BEEF HEART COMPLEX III: ISOLATION AND CHARACTERIZATION OF CYTOCHROME *b*

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1. Introduction

Cytochrome *b* has been isolated in homogenous form only from *Neurospora crassa* [1] and *Locusta migratoria* [2]. In the procedures [1,2] cholate and deoxycholate were used as detergents. The isolation of complex III from beef heart by a hydroxyapatite chromatography in Triton X-100 [3,4] made possible the separation of its polypeptide subunits in the mild detergent Triton X-100. The present report describes briefly a simple large-scale purification method for cytochrome *b*. A short characterization of its properties will be given.

2. Methods and isolation procedure

Isolation of the antimycin-loaded complex III, protein and heme determinations, polyacrylamide gel electrophoresis in sodium dodecylsulfate (SDS) and analytical ultracentrifugal studies were performed as in [4].

Redox titrations were as in [5]. Triton binding was determined with ^{3}H-labeled Triton X-100 by gel chromatography on Sephadex G-200. The amino acid composition of the isolated cytochrome *b* was determined from samples hydrolyzed in 5.7 N HCl at 105°C for 24 h, 48 h, 96 h and 120 h on a Durrum D-500 amino acid analyzer. Cysteine and methionine were estimated after performic acid oxidation. Tryptophan was determined spectrophotometrically and after hydrolysis in 4 N toluene sulfonic acid [6].

For amino-end-group determination the protein dissolved in SDS was covalently attached to *p*-phenylene diisothiocyanate-activated porous glass beads [7] and degraded in a solid-phase sequencer [8]. The resulting phenylthiohydantoin (PTH) amino acids were identified by gas–liquid chromatography.

The isolation procedure and the application of the various buffers are summarized in scheme 1 (cf. table 1). The antimycin-loaded complex III (760 mg protein) is bound on a hydroxyapatite column in the first step. Then it is dissociated into its individual subunits by 70 ml of a buffer containing 1.5 M guanidine (buffer (i) in the scheme). Subsequently the buffer (ii) is used for complete elution of the proteins. Under this procedure cytochrome c_1 remains bound to the column, whereas cytochrome *b* and the other polypeptides elute in the pass through fractions. They seem to be present in dissociated state.

Isolation of cytochrome c_1 and the other subunits which remain bound on the various hydroxyapatite columns will be described in a later paper. In step 3 guanidine is removed by passing the preparation (in ~ 100 ml) through a Sephadex G-25 column. The following two hydroxyapatite chromatographies (steps 4, 6) which are interrupted by a desalting step (step 5) separate the core proteins and the 12 000 mol. wt protein from cytochrome *b*.

Table 1 lists the enrichment and yields of the subsequent preparation steps. Separation of cytochrome c_1 only slightly improves the specific heme

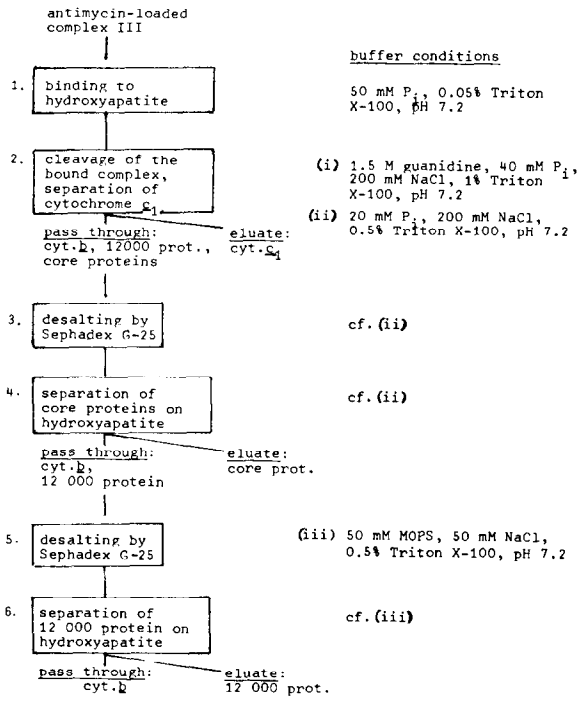

Fig.1. Flow scheme for the isolation of cytochrome *b* from beef heart. The first and second hydroxyapatite columns each have vol. 150 ml, the third 250 ml. The Sephadex G-25 columns each have vol. 600 ml. All buffers contain 5 mM sodium azide. The hydroxyapatite was prepared according to [15].

content. The essential enrichments occur during the following two hydroxyapatite chromatographies. The preparation yield is on the average about 40%. The final concentration of cytochrome *b* is about 10 μM. It is 90% pure and stable for weeks at 4°C.

3. Properties of cytochrome *b*

3.1. *Molecular weight and state of aggregation*

The minimum molecular weight as calculated from the heme content is 33 000. The preparation reveals in SDS–polyacrylamide gel electrophoresis only one band in the 31 000 mol. wt region. Traces of core protein in the 45 000 mol. wt region are sometimes visible. The molecular weight and state of aggregation of the isolated protein were investigated by high-speed ultracentrifugal velocity and sedimentation equilibrium runs. They were performed at room temperature in the presence of 0.5% Triton X-100.

Figure 2 depicts a curve of log concentration

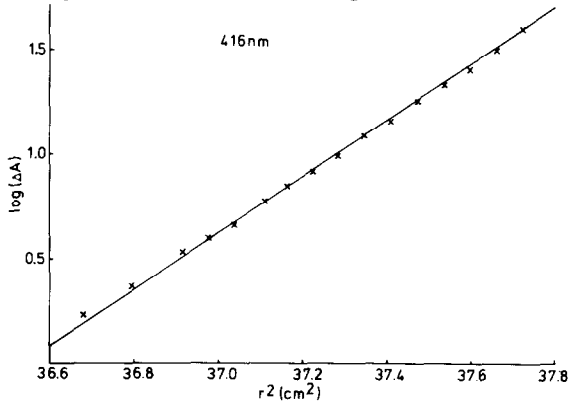

Fig.2. Sedimentation equilibrium run of cytochrome *b* in Triton X-100. The logarithm of recorder deflection in mm (proportional to the absorption) is plotted versus the square of the distance to the center of rotation r^2 (cm^2). The rotor speed was 24 000 rev./min, the protein conc. 1.5 mg/ml. After 24 h centrifugation, equilibrium was reached. Light of 415 nm was used, temp. 22.5°C.

Table 1
Table of enrichment and yield

Step	Fraction	Protein (mg)	Heme *b* (nmol)	Heme *b*/prot. (nmol/mg)	Yield (%)
1.	bc_1 complex	760	6080	8.0	100
2.	After 1st HTS	527	4864	8.5	80
3.	After 1st gel	515	4378	8.5	72
4.	After 2nd HTS	209	3770	18.0	62
5.	After 2nd gel	172	3101	18.0	51
6.	After 3rd HTS	81	2432	30.0	40

Abbreviation: HTS, hydroxyapatite self-prepared

The various steps are described by scheme 1

versus r^2 (the distance to the center of rotation) of a sedimentation equilibrium run in which heme *b* was monitored at 415 nm. A straight line is obtained, supposing the homogeneity of the preparation. After correction for the specifically bound Triton (1.1 g Triton X-100/g protein) the results of 5 runs yield mol. wt 62 000 ± 3000 (SEM, n=5). From this result it was concluded that the isolated cytochrome *b* is present in a dimeric aggregation state.

3.2. *Amino acid composition and amino-end group*

The amino acid composition (table 2) shows a high content of nonpolar amino acids, especially leucine and isoleucine, and a low content of lysine and arginine. The low polarity [9] of 0.32 is characteristic of an integral membrane protein. Solid-phase Edman degradation of the glass-bound protein over 15 steps resulted in an increasing unspecific background of PTH amino acids as expected for an amino-end blocked protein. After incubation in methanol–HCl (1.5 h in 0.5 M HCl at 25°C) a single methionine was released in the first step of the degradation followed by glycine in the second step. These results suggest that the N-terminal residue of the subunits is N-formyl methionine [10]. The amino acid sequence obtained in these degradation experiments will be reported later.

Table 2
Amino acid composition

Amino acid	mol/100 mol
Aspartic acid	7.48
Threonine[a]	7.61
Serine[a]	5.94
Glutamic acid	3.50
Proline	5.92
Glycine	6.63
Alanine	7.22
Cysteine	0.96
Valine[b]	4.72
Methionine	3.77
Isoleucine[b]	9.42
Leucine[b]	15.68
Tyrosine	3.97
Phenylalanine	6.21
Tryptophan	3.03
Lysine	2.70
Histidine	3.09
Arginine	2.16

[a] Corrected for destruction by zero-time extrapolation
[b] From 120 h hydrolysis

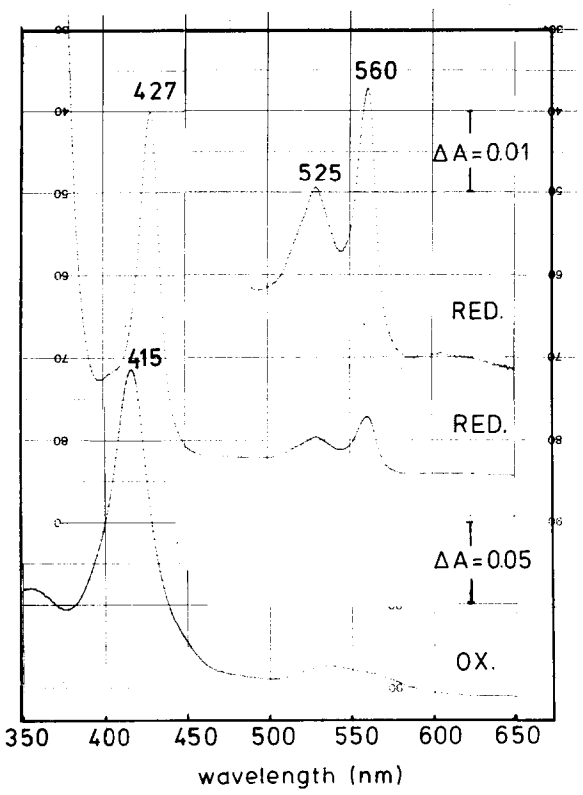

Fig.3. Absolute absorbance spectra of isolated cytochrome *b* from beef heart. The spectra were performed with a Cary 118 spectrophotometer in a buffer containing 0.5% Triton X-100 with 10 mm light path at 25°C. Cytochrome *b* was reduced by addition of a few grains of sodium dithionite.

3.3. *State of nativity*

Figure 3 shows the absolute absorbance spectra of cytochrome *b*. In the oxidized state (the lower curve) it has only in the γ-region a prominent band with an A_{415} max. In the dithionite-reduced state (the upper two curves) it has in the α-region an A_{560} max, in the β-region an A_{525} max and in the γ-region an A_{427} max. At low temperature the absorbance maxima shift 2 nm to the blue (not shown). The isolated cytochrome *b* reacts with carbon monoxide. This was indicated by an abolition of the α- and γ-absorbance bands of ferrocytochrome *b*. It is autoxidizable. A cytochrome *b* preparation reduced by dithionite

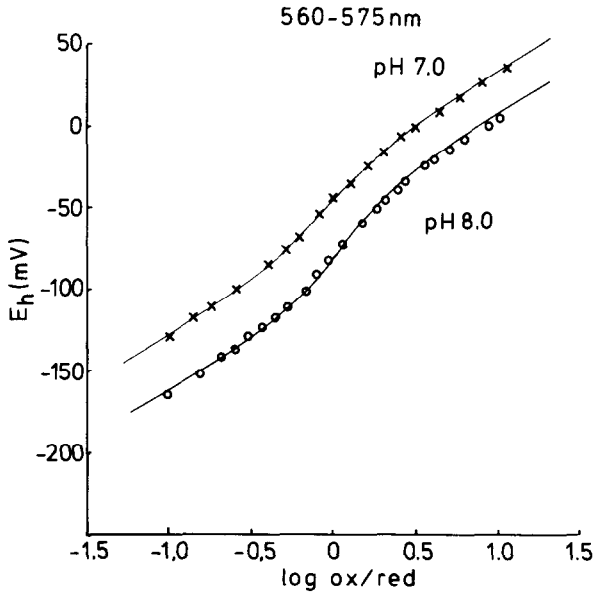

Fig.4. Nernst plot of redox titrations of the isolated cytochrome *b* dimer, at pH 7.0 and pH 8.0, respectively. The titrations were performed according to [11] in negative potential direction by addition of dithionite, in positive potential direction by addition of ferricyanide. The incubation buffer contained 0.5% Triton X-100, 100 mM Tris–HCl, 100 mM MOPS, light path 5 mm, temp. 25°C.

becomes reoxidized during chromatography on Sephadex G-25.

The redox titrations were performed according to [11] under application of redox mediators and electrodes. They give sigmoidal curves in the Nernst plot (cf. fig.4). In the analysis according to [12] two components were estimated to contribute equally to the absorbance changes. At pH 7.0 the midpoint potentials are calculated to be −5 mV for the high potential component and −85 mV for the low potential component, respectively. The midpoint potentials are about 100 mV more negative compared to cytochrome *b* integrated in complex III [5].
At pH 8.0 the apparent midpoint potentials of the isolated cytochrome *b* dimer are about 40 mV more negative than at pH 7.0. They are calculated to be −35 mV and −125 mV (cf. lower curve, fig.4). The pH dependence of the midpoint potentials demonstrates that the isolated cytochrome *b* dimer possesses in a similar manner as cytochrome *b* integrated in the mitochondrion a so-called 'Bohr effect' [13].

4. Discussion

Cytochrome *b* from beef heart when isolated in Triton X-100 has a dimeric aggregation state with mol. wt 62 000 in analogy to cytochrome *b* of *Neurospora crassa* when isolated in cholate, deoxycholate [14]. It is not yet known whether it consists of two equal or two different polypeptide chains. The question arises as to whether the dimer reflects the native aggregation state. However, a tetrameric natural aggregation state of cytochrome *b* must be considered, since in Triton X-100 the isolated complex III is present as a dimer [4] and the complex contains 2 cytochrome *b* molecules/monomer. The Bohr effect of the isolated cytochrome *b* makes the heme *b*-carrying polypeptide subunits themselves likely candidates as proton transporter of complex III, the locus of the second phosphorylation site.

Acknowledgements

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