Volume 249, number 1, 67-69 FEB 07180 May 1989

$\alpha_3\beta_3$ complex of thermophilic ATP synthase

Catalysis without the γ-subunit

Yasuo Kagawa, Shigeo Ohta and Yoko Otawara-Hamamoto

Department of Biochemistry, Jichi Medical School, Minamikawachi-machi, Tochigi-ken, 329-04, Japan

Received 29 March 1989; revised version received 12 April 1989

A complex of the α- and β-subunits of thermophilic ATP synthase showed about 25% of the ATPase activity of the $\alpha\beta\gamma$ complex. The $\alpha_3\beta_3$ hexamer structure was analyzed by sedimentation (11.2 S) and gel filtration (310 kDa). Dilution of the $\alpha\beta$ complex caused dissociation of the complex and rapid loss of ATPase activity which was restored by addition of the γ-subunit. A previous method using urea for isolating the subunits resulted in an $\alpha\beta$ complex with lower activity than that prepared by over-expression of the genes. The $\alpha\beta$-ATP complex was formed from the $\alpha\beta$ complex, ADP and P_i in the presence of dimethyl sulfoxide.

F_1-ATPase; Oligomer, $\alpha_3\beta_3$; ATPase; ATP synthesis; (Thermophilic bacteria)

1. INTRODUCTION

ATP synthase is a major enzyme for the energy supply of cells. Its catalytic portion is termed F_1 and consists of the α, β, γ, δ and ϵ subunits [1-3]. On reassembly of purified subunits, only the complexes containing $\alpha\beta\gamma$ of *E. coli* F_1 (EF_1) ATPase showed activity [3]. As truncation [3] and cross-linking [4] of γ resulted in loss of ATPase activity, the essential role of γ during ATP synthesis was proposed [4]. In contrast to these results obtained with EF_1, a mixture of α and β of thermophilic F_1 (TF_1) showed weak ATPase activity [2,5], complex formation [5] and conformational $\alpha\beta$ interactions detected by H-D exchange reactions [6]. These results on the $\alpha\beta$ complex have been neglected by others working on F_1. Recently, the subunits of TF_1 were sequenced [7] and prepared by over-expression of their respective genes [7]. The over-expressed TF_1 subunits were more active than urea-treated subunits (uα, uβ) prepared according to classical methods [2,8]. The ATPase activity of the $\alpha\beta$ complex, prepared with our genes [7], showed asymmetric and allosteric properties [9]. As enzyme-bound ATP is synthesized by TF_1 from ADP and P_i [10], the $\alpha\beta$ complex is expected to display this activity.

This paper reports determination of the hexamer structure and activity of the $\alpha_3\beta_3$ complex which dissociates easily.

Correspondence address: Y. Kagawa, Department of Biochemistry, Jichi Medical School, Kawachi-gun, Tochigi 329-04, Japan

Abbreviations: F_1, catalytic portion of ATP synthase; TF_1, F_1 from thermophilic bacterium PS3; EF_1, F_1 from *E. coli*; AMPPNP, adenyl-5'-yl imidodiphosphate; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; Mes, 4-morpholineethanesulfonic acid; α, β and γ, subunits of F_1; HPLC, high-performance liquid chromatography

2. MATERIALS AND METHODS

TF_1, uα and uβ were prepared by the urea method [8]. Thermophilic α, β and γ were obtained by over-expression of the respective genes [7], in *E. coli* strain DK8 lacking all EF_1 genes. Further purification of α and β to microcrystals, using a Green A column, was carried out as described (Shirakibara, Y., to be reported elsewhere). Gel filtration and sedimentation analysis of the $\alpha\beta$ mixture were performed as described in the legends to figs 1 and 2, respectively, and for ATPase activity, according to

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

the legends to figs 3 and 4. ATP synthesis was measured by adding the $\alpha\beta$-ADP complex (final 2 mg/ml) to 20 mM $^{32}P_i$ (8200 cpm/nmol), 40% DMSO, 80 mM Mes-NaOH (pH 6.0) and 2 mM $MgSO_4$ as in [10].

3. RESULTS AND DISCUSSION

3.1. *Association of the α- and β-subunits into the $\alpha_3\beta_3$ complex*

The mixture of α and β eluted to give two distinct peaks corresponding to 310 and 53 kDa, respectively, on gel filtration (fig.1). The larger peak corresponded to $\alpha_3\beta_3$ (319 582 Da from the DNA sequence [7]), which eluted soon after that of TF_1 (380 kDa by HPLC [11]). The smaller peak contained both α and β. When analyzed separately, both α and β gave only a single peak at about 53 kDa. Similarly, two components were observed during sedimentation analysis (fig.2). The rapidly sedimenting component, 11.2 S, corresponded to $\alpha_3\beta_3$, which sediments more slowly than F_1 (11.9 S [12]). The s value of the smaller component, 4.1 S, corresponded to that of the mixture of free α and β. Polyacrylamide gel electrophoresis of the mixture of α and β [2] revealed no additional band apart from those of α and β due to dilution for 2 h. When the mixture of α and β was diluted from 5 to 1 mg/ml, the $\alpha\beta/(\alpha+\beta)$ ratio in the HPLC elution curve decreased (fig.1). A symmetrical arrangement of deuterated α- and β-subunits in the hexamer was shown by cold neutron scattering (with Ito, Y., Harada, M., Schefer, J. and Schoenborn, B.P., in preparation).

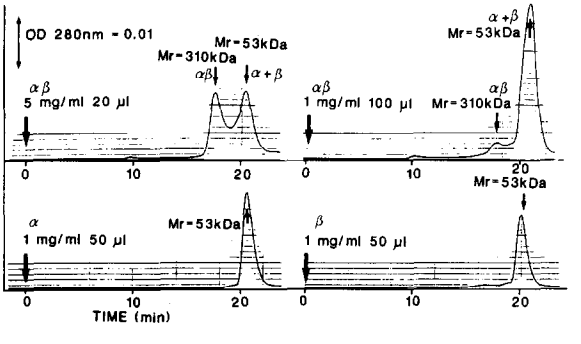

Fig.1. Gel filtration of the $\alpha\beta$ mixture. The indicated amounts of samples were analyzed in a Waters model 441 HPLC apparatus with a column of TSK gel G 400 SW (7.5 × 600 mm) at 22°C, and samples eluted with 0.1 M NaCl and 0.1 M Tris-Cl (pH 7.8) at a rate of 1 ml/min.

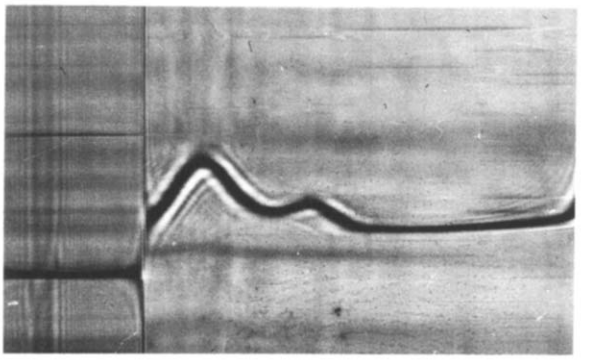

Fig.2. Sedimentation pattern of a mixture of α- and β-subunits at 40 min. The mixture of α and β [(1:1, 6.3 mg/ml, in 50 mM Tris-HCl (pH 7.75), 0.1 mM EDTA and 0.1 mM DTT] was analyzed in a Beckman Spinco model E with rotor An-D, at 48 000 rpm and 20.0°C. s(20,water) values were 11.210 S (right) and 4.091 S (left).

3.2. *Catalytic activities of the easily dissociating $\alpha_3\beta_3$ complex*

The low ATPase activities were reconstituted when subunits were added directly to a cuvette (fig.3). The very weak ATPase activity of mesophilic β [13] was also detected even in β of crystalline purity, but was removed by site-directed

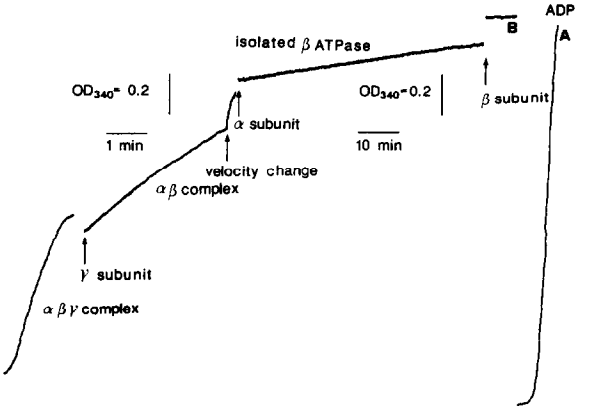

Fig.3. ATPase activity of the β-subunit, and mixtures of the α- and β-, and α-, β- and γ-subunits. Activity was measured by coupling to the oxidation of NADH in a Beckman model 35 spectrophotometer (at 340 nm) in the presence of 4 mM ATP, 5 mM phospho*enol*pyruvate, 0.3 mM NADH, 50 mM Tris-SO_4 (pH 7.9) and 4 µg/ml each of lactate dehydrogenase and pyruvate kinase in a final volume of 0.25 ml at 22°C. (A) 5 µl of 0.2 M ADP. (B) At the times indicated by arrows, 80 µg subunit β (20 µl), 80 µg subunit α (20 µl) and 20 µg subunit γ (20 µl, turbid suspension) were added; ATPase activities were 1.6, 81 and 320 nmol/min per mg β, respectively.

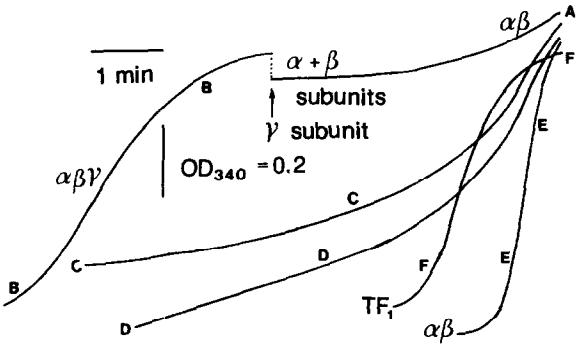

Fig.4. Inactivation of the $\alpha\beta$ ATPase by dilution, and its reactivation by the γ-subunit. Using a Beckman model DU70 spectrophotometer, the following amounts of the mixture of α and β (1:1, 4 mg/ml) or TF_1 were added to 50 μl of the reaction medium described in the legend to fig.3. (A) 2 μg mixture (0.5 μl, final 39.6 μg/ml), (B) 20 μg subunit γ (1 μl) added to (A), (C) 5.6 μg mixture (1.4 μl), (D) 8 μg mixture (2 μl), (E) 20 μg mixture (5 μl, final 363.6 μg/ml), and (F) 2 μg TF_1 (5 μl, final 36.4 μg/ml).

mutagenesis of the catalytic site of β [14]. After preincubation of α with β (4 mg/ml each, 22°C), the specific ATPase activity of the $\alpha\beta$ mixture was 556 nmol/min per mg (K_m = 0.36 mM) (fig.4E), while that of $\alpha\beta\gamma$ amounted to 1.9 μmol/min per mg (K_m = 0.28 mM). Dilution of the mixture of α and β caused rapid inactivation, recovery being achieved by the addition of γ (fig.4A,B). Previous ATPase assays ($\alpha\beta < 40$ μg/ml, 10 min incubation) [2,5] underestimated the activity of $\alpha\beta$. The $\alpha\beta$ was AMPPNP-sensitive and azide-insensitive. The mixture of uα and uβ showed low ATPase activity (11–34 nmol/min per mg β). Moreover, once uα had been mixed with β, the addition of α did not reconstitute the activity of the mixture of α and β. Similarly, restoration of the ATPase activity of the uβ+α mixture by the addition of β was poor, indicating the formation of less active uαuβ, u$\alpha\beta$ and u$\beta\alpha$ complexes. Enzyme-bound [^{32}P]ATP [10] (0.202 mol ATP/mol $\alpha_3\beta_3$) was synthesized by $\alpha\beta$ in 40% DMSO. The extent of carbamoylation of the subunits with 8 M [^{14}C]urea (185 kBq/mmol) during the isolation procedure [8] was slight (1.02 mol/mol α and 0.54 mol/mol β).

Studies on EF_1 [3,4], in contrast to our findings with TF_1-$\alpha\beta$, indicated that γ is essential for ATPase activity. These variations may be due to differences between unstable EF_1 and stable TF_1 [1]. Considering the highly conserved structures of both the α- and β-subunits in different species [1–3,7], the role of the γ-subunit [3,4] should be reassessed.

Acknowledgements: We thank Professor T. Takagi and Ms M. Sakai, Protein Institute, Osaka, for sedimentation analyses. This work was supported by Special Grant no. 62617006 from the Ministry of Education, Science and Culture of Japan for studies on bioenergy.

REFERENCES

[1] Kagawa, Y. (1984) in: Bioenergetics (Ernster, L. ed.) pp. 149–186, Elsevier, Amsterdam.
[2] Yoshida, M., Sone, N., Hirata, H. and Kagawa, Y. (1977) J. Biol. Chem. 252, 3480–3485.
[3] Futai, M. and Kanazawa, H. (1983) Microbiol. Rev. 47, 285–312.
[4] Kandpal, R.P. and Boyer, P.D. (1987) Biochim. Biophys. Acta 890, 97–105.
[5] Kagawa, Y. and Nukiwa, N. (1981) Biochem. Biophys. Res. Commun. 100, 1370–1376.
[6] Ohta, S., Tsuboi, M., Yoshida, M. and Kagawa, Y. (1980) Biochemistry 19, 2160–2168.
[7] Ohta, S., Ishizuka, M., Yohda, M., Hirata, H., Hamamoto, T., Otawara-Hamamoto, Y., Matsuda, K. and Kagawa, Y. (1988) Biochim. Biophys. Acta 933, 141–155.
[8] Kagawa, Y. and Yoshida, M. (1979) Methods Enzymol. 55, 781–787.
[9] Yoshida, M., Miwa, K., Ohtsubo, M., Denda, K., Date, T. and Allison, W.S. (1988) Abstr. 11th Annu. Meet. J. Soc. Mol. Biol. (in Japanese) 156.
[10] Yohda, M., Kagawa, Y. and Yoshida, M. (1986) Biochim. Biophys. Acta 850, 429–435.
[11] Yoshida, M., Sone, N., Hirata, H., Kagawa, Y. and Ui, N. (1979) J. Biol. Chem. 254, 9525–9533.
[12] Penefsky, H.S. and Warner, R.C. (1965) J. Biol. Chem. 240, 4694–4702.
[13] Harris, D.A., Boork, J. and Baltscheffsky, M. (1985) Biochemistry 24, 3876–3883.
[14] Yohda, M., Ohta, S., Hisabori, T. and Kagawa, Y. (1988) Biochim. Biophys. Acta 933, 156–164.