DISTINCTION BETWEEN OXIDIZING AND REDUCING SITES OF CYTOCHROME *C* BY CHEMICAL MODIFICATION WITH PYRIDOXAL PHOSPHATE

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1. Introduction

It was recently reported that antibodies to cytochrome *c* inhibit selectively the oxidation of the enzyme by cytochrome oxidase and its reduction by succinic dehydrogenase [1]. However, attempts to differentiate between oxidizing and reducing sites of cytochrome *c* through chemical modification have been hitherto inconclusive.

In this communication, we describe the preparation of cytochrome *c* derivatives obtained by covalent binding of pyridoxal phosphate, with normal physicochemical properties and reactivity with cytochrome oxidase, but with impaired enzymic reducibility.

2. Materials and methods

2.1. *Materials*

Horse heart cytochrome *c* (Type III), NADH, NADH–cytochrome *c* reductase, and pyridoxal phosphate (PLP) were from Sigma Chemical Co. Beef-heart cytochrome oxidase was prepared according to Yonetani [2]. All other chemicals were commercial preparations of the best degree of purity available.

2.2. *Methods*

The enzymic activity of native and modified cytochrome *c* was estimated by measuring their ability to restore the respiratory activity to cytochrome *c* depleted rat liver mitochondria [3]. Spectrophotometric measurements of ferrocytochrome *c* oxidation by cytochrome oxidase were performed as described by Smith [4]. Initial rates of reduction of ferricytochrome *c* by rat liver mitochondrial succinic dehydrogenase were followed at 550 nm, in the presence of 1 mM KCN [5]. NADH–cytochrome *c* reductase activity was assayed according to Mahler [6]. Covalent binding of PLP to cytochrome *c* was performed in the following way: a solution of ferric cytochrome *c* (5 mg/ml) in 0.05 M Tris–cacodylate buffer, pH 7.0 was incubated for 5 min in the presence of 1–1.5 mM PLP. A solution of 0.4 M $NaBH_4$ in 0.02 M NaOH was added, to make a final concentration of 25 mM borohydride. After 10 min at room temperature, the solution was diluted 10-fold and oxidized with potassium ferricyanide. Chromatography on Amberlite CG-50 using a NaCl gradient as eluent resolved the products into 3 peaks (fig. 1). The slowest peak was native cytochrome *c*. The first and second fractions showed an absorption band at 325 nm. The absorbance at 325 nm indicated that the first fraction contained two residues of PLP, and the second fraction only one. They were labelled $(PLP)_2$-*c* and $(PLP)_1$-*c* respectively.

3. Results

The two derivatives showed the characteristic properties of native cytochrome *c*. In the ferric form, the 695 nm band was present, indicating coordination of the iron by a methionine sulfur. The rate constants for the reduction with ascorbate at pH 7.0 and 23°C in 0.1 M phosphate buffer were: 20, 20 and 19 M^{-1} sec^{-1} for native cytochrome *c*, $(PLP)_1$-*c* and (PLP)-*c* respectively. The ferrous forms of $(PLP)_1$-*c* and

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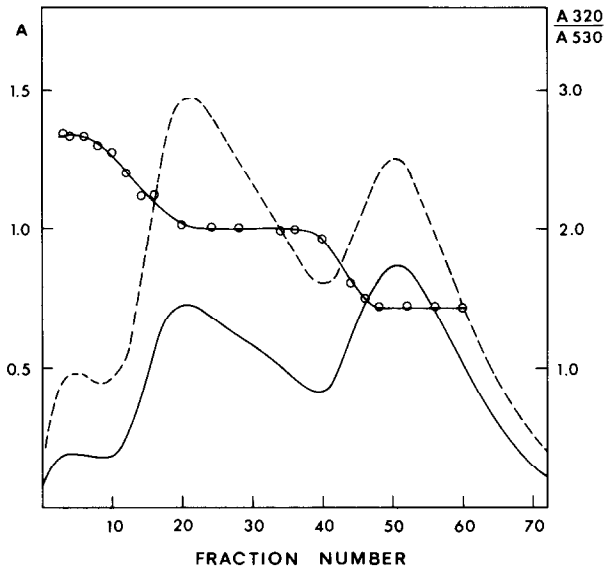

Fig. 1. Ion exchange chromatography of the reaction products on an Amberlite CG-50 column with a linear gradient from 0 to 0.6 M NaCl in 0.02 M phosphate buffer pH 8.0 (200 ml each) 2.9 ml fractions collected. Order of elution: $(PLP)_2$-*c*; $(PLP)_1$-*c*; cytochrome *c*. (——) *A* at 530 nm; (– – –) *A* at 320 nm; (o–o–o) A_{320}/A_{530}.

$(PLP)_2$-*c* were not autoxidizable, the ferric forms reacted with cyanide at pH 7, with rates identical to those of native cytochrome *c*. All these results indicated that in $(PLP)_1$-*c* and $(PLP)_2$-*c* the heme environment remained the same as in native cytochrome *c*, and that the chemical modifications did not cause major alterations in the tertiary structure of the protein.

The restoration of respiratory activity of cytochrome *c* depleted mitochondria by $(PLP)_1$-*c*, $(PLP)_2$-*c* and the native protein is shown in fig. 2. $(PLP)_1$-*c* is only partly active; $(PLP)_2$-*c* was almost entirely inactive. On the other hand, the two derivatives were oxidized by a purified preparation of beef heart cytochrome oxidase at identical rates (table 1). This indicated that the reducibility of $(PLP)_1$-*c* and $(PLP)_2$-*c* by enzyme systems was impaired and, indeed, this was found to be so for succinic dehydrogenase of the respiratory chain and for NADH cytochrome *c* reductase (table 1).

In order to determine the sites of covalent binding of PLP to the cytochrome *c* protein, $(PLP)_1$-*c* and $(PLP)_2$-*c* were digested with chymotrypsin, and chromatographed on Dowex 50-X4 at 55°C with pyridine–acetate buffers. The fractions showing absorption peaks at 325 nm were further purified by paper chromatography and analyzed for amino acid content. The compositions of the PLP-bound peptides permit to identify them with the following partial sequences: 1–10; 68–74, and 83–94. The main heme peptide isolated showed an increase in absorbance in the 325 nm region, although not a definite peak. The possibility that PLP is bound to it is being investigated. For this reason, the relative yields of the different PLP-peptides in the two modified proteins could not be estimated.

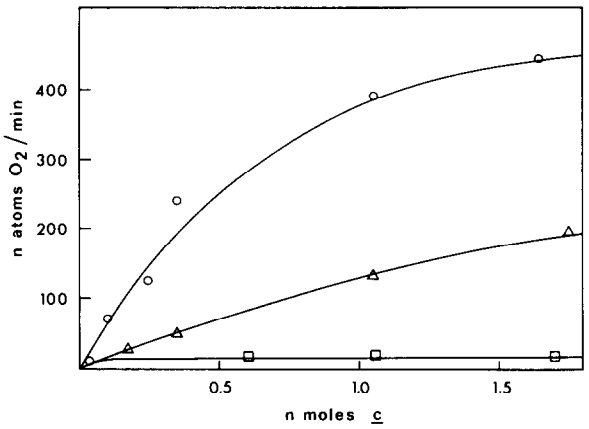

Fig. 2. The increase of oxygen consumption of cytochrome *c* depleted rat liver mitochondria upon addition of: (o–o) native cytochrome *c*; (△–△) $(PLP)_1$-*c* and (□–□) $(PLP)_2$-*c*. Reaction mixture: (3.6 ml) 6 mM succinate; 5 mM phosphate, pH 7.0; 0.25 M sucrose; 4.8 mg mitochondrial protein.

4. Discussion

The covalent binding of one or two PLP molecules affects the enzymic reducibility of cytochrome *c*, while its enzymic oxidizability and its reduction with ascorbate are not impaired. This is, to our knowledge, the first instance of a modification of the cytochrome *c* protein that distinguishes between reductase and oxidase sites of the enzyme, without, at the same time, causing major changes in the physicochemical properties of the molecule.

In cytochrome *c*, the amino terminal end is acetylated, hence only lysine residues are susceptible to

Table 1
Enzymic properties of $(PLP)_1$-*c* and $(PLP)_2$-*c*.

	Cytochrome oxidase activity* k (sec^{-1})	NADH-cytochrome *c* reductase at V_{max}** (mM/min)	Initial rates of reduction in succinic dehydrogenase*** (mM/min)
Cytochrome *c*	1.1×10^{-2}	4.0×10^{-2}	4.4×10^{-3}
$(PLP)_1$-*c*	1.0×10^{-2}	1.0×10^{-2}	3.7×10^{-3}
$(PLP)_2$-*c*	1.0×10^{-2}	0.4×10^{-2}	0.9×10^{-3}

* Measured at 2.3×10^{-5} M cytochrome *c*, 3.8×10^{-8} M oxidase [4].
** Determined at an enzyme concentration of 2×10^{-3} units/ml [6].
*** 0.06 mg mitochondrial protein; 4.5×10^{-5} M cytochrome *c* [5].

modification by PLP and borohydride. The PLP-bound peptide 1–10 has three lysines, at positions 5, 7 and 8; the peptide 83–94 has lysines at positions 86, 87 and 88. It is noteworthy that the ϵ-amino groups of all these lysine residues define a positive region, at the top portion of the molecule [7]. The PLP-bound peptide 68–74 contains lysines 72 and 73, that belong to the invariant 70–80 sequence, and are located at a different position in the molecular surface [7].

The following conclusions can be reached: first, that the chemical modification with PLP has singled out two regions of cytochrome *c* as possible sites for its attachment to the mitochondrial reductases; second, that these regions cannot be involved in the reaction with cytochrome oxidase. Studies now in progress will hopefully define the exact location of the reductase site of cytochrome *c*.

References

[1] Smith, L., Davies, H.C., Reichlin, M. and Margoliash, E. (1973) J. Biol. Chem. 248, 237.
[2] Yonetani, T. (1960) J. Biol. Chem. 235, 845.
[3] Jacobs, E.E. and Sanadi, D.R. (1960) J. Biol. Chem. 235, 531.
[4] Smith, L. (1955) in: Methods of Biochemical Analysis (Glick, D., ed.), Vol. 2, p. 427, Interscience, New York.
[5] Green, D.E., Mii, S. and Kohout, P.M. (1955) J. Biol. Chem. 217, 551.
[6] Mahler, H.R. (1955) in: Methods in Enzymology (Colowick S.P. and Kaplan, N.O., eds.), Vol. 2, p. 688, Academic Press, New York.
[7] Dickerson, R.E., Takano, T., Eisenberg, D., Kallai, O.B., Samson, L., Cooper, A. and Margoliash, E. (1971) J. Biol. Chem. 246, 1511.