of them probably contributes, at least, to maintain secondary and tertiary structures for active conformation of the protein.

The authors are indebted to Sankyo Co. for the supply of the crystalline yeast cytochrome *c* and to Miss S. SAKAKI for her help in some of this work. They are also grateful to Dr. E. MARGOLIASH for providing them the information of the amino acid sequences of swine and chicken cytochromes *c* before publication.

Institute for Protein Research,
Osaka University,
Kita-ku, Osaka (Japan)

KOZO NARITA
KOITI TITANI
YOSHIHITO YAOI
HIRONORI MURAKAMI

[1] K. NARITA, K. TITANI, Y. YAOI, H. MURAKAMI, M. KIMURA AND J. VANĚČEK, *Biochim. Biophys. Acta*, 73 (1963) 670.
[2] E. GROSS AND B. WITKOP, *J. Biol. Chem.*, 237 (1962) 1856.
[3] K. TITANI, K. NARITA AND K. OKUNUKI, *J. Biochem. (Tokyo)*, 51 (1962) 350.
[4] E. MARGOLIASH, E. L. SMITH, G. KREIL AND H. TUPPY, *Nature*, 192 (1961) 1126.
[5] H. MATSUBARA AND E. L. SMITH, *J. Biol. Chem.*, 237 (1962) PC 3575.
[6] E. MARGOLIASH, S. B. NEEDLEMAN AND J. W. STEWART, *Acta Chem. Scand.*, 17 (1963) 250.
[7] J. I. HARRIS, *Biochem. J.*, 71 (1959) 451.
[8] J. I. HARRIS AND P. ROOS, *Biochem. J.*, 71 (1959) 434.

Received October 14th, 1963

Biochim. Biophys. Acta, 77 (1963) 688–690

PN 10080

Soluble cytrochromes in *Escherichia coli*

It is well known that four cytochrome pigments occur in *Escherichia coli*, *i.e.* membrane-bound cytochromes b_1, a_1 and a_2 (ref. 1) and a CO-binding pigment or cytochrome *o* of unknown intracellular localization[2,3]. Recently a fifth pigment has been added to this list by GRAY *et al.*[4,5] who showed that a soluble cytochrome of the *c* type ("cytochrome *c*-551") is synthesized in *E. coli* and related facultative anaerobes when they are grown anaerobically. While studying the enzyme system involved in nitrate respiration[6], we have also noticed the presence of a closely similar cytochrome ("cytochrome *c*-552") in a soluble fraction obtained from *E. coli* (Yamagutchi) grown anaerobically in the presence of nitrate. Furthermore, it has been disclosed that cytochrome *c*-552 was always accompanied by smaller amounts of a second cytochrome of the *c* type ("cytochrome *c*-550") and a pigment of the *b* type ("cytochrome *b*-562"). This paper describes briefly the purification and properties of these soluble cytochromes.

E. coli (Yamagutchi) was cultivated anaerobically in a medium containing glucose, inorganic salts (including 0.1% $NaNO_3$) and 0.05% yeast-extract powder, and the cells harvested at the log phase were disrupted in 0.1 M phosphate (pH 7.0) by sonic oscillation (9 kcycles) for 10 min. When the sonicate was centrifuged at 105 000 × *g* for 3 h, practically all of the cytochrome b_1 as well as the cytochromes of the *a* type were recovered in the particulate sediment; the cytochrome b_1 content in the intact cells was determined by the previously reported method[7] to be 0.34 mμmole/mg of

protein. The soluble supernatant fraction, on the other hand, contained cytochrome *c*-552 as evidenced by absorption peaks at 552, 523 and 420 mμ in the dithionite-reduced minus oxidized difference spectrum. Assuming appropriate molar extinction coefficients (see below), its content in the intact cells was estimated to be about 0.06 mμmole/mg of protein. This value was considerably higher than those reported by GRAY *et al.*[4] for cytochrome *c*-551 in other strains of *E. coli*.

For purification of cytochrome *c*-552, the well-packed cells were treated in a blendor for 3 min with 1 vol. of *n*-butanol, and after separation of layers the upper butanol layer was discarded by careful decantation. The residue was homogenized with 1 vol. of water and dialysed overnight against a large amount of water at 0°. The dialysed suspension was centrifuged at 78 000 × *g* for 30 min and the resultant supernatant was fractionated at pH 8.0 with ammonium sulfate in the presence of 0.3% basic lead acetate. The precipitate collected between 0.50 and 0.95 saturation was dialysed against water and applied to a hydroxylapatite column equilibrated with 0.01 M phosphate (pH 7.0). A small amount of a red pigment thereby passed through the column and was identified as a cytochrome with a reduced α-band at 550 mμ (cytochrome *c*-550). On washing the column with phosphate buffer (pH 7.0) of increasing concentration, the main red band containing cytochrome *c*-552 was eluted out at a concentration of 0.05 M. A third red band still remaining on the top of the column was finally eluted out with 0.25 M pyrophosphate (pH 7.0); this fraction contained cytochrome *b*-562. The eluate containing cytochrome *c*-552 was dialysed against water and adsorbed on a DEAE-cellulose column equilibrated with 0.01 M phosphate (pH 7.0). On washing with the same buffer, the pigment migrated as a broad band and was eluted out. The diluted eluate thus obtained was further purified and concentrated by gel filtration through a Sephadex G-75 column. In a typical experiment about 6 mg of purified cytochrome *c*-552 was thus obtained from 250 g of wet cells in a yield of 12%.

As shown in Fig. 1, the spectra of purified cytochrome *c*-552 were characteristic of *c*-type pigments, with absorption peaks at 552 mμ (α), 523 mμ (β) and 420 mμ

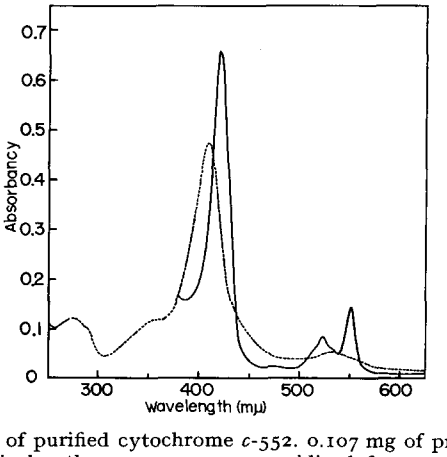

Fig. 1. Absorption spectra of purified cytochrome *c*-552. 0.107 mg of protein/ml of 0.05 M phosphate buffer (pH 7.0). Optical path, 1.0 cm. ·····, oxidized form; ———, dithionite-reduced form.

(Soret) in the reduced form and at 532 mμ, 409 mμ (Soret) and 355 mμ in the oxidized form. It is most likely from these spectra that this cytochrome is identical with cytochrome *c*-551 reported by GRAY *et al.*[4]. Although the purity of the preparations has not yet been determined, the fact that the reduced α-band was 1.2–1.5-fold as high as the protein peak at 280 mμ suggested satisfactorily high purity of the preparations. The purified pigment gave a spectrum with maxima at 550, 520 and 414 mμ when converted into the reduced pyridine hemochromogen form, and the heme moiety was not split off from the protein by acid acetone. The molar extinction coefficients employed above for estimation of this pigment were calculated from Fig. 1 and the known molar extinction coefficients for pyridine hemochromogen of heme *c* (ref. 8). A minimum molecular weight of about 26 000 was obtained for the best preparation of cytochrome *c*-552 from its heme *c* content.

Purified cytochrome *c*-552 could be reduced by dithionite, but borohydride, cysteine, ascorbate and ferrocyanide failed to reduce the pigment even under anaerobic conditions. The standard oxidation–reduction potential of the pigment at pH 7.0 was estimated roughly to be about −200 mV by the use of a redox dye, phenosafranin. It was rapidly reducible by NADH or NADPH in the presence of a soluble protein fraction from anaerobically grown *E. coli* cells, and also slowly reducible by NADPH in the presence of a NADPH-oxidizing flavoprotein purified from rabbit-liver microsomes[9]. The cytochrome reduced enzymatically or by dithionite was reoxidized very rapidly by bubbling air, indicating its autoxidizability. However, the pigment combined with neither cyanide nor CO in both the oxidized and reduced states at a pH range between 5.0 and 8.0.

The second cytochrome of the *c* type (cytochrome *c*-550), obtained in a small amount in the hydroxylapatite column chromatography, showed absorption peaks at 550 mμ (α), 521 mμ (β) and 418 mμ (Soret) in the reduced form and at 408 mμ (Soret) in the oxidized form. Spectrophotometrically, this pigment was different clearly from cytochrome *c*-552 and rather similar to mammalian cytochrome *c* or to a pigment recently reported in *Salmonella typhimurium*[10]. This cytochrome showed no affinity for cyanide and CO.

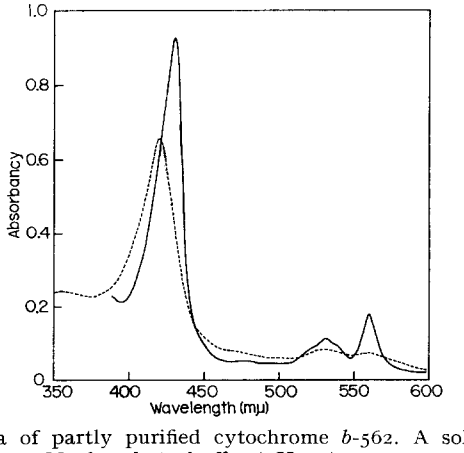

Fig. 2. Absorption spectra of partly purified cytochrome *b*-562. A solution of undetermined protein concentration in 0.05 M phosphate buffer (pH 7.0). · · · · · · · , oxidized form; ———, dithionite-reduced form.

The third hemoprotein isolated in the hydroxylapatite chromatography (cytochrome *b*-562) could better be purified from the soluble fraction obtained from the sonicate of intact cells by DEAE-cellulose column chromatography followed by ammonium sulfate fractionation and hydroxylapatite column chromatography. As shown in Fig. 2, the pigment thus purified showed absorption bands at 562 mμ (α), 532 mμ (β) and 427 mμ (Soret) in the reduced form and at 420 mμ (Soret) in the oxidized form. These spectra resembled those reported for "cytochrome *b*" of *Bacterium anitratum*[11]. The prosthetic group of this cytochrome was identified as protoheme from its pyridine hemochromogen spectrum. This pigment was definitely different from cytochrome b_1 (ref. 7) not only in its spectral properties but also in its unusual non-autoxidizability. Cyanide and CO again showed no affinity to this cytochrome.

The three soluble cytochromes described in this paper seemed to be present also in *E. coli* B, *E. coli* var communior and *Serratia marcescens* grown under similar conditions.

Institute for Protein Research,
Osaka University,
Kita-Ku, Osaka (Japan)

TAKESHI FUJITA
RYO SATO

[1] A. TISSIÈRES, in J. E. FALK, R. LEMBERG AND R. K. MORTON, *Haematin Enzymes*, Pergamon Press, Oxford, 1961, p. 218.
[2] B. CHANCE, *J. Biol. Chem.*, 202 (1953) 383.
[3] L. N. CASTOR AND B. CHANCE, *J. Biol. Chem.*, 234 (1959) 1587.
[4] C. T. GRAY, J. W. T. WIMPENNY, D. E. HUGHES AND M. RANLETT, *Biochim. Biophys. Acta*, 67 (1963) 157.
[5] J. W. T. WIMPENNY, M. RANLETT AND C. T. GRAY, *Biochim. Biophys. Acta*, 73 (1963) 170.
[6] E. ITAGAKI, T. FUJITA AND R. SATO, *J. Biochem. (Tokyo)*, 52 (1962) 131.
[7] T. FUJITA, E. ITAGAKI AND R. SATO, *J. Biochem. (Tokyo)*, 53 (1963) 282.
[8] D. L. DRABKIN, *J. Biol. Chem.*, 146 (1942) 605.
[9] H. NISHIBAYASHI, T. OMURA AND R. SATO, *Biochim. Biophys. Acta*, 67 (1963) 520.
[10] M. RICHMOND AND N. O. KJELDGAARD, *Acta Chem. Scand.*, 15 (1961) 226.
[11] J. G. HAUGE, *Arch. Biochem. Biophys.*, 94 (1961) 308.

Received September 16th, 1963

Biochim. Biophys. Acta, 77 (1963) 690–693

PN 10077

Citryl-CoA and the citrate condensing enzyme

Previous work from this laboratory[1,2] has shown that a preparation of synthetic citryl-CoA which was cleaved by the citrate cleavage enzyme to acetyl-CoA and oxaloacetate, inhibited the citrate condensing enzyme. It is the purpose of this paper to extend our earlier observations and present evidence which indicates that the inhibition by citryl-CoA is competitive for both acetyl-CoA and oxaloacetate. It is further shown that the slow cleavage of citryl-CoA catalyzed by condensing enzyme, which was first reported by EGGERER AND REMBERGER[3], occurs at the same site as acetyl-CoA and oxaloacetate condensation.

Abbreviation: DTNB, 5,5′-dithio-*bis*-2-nitrobenzoic acid.