was 1.2×10^{-5} M and the pH was 6.5 (0.01 M phosphate buffer).

transitions, one with a pK of 5.8, have been described for the unadeca peptide of cytochrome c [13]. The transition near pH 6 is thought to result from the intermolecular binding of the α-NH_2 group of valine to the heme iron. In the case of the peptide from cytochrome c_1, this optical transition, reflecting a change in aggregation state, could result from the binding of either the α-, or ε-NH_2 group of lysine. It is most probably that the α-NH_2 group is involved since its intrinsic pK is lower. Since the aggregated state is characterized by hyperchromicity, it is likely that, as in the unadeca peptide of cytochrome c, the heme groups in the aggregated state are arranged in a head to tail manner (14). In native cytochrome c the fifth and sixth iron ligands are known to be a histidine and a methionine residue. Previous studies with cytochrome c heme peptides (15,16) have shown that the histidine residue proximal to one of the cystines is the fifth iron ligand. The sixth ligand position in this case is occupied by different moieties under different solvent conditions (usually NH_4^+, pyridine, the α-NH_2 group, or the ε-group of lysine). Since the cytochrome c_1 heme undecapeptide contains a methionine residue the possibility exists that this residue could serve as a sixth ligand. Methionine liganded to heme iron gives a characteristic absorption band at 695 nm (17). The extinction coefficient at this wave length is very low and unfortunately not enough material was available to make a spectroscopic study feasible. However, the observed aggregation is consistent with the hypothesis that methionine is sterically hindered from binding to the iron in this peptide.

The peptic heme peptide of cytochrome c_1 appears to be similar in many respects to the previously described tryptic peptide (4), which consisted of 14 amino acids (ten of which are common between the two peptides). The tryptic peptide also exhibited aggregation, a phenomenon that was found here with the peptic heme peptide. The many similarities of this peptide with the peptide from cytochrome c (i.e. amino acid composition, spectra and chromatographic properties) suggest sequence homology. Whether this homol-

ogy holds for other regions of the polypeptide chains of cytochrome c and c_1, or merely reflects the requirements of a heme binding region, is not known at present and will be resolved when sequence data for cytochrome c_1 becomes available.

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