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In vitro heat effect on heterooligomeric subunit assembly of thermostable indolepyruvate ferredoxin oxidoreductase

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Abstract Indolepyruvate ferredoxin oxidoreductase (IOR) from hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1 catalyzes the oxidative decarboxylation of arylpyruvates by forming a heterooligomeric complex ($\alpha_2\beta_2$). The genes *iorA* and *iorB* which encode respective α and β subunits, were coexpressed heterologously in *Escherichia coli* cells under anaerobic conditions. IOR activity was detected from the cell extract containing both subunits and its activity was enhanced by in vitro heat treatment prior to the assay. The *iorA* and *iorB* were expressed individually and each subunit was examined for enzymatic activity with and without heat treatment. IOR activity was detected neither from the extract of α subunit nor β subunit. The α and β subunits were mixed and then IOR activity was examined. Weak IOR activity was detected without heat treatment, however, upon heat treatment its activity was enhanced. The mixture of individually heat treated α and β subunits did not possess any IOR activity even though the mixed sample was heat treated again. IOR α and β subunits were individually purified to homogeneity, mixed with or without heat treatment and subunit assembly was examined by determining molecular mass. Upon heat treatment, inactive α and β were converted to an active high molecular weight complex (195 kDa) which corresponds to the $\alpha_2\beta_2$ structure. However, the active complex was not formed without heat treatment, suggesting that high temperature environments are important for the heterooligomerization of IOR subunits.

Key words: Oxidoreductase; Structural maturation; Hyperthermophile; Archaea; *Pyrococcus*

1. Introduction

The amino acid sequence possesses the information for specifying the three dimensional structure of a protein. Besides sequence information, some accessory factors may play an important role for proper folding. In hyperthermophilic microorganisms which can grow above 90°C, additional factors such as high concentrations of potassium ion [1] and the novel sugar di-inositol-1′-phosphate, 2,3-diphosphoglycerate [2] or molecular chaperones [3,4] are considered to play important roles for exact tertiary structure formation. Furthermore, recent studies indicate that high temperature itself has significant effects on the proper folding of thermostable proteins. Effect of heat treatment on proper oligomerization was well studied for homohexameric thermostable glutamate dehydrogenase (*Pk*-GDH) from *Pyrococcus kodakaraensis* [5,6] and other thermostable GDHs [7–9]. Recombinant *Pk*-GDH heterologously expressed in *Escherichia coli* formed a different structure from that of natural GDH. Upon in vitro heat treatment, the structure of recombinant *Pk*-GDH was converted to a different form which was closer to the natural form. Such an in vitro structural conversion induced by heat was also reported from various kinds of thermostable proteins. D-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Thermotoga maritima*, which possesses a homotetrameric structure, needs a relatively high temperature for efficient subunit assembly for in vitro reconstitution [10]. When reconstitution was performed at low temperature (0°C) by using a chemically denatured fraction, GAPDH exists as an inactive intermediate. However, high temperature environment induced structural conversion to form proper tetrameric structure [11]. In the case of a low molecular weight monomeric protein such as a ferredoxin, structural conversion by heat was also observed [12]. These observations indicate a close relationship between high temperature and proper assembly for thermostable proteins. In spite of various analyses of heat effect on homooligomeric structures, a limited number of studies was reported for heterooligomeric proteins. Especially how heterooligomeric enzymes from hyperthermophiles form a proper tertiary structure during exposure to heat has been unclear. In this report, heat inducible assembly of a heterooligomeric enzyme has been studied using indolepyruvate ferredoxin oxidoreductase (IOR) as a model protein.

The 2-keto acid oxidoreductases involved in different metabolic reactions are reported from various microorganisms [13–19]. Indolepyruvate ferredoxin oxidoreductase (IOR) is one among the three known ferredoxins and coenzyme A dependent 2-keto acid oxidoreductases involved in novel amino acid metabolic pathways found only in peptide utilizing hyperthermophilic archaea [15–18]. IOR oxidizes arylpyruvates such as indolepyruvate, phenylpyruvate, and *p*-hydroxyphenylpyruvate, which originate from the aromatic amino acids tryptophan, phenylalanine and tyrosine, respectively, through the reaction of aromatic aminotransferases. IOR is known to form a heterooligomeric structure composed of two kinds of subunits, α and β [17]. The genes *iorA* and *iorB* which encode respective α and β subunits, have been cloned and sequenced from *Pyrococcus kodakaraensis* KOD1 [18]. It has been reported that the α subunit of *Pk*-IOR has a mosaic structure composed of features of various oxidoreductase subunits from pyruvate ferredoxin oxidoreductase (POR) and 2-ketoisovalerate ferredoxin oxidoreductase (VOR) [18].

In the present study, *iorA* and *iorB* genes were expressed individually in *E. coli* and each subunit was purified. α and β subunits were combined in vitro and the effect of heat on subunit association to form the proper oligomeric structure was examined.

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2. Materials and methods

2.1. Host bacterial strains, plasmids and media

Pyrococcus kodakaraensis KOD1 was isolated from a solfatara at a wharf on Kodakara Island, Kagoshima, Japan [20,21]. The *Escherichia coli* strains used in DNA manipulations were: JM109 (*recA1-supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]), and TGI (*supE hsd* Δ*5 thi* Δ (*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]). *E. coli* BL21 (DE3) (*hsdS gal* λ*lc*I*ts*857 *ind1 Sam*7 *nin*5 *lacUV5*-T7 gene *1*) was used to overexpress *iorA* and *iorB* genes. *E. coli* strains TG1 and JM109 were cultivated in Luria-Bertani medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl in 1 l deionized water, pH 7.0) at 37°C. NZCYM medium (10 g NZ amine, 5 g yeast extract, 1 g casamino acids, 5 g NaCl and 2 g $MgSO_4 \cdot 7H_2O$ in 1 l deionized water, pH 7.0) was used for BL21 (DE3) cultivation. Plasmids pUC18/pUC19 and pET-8c were used as cloning vectors. Ampicillin (Meiji Seika, Tokyo, Japan) was used routinely at a final concentration of 50 μg/ml.

2.2. Recombinant DNA manipulations

DNA manipulation was performed by standard methods as described previously [22]. Restriction enzymes and other modifying enzymes were purchased from Takara Shuzo, Kyoto, Japan. Chromosomal DNA from *Pyrococcus kodakaraensis* KOD1 was prepared by the sarcosyl method and purified by CsCl equilibrium density gradient ultracentrifugation [23]. Small scale preparation of *E. coli* plasmid DNA was achieved by using Wizard Miniprep DNA purification kit (Promega Japan, Tokyo, Japan) and large scale plasmid DNA preparation was performed by Qiagen plasmid Maxi kit (Qiagen, Chatsworth, CA, USA).

2.3. Expression of ior genes and crude enzyme preparation

The regions for genes *iorA*, *iorB* and *iorA-iorB* were amplified by polymerase chain reaction (PCR) using respective forward and reverse primers (IOR-3–6). IOR-3 (5′-GCG AAA GTT ACC GAC ATA GTG TTG TGG GA-3′) and IOR-5 (5′-TAC TCC TAG ATC T CA CTC ACC CTC CTT-3′) were used for the amplification of *iorA* gene, and IOR-4 (5′-CGA GAT CTC AGT CCG TCC GTC ATT TGA GA-3′) and IOR-6 (5′-AAG GAG TAC AAC ATC GTT ATC ACC GGA-3′) were used for the amplification of *iorB* region. The 1938-bp and 675-bp fragments were obtained for *iorA* and *iorB* regions, respectively. The plasmid pET-8c (Novagen, Madison, WI, USA) was digested with respective *Nco*I, treated with Klenow polymerase to fill in the cohesive end and again digested with *Bam*HI. The amplified DNA for *iorA* and *iorB* were digested with *Bgl*II (primers IOR-4 and -5 possess *Bgl*II sites as underlined), phosphorylated with T4 kinase and ligated to the prepared pET-8c vector, and the resulting plasmids were designated as pIORα and pIORβ, respectively. Confirmation of *iorA* and *iorB* genes cloned into pET-8c vectors was performed by sequence determination using the dideoxy chain termination method [24]. For coexpression of *iorA* and *iorB* genes, primers IOR-3 and IOR-4 were used and DNA fragments harboring two genes were obtained. Other procedures were carried out by the same methods as for pIORα and pIORβ constructions. The constructed plasmid was designated as pIOR. *E. coli* BL21(DE3) cells were transformed with, respectively, pIORα, pIORβ, pIOR and pET-8c (control) and incubated at 37°C

Recombinant proteins were overproduced in anaerobically cultured *E. coli* BL21(DE3) cells harboring plasmids at 37°C. When optical density (OD_{660}) reached 0.35, gene expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h. The cells from a 100-ml culture were harvested by centrifugation and disrupted by sonication in 3 ml of 50 mM Tris-HCl (pH 8.0) buffer with 2 mM sodium dithionite and 2 mM dithiothreitol (DTT) in an anaerobic chamber (Tabai Espec, Osaka, Japan) filled with an anaerobic gas (H_2, 5%; N_2, 90%; and CO_2, 5%). The supernatant was recovered after centrifugation at 27 000×*g* for 20 min at 4°C and used for crude enzyme assay.

2.4. Purification of IOR subunits

In order to purify α and β subunits of IOR, all procedures were carried out under strictly anaerobic conditions. Two kinds of buffers were used for purification: buffer A: 50 mM Tris-HCl, pH 8.0; buffer B: 50 mM sodium acetate, pH 4.5. Both buffers contained 2 mM sodium dithionite and 2 mM dithiothreitol (DTT) to protect against trace O_2 contamination. The buffers were repeatedly degassed and flushed with argon. All chromatography columns were run using a fast protein liquid chromatography system (Amersham Pharmacia Biotec). The cells from a 500-ml culture were harvested by centrifugation and disrupted by sonication in 10 ml of buffer A in an anaerobic chamber filled with an anaerobic gas. The supernatant was brought to 70% saturation with solid ammonium sulfate followed by stirring at 4°C for 4 h. The suspension was centrifuged at 27 000×*g* for 20 min at 4°C and the resulting pellet was dissolved in 10 ml of buffer A. The solution was dialyzed overnight against the same buffer. Further chromatographies were performed as described below.

For α subunit purification, all columns were equilibrated with buffer A. The crude extract (10 ml) was applied to an anion-exchange column (HiTrapQ, Amersham Pharmacia Biotec) and α subunit was eluted with a linear gradient of 0–1 M NaCl at a flow rate of 1 ml/min. The fractions containing α subunit were combined and dialyzed against buffer A. The dialyzed solution (10 ml) was applied to a hydroxyapatite column (∅1.5×10 cm) and eluted with a linear gradient from 0 to 0.2 M KCl at a flow rate of 0.2 ml/min. Positive fractions were then combined, concentrated by Centriprep 10 (Amicon Japan, Tokyo, Japan) and finally applied to a Superdex 200 column (Amersham Pharmacia Biotec) equilibrated with buffer A containing 100 mM NaCl.

For β subunit purification at first the crude extract (10 ml) was applied to a HiTrapQ column using buffer A as eluent buffer. Most of the contaminated proteins bound to the column, but β subunit was eluted in the unbound fractions. Flow through fractions were combined and dialyzed against buffer B. The obtained solution was applied to HiTrapSP column (Amersham Pharmacia Biotec) with a linear gradient of 0–1 M NaCl. Eluted fractions were then combined and the solution was dialyzed against buffer A containing 100 mM NaCl. The dialyzed sample was concentrated by Centriprep 10 and applied to a Superdex 200 column.

2.5. Enzyme assay

IOR activity was determined by the indolepyruvate dependent reduction of methyl viologen as previously described [17]. The standerd assay mixture contained indolepyruvate 5 mM, thiamine pyrophosphate (TPP) 0.4 mM, coenzyme A 0.1 mM, and methyl viologen 1 mM

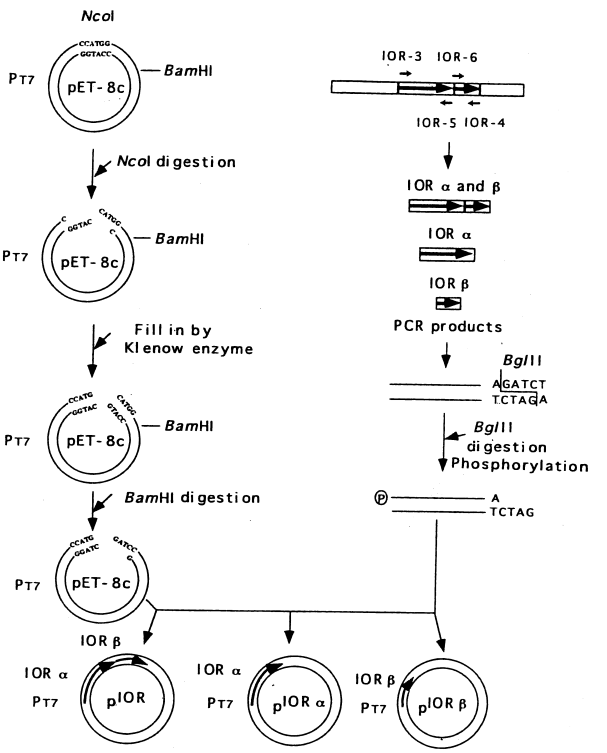

Fig. 1. The strategy for constructing the plasmids pIOR, pIORα and pIORβ.

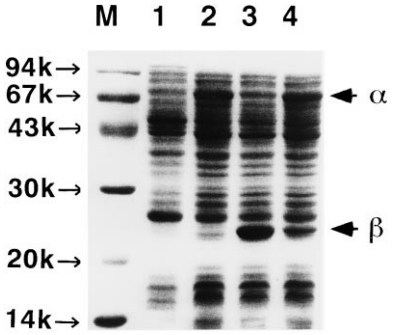

Fig. 2. The 0.1% SDS-15% polyacrylamide gel electrophoresis of *E. coli* extracts harboring various recombinant plasmids. Lanes: 1: whole cell fraction from *E. coli* (pET-8c); 2: whole cell fraction from *E. coli* (pIORα); 3: whole cell fraction from *E. coli* (pIORβ); 4: whole cell fraction from *E. coli* (pIOR); M: molecular weight markers (phosphorylase *b*, 94 000; albumin, 67 000; ovalbumin, 43 000; Carbonic anhydrase, 30 000; Trypsin inhibitor, 20 100).

in 50 mM EPPS buffer, pH 8.4. Absorbance changes at 600 nm were measured at 70°C using a Shimazu UV-160 spectrophotometer (Shimazu, Kyoto, Japan). For crude enzyme assay, 100 μl of extract was utilized. One unit of IOR activity is defined as the oxidation of 1 mmol of indolepyruvate per minute.

3. Results and discussion

3.1. Heterologous expression of *iorA* and *iorB*

Two genes, *iorA* and *iorB*, encoding α and β subunits of IOR, respectively, were found to be tandemly arranged, which suggests that gene expression is translationally coupled in vivo [18]. In order to coexpress both *iorA* and *iorB* genes in *E. coli*, a DNA fragment harboring both genes was cloned into plasmid pET8c as shown in Fig. 1. The constructed plasmid was designated as pIOR. Furthermore, to achieve individual expression, *iorA* and *iorB* were separately cloned into plasmid pET8c and constructed plasmids were designated as pIORα and pIORβ, respectively. *E. coli* BL21(DE3) cells harboring, respectively, pIOR, pIORα and pIORβ were cultured anaerobically and disrupted by sonication in an anaerobic chamber. After cell disruption by sonication, the cytoplasmic supernatant fraction was recovered by centrifugation. As shown in Fig. 2, coexpression and individual expression were achieved satisfactorily. The obtained extracts were used for enzyme assay for IOR by following the indolepyruvate-dependent reduction of methyl viologen.

3.2. Heat effect on IOR activity

The enzyme activity of the cytoplasmic extract harboring pIOR was 0.04 units ml^{-1} and no activity was detected from the extract of cells harboring pET-8c. In order to examine the effect of heat treatment on enzymatic activity, the crude extract (1000 μl) was heat treated for 20 min at various temperatures in an anaerobic chamber and remaining enzyme activity was measured using 100 μl of each extract at 70°C. Most enzymes from mesophilic microorganisms are generally thermolabile and enzyme loses its activity by heat treatment. However, IOR activity of pIOR extract was enhanced by heat treatment and the treatment at 70°C was most effective as shown in Fig. 3a. Crude extracts of pIORα and pIORβ were also obtained, and examined for IOR activity as shown in Fig. 3b. However, no activity was detected from the extracts for α and β subunits in the presence or absence of heat treatment. These results indicated that both α and β subunits are necessary for IOR activity. Coexpressed α and β subunits might be associated incompletely in *E. coli* cells, and upon heat treatment the incomplete form might be converted to a more active one.

In order to analyze the effect of heat treatment on active complex formation, crude extracts of α and β subunits (500 μl each) were combined in the anaerobic chamber and heated at various temperatures at 45–80°C for 20 min. The mixture was then examined for IOR activity at 70°C. The heat treated extract at 70°C showed the highest activity, the value of which (0.36 units ml^{-1}) was almost the same as that of a coexpressed one (0.32 units ml^{-1}). The enhanced activity of the subunit mixture seems to be due to active complex formation by heat. In order to obtain further information, each subunit was heat treated at 70°C for 20 min and then mixed. The mixture was reheated at various temperatures and examined for IOR activity. As shown in Fig. 3c, no activity was detected from the mixture. This result indicates that once heat treatment was performed before mixing, each subunit could not assemble properly to form an active heterooligomeric complex. It means that aberrant forms of subunits are not used for the association of subunits. Proper oligomerization seems to be

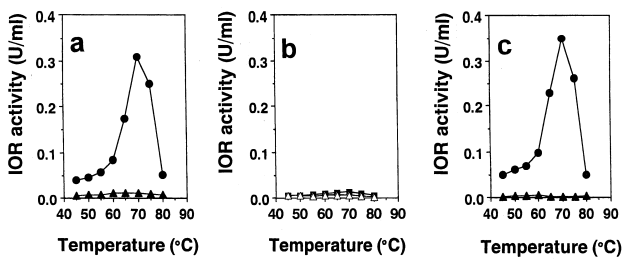

Fig. 3. Effect of heat treatment on enzymatic activities of various crude extracts. Crude cell extracts were heat treated at indicated temperatures (45–80°C) for 20 min and IOR activity was examined at 70°C. a: Enzymatic activities of crude extracts of *E. coli* harboring pET-8c (closed boxes) and pIOR (closed circles). b: Enzymatic activities of crude extracts of *E. coli* harboring pIORα (open triangles) and pIORβ (closed circles). c: Enzymatic activity of combined crude extracts of *E. coli* harboring pIORα and pIORβ with (closed triangles) or without (closed circles) heat pretreatment at 70°C for 20 min before mixing.

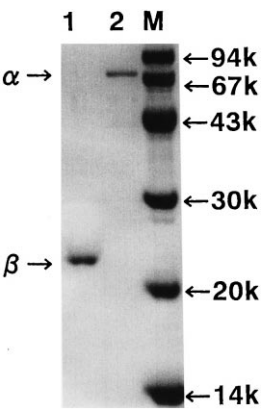

Fig. 4. The 0.1% SDS-15% polyacrylamide gel electrophoresis of purified α and β subunits of IOR. Lanes: 1: purified β subunit; 2: purified α subunit; M: molecular weight markers. The markers are the same as indicated in Section 2.

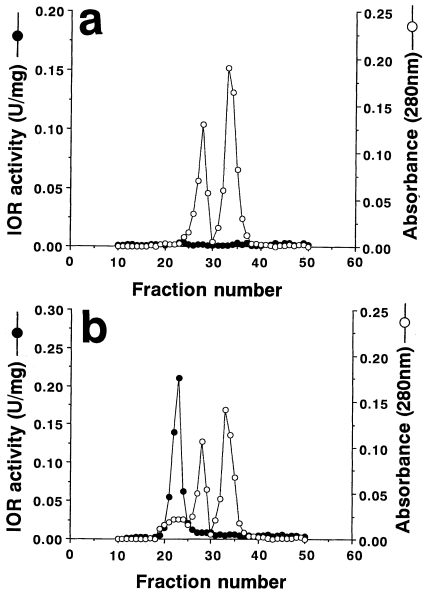

Fig. 5. Effect of heat treatment on subunit assembly. Gel filtration profile of mixed α and β subunits of IOR with (b) or without heat treatment (a). The closed and open circles indicate IOR activity and absorbance at 280 nm, respectively.

coupled with proper structure formation of each subunit during exposure to heat.

3.3. In vitro heat effect on subunit assembly

In vitro subunit assembly induced by heat was reported from various homooligomeric thermostable enzymes [7–12]. Heterooligomeric assembly of which the action is dependent on heat was reported only from histones of *Methanothermus fervidus* [25]. Histones from *M. fervidus* (HMF) are composed of two kinds of subunits HMfA and HMfB. Heat treatment of a mixture of recombinant HMfA and HMfB homodimers at 95°C for 5 min generates heterodimers (HMfA-HMfB complex), indicating a close relationship between high temperature and proper oligomerization for thermostable proteins.

In order to confirm the result that the activity enhancement of IOR is due to heat induced subunit assembly, α and β subunits were individually purified and in vitro protein assembly was performed. IOR is known to be very sensitive to oxygen and loses its activity in the presence of air [17,18]. The α and β subunits were purified (Fig. 4) under strictly anaerobic conditions as described in Section 2. Equal amounts (500 pmol each) of α and β subunits were mixed in 100 μl of buffer A (50 mM Tris-HCl, pH 8.0), kept at room temperature (20°C) for 20 min and applied to a gel filtration column. As shown in Fig. 5a, two major peaks of Ab280 were detected, which corresponded to the monomeric molecular weights of α (71 kDa) and β (24 kDa) subunits. However, no detectable peak was observed at the position of the heterooligomeric complex $\alpha_2\beta_2$ (192 kDa). In addition, IOR activity was not detected in any fractions, showing that mixing only without heat treatment does not induce subunit assembly. Next the same amounts of α and β subunits were mixed and the mixture was heat treated at 70°C for 20 min. The sample was applied to the gel filtration column and the eluted fraction was examined for IOR activity. The highest activity (0.21 units mg^{-1}) was detected at the position of 200 kDa,

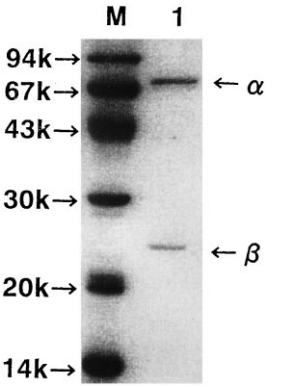

Fig. 6. 0.1% SDS-15% polyacrylamide gel electrophoresis demonstration of active fractions. The peak fraction of IOR activity (no. 23 of Fig. 5b) was applied. The markers are the same as indicated in Fig. 2.

which corresponds to the deduced molecular weight of the $\alpha_2\beta_2$ complex (192 kDa). The active peak fraction was then analyzed by SDS-PAGE. As shown in Fig. 6, both α and β subunits were identified, indicating that the active fraction possessed the assembled complex. However, most of the α and β subunits still remained as monomeric form (Fig. 5b). The total protein amount of the assembled peak fraction was 3.8 μg which corresponds to 20 pmol of $\alpha_2\beta_2$ complex. If subunit assembly was completely achieved, 250 pmol of the $\alpha_2\beta_2$ complex would be expected to form from 500 pmol of α and β subunits. In the present system, the proportion of assembled complex was calculated as less than 10% of the expected amount. This incompleteness provides us with the idea that besides heat some cytoplasmic factors might be involved in the complete protein assembly in *P. kodakaraensis*. Additional factors like molecular chaperones [3,4] may be involved in protein assembly in vivo. Furthermore, both α and β subunits are oxygen sensitive and subunits might be partially oxidized during purification, resulting in unsuitable conformation for the assembly. When the sample for gel filtration was prepared using preheated α and β subunits, active IOR complex was not obtained even though heat treatment was performed again after mixing (data not shown).

In hyperthermophiles, high temperature seems important for the protein assembly of oligomeric protein. In the present study, heat induced subunit assembly was introduced by using indolepyruvate ferredoxin oxidoreductase (IOR) as a model protein. As we mentioned above, once heat treated α and β subunits were not assembled by additonal heat treatment. A tentative model of subunit assembly of IOR was shown in Fig. 7. Individual heat treatment might form an aberrant

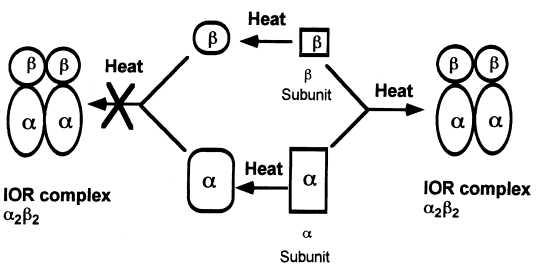

Fig. 7. The tentative model for subunit assembly of IOR induced by heat treatment.

structure of subunits and heated subunits could not be utilized for the assembly. For proper quaternary structure formation, each subunit is required to form a proper tertiary structure. Previous investigations have indicated that the thermostability of a protein arises from the simultaneous effects of several forces, including hydrogen bonds, ion pairs, or hydrophobic bonds [26]. Each subunit of IOR might not be suitably arranged for such bond formation at lower temperature. However, when heat treatment was performed, tertiary structure of each subunit was modified through temperature shift and then a proper quarternary structure could be formed.

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