Biochimica et Biophysica Acta, 545 (1979) 424—428

BBA 47621

ISOLATION OF *DESULFUROMONAS ACETOXIDANS* CYTOCHROME *c*-551.5 FROM THE MIXED CULTURE *'CHLOROPSEUDOMONAS ETHYLICA'*

MICHAEL D. FIECHTNER and RICHARD J. KASSNER *

Department of Chemistry, University of Illinois at Chicago Circle, Chicago, IL 60680 (U.S.A.)

(Received June 27th, 1978)

Key words: Cytochrome c-551.5; Triheme protein; (Desulfuromonas acetoxidans, Isolation)

Summary

The mixed culture *'Chloropseudomonas ethylica'* strain 2K has been grown on a medium which enhanced the yield of cytochrome *c*-551.5 from *Desulfuromonas acetoxidans*. The cytochrome was purified to homogeneity and an isoelectric point of 8.40 was determined. A determination of the amide content indicated that the cytochrome contains two more amides than previously reported.

Introduction

Desulfuromonas acetoxidans cytochrome *c*-551.5 is a unique protein of low molecular weight containing three hemes [1]. Prior to the isolation of a pure culture of *D. acetoxidans* by Pfennig and Biebl [1], cytochrome *c*-551.5 had been isolated from mixed cultures (*D. acetoxidans* and a phototrophic green sulfur bacterium, *Prosthecochloris aestuarii*) then called *'Chloropseudomonas ethylica'* [2—4].

Attempts in this laboratory to culture *'C. ethylica'* as a source of cytochrome *c*-551.5 according to the method of Bose [5] have consistently yielded large amounts of the green sulfur phototroph relative to *D. acetoxidans*, as evidenced by the large amounts of cytochrome *c*-555 (from *P. aestuarii*) isolated relative to cytochrome *c*-551.5. In addition, the large number of chromatographic steps in previously published isolation procedures for cytochrome *c*-551.5 [3,4], along with discrepancies between reported isoelectric points and that observed by us, has led us to reevaluate the growth conditions, isolation procedure, and the amide content.

* To whom communications regarding this manuscript should be addressed.

In the present report, we wish to suggest an alternative growth medium and describe a simplified isolation procedure which we have found successful in yielding large quantities of cytochrome *c*-551.5 from the mixed culture *'C. ethylica'*. The wide availability of the *'C. ethylica'* cultures, along with the possibility that the two organisms may grow better syntrophically than in pure cultures, points to the desirability of the present procedure.

Methods and Results

Growth conditions. Cultures of *'C. ethylica'* strain 2K (kindly provided by Prof. J. Olson) were grown anaerobically at 28°C in 4 l reagent bottles, illuminated by banks of 40 W incandescent lamps providing an average light intensity of about 10^{-2} joules $\cdot$ cm^{-2} $\cdot$ s^{-1} measured with a YSI-Kettering Model 65 radiometer at the surface of the bottles. 200 ml of a 48 h growth culture were used to inoculate 3800 ml of media and the cells harvested after 48 h with a Sharples Supercentrifuge, resulting in a cell yield of 0.9 g wet paste/l. Upon the suggestion of Prof. Norbert Pfennig (personal communication), the mixed culture was grown on a medium recently described by Pfennig and Biebl [1] for culturing the pure strain *D. acetoxidans*. Sodium malate (2.3 g/l) was added as an electron acceptor, and sodium pyruvate (o.5 g/l) as the carbon source and electron donor for *D. acetoxidans*. The medium also contained 21 μg biotin, 4 g $NaHCO_3$, and 0.3 g $Na_2S \cdot 9H_2O$ per l. Basal salts, sulfide/bicarbonate, pyruvate and malate solutions were autoclaved separately, cooled and mixed resulting in a final pH of 7.2.

Isolation of cytochrome c-551.5. All operations were carried out at 4°C. 170 g of frozen cell paste were thawed and suspended in 0.10 M potassium phosphate buffer, pH 7.0 (hereafter referred to as standard buffer) to give a final volume of 600 ml. After the addition of deoxyribonuclease, the cells were broken by sonication of 200-ml batches for 20 min with a Bronwill Biosonik III using a 3/8 inch diameter probe tip operating at 180 W. The cell suspension was mixed with a magnetic stirrer and cooled with an ice-water bath. The broken cell suspension was centrifuged for 10 min at 95 500 $\times g$ to remove cell debris. The pellet was resuspended in standard buffer, sonicated and centrifuged as before. The combined red-brown supernatant was passed through a DEAE-cellulose column (2.5 X 90 cm) equilibrated with standard buffer. The pink eluate (about 1 l) containing the unadsorbed cytochromes was brought to 40% saturation with ammonium sulfate and centrifuged for 10 min at 16 000 $\times$ *g*. The blue-green pellet was discarded. The supernatant was then brought to 100% saturation with ammonium sulfate and the fine protein precipitate was collected by filtering through a DEAE-cellulose column (7 X 5 cm) equilibrated with standard buffer saturated with ammonium sulfate. The precipitated cytochromes adhered to the top of the column and were subsequently eluted with standard buffer. The cytochrome eluate (350 ml) was concentrated by saturation to 100% with ammonium sulfate and centrifugation for 1 h at 95 500 $\times g$. The red pellet was dissolved in 0.05 M Tris buffer (pH 7.5) containing 0.2 M NaCl and chromatographed on a G-75 Sephadex column (2.5 X 100 cm) equilibrated with the same buffer. The cytochrome fraction was dialyzed versus 0.01 M potassium phosphate buffer (pH 7.0) and applied to a

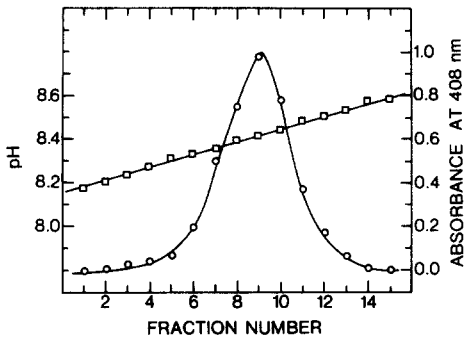

Fig. 1. Elution diagram of cytochrome *c*-551.5 after isoelectric focusing. ○, absorbance at 408 nm; □, pH measured at 4°C. Fraction volumes were approximately 1 ml.

Biorex-70, 100—200 mesh, column (0.9 X 5 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.0). Under these conditions the cytochrome *c*-551.5 bound tightly to the top of the column, whereas the cytochrome *c*-555 passed through the column. Cytochrome *c*-551.5 was eluted with 0.5 M potassium phosphate buffer (pH 7.0), containing 0.5 M NaCl, and dialyzed versus 1 mM NH_3. Subsequent isoelectric focusing resulted in the formation of a single band.

Isoelectric point determination. The isoelectric point, p*I*, was determined by isoelectric focusing using a 110 ml Ampholine column and carrier ampholyte (pH range 7—9, LKB Produkter, AB, Stockholm-Bromma, Sweden) at 4°C for 7 days. pH measurements were made at 4°C using a Radiometer PHM 65 pH meter. Absorbance at 408 nm was monitored with a Cary 14R spectrophotometer. As shown in Fig. 1, cytochrome *c*-551.5 exhibits a p*I* of 8.40.

Yield and purity. The present isolation procedure yielded 1.7 μmol cytochrome *c*-551.5 and 1.5 μmol cytochrome *c*-555 as determined spectrophotometrically using the extinction coefficients of Shioi et al. [4]. The electrofocused cytochrome *c*-551.5 exhibited the following absorbance ratios: $A_{\alpha(red)}/A_{280(ox)} = 3.22$; $A_{\gamma(red)}/A_{\gamma(ox)} = 1.63$; $A_{\gamma(red)}/A_{\alpha(red)} = 6.44$. These values are in good agreement with those previously reported [3,4,6].

Amino acid analysis. Samples of the cytochrome were hydrolyzed anaerobically in 6 N HCl at 110°C for 18 and 40 h. After hydrolysis the acid was removed under vacuum. The amino acid composition was determined with a Durrum model D-5 amino acid analyzer. With the exception of cysteine and proline, which were not assayed, the composition was found to be identical to the sequence data reported by Ambler [7] for cytochrome *c*-551.5 from *C. ethylica.*

Amide determination. The total amide content (glutamine + asparagine) of cytochrome *c*-551.5 was determined by acid hydrolysis followed by a photometric ninhydrin ammonia assay. The techniques used were those described by Wilcox [8], and Moore and Stein [9]. The determinations were corrected by using blank solutions which were handled in an identical manner as the samples, and by measuring the free ammonia in each sample. The accuracy of the method was confirmed by assaying commercial samples of cytochrome *c* (Sigma Type VI) and glutamine (Sigma, NH_3 free). The results of five separate

determinations indicated an amide content of 6 residues per molecule of cytochrome *c*-551.5.

Heme determination. The heme content of cytochrome *c*-551.5 was determined spectrophotometrically for an accurately weighed protein sample. The cytochrome was completely desalted by chromatography (2X) on a 35 X 2.5 cm G-25-80 Sephadex column equilibrated with doubly distilled water, and thoroughly dessicated prior to weighing. A value of 3 hemes per molecule was determined using an extinction coefficient of 9.6 $mM^{-1} \cdot cm^{-1}$ per heme [3] for the α-peak of the oxidized cytochrome.

Discussion

The present results indicate that the medium used to grow the mixed culture yields large and approximately equimolar amounts of cytochromes *c*-551.5 and *c*-555. In contrast, mixed cultures grown on the medium of Bose [5], yielded approximately the same amount of cytochrome *c*-555, but only about 1/4 the amount of cytochrome *c*-551.5. These observations suggest an advantage of the present method over that of Bose [5] for the preparation of cytochrome *c*-551.5.

The simplified isolation procedure described is based on a newly determined value of the isoelectric point. Meyer et al. [3] have reported an isoelectric point of 4.1 for cytochrome *c*-551.5 and have observed binding of the protein to DEAE-cellulose, an anion exchange resin, at pH 7.3. These observations are in agreement with those of Olson and Shaw [2] who found that the elution of the cytochrome from DEAE-cellulose at pH 7.8 required approximately 0.2 M NaCl. In contrast, Shioi et al. [4] reported an isoelectric point of 7.77 and observed the cytochrome to adsorb on Amberlite CG-50, a cation exchange resin, at pH 7.0. We have found that, at pH 7.3, cytochrome *c*-551.5 binds weakly to DEAE-cellulose and only at low buffer concentration (1 mM Tris). However, the protein adsorbs very strongly on Biorex-70, a cation exchange resin, at pH 7.0. These latter observations suggest that cytochrome *c*-551.5 has a basic isoelectric point and are consistent with a measured value of 8.40. The discrepancy between this value and that reported by Shioi et al. [4], may indicate that the present purification procedure yields a protein which has undergone the least deamination.

The reported amino acid sequence of *'C. ethylica'* cytochrome *c*-551.5 [7] shows 5 Asp, 3 Glu, 4 Asn, 12 Lys, and 6 His which are coordinated to the 3 heme irons [8]. In addition to the above acidic and basic amino acid residues, there are 6 propionic acid groups associated with the 3 hemes. Including the terminal amino and carboxyl groups, there would be 13 free amino groups and 15 carboxyl groups. On this basis one would expect a neutral or slightly acidic isoelectric point, even allowing for the small perturbations of pK_a values by the protein environment. The observed basic isoelectric point suggests that a greater number of carboxyl groups than previously reported [7], may be amidated in the native protein. This suggestion is supported by the results of the present amide determination which indicate that the cytochrome contains two more amides than reported in the amino acid sequence [7]. These additional amides would indicate the presence of 13 free amino and 13 carboxyl

groups. Considering that one or more of the propionyl side chains of the three heme groups may be unionized as in the monoheme *c*-type cytochromes [10], the number of free amino groups may actually exceed the number of ionizable carboxyl groups. These observations appear to be fully consistent with the somewhat basic isoelectric point observed.

The function of cytochrome *c*-551.5 remains to be elucidated. Its structural similarity to the 4 heme cytochrome c_3 from *Desulfovibrio vulgaris* [11] suggests a possible role in sulfur metabolism. A significant difference between *D. vulgaris* and *D. acetoxidans*, however, is that while the latter can grow using colloidal sulfur as a terminal electron acceptor, the former cannot [1,6]. As Probst et al. [6] have speculated, the structural differences between cytochromes c_3 and *c*-551.5 may account for this behavior. The availability of these multiheme cytochromes will certainly be important in constructing the sequence of reactions involved in bacterial sulfur metabolism, as well as in the study of electron transfer mechanisms involving heme in both intra and intermolecular reactions.

It is the intent of this communication to help improve the availability of cytochrome *c*-551.5.

Acknowledgements

We wish to thank Dr. Emmanuel Margoliash and Dr. Dennis Borden for the amino acid analysis, and Prof. Norbert Pfennig for the helpful suggestion about the culture medium.

References

1 Pfennig, N. and Biebl, H. (1976) Arch. Microbiol. 110, 3—12
2 Olson, J.M. and Shaw, E.K. (1969) Photosynthetica 3, 288—290
3 Meyer, T.E., Bartsch, R.G. and Kamen, M.D. (1971) Biochim. Biophys. Acta 245, 453—464
4 Shioi, Y., Takamiya, K. and Nishimura, M. (1972) J. Biochem. 71, 285—294
5 Bose, S.K. (1963) in Bacterial Photosynthesis (Gest, H., San Pietro, A., Vernon, L.P., eds.), pp. 501—510, Antioch Press, Yellow Springs, Ohio
6 Probst, I., Brushi, M., Pfennig, N. and LeGall, J. (1977) Biochim. Biophys. Acta 460, 58—64
7 Ambler, R.P. (1971) FEBS Lett. 18, 351—353
8 Wilcox, P.E. (1967) Methods in Enzymology 11, 63—93
9 Moore, S. and Stein, W.H. (1954) J. Biol. Chem. 211, 907—913
10 Timkovich, R. and Dickerson, R.E. (1976) J. Biol. Chem. 251, 4033—4046
11 Dobson, C.M., Hoyle, N.J., Geraldes, C.F., Wright, P.E., Williams, R.J.P., Bruschi, M. and LeGall, J. (1974) Nature 249, 425—429