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OBSERVATIONS ON THE c-TYPE CYTOCHROMES OF THE EXTREME THERMOPHILE, Thermus thermophilus HB8: CYTOCHROME c_{552} IS LOCATED IN THE PERIPLASMIC SPACE*

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SUMMARY. We have shown that cytochrome c_{552} of Thermus thermophilus HB8, isolated previously by Hon-nami and Oshima (K. Hon-nami and T. Oshima (1977) J. Biochem. (Tokyo) 82, 769-776), resides in the periplasmic space of this gram-negative organism. As much as 90% of this protein was released from cells having the outer membrane disrupted by lysozyme. By contrast less than 1% of the cytoplasmic glucose-6-phosphate dehydrogenase and glutamate dehydrogenase were released by this treatment. It was further observed that the two other c-type cytochromes, c_{549} and $c_{549,555}$ (split alpha band) are membrane bound proteins. The amount of each of the c-cytochromes present in the bacteria was shown to depend strongly on the carbon source in the culture medium.

INTRODUCTION

Thermus thermophilus HB8 (1) is a strict aerobe, classified as an extreme thermophile (2,3), because it can only be grown at high temperatures (50 - 85 °C). The organism is gram negative and has a double membrane structure with a periplasmic space similar to enterobacteria (1,4,5). Recent studies have revealed many similarities between the electron transport chain of Thermus and that of mitochondria. Thus, the isolated plasma membranes were shown to contain a-, b-, and c-type cytochromes (6), and a terminal oxidase has been isolated which is similar to mitochondrial cytochrome oxidase (7,8).

The organism contains three c-type cytochromes which are distinguished by the absorption maxima of the reduced proteins in the visible region:

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$\underline{c}_{549}$, $\underline{c}_{555,549}$, and $\underline{c}_{552}$. Cytochrome $\underline{c}_{549}$ is tightly associated with the cytochrome $\underline{aa}_3$ complex and has previously been termed $\underline{c}_1$ (7), the split-alpha c-cytochrome, $\underline{c}_{555,549}$ (9), which we will show here to be associated with the plasma membrane, was first isolated by Hon-nami and Oshima (8-10); and $\underline{c}_{552}$ which was isolated and studied in some detail by Hon-nami and Oshima and their co-workers (11-14). The functions of the three different cytochromes are not presently known although Hon-nami and Oshima (8) demonstrated that reduced $\underline{c}_{552}$ was efficiently oxidized by catalytic amounts of the cytochrome $\underline{c}_1$ $\underline{aa}_3$ complex. As a step toward elucidating the functions of the c-cytochromes in Thermus we report our preliminary results on the cellular distribution of these proteins and their synthesis by the cells under differing growth conditions.

MATERIALS AND METHODS

Optical spectra were recorded on a Perkin-Elmer Model 320 UV/Vis spectrophotometer. Protein concentrations were determined by the method of Lowry et al (15) using bovine serum albumin as a secondary standard. Lysozyme was obtained from Calbiochem-Behring; glucose-6-phosphate, deoxyribonuclease I, and $NADP^+$ were purchased from Sigma Chemical Co. DEAE-cellulose (DE-52) anion exchange resin was obtained from Whatman, and all other reagents were of the highest quality commercially available.

Thermus thermophilus HB8 cultures were obtained from the American Type Culture Collection (No. 27634), grown at 70 °C on a purely synthetic medium containing glutamate or glucose plus NH_4Cl as the C and N sources, and the cells were harvested from the late log phase of growth. Cells grown under these conditions show no "rotund bodies" or filaments (4), and a suspension having an O.D. of 1 at 600 nm contains 1.8×10^9 viable cells per ml (K. L. Findling, et al, In preparation).

Cell fractionation. The cells (2.5×10^{13} cells in 60 g wet weight) were washed once with pH 7.8, 20 mM Tris.HCl buffer (used throughout) and spheroplasts were prepared by suspending the cells in 200 ml buffer containing 120 mg lysozyme and heating for 50 min at 70 °C. Spheroplast formation was confirmed by phase contrast microscopy, and the suspension was then centrifuged at 37,000 x g for 2 hours to obtain the spheroplast pellet and the "lysozyme supernatant." The pellet was gently suspended into buffer and centrifuged again to obtain the "spheroplast wash" supernatant. This pellet was suspended in 10 ml buffer per gram wet weight cells and deoxyribonuclease added to a final concentration of 20 μg/ml. A portion of this suspension and of whole cells was set aside for sonication.

The spheroplasts were disrupted in a pressure cylinder (16) at 2,350 psi and the membrane and cytosolic fractions separated by centrifugation at 113,000 x g for 5 hours to obtain the "pressure cylinder supernatant." A portion of washed spheroplasts was sonicated (140 watts for 15 min) at ice temperature then centrifuged at 37,000 x g to obtain the "sonication supernatant" and a precipitate. The precipitate was solubilized in 20 mM buffer containing 2% Triton X-100.

Enzyme assays. The activities of glucose-6-phosphate and glutamate dehydrogenases were measured spectrophotometrically by the method of Malamy and Horecker (17). Both enzymes were assayed by determining the rate of absorbance change at 340 nm resulting from the reduction of $NADP^+$. After allowing time for the consumption of endogenous substrate the reactions were started by addition of substrate. All fractions were subjected to the same sonication procedure and centrifugation prior to assay.

After the enzyme assays were completed, the whole cell wash, lysozyme supernatant, spheroplast wash, cytosolic supernatant, and spheroplast sonication supernatant were concentrated by freeze-drying and the material redissolved into buffer.

Cytochrome determination. Cytochromes c_{552} and $c_{555,549}$ were determined spectrophotometrically from the difference spectrum: dithionite reduced minus ferricyanide oxidized using $\Delta\varepsilon_{552}$ = 14.3 (8) and $\Delta\varepsilon_{555}$ = 15.2 $mM^{-1}cm^{-1}$ (9), respectively. Interference of small amounts of $c_{555,549}$ in the assesment of c_{552} concentrations was eliminated by suspending some DE-52 into the solution to adsorb the acidic $c_{555,549}$ and then removing the ion exchange resin with a brief centrifugation. Cytochrome c_{549} was estimated by measuring the amount of cytochrome a ($\Delta\varepsilon_{605}$ = 11.7 $mM^{-1}cm^{-1}$) and assuming a c_{549} to a ratio of 0.5 (7).

RESULTS AND DISCUSSION

Cytochrome c_{552} is a small, basic (pI = 10.8) protein (11) which can be partially purified from the lysozyme supernatant of HB8 cells by adsorption to a cation exchanger and further purified by gel filtration. In contrast, the split-alpha cytochrome, $c_{549,555}$, is an acidic (pI = 4.0) protein (9) extrinsically associated with the membrane (see below), but is easily dislodged and subsequently purified as a soluble protein by a combination of ion exchange and gel filtration procedures (R. Lorence et al, In preparation). Cytochrome c_{549} can be isolated as a complex with cytochrome aa_3 (7,8, T. Yoshida, et al, In preparation). The reduced minus oxidized optical spectra of these proteins are presented in Fig. 1. Shown in Fig. 2 are similar spectra of cell fractions containing c_{552} and $c_{555,549}$ before and after treatment with a small amount of DE-52 to remove the acidic $c_{555,549}$. In this fashion c_{552} can be quantified without interference from the split-alpha cytochrome. Cytochrome a was observed only in the detergent extract of the membrane fraction, and the contribution of c_{549} to the absorbance at 555 nm was subtracted before calculating the concentration of $c_{555,549}$. Table I summarizes the enzyme levels and cytochrome content of several cellular fractions obtained from T. thermophilus grown on glutamate as the C/N source.

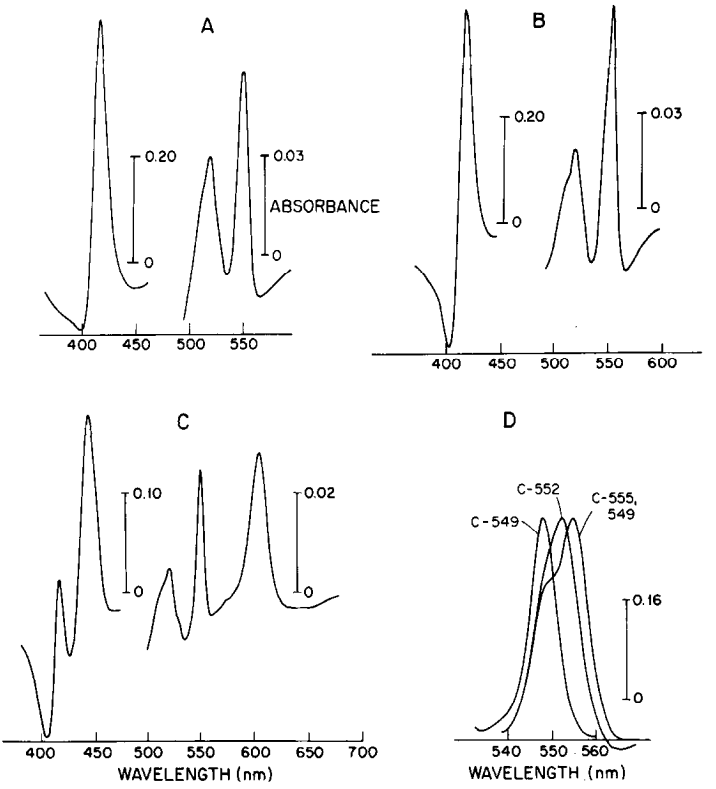

Fig. 1. Reduced minus oxidized difference spectra of the c-type cytochromes of Thermus thermophilus HB8. All samples were maintained in 20 mM Tris-HCl, pH 7.8. The reduced form was obtained by treatment with a small excess of dithionite and the oxidized form by including 10 μM ferricyanide. A. Cytochrome c_{552} partially purified from the lysozyme supernatant by adsorption to a cation exchanger followed by gel filtration. B. Cytochrome $c_{555,549}$ purified by a combination of anion exchange and gel filtration. C Cytochrome c_{549} as the aa_3 complex (Ref. 7) and solubilized in 2% Triton X-100. D. Comparison of the alpha peaks of the reduced minus oxidized forms of the three cytochromes.

Although the spheroplasts of Thermus cells have been shown by Oshima and Imahori (1) to be very resistant to osmotic lysis we have tested our preparation for possible leakage of intracellular materials by measuring the activities of two enzymes known to be in the cytosol of bacteria (18): glucose-6-phosphate dehydrogenase and glutamate dehydrogenase. The data given in Table I indicate that these enzymes are present in the lysozyme supernatant at extremely low levels compared to the sonicates of either whole cells or spheroplasts, and they are, to a large extent, released into the soluble

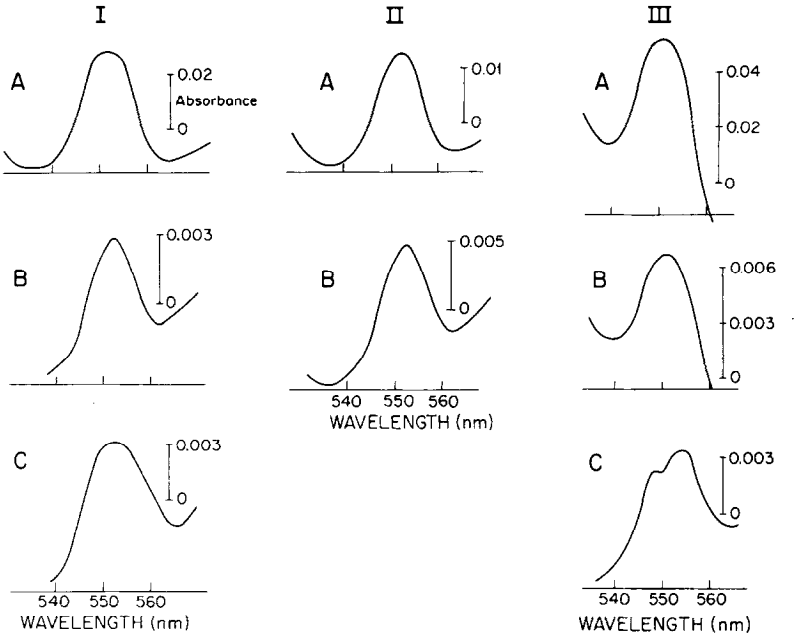

Fig. 2. Reduced minus oxidized optical spectra of cytochromes found in various cell fractions demonstrating removal of $c_{555,549}$ by anion exchange resin. I, supernatant from pressure cylinder treatment; II, lysozyme supernatant; and III, supernatant from sonicated spheroplasts. Cytochrome $c_{555,549}$ was removed from solution by addition of ∿20 mg dry DE-52 to the original fraction (A) to yield solution (B) after centrifugation with a two-fold increase in the original volume. The resin was washed once with buffer then treated with buffered 0.7 M NaCl to obtain solution (C) at three times the volume of (A). Control experiments with purified cytochromes c_{552} and $c_{555,549}$ showed that the former did not associate with the resin and the latter could be recovered from the resin in ∿70% yield.

phase by the pressure cylinder treatment. The lower activity of these two enzymes solubilized by the decompression method (50%) when compared to sonication, attests to the sturdiness of the Thermus protoplast reported earlier. When the same experiment was done with cells which had previously been frozen in buffer a small amount (<5%) of the total activity of both enzymes was found in the lysozyme supernatant. This is attributed to lysis induced by the freeze-thaw cycle. The data of Table I support the microscopic observation that cells treated with lysozyme by our protocol are stable and have not released intracellular components.

The remainder of the data in Table I suggests two facts: Cytochrome c_{552}, which is a soluble protein in the usual sense, resides in the periplasmic

TABLE I: Cellular distribution of the c-type cytochromes, glucose-6-phosphate dehydrogenase and glutamate dehydrogenase in Thermus thermophilus grown on glutamate.

Fraction	Protein		c_{552}		$c_{555,549}$		c_{549}		G6PDH		Glu DH	
	mg	%	nmoles	%	nmoles	%	nmoles	%	Activity[a]	%	Activity	%
Whole Cell Wash	60	0.7	3.7	12		∿0		∿0	∿0	0	∿0	0
Whole Cells[b]	8500	100							32	100	3.2	100
A. Lysozyme Supernatant	70	1.3[c]	14.7	60[d]		∿0		∿0	∿0	0	0,0.5[e]	0
B. Spheroplast Wash	40		3.7						∿0		0	
C. Spheroplasts	7150	85										
1) Pressure Cylinder Supernatant	1100	13	7.4	24	18	3		∿0	11	35	1.7	53
2) Sonication Supernatant	1000	12	8.3	27	55	9		∿0	24	73	3.4	104
3) Sonication Precipitate, Triton X-100 Solubilized					540	91	98	100				
Sum of Parts			30.4	100	590	100	98	100				

(a) Rate given as ΔA_{340}/min (total activity)

(b) 2.5×10^{13} cells

(c) Corrected for added lysozyme

(d) Treatment with higher salt (200 mM Tris/Cl, pH 7.9) released 90% of cytochrome c_{552} in the lysozyme supernatant and superoplast wash

(e) Due to the large error limits under our conditions of assay, this value is not different from zero

TABLE II: Comparison of the number of the c-type cytochromes per cell for glutamate-grown and for glucose-grown T. thermophilus.

Cytochrome	Glucose-grown	Glutamate-grown
c_{552}	3400	700
$c_{555,549}$	5000	14,000
c_{549}	< 400	2,300

space, while cytochromes $c_{555,549}$ and the c_1aa_3 complex (c_{549}) are associated with the membrane fraction. Thus, c_{552} is found to a small extent in the wash fluid of whole cells and a large fraction of this cytochrome is released from the cells upon treatment with lysozyme. The lesser amount which remains with the spheroplast fraction is probably due to incomplete removal of the cell wall. By contrast, $c_{555,549}$ and c_{549} are not solubilized by spheroplast formation or disruption of the spheroplasts by either sonication or the pressure release method. However, the data of Table I show that these proteins can be solubilized from a membrane pellet by treatment with detergents. As mentioned above, c_{549} can be purified from this solution as a detergent soluble complex with cytochrome aa_3 (7,8), and $c_{555,549}$ can be further purified as a soluble protein (9, Lorence et al, In preparation). These results indicate that both $c_{555,549}$ and c_{549} are bound to the membrane.

A similar set of experiments was carried out with cells grown on glucose and NH_4Cl as the C/N source, and the c-cytochrome compositions of cells grown in the two media are compared in Table II. The cytochrome content clearly depends on the nature of the carbon source with the most striking result being the strong depression of cytochrome a in cells grown on glucose. The split-alpha cytochrome also decreases in this medium while c_{552} is increased approximately 5-fold.

What is the function of the periplasmic cytochrome c? A cytochrome c_{552} having distinctly different physical properties from the c_{552} of Thermus

has been isolated from the periplasmic space of E. coli (18) where it plays an essential role in the coupling of CO_2 evolution and nitrite reduction under anaerobic conditions (19). Cytochromes c have also been isolated from the periplasmic space of several other bacteria (20-23) but the functions of these proteins have not been elucidated. In the case of Thermus, Non-nami and Oshima (8) have shown and we have confirmed that reduced c_{552} is rapidly reoxidized by catalytic amounts of cytochrome c_1aa_3 in the presence of oxygen. It is thus possible that c_{552} is the physiological donor of electrons to c_1aa_3. However, because c_{552} is quite basic (pI = 10.8) and c_1aa_3 is fairly acidic (pI = 6.5, Yoshida et al, In preparation), non-specific electron transfer between these proteins cannot be excluded. Nevertheless, there are some striking analogies between the mitochondrial cytochrome c/cytochrome oxidase arrangement as elucidated by various topographical studies (24) and that present in Thermus. While the ratio of c to aa_3 is substantially different in mitochondria, 2, compared to the ratio of c_{552} to c_1aa_3 in Thermus, 0.3 when grown on glutamate and ∿9 when grown on glucose, the rather similar physical properties and cellular localization of mitochondrial cytochrome c and bacterial c_{552} suggest but do not prove an analogous function of the two proteins. Hon-nami and Oshima (8) also showed that $c_{555,549}$ could serve as an electron source to the oxidase in the presence of catalytic amounts of c_{552}, but the relevance of this observation to the events occurring in the cell is not evident since it is not known to which side of the membrane $c_{555,549}$ is attached.

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