Nitrogen Isotope Labeled Tetraheme Cytochrome c₃ on a Defined Medium

Andre Kim and Jang-Su Park*

Department of Chemistry and Center for Innovative Bio-physio Sensor Technology, Pusan National University, Busan 609-735, Korea. E-mail: jaspark@pusan.ac.kr

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To obtain cytochrome c₃ labeled with a stable isotope, the conditions of cultivation and the composition of medium for DvMF were examined. The growth of DvMF was steady and reproducible under purging with N₂ and under pH control. DvMF was able to go on a defined medium without natural products. The composition of medium containing a small amount of NH₄Cl as sole nitrogen source was established. Then, uniformly ¹⁵N-labeled cytochrome c₃ was obtained during the culture of DvMF in a defined medium with ¹⁵NH₄Cl; it was confirmed by ¹H-¹⁵N HMQC.

Key Words: Cytochrome c₃, ¹H-¹⁵N HMQC, Stable isotope

Introduction

Cytochrome c₃, found in sulfate-reducing bacteria of the genus Desulfovibrio,¹ is a small (14 kDa) tetraheme protein and functions as an electron carrier for hydrogenase.² This cytochrome c₃ is a great interesting protein.³ Especially, the relationship between its structure and its redox behavior has been the subject of several physicochemical and spectroscopic studies.⁴⁻⁹ The determination of the solution structure of small protein has been done by isotope-edited NMR experiments.¹⁰⁻¹¹ However, there are no reports of stable isotope uniformly labeled cytochrome c₃. Therefore, it is necessary to establish methods for obtain enough uniformly ¹⁵N-labeled cytochrome c₃. Desulfovibrio sp. were grown on the published media containing natural product, e.g., yeast extract and polypeptone.¹,¹² Natural products consist of various amino acids, consequently, those media have not been suitable for stable isotope uniform labeling. In this work, we have examined the conditions of cultivation and the composition of medium with the intention of obtaining uniformly ¹⁵N-labeled cytochrome c₃.

Experimental Section

Bacteria Strain. Desulfovibrio vulgaris Miyazaki F (DvMF) is the strain isolated by Iwasaki¹⁰ and obtained from Prof. T. Yagi of Shizuoka University.

Culture Media. The media had the following composition (per liter distilled water): 0.5 g of KH₂PO₄; 1 g of NH₄Cl; 10 g of 70% (w/v) sodium lactate; 4.5 g of Na₂SO₄0.04 g of CaCl₂·H₂O; 0.06 g of MgSO₄·7H₂O; 0.004 g of FeSO₄·7H₂O; and 1 mL of resazurin solution (1 g/L). NH₄Cl was changed in the range of 0.1 to 1.3 g as required. Filtersterilized 10% (w/v) yeast extract (Difco) solution or vitamin solution was added (10 mL/L) where indicated. The vitamin solution contained (per liter distilled water): 2 mg of biotin; 1 mg of pyridoxine HCl; 5 mg of thiamine HCl; 5 mg of cyanocobalamin; 5 mg of p-aminobenzoic acid; 5 mg of thiotic acid. The pH was adjusted to 7.4 with 5 M NaOH.

Cultivation of Bacteria. DvMF was grown anaerobically in a 1 L and a 70 L fermenter containing 900 mL and 50 L of medium, respectively. The growth of DvMF in the test medium was examined after at least two serial transfers of the cells at the early stationary phase in the same medium. After inoculation (5% inoculum) the culture was continuously stirred at 100 rpm, purged with N₂ at 0.04 VVM (gas supply per media volume per min), and kept at 37 °C.

Estimation of Growth. The estimation of growth by measuring the optical density was difficult since the culture was blackening by FeS precipitation. The growth was denoted by grams of protein from one liter of culture. The total growth was obtained by subtracted Xi from Xf. Xi was the maximum value of the concentration of cell protein. The concentration of cell protein was measured after these cultures were hydrolyzed in 0.2 M NaOH.

NMR Measurement. Cytochrome c₃ was purified from DvMF by the procedure described previously.¹³ The purity was confirmed by SDS-polyacrylamide gel electrophoresis. NMR spectra were obtained at pH 5.0 (30 mM phosphate buffer) and 30°C with a Bruker AM400 NMR spectrometer. The pulse sequence for heteronuclear multiple-quantum spectroscopy (HMQC) was referenced to Bax et al.¹⁴ Water suppression was achieved by presaturation during the relaxation delay.

Results and Discussion

Conditions of Cultivation. Figure 1 shown the effects of purging with N₂ on the DvMF growth. The specific growth rate is about to double and the total growth is 3 times, when the culture has been purged with N₂. The results are steady and reproducible. Reis et al. has reported that H₂S has a direct and reversible toxic effect on the other sulfate reducing bacteria.¹⁵ Above phenomenon is due to the elimination of H₂S by purging with N₂.

On the other hand, the pH of the culture is led to alkalinity by eliminating H₂S. The influence of pH on the growth was
examined by using an automatic pH-stat (1 M HCl) under N2 purging. The dependence of specific growth rate and the total growth on the pH of the culture are given in Figure 2. As it can be observed, the total growth increases with the pH, reaching its maximum value, 0.448 g/L, at pH 7.4. The elevation of the specific growth rate with pH has the same pattern as the total growth. In fact, the maximum value of the specific growth rate is obtained at pH 7.4, \( \mu = 0.24 \text{ h}^{-1} \), equal to a mean doubling time of 2.9 h.

As it can be observed in Figure 2, a good fit (full line) of experimental results for the specific growth rate is obtained by using the inhibition model reported by Tang et al.\(^{16}\). The results suggest that hydrogen ion, H\(^+\), is a growth inhibitor for the culture of DvMF and the pH control is important for the DvMF growth. Therefore, high cell densities are only reached by pH control and by the elimination of H\(_2\)S by purging with N\(_2\).

**Composition of Medium.** DvMF was well grown in the presence of yeast extract.\(^{13}\) Yeast extract consists of various amino acids and vitamins, consequently, the medium containing yeast extract has not been suitable for uniform labeling. It is necessary to determine the compositions of medium as sole and small nitrogen sources for the purpose of the efficiency of \(^{15}\)N uniform labeling. The effects of adding yeast extract to medium on the growth are examined as further details on the requirement of yeast extract is not examined for DvMF (Table 1). In the presence of yeast extract, the specific growth rate and the total growth are about 1.4 times that of the culture without yeast extract. While the specific growth rate and the total growth of the culture with vitamins is almost same to that of the culture without additives, it is not clear why yeast extract improves the growth; however, two interpretations can be offered: (1) To facilitate the synthesis of amino acids by the cells, yeast extract provides an exogenous supply of these acids: hence this enormously improved specific growth rate over the growth rate in the medium without yeast extract. (2) The presence of substances in the various amino acids, other acids which can be used as an energy source in addition to the lactate. The use of amino acids as energy substrates by *Desulfovibrio* strain has been especially reported.\(^{17}\) As DvMF does not essentially require yeast extract, we would hereafter grow DvMF on the medium without yeast extract.

The cultures were grown under pH control (pH 7.4) in an anaerobic fermentor on 62.5 mM lactate and 31.7 mM sulfate medium (containing 0.8 mg Fe\(^{2+}\) ion/L) with varying concentrations of ammonium chloride (Fig. 3). Ammonium chloride was found to be limiting for concentration up to 0.3 g/L. In these experiments, the ratio used on sulfate to lactate was 1 : 2. This value is keeping up with the overall equation for the oxidation of lactate by sulfate.\(^{16}\)

\[
2\text{CH}_3\text{CHOHCOO}^- + \text{SO}_4^{2-} \rightarrow 2\text{CH}_3\text{COO}^- + 2\text{CO}_2 + 2\text{H}_2\text{O}
\]

In addition, the growth of is reproducible. Therefore, 0.3 g/L is the concentration of ammonium chloride that was the minimum requirement of nitrogen substrate for the cells to grow under N\(_2\) purging and under pH control.

We decided the composition of medium and the conditions of cultivation from these results. The defined medium has the following composition (per liter distilled water): 0.5 g of KH\(_2\)PO\(_4\); 0.3 g of NH\(_4\)Cl; 10 g of 70% (w/w) sodium lactate;
Uniformly 15N-labeled cytochrome c3 and kept at pH 7.4 and 37 °C.

The uniformly 15N-labeled cytochrome c3 is composed of 107 amino acids containing 4 proline, had 102 backbones NH. This result indicates that cytochrome c3 is uniformly labeled with 15N.

The conditions of cultivation and the composition of medium to obtain uniformly 15N-labeled cytochrome c3 from Desulfovibrio sp. are established in this work. Our next target is to make sequential assignment of cytochrome c3 on the basis of uniformly 15N-labeled cytochrome c3 obtained in this work.

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References


Figure 3. Effects of ammonium chloride concentration on total growth in defined medium.

Figure 4. Section of a 1H-15N HMQC spectrum of ferricytochrome c3. The spectrum was acquired with spectral widths of 4000 Hz for 1H and 5000 Hz for 15N, 4K data points in t2, and 32 transients for each of 512t1 points. 1H chemical shifts were referenced to the 1H2O signal at 4.73 ppm. 15N chemical shifts were referenced to external saturated NH4Cl at 27.34 ppm.

4.5 g of Na2SO4; 0.04 g of CaCl2·2H2O; 0.06 g of MgSO4·7H2O; 0.004 g of FeSO4·7H2O. The culture was continuously stirred at 100 rpm, purged with N2 at 0. 04 VVM and kept at pH 7.4 and 37 °C.

Production of Uniformly 15N-labeled Cytochrome c3. Uniformly 15N-labeled cytochrome c3 has never been obtained. The uniformly 15N-labeled cytochrome c3 would be obtained by replacing nitrogen in the above defined medium with 15NH4Cl (15N; 99.7% Isotec). We obtained 1.82 μmol (25.3 mg) of cytochrome c3 from 97.6 wet grams of DvMF grown in 50 L of the defined medium. Tsuji and Yagi have reported that the yields of cells and proteins were 17.7-26.0 wet grams and 55.5-70.9 nmol in 26 L of the peptone medium. Therefore, our results are satisfactory. Figure 4 showed the 1H-13N HMQC spectrum of uniformly 15N-labeled cytochrome c3. We observed at least 102 signals in Figure 4. DvMF cytochrome c3 was obtained by replacing nitrogen in the above defined medium. Therefore, our results are satisfactory.