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PYRIDOXAL PHOSPHATE MODIFIED CYTOCHROMES *c*

IDENTIFICATION AND ELECTRON TRANSFER PROPERTIES

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The preparation, purification and characterization of the three singly, three doubly and one triply substituted derivatives of cytochrome *c* modified by pyridoxal phosphate (PLP) at lysine residues are reported. The PLP positions in PLP derivatives were determined by the amino acid analysis and sequence of PLP peptides. The results identified the lysine at position 86 in one of the singly substituted, lysine 79 in the other singly substituted and lysines 86 and 79 in the third doubly substituted cytochrome *c* derivatives. The area surrounding phenylalanine 82 forms the predominant PLP binding site on the cytochrome *c* molecule. The visible, CD and proton NMR spectra, the full intensity of the conformation-sensitive 695 nm band and the oxidation-reduction properties provide evidence to confirm the conclusion that singly and doubly substituted PLP cytochromes *c* retain the native conformation. The ability to restore both succinate and ascorbate/TMPD oxidation in cytochrome *c*-depleted mitochondria decreases in the order: native cytochrome *c* > PLP-Lys-79-cytochrome *c* > PLP-Lys-86-cytochrome *c* > PLP-Lys-79,86-cytochrome *c* > triply substituted derivative.

Introduction

Different chemical modifications of ε-amino groups of cytochrome *c* lysine residues have been carried out and most of them have been reviewed in Refs. 1–5. These derivatives were applied to analyse the problems of intramolecular interaction and electron transfer between cytochrome *c* and its biological donors and acceptors.

Aviram and Schejter [3] have described the use of PLP in preparing one doubly substituted and one singly substituted modified derivatives of cytochrome *c* at lysine residues. Three fluorescence PLP peptides have been detected and allocated to the 1–10, 68–74 and 83–94 positions. However, the real reaction positions for PLP were not clear from these data. Furthermore, the doubly substituted derivative formation suggests the possibility of the existence of two singly substituted derivatives which were not reported in Ref. 3, but are established in this work.

The purpose of the present study is to identify the sequence position of the lysine residues in two

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Abbreviations: PLP, pyridoxal phosphate; PLP-Lys-86-cyt. *c*, the first singly substituted cytochrome *c* modified by PLP at lysine 86; PLP-Lys-79-cyt.*c*, the second singly substituted cytochrome *c* modified by PLP at lysine 79; PLP-Lys-79, 86-cyt.*c*, doubly PLP substituted cytochrome *c* at lysine 79 and lysine 86; $(PLP)_3$-cyt.*c*, triply PLP substituted cytochrome *c*; ITH, diphenylindenonylthiohydantoin; TMPD, *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine dihydrochloride.

strictly homogeneous, singly substituted cytochromes *c*, modified by PLP as well as their common doubly modified product, and to determine their redox potentials and spectral and electron transfer properties in cytochrome *c*-depleted mitochondria.

Materials and Methods

Horse-heart cytochrome *c* was obtained from Miles Seravac. For electron transfer experiments, cytochrome *c* was from Sigma (type VI). Pyridoxal phosphate, Tris, cacodilic acid and pyridine were purchased from Fluka. Potassium ferrocyanide, sodium borohydride, cellulose MN 300 were obtained from Merck. Amberlite CG-50 (type II, 200 mesh) was a product of British Drug Houses. Chymotrypsin was obtained from Worthington Biochemicals. All they were applied without further purification as described below. Sometimes an independent column chromatography control on the purity (95%) of monomeric cytochrome *c* in different purchased lots was carried out. Succinate and rotenone (Sigma), 2,4-dinitrophenol and TMPD (Fluka) were recrystallized. All other chemical were of the best quality available. All solutions were prepared with doubly distilled water.

Preparation of pyridoxal phosphate cytochrome c derivatives. Cytochrome *c* was modified by covalent binding of PLP to lysine residues by the method of Aviram and Schejter [3]. The procedure described here and the reagent ratio lead to three different singly substituted and three different doubly substituted derivatives and one new triply substituted derivative being obtained.

A solution of ferricytochrome *c* (100 mg/ml) in 0.05 M Tris-cacodylate buffer (pH 7.0) was incubated for 20 min with PLP (cytochrome *c*/PLP = 1 : 3). A solution of 0.04 M $NaBH_4$ in 0.02 M NaOH was then added to make a final concentration of 25 mM borohydride. After 20 min at room temperature, the products were oxidized with an excess of potassium ferricyanide. The low-molecular-weight reagents were separated on a Sephadex G-15 volume (1.5 × 30 cm). The modified derivatives of cytochrome *c* were fractioned on Amberlite CG-50 column (1.5 × 20 cm) and had been stabilized previously with 0.005 M phosphate buffer (pH 8.0). The different cytochrome *c* fractions were eluted by a stepwise gradient with NaCl in the same buffer. All fractions were rechromatographed precisely. To identify the different fractions, the theoretical ratio, based on the additive scheme, is calculated from the following equation [6,7]:

$$A_{325}/A_{530} = (17.4 + a \cdot 9.7) : 11.1$$

where A_{325} and A_{530} are the absorptions at 325 and 530 nm; 17.4 and 11.1 are the millimolar extinction coefficients of ferricytochrome *c* at 325 and 530 nm, respectively, in 1 cm optical cell [6]; 9.7 is the millimolar extinction coefficient of pyridoxallysine at 325 nm [7]; *a* takes values 3, 2, 1 and 0 for the trisubstituted, disubstituted, monosubstituted and unmodified cytochrome *c*, respectively. We have used the coefficient of pyridoxallysine at 325 nm [7], instead of the coefficient of pure PLP at 320 nm [3]. Different PLP-modified cytochrome *c* were compared following assumption that they all had equal extinction-coefficients at 530 nm.

Peptide mapping. The hydrolysis of the two singly substituted derivatives (30 mg of each) of cytochrome *c* was catalyzed by chymotrypsin and carried out as described in Ref 2. The chymotryptic peptides of components I_a and I_c were separated by paper chromatography (Whatman 3) using buffer pyridine/ butanol/ acetic acid/ water (2 : 3 : 1 : 4). The fluorescent fractions were eluted with 10% acetic acid. The fractions were additionally purified by thin-layer electrophoresis on cellulose (MN 300) at 40 V/cm for 30 min using a buffer pyridine/acetic acid/water (20 : 9 : 971), pH 5.6. Because of their low concentration, the peptide bands were additionally detected with fluorescamine and were extracted three times with 10% pyridine.

Hydrolysis of the isolated peptides with 6 M HCl for 24 h at 110 °C was followed by analysis on an amino acid analyzer D-500 (Durrum).

The amino acid sequences of the purified PLP-Lys containing peptides were determined using the coloured reagent of Ivanov and Mancheva [8] 2-*p*-isothiocyanophenyl-3-phenylindenone. A modified procedure for the sequencing of microquantities (about 1 nmol) was applied. The cleaved N-terminal amino acids in the form of diphenylindenon-

ylthiohydantoin (ITH) derivatives were identified by using high-performance liquid chromatography under conditions described in Ref. 9. In the investigation, a chromatography series 2/2 combined with a UV/VIS variable wavelength detector model LC-55 (both from Perkin Elmer) and a Honeywell Model 194 recorder were used. A prepacked column 25 × 0.46 cm i.d. with 10 μm Lichrosorb RP-18 particles (Knauer) was used for separation. Most of the ITH-amino acids were separated with 0.01 N sodium acetate (pH 4.80) and a linear gradient elution from 44 to 94% acetonitrile in 2%/min, the flow velocity being 2 ml/min. The separation of ITH-Leu and ITH-Ile was performed using an isocratic elution with acetonitrile/ethyl acetate (78 : 22, v/v). The concentration of the combined organic solvent was 44% with respect to the mixture of organic solvent and sodium acetate buffer (pH 4.80), (see above). The separated ITH-amino acids were detected at 267 nm.

Pyridoxal phosphate-ε-*N*-lysine was synthesized by mixing lysine and pyridoxal phosphate in 1 : 1 molar ratio at pH 9.0 followed by borohydrate reduction. The main component, ε-*N*-lysine, was fractionated from the mixture by preparative paper chromatography (Whatman 3) using the above-mentioned system ($R_F = 0.4$). The corresponding ITH-derivative was prepared according to the method for obtaining ITH-amino acids [8].

Redox potential and spectral measurements. The redox potentials of the native and PLP modified cytochromes *c* were determined by spectrophotometric titration with potassium ferrocyanid applying the method described in Ref 10 and were calculated by the following equation [11]:

$$E_m = E_0 + 0.0275 \log \frac{[\text{K ferricyanide}]}{[\text{K ferrocyanide}]} - 0.055 \log \frac{[\text{ferricyt. } c]}{[\text{ferrocyt. } c]}$$

in which $E_0 = 0.355 \pm 0.001$ V.
E_0 is the standard redox potential of the couple in the equation:

$$Fe(CN)_6 + \tfrac{1}{2}H_2 = Fe(CN)_6^{4-} + H^+$$

Miscellanea. Rat liver mitochondria were isolated using the method of Johnson and Lardy [12] in a medium containing 0.25 M sucrose and 5 mM EDTA-KOH (pH 7.4) and were washed twice with 0.25 M sucrose. Cytochrome *c*-depleted mitochondria were prepared according to Jacobs and Sanadi [13]. Mitochondrial protein was determined using the method of Lowry et al. [14]. The cytochrome content of cytochrome *c*-depleted mitochondria was determined from reduced-minus-oxidized difference spectra [15,16]. Typical values, in nmol/mg of protein were: cytochrome aa_3, 0.238; cytochrome *b*, 0.229; cytochrome c_1, 0.130; cytochrome *c*, 0.022. Rates of oxygen uptake were measured polarographically as described by Ferguson-Miller et al. [17]. Rates of oxygen uptake were calculated assuming 240 nmol O_2/ml of buffer [18]. The respiratory control ratio was determined according to the method of Estabrook [19].

Instruments. Absolute and difference absorption spectra were recorded on double-beam automatic spectrophotometer SPECORD UV/VIS (Carl Zeiss-Jena, G.D.R.). Comparative circular dichroism spectra of all cytochromes studied were recorded by a Jobin Ivon Mark III Dichrographe (France) in 190–240 nm and 370–600 nm regions in 1 nm and 10 nm optical cells, respectively. The solutions for the differential spectrophotometry were prepared with correct adjustment of the absorption at 530 nm, to be equal for all samples. All buffers were prepared by using a digital MV-87 pH-meter (G.D.R.) with ±0.01 accuracy.

Results

Preparation of pyridoxal phosphate derivatives of cytochrome c

Three lysine residues from a cytochrome *c* molecule can react with the aldehyde group of PLP under the experimental conditions employed.

$$\text{Cyt.}c\text{-(Lys-NH}_2)_m + m\text{OHC-PLP}$$

$$\begin{array}{l} \overset{-H_2O}{\underset{H_2O}{\rightleftarrows}} \text{Cyt.}c\text{-(Lys-N = CH-PLP)}_m \\ \text{pH = 7} \quad \downarrow NaBH_4 \\ \text{Cyt.}c\text{-(Lys-NH-CH}_2\text{-PLP)}_m \end{array} \qquad m = 1, 2, 3$$

According to the number of bonds with PLP, singly, doubly and tryply substituted derivatives of cytochrome *c* may be obtained. The yields and distribution of fractions depend on the molar ratio of the reaction reagents and on the reaction time.

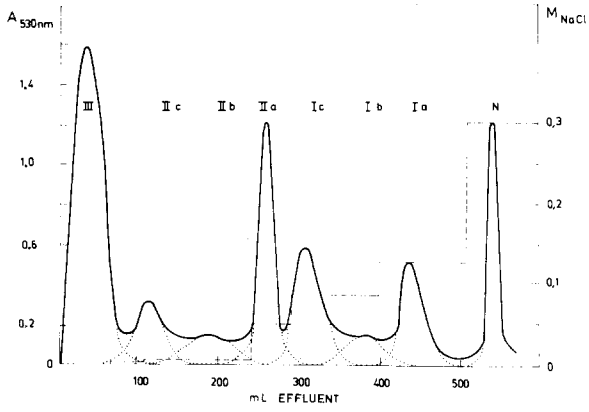

Fig. 1. Ion-exchange chromatography of the PLP – modified cytochromes *c*. PLP-modified cytochromes *c* were separated on Amberlite CG-50 column (15 × 150 mm) previously stabilized with 0.01 M phosphate buffer (pH 8.0). Step-wise gradient of NaCl in the same buffer was applied as indicated by - - - - - -. The elution curve (———) was obtained by measuring absorption at 530 nm. The fractions were pooled as shown by · · · · · ·.

The PLP-cytochromes *c* were separated by ion-exchange chromatography on Amberlite CG-50 into fractions, depending on the number of lysines substituted. An example of the typical elution profile of a PLP-cytochromes *c* preparation is shown in Fig. 1. The products of the reaction were eluted with a gradient of NaCl (from 0.01 M to 0.3 M), pH 8.0. Eight fractions were obtained and identified by the ratio A_{325}/A_{530} (Table I). All fractions were purified chromatographically. The analysis of the purified fractions indicates that fractions I_a, I_b, I_c were mono-PLP-substituted fractions, II_a, II_b, II_c were di-PLP-substituted and fraction III was tri-PLP-substituted. Fraction N was unmodified native cytochrome *c*. As fractions I_b, II_b, II_c had a low yield, they were not analysed further. The composition of the fractions under the reaction conditions employed (cytochrome *c*/PLP = 1 : 3, reaction time 20 min) are presented in Table I.

Identification of PLP-modified lysine residues in PLP-cytochromes c

The sequence location of the modified lysine was determined by a semi-automated Edman degradation of the chymotryptic peptides of the PLP-cytochrome *c* derivative as described below. The peptides that contained PLP-lysine were preparatively recovered from the chymotryptic digestion of each of the highly purified derivatives. This was facilitated by the fluorescence of the modifying PLP reagent. Components I_a and I_c showed a major fluorescent peptide fraction in the preparative paper chromatography (see Methods) with $R_F = 0.16$ and $R_F = 0.28$, respectively. Component II_a had two major fluorescent peptide fractions with the same R_F values (0.16 and 0.28). All of these PLP-peptides were purified by cellulose

TABLE I

ABSORPTION (THEORETICAL AND EXPERIMENTAL) RATIO A_{325}/A_{530} AND YIELD OF INDIVIDUAL CYTOCHROMES *c*

I_a, I_c and II_a have been studied further in this paper. The standard error of the experimental values varied from 0.08–0.12

Fraction		A_{325}/A_{530}			
	Molar ratio $M_{PLP}/M_{cyt.c}$	Theoretical	Experimental	Lysine modified	Yield (%)
Untreated cyt.*c*	0	1.46	1.45	–	
Unmodified cyt.*c*	0	1.46	1.52	–	12
I_a	1	2.34	2.38	79	9.9
I_b	1	2.34	2.39		5.4
I_c	1	2.34	2.40	86	12.4
II_a	2	3.22	3.28	79, 86	14.4
II_b	2	3.22	3.34		5.5
II_c	2	3.22	3.40		7.2
III	3	4.09	4.24		33.2

TABLE II

AMINO ACID COMPOSITION OF PLP-PEPTIDES OF SINGLY MODIFIED CYTOCHROMES *c*

Amino acid	I_c component		I_a component	
	nmol	Molar ratio	nmol	Molar ratio
PLP-Lys	2.10	0.87	1.90	0.90
Aspartic acid	2.40	1.00	–	–
Glutamic acid	2.28	1.78	–	–
Threonine	2.12	0.88	2.04	1.03
Proline	–	–	1.84	0.89
Glycine	3.05	1.23	2.36	1.12
Alanine	2.63	1.10	–	–
Methionine	–	–	1.80	0.86
Leucine	2.02	0.84	–	–
Isoleucine	2.40	1.00	2.11	1.00
Lysine	3.89	0.85	–	–
Arginine	2.08	0.85	–	–

thin-layer electrophoresis. The identification of these peptide containing ε-PLP-lysine residues was confirmed by amino acid analysis after 24 h hydrolysis with 6 M HCl (Table II). The exact position of the modified residues was established by Edman sequence degradation by using a coloured reagent with high sensitivity – 2-*p*-isothiocyanophenyl-3-phenylindenone [8,9]. The results are presented in Table III. These data show that the modified residues in components I_a and I_c are lysines at positions 86 and 79, respectively. The presence of the same peptides as major PLP-labeled peptides in component II_a shows that the doubly modified groups in it are also Lysine 79 and Lysine 86.

Spectral and redox properties of PLP-cytochromes c

In accordance with Aviram and Schejter [3], all derivatives have a preserved 695 nm band with the same extinction coefficient. Native conformation of modified proteins was proved once more by recording the proton NMR spectra [20] and the total circular dichroism spectra of each fraction in the three spectral ranges (see Methods). It was

TABLE III

AMINO ACID SEQUENCE OF THE ISOLATED PLP-PEPTIDES OF SINGLY MODIFIED CYTOCHROMES *c*

Partial amino acid sequence of cytochrome *c*	Tyr	Ile	Pro	Gly	Thr	Lys	Met	Ile
Number	74	75	76	77	78	79	80	81
N-terminal sequence of I_a component		Ile	Pro	Gly	Thr	Lys-PLP		
Partial amino acid sequence of cytochrome *c*	Phe	Ala	Gly	Ile	Lys	Lys	Lys	Thr
Number	82	83	84	85	86	87	88	89
N-terminal sequence of I_c component		Ala	Gly	Ile	Lys-PLP			

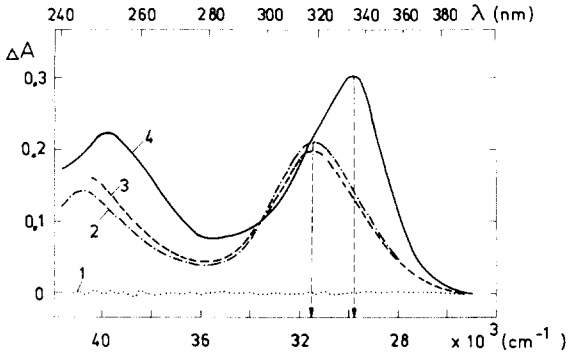

Fig. 2. Difference spectra of PLP – modified ferricytochromes *c* against native ferricytochrome *c*. All solutions were prepared so as to have equal absorption at 530 nm. Absolute concentration of each cytochrome *c*, $2.25 \cdot 10^{-5}$ M (pH 8.0). 1, (· · · · · ·), Intact cytochrome *c*; 2, (– · – · –), PLP-Lys-79-cyt.*c*; 3, (– – – – –), PLP-Lys-86-cyt.*c*; 4, (———), PLP-Lys-79, 86-cyt.*c*.

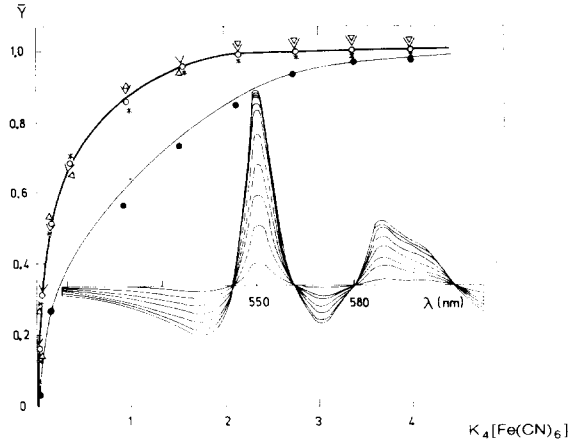

Fig. 3. Determination of the redox potentials of the native and PLP-cytochromes *c* by spectrophotometric titration with increasing concentrations of potassium ferrocyanide at pH 7.0, 25 °C. Cytochrome *c* concentration, 0.011 mM in 0.01 mM tris-phosphate buffer, pH 7.0. Concentration of potassium ferrocyanide, 0.01–4 mM, ○———○, native cytochrome *c*; △———△, PLP-Lys 86-cyt.*c*; v—v, PLP-Lys 79-cyt.*c*; * — *, PLP-Lys 79, 86-cyt.*c*; ●———●, $(PLP)_3$-cyt.*c*.

found that all singly and doubly PLP-modified cytochromes *c* have identical spectra with the earlier reported [21] spectrum of native cytochrome *c*.

The difference spectra of PLP-modified cytochromes *c* against native cytochrome *c* in the region of PLP absorption (250–390 nm) are presented in Fig. 2. Differences due to absorption of PLP were registered in this region. PLP-Lys-79-cyt.*c* and PLP-Lys-86-cyt.*c* (curves 2 and 3) have almost the same difference spectra with an absorbance maximum at approx. 517 nm and a millimolar absorption coefficient $\Delta\epsilon_{mM} \approx 9.5$. Those values are quite close to the value for PLP-Lys $\Delta\epsilon_{mM} = 9.7\ mM^{-1} \cdot cm^{-1}$ at 325 nm [7]. The difference spectrum of PLP-Lys-79, 86-cyt.*c* (curve 4) exhibited a bathochromic shift to 340 nm ($\Delta\lambda = 23$ nm or 1800 cm^{-1}) and $\Delta\epsilon_{mM} \approx 13.8\ mM^{-1} \cdot cm^{-1}$ was obtained. Thus, the additive scheme in this case was not supported ($\Delta\epsilon_{mM}$ is not $19.4\ mM^{-1} \cdot cm^{-1}$) and a hypochromism of about 25% was registered.

The redox potentials of PLP-cytochromes *c* in pH range 6–8 were identical with those of native cytochrome *c* (Fig. 3).

Interaction of PLP-modified cytochromes c with cytochrome c-depleted mitochondria

The reaction of PLP-modified derivatives with

TABLE IV

EFFECT OF ASCORBATE AND TMPD CONCENTRATIONS ON THE ACTIVITY OF ASCORBATE-TMPD OXIDASE WITH NATIVE AND PLP-MODIFIED CYTOCHROMES *c*

The incubation medium for measuring oxygen consumption and experimental details are as in Fig. 4. Concentration of each cytochrome *c*, 7.5 μM.

Ascorbate (mM)	TMPD (mM)	Oxygen consumption (nmol O_2/min)		
		Native cyt. *c*	PLP-Lys 79--cytochrome *c*	PLP-Lys 86--cytochrome *c*
10	0	60	20	5
1	0.1	175	45	30
10	1	425	120	30

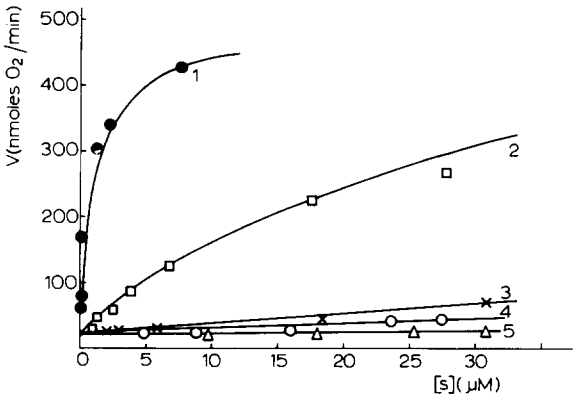

Fig. 4. Effect of native and PLP modified cytochromes *c* on the ascorbate/TMPD oxidation in cytochrome *c*-depleted mitochondria. The rate of oxygen consumption (V) was measured in a medium comprising 0.2 M sucrose/30 mM Tris-HCl (pH 7.4)/10 mM KH_2PO_4/20 mM KCl/2.5 mM $MgSO_4$/0.25 mM EDTA. In presence of 1 μM rotenone and 0.1 μg/mg of protein antimycin A, 10 mM sodium ascorbate and 1 mM TMPD were the reducing agents. Total volume, 2 ml; mitochondrial protein, 2 mg; 25 °C; [S], concentration of added cytochrome *c* in μM. 1, (●), native protein; 2, (□), PLP-Lys 79-cyt.*c*; 3, (×), PLP-Lys 86-cyt.*c*; 4, (○), PLP-Lys-79, 86-cyt.*c*; 5, (△), $(PLP)_3$-cyt.*c*.

ascorbate-TMPD oxidase of cytochrome *c*-depleted mitochondria was assayed by measuring oxygen uptake (Fig. 4). While native cytochrome *c* restores respiration at a very low concentration. considerably higher concentrations of PLP-Lys-79-cyt.*c* are necessary. The activities of PLP-Lys-86-cyt.*c*, PLP-Lys-79, 86-cyt.*c* and $(PLP)_3$-cyt.*c* are very low.

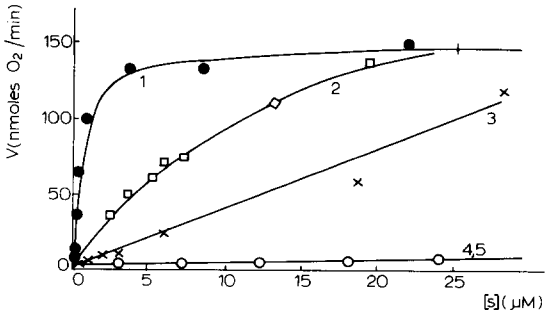

Fig. 5. Effect of native and PLP-modified cytochromes *c* on the succinate oxidation in cytochrome *c*-depleted mitochondria. The incubation medium was as in Fig. 4. Concentration of rotenone, 1 μM; concentration of sodium succinate, 10 mM. Other experimental conditions as in Fig. 4, 1, (●), native cytochrome *c*; 2, (□), PLP-Lys 79-cyt.*c*; 3, (×), PLP-Lys 86-cyt.*c*; 4, (○), PLP-Lys 79, 86-cyt.*c*; 5, (○), $(PLP)_3$-cyt.*c*.

To understand the reason for the impaired interaction of the PLP-derivatives with the oxidase, experiments with different ascorbate and TMPD concentrations were done. The results are presented in Table IV. They indicate that increasing the concentrations of the reducing agents increases the velocities of native cytochrome *c* and that PLP-Lys-79-cyt.*c*. PLP-Lys-86-cyt.*c* is not sensitive to changes in ascorbate and TMPD concentrations.

The reaction of PLP-modified derivatives with the entire succinate oxidase system of cytochrome *c*-depleted mitochondria was also studied polarographically (Fig. 5). with sodium succinate as reducing agent. The respiration is restored by both

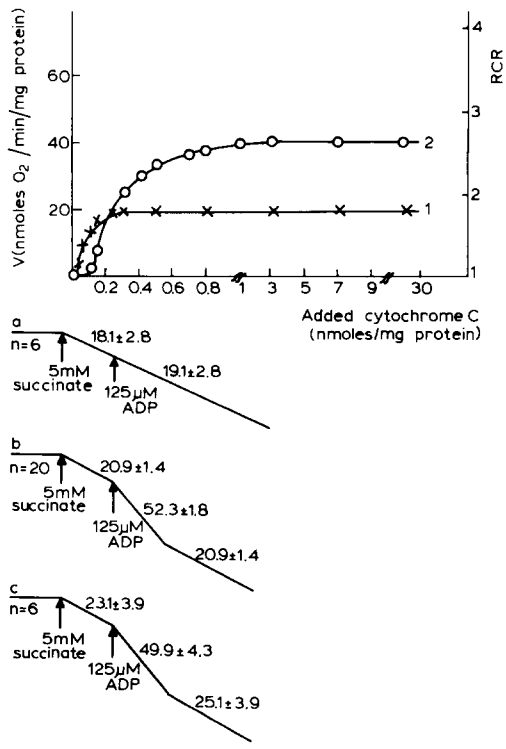

Fig. 6. Effect of native cytochrome *c* and PLP-Lys 79, 86-cyt.*c* on oxygen consumption and respiratory control ratio (RCR) in cytochrome *c*-depleted mitochondria. Incubation medium as in Fig. 4; sodium succinate, 5 mM; rotenone, 1 μM. A. Restoration of oxygen consumption (1; ×) and RCR (2; ○) by increasing concentrations of native cytochrome *c*. B. Polarographic traces on which the additions of 5 mM succinate and 125 μM ADP are indicated by arrows. The numbers above the separate parts of the traces represent nmol O_2/min per mg of mitochondrial protein, expressed as the mean ± S.E.; *n*, number of experiments.

mono-derivatives, but at considerably higher concentrations than native cytochrome *c*. Both PLP-Lys-79, 86-cyt.*c* and $(PLP)_3$-cyt.*c* are entirely ineffective.

In view of the interesting effect of the cytochrome *c* 'dimer' which was relatively ineffective as an electron carrier, but was as effective as native protein in the reconstitution of oxidative phosphorylation [22], we studied the activity of PLP-Lys-79, 86-cyt.*c* in the restoration of the respiratory control ratio. This derivative is entirely inactive in the restoration of the succinate respiration in cytochrome *c*-depleted mitochondria (Fig. 5) but has native overall conformation and redox properties, as shown above. It is known that different amounts of native cytochrome *c* are necessary for the restoration of the electron transfer and the P/O ratio [22]. Fig. 6A shows this dependence on our experimental conditions for the restoration of electron transfer and the respiratory control ratio by native protein. Fig. 6B shows the ability of the di-PLP-derivative to restore the respiratory control ratio provided that there is a minimal amount of native cytochrome *c* to support respiration. On trace a it is seen that the addition of a minimal amount of native cytochrome *c* (0.133 ± 0.021 nmol/mg of protein) to *c*-depleted mitochondria ($n = 6$) restores respiration only and not the respiratory control (respiratory control ratio = 1.07 ± 0.03, $n = 6$). On trace b the respiratory control is restored by native cytochrome *c* (above 1 nmol/mg of protein). The respiratory control ratio is equal to 2.50 ± 0.09, $n = 20$. On trace c the same low amount of native cytochrome *c* as in trace a (0.133 ± 0.021 nmol/mg of protein) was added to restore respiration. The addition of PLP-Lys-79,86-cyt.*c* (above 4 nmol/mg protein) restores the respiratory control (respiratory control ratio-2.15 ± 0.05, $n = 6$); n is the number of experiments.

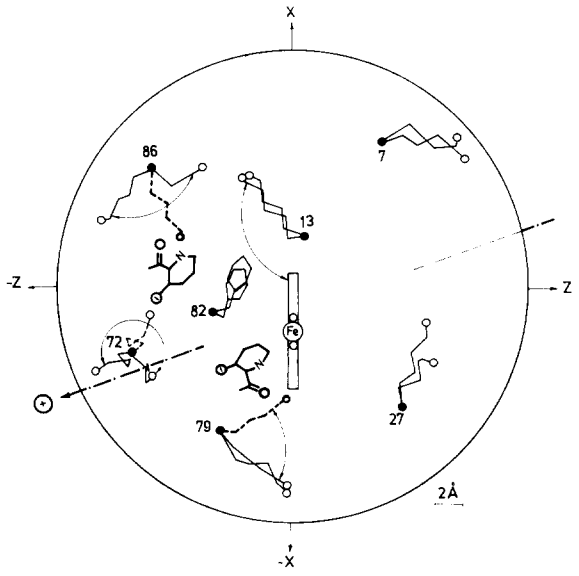

Fig. 7. A projection of some of the amino acid residues of cytochrome *c* with two possible binding sites for PLP. (The plane is exactly perpendicular to the heme and passes through N(A)-Fe-N(C) (X-axis). The geometric centre is placed in the centre of the molecular mass. All positions are recalculated from the atomic coordinates of [26]. The dipole axis is indicated by -·-·-. The arcs represent schematically the possible declinations of the lysine residues. The PLP projections are only tentative.

Discussion

The modification of cytochrome *c* with PLP was made first by Aviram and Schejter [3] as mentioned in Introduction. However, they did not establish the position of lysine residues modified by PLP in their isolated monosubstituted and disubstituted derivatives. The presence of three PLP-containing peptides in their analysis clearly shows that these PLP-modified derivatives were not completely purified. Indeed, we assume this hypothesis to be true because we separated and purified PLP-cytochrome *c* many times [23] and have always obtained appreciable amounts of two monosubstituted derivatives and a small amount of a third mono-derivative. Amino acid analysis and sequences of PLP-containing peptides in fraction I_a and I_c indicate that the modified lysine residues are at positions 86 and 79, respectively. The analysis of fraction II_a indicates that this doubly substituted derivative was modified at lysine residues in the same positions of 86 and 79. The results reported here show that PLP-Lys-86 cyt.*c* probably corresponds to the third peptide suggested in Ref. 3. The other singly substituted PLP – Lys-79-cyt.*c*, isolated and characterized by us in this work, does not correspond to any other peptides suggested in Ref. 3. The total analysis of the other double and triple derivatives of cytochrome *c* is an object of future study.

Reactivity of PLP with lysine residues

On the basis of the relative yields of the reaction products, one gets some idea about the mechanism of the reaction of PLP with cytochrome *c*. The lysine residues of haem proteins show different specific reactivity to PLP (Refs. 3, 24, 25 and this paper). The PLP-Lys-86-cyt.*c* and PLP-Lys-79-cyt.*c* were isolated in approximately equal amounts (Table I). However, from the kinetics of the reaction of cytochrome *c* with PLP it may be suggested that Lys-79 is slightly more reactive than Lys-86. The yield of the third, unidentified lysine residue (X) was about 2-times smaller than that of PLP-Lys-86-cyt.*c* and PLP-Lys-79-cyt.*c* because it has a limited reactivity to PLP. These singly substituted derivatives reacting with PLP are subsequently converted to doubly substituted ones: Lys-79 or Lys-86 from PLP-Lys-79-cyt.*c* and PLP-Lys-86-cyt.*c*, respectively, react with the PLP molecule, the result of which is the formation of PLP-Lys-79, 86-cyt.*c*. The ratio of the products of this reaction is as follows: PLP-Lys-79, 86-cyt.*c*/PLP-Lys-86, Lys X-cyt.*c*/PLP-Lys-79, Lys X-cyt.*c* ≃ 2 : 1 : 1. It is the same as expected on the bias of equal reactivity of both centres and the reduced reactivity of the third.

A more detailed explanation of the specific reactivity of the lysine residues to PLP is possible under our experimental conditions. It is based on the examination of the protein structure by using a self-constructed model of cytochrome *c* (Nicolson's Labquip molecular models and the atom coordinates of cytochrome *c* received from Protein Data Bank) [26]. In accordance with Brautigan et al. [1] it may be suggested that the area surrounding phenylalanine 82 forms the predominant hydrophibic PLP-binding sites on the cytochrome *c* molecules (fig. 7).

Inspection of cytochrome *c* structure with regard to PLP dipole orientation shows two possible binding sites for PLP. These sites are situated on the positively charged end of the dipole axis of the cytochrome *c* molecule at both sides of phenylalanine 82 [27]. Thus, the PLP aldehyde group can be in close proximity to ε-amino groups of Lys-86 or Lys-79, respectively. In both cases, the phosphate group is orientated to Lys-72 and probably forms a salt bridge with it. This will fix the PLP to the cytochrome *c* molecule at two different orientations and the reaction of the aldehyde group with Lys-86 and Lys-79 would be facilitated. The interaction of Lys-72 with the PLP aldehyde group is possible if the PLP phosphate group electrostatically binds directly to ε-amino groups of Lys-86 or Lys-79 (equivalent to PLP reorientation at about 180 °C (Fig. 7). The suggestion made above could be confirmed by the fact that Lys-72 was involved in PLP-containing peptides [3], and that Lys-72 is the possible site 'X', described here as the third reaction centre. But in all cases, the PLP interaction with Lys-13 is not possible in space when PLP is bound in these two sites. The structural positions of the rest of the lysine residues are far from these PLP binding centres. The above description explains the preferred amounts of PLP-Lys-86-cyt.*c*, PLP-Lys-79-cyt-*c* and one disubstituted PLP-Lys-79, 86-cyt.*c*. As a matter of fact, no more than triply PLP-modified derivatives of cytochrome *c* have been obtained under our experimental conditions, which indicates that PLP binds in these two positions. At the analogical sites of another reagent 4-chloro-3,5-dinitrobenzoate, the reactive group has an opposite orientation [1]. This was used to explain the increased reactivity of Lys-13 towards 4-chloro-3,5-dinitrobenzoate [1].

Ion-exchange chromatography and charge properties of PLP-modified cytochromes c

The chromatographic behaviour of PLP-cytochrome *c* on Amberlite CG-50 depends on the net charge of the protein molecule and on the surface charge of the modified lysine. The change of +1 to −2, −4, −6 in the mono-, di- and triderivatives, respectively, allows chromatographic separation of the modified products depending on the PLP-Lysine content (Fig. 1). The dipolar charge topography of cytochrome *c* orients the molecule so as to present the strongly basic patches from the lysine residues around the positive end of the dipole axis (as proposed by Koppenol et al. [27]) towards the negatively charged surface of the cation exchange resin Amberlite CG-50 [1]. Modification of one of the lysines involved in the more favourable area for binding to resin could have a major effect on the chromatographic mobility of PLP cytochromes *c*. Lys-86 is nearer the dipole axis than Lys-79 and probably the modification of this residue leads to more considerable changes in

charge distributions and it is probably for that reason that PLP-Lys-86-cyt.*c* has higher chromatographic mobility than the PLP-Lys-79-cyt.*c*. Our results are in agreement with the explanation in Ref. 1 concerning the chromatographic behaviour of other modified cytochromes *c*.

Spectral and oxidative reduction properties of PLP-modified cytochromes c

PLP-cytochromes *c* have the same visible spectrum as unmodified protein. The absence of any change in the conformation-sensitive 695 nm absorption band is indicative of methionine-80 ligation of the haem prosthetic group [21].

Although the position of the PLP-chromophores is different, both PLP-Lys-86-cyt.*c* and PLP-Lys-79-cyt.*c* exhibited almost the same difference spectra. The structure analysis of a self-constructed model of cytochrome *c* and its PLP-Lys-79, 86-diderivative shows that both PLP moieties are large enough to be in contact with their pyridine rings. The observed long-wave shift to 340 nm, accompanied with about 25% hypochromism for the pyridoxal phosphate absorption band (fig. 2), may be a consequence of 'head-to-tail' interaction between PLP chromophores. In such orientation, a direct electron interaction between both PLP moieties arose. From the difference ($\nu_1 - \nu_2$) and the areas under the differential spectra (Fig. 2), the distances between the centres of pyridine rings of PLP were calculated using the approach used in Refs. 28 and 29 as follows:

$$R_{ij} \simeq D_i C_{ij} / h(\nu_1 - \nu_2)^{1/3}$$

and

$$D_i = 1.63 \cdot 10^{-38} \left(\Delta_i \epsilon_i / \lambda_i^0\right),$$

where R_{ij} is the distance between the two PLP chromophores in Å, Δ_i and ϵ_i are the halfwidth (in nm) and the extinction coefficient (in $M^{-1} \cdot cm^{-1}$), respectively, at λ_i^0 (max.) in nm; C_{ij} is a constant near to 1; h is the Planck constant. It was calculated that $R_{(min)} = 3.5$ Å and $R_{(max)} = 5$ Å.

The change of the net charge and charge distribution in PLP-Lys-86-cyt.*c*, PLP-Lys-79-cyt.*c* and PLP-Lys-79, 86-cyt.*c* do not alter ther redox potentials. This may be due to the total exposure of these lysine residues to the solvent groups. For that reason, the charge effect is lowered greatly as a result of the water environment (high dielectric constant). Obviously the modification of such surface groups does not change the hydrophobic haem environment that is important for the cytochrome *c* redox properties. Probably the strong decrease of the net charge in $(PLP)_3$-cyt.*c* leads to the decrease of its redox potential with 10 mV.

Thus, the visible, CD and proton NMR spectra, the full intensity of the conformation sensitive 695 nm band and the oxidation reduction properties provide evidence to confirm the conclusion that singly and doubly substituted PLP-cytochromes *c* retain the native conformation. Hence, these modified PLP-cytochromes *c* are suitable for assessing the mechanism of cytochrome *c* interactions with its biological redox partners.

Interaction of PLP-modified cytochromes c with cytochrome c-depleted mitochondria

Aviram and Schejter [3], being the first to describe the PLP modification of cytochrome *c*, obtained one diderivative and one monoderivative. Their mono-derivative was partially active, while their diderivative was entirely inactive in the restoration of the succinate respiration of cytochrome *c*-depleted mitochondria. These derivatives were reported as being oxidized by isolated cytochrome *c* oxidase with the same rate constants as native cytochrome *c* [3].

As shown above, there is not one but three monoderivatives, and not one but three diderivatives. Thus, agreement between our results and those of Aviram and Schejter [3] is not total. Our results (Fig. 4 and Fig. 5) show, in contrast to theirs, that the PLP modification affects the interaction of cytochrome *c* not only with the oxidase, but also with its reductase. On both substrates, succinate and ascorbate + TMPD, the activity of cytochromes *c* decreases in the order: native protein > PLP-Lys-79-cyt.*c* > PLP-Lys-86-cyt.*c* > di- and tri-derivatives. Maybe the discrepancy between their [3] and our results is due also to differences in ionic strength. It is shown that ionic strength strongly influences cytochrome *c*-oxidase interaction [30].

Our results with the ascorbate-TMPD oxidase show that the mono-derivatives, though with an

equal net charge, have different electron transfer properties (Figs. 4, 5). The reason for this may be due to a different orientation or shielding of the modified proteins in the membrane. As a consequence of the different orientation, the reduction of the derivatives by ascorbate/TMPD would not proceed to the same degree. If derivatives were reduced in solution, previously to binding, no difference should exist between them. The marked difference found experimentally supports the concept that, in the polarigraphic assay, TMPD reduces mainly bound and not free cytochrome *c* [31] and is evidence that the two mono-derivatives are oriented in a different way in the membrane and hence have different accessibility for ascorbate/TMPD. This conclusion is also supported by the experiments done with different concentrations of ascorbate and TMPD (Table IV). The increase of these concentrations increases the degree of reduction of native protein and PLP-Lys-79-cyt.*c* but does not affect PLP-Lys-86-cyt.*c*. Therefore, impaired interaction of modified cytochrome *c* with its redox partners may be due, not only to the shielding of an amino group between the partners, but to a strong reorientation of the molecule in the membrane as a result of a change of the charge multipole.

The results in Fig. 6 support the data of Shur-Perek and Avi-Dor [22] that cytochrome *c* is necessary in mitochondria, not only as an electron carrier, but also for the process of energy coupling. Most probably this is not related to artificial factors, since the diderivative has native overall conformation and redox properties. In this sense, it is a better tool for discriminating between the two functions of cytochrome *c* than its 'dimer' [22], in which the lack of the 695 nm band shows that the native conformation is not retained.

The results support the concept that there is probably an excess of cytochrome *c* in the membrane which is not directly necessary for electron transfer. Maybe it is essential for the membrane structure and is associated with energy coupling.

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