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The Dual Functions of *Tetrahymena* Citrate Synthase Are Due to the Polymorphism of Its Isoforms[1]

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Previous studies have shown that *Tetrahymena* citrate synthase and the *Tetrahymena* 14-nm filament protein are encoded by a single gene and translated from one species of mRNA, and that they are identical in terms of molecular weight, antigenicity, and some enzymatic properties. In this study, using two-dimensional gel electrophoresis, we demonstrated that the citrate synthase comprised pI 7.7 and 8.0 isoforms, while the 14-nm filament protein comprised three isoforms with isoelectric points of 7.7, 8.0, and 8.4. The amino acid sequences of the NH_2-terminal portions of all isoforms were identical and the peptide maps with V8 protease were almost the same. In addition, when the citrate synthase activity of each isoform was measured after separation by non-urea isoelectric focusing without denaturing treatment, the pI 7.7 and/or pI 8.0 isoforms exhibited the citrate synthase activity, but the pI 8.4 isoform only found for the 14-nm filament protein did not possess this activity. These results suggest that the polymorphism of these isoforms is caused by some posttranslational modifications, and that it may have resulted in the different compartmentalization and functions of *Tetrahymena* citrate synthase and the 14-nm filament protein.

Key words: 14-nm filament protein, citrate synthase, multifunctional protein, posttranslational modification, *Tetrahymena*.

Immunofluorescence studies have shown that the *Tetrahymena* 14-nm filament protein is localized in the cytoplasm as filamentous structures and in mitochondria. The cytoplasmic 14-nm filament protein is involved in oral morphogenesis (*1, 2*), and in pronuclear behavior during conjugation (*3, 4*). Cloning and sequencing of the 14-nm filament protein gene from a *Tetrahymena thermophila* cDNA library revealed that the deduced amino acid sequence of the 14-nm filament protein, with a presequence peptide (21-residues) at its NH_2-terminus, exhibits high sequence identity (51.5%) with porcine heart mitochondrial citrate synthase, and that the functional domains of citrate synthase are conserved (*5*). Indeed, the purified 14-nm filament protein was demonstrated to have citrate synthase activity, it being the TCA cycle enzyme within mitochondria (*5*).

Furthermore, we examined whether the mitochondrial citrate synthase and the 14-nm filament protein are identical. A comparison of some properties of the purified citrate synthase and the purified 14-nm filament protein gave the following results (*6*). (i) The molecular weights of the citrate synthase and 14-nm filament protein were both 49,000, and an anti-14-nm filament protein antiserum cross-reacted with the citrate synthase. (ii) The purified citrate synthase showed much the same enzymatic properties as the purified 14-nm filament protein, in terms of optimum pH, optimum KCl concentration, effects of substrate concentrations (acetyl-CoA and oxaloacetate), and inhibitory effect of ATP. (iii) An anti-14-nm filament protein monoclonal antibody strongly suppressed the enzymatic activity of the purified citrate synthase. In addition, the results of Southern and Northern blotting involving cDNA encoding the 14-nm filament protein as a probe indicated that the citrate synthase and 14-nm filament protein are encoded by a single gene and translated from one species of mRNA (*7*). Although a number of studies have been performed on the identity between the citrate synthase and the 14-nm filament protein, the regulatory mechanisms underlying their cellular compartmentalization and functions are unclear.

In this study, we found that the citrate synthase comprises pI 7.7 and 8.0 isoforms, while the 14-nm filament protein comprises pI 7.7, 8.0, and 8.4 isoforms. For these isoforms, the amino acid sequences of the NH_2-terminal portions and peptide maps with V8 protease were almost the same. Using the new technique of non-urea isoelectric focusing, we succeeded in measuring the citrate synthase activities of these isoforms. Here we show that the pI 7.7 and 8.0 isoforms of the citrate synthase and 14-nm filament protein possess citrate synthase activity, but that the pI 8.4 isoform of the 14-nm filament protein possessed no activ-

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Abbreviations: PMSF, phenylmethanesulfonyl fluoride; TLCK, *Nα*-tosyl-L-lysylchloromethyl ketone; MES, 2-(*N*-morpholino)-ethanesulfonic acid.

ity. Finally, we discuss the possibilities that the polymorphism of the isoforms is caused by some posttranslational modifications and that it gives rise to the differences in the functions and localization of the citrate synthase and 14-nm filament protein.

MATERIALS AND METHODS

Cell Culture—Cultivation of *Tetrahymena pyriformis* (strain W) was performed as described previously (*8*).

Purification of Citrate Synthase—The citrate synthase was extracted from *Tetrahymena* mitochondria and then purified by ammonium sulfate fractionation, and Butyl-Toyopearl PAK 650S column (2.2×20 cm; Tosoh, Tokyo) and SP-Toyopearl PAK 650S column (2.2×20 cm; Tosoh) chromatographies, with monitoring of the enzymatic activity. These methods were described in detail previously (*6*).

Preparation of the 14-nm Filament Protein—The 14-nm filament protein was prepared from *Tetrahymena* acetone powder by the assembly and disassembly procedure, as described previously (*9*).

Two-Dimensional Electrophoresis with an Immobiline System—Immobiline isoelectric focusing electrophoresis was performed for the first dimension. Immobiline DryStrips (pH 3-10 L; Pharmacia Biotech, Uppsala, Sweden) were rehydrated with 20 ml of 5 M urea, 2 M thiourea, 0.5% Triton X-100, 0.04% acetic acid, and a few grains of DTT for 2 h at room temperature. Proteins were dialyzed against the same solution as described above and then subjected to isoelectric focusing. Isoelectric focusing was performed at 3,500 V for 16 h, and then the gels were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (*10*).

Non-Urea Isoelectric Focusing—The new technique of isoelectric focusing (IEF) was developed from the method of Hirabayashi (*11*). The purified citrate synthase and purified 14-nm filament protein were dialyzed against 5 mM MES (pH 7.0), 100 mM KCl, 0.5 mM oxaloacetic acid, and 10% glycerol, and then applied to agarose IEF gels consisting of 1% agarose-IEF, 12.5% sorbitol, 20% glycerol, and Pharmalyte (Pharmacia Biotech). Ten milliliters of the gel contained 75, 400, 200, and 150 μl of Pharmalyte of pH 2.5-5, 3-10, 4-6.5, and 8-10.5, respectively. After focusing at 4°C for 16 h at 500 V, each agarose gel was sliced into 2 mm sections, and the protein contained within each slice was extracted with 20 μl of 5 mM MES (pH 7.0), 100 mM KCl, 0.5 mM oxaloacetic acid, and 10% glycerol. The citrate synthase activity of each extracted protein was assayed by the DTNB method, as described previously (*6*). For the second dimensional gel electrophoresis, an agarose gel, not sliced, was fixed with 10% trichloroacetic acid and 5% sulfosalicylic acid, and then subjected to SDS-PAGE.

Subcellular Fractionation—*Tetrahymena* cells were washed with solution A (0.3 M sucrose, 10 mM triethanolamine, 2 mM EDTA, pH 7.4), and then suspended in 1 mg/ml digitonin, 5 μg/ml leupeptin, 0.1 mM PMSF, and 0.01 mM TLCK in solution A. After 5 min at 0°C, undisrupted cells and some insoluble substances were removed from the mixture by low speed centrifugation, and then the supernatant was re-centrifuged for 10 min at 10,000×*g* to obtain the cytoplasmic fraction (the supernatant) and the mitochondria-rich fraction (the pellet). To purify mitochondria, the pellet layered on a 1 M sucrose cushion was centrifuged and then the interface layer was collected. The oral apparatus fraction was prepared according to the methods of Vaudax and Williams *et al.* (*12, 13*). All the samples were solubilized with a 8 M guanidine-HCl solution containing 10% (v/v) 2-mercaptoethanol and 0.1 M Tris buffer, pH 7.5, and then dialyzed against 7 M urea and subjected to two-dimensional gel electrophoresis with an Immobiline system. After electrophoresis, they were transferred to a polyvinylidene difluoride (PVDF) filter and probed with anti-14-nm filament protein antibodies by the method of Towbin *et al.* (*14*) with some modification (*15*).

Amino Acid Sequencing—The amino acid sequences of the NH_2-terminal portions of all isoforms separated by two-dimensional gel electrophoresis were determined by automated Edman degradation with an Applied Biosystems (Foster City, CA, USA) gas-phase sequencer.

Peptide Mapping by V8 Protease Digestion—Peptide mapping was performed according to the method of Cleaveland *et al.* (*16*). The isoforms of the purified citrate synthase and the purified 14-nm filament protein were cut out from the gel after two-dimensional gel electrophoresis. Each gel slice was placed in a sample well and then *Staphylococcus aureus* V8 protease was overlaid on each slice. The digestion proceeded directly in the stacking gel during the electrophoresis. The electrophoresis was performed in almost the normal manner except for turning off of the current for 30 min when the bromophenol blue dye neared the bottom of the stacking gel.

Antibody—The anti-14-nm filament protein polyclonal antibody (pAb49KI) used in the present paper was the same as that used in our previous studies (*4, 6, 15*).

RESULTS

Comparison of the Two-Dimensional Gel Electrophoresis Patterns of the Citrate Synthase and 14-nm Filament Protein—To ascertain whether a difference between *Tetrahymena* citrate synthase and the 14-nm filament protein exists or not, we compared these proteins by two-dimensional gel electrophoresis. The citrate synthase purified from a mitochondrial extract comprised two isoforms, whose isoelectric points were 8.0 and 7.7 (A and a in Fig. 1, I). The purified 14-nm filament protein comprised pI 8.0 and 7.7 isoforms (A and a in Fig. 1, III), and an additional isoform whose isoelectric point was more basic, *i.e.*, pI 8.4 (B in Fig. 1, III). When two-dimensional gel electrophoresis of a mixture of the citrate synthase and the 14-nm filament protein was carried out, it was found that the mixture contained three isoforms. The amounts of the pI 8.0 and 7.7 isoforms in the mixture were increased roughly twofold comprised to those of the corresponding isoforms of the citrate synthase or the 14-nm filament protein (A and a in Fig. 1, II), while the amount of the pI 8.4 isoform did not increase (B in Fig. 1, II). Therefore the pI 8.0 and the pI 7.7 isoforms are common isoforms of the citrate synthase and the 14-nm filament protein, but the pI 8.4 isoform is a specific isoform of the 14-nm filament protein. Thus, this was the first evidence of a difference between the citrate synthase and the 14-nm filament protein.

The existence of these isoforms *in vivo* was examined by two-dimensional gel electrophoresis using a mitochondrial fraction and a cytoplasmic fraction. Cells were disrupted by digitonin treatment, and then separated into cytoplasmic

fraction and mitochondrial fractions by centrifugation. The isoforms of the citrate synthase and 14-nm filament protein in each fraction were detected by immunoblot analysis with an anti-14-nm filament protein antibody after two-dimensional gel electrophoresis. In the whole cell extract, three isoforms were observed (Fig. 2, I). The two-dimensional gel electrophoretic pattern of the mitochondrial fraction, which was expected to contain two isoforms, was the same as the pattern of the purified citrate synthase fraction (Fig. 2, II). However, there was no isoform in the cytoplasmic supernatant fraction (Fig. 2, III). The cytoplasmic 14-nm filament protein may form 14-nm filaments in the cytoplasm or associate with cytoplasmic structures such as the oral apparatus. Since a previous study has shown that the 14-nm filament protein is involved in oral morphogenesis (*1*), we prepared an oral apparatus fraction and examined the 14-nm filament protein isoforms in this fraction by immunoblot analysis. As shown in Fig. 2, IV, three isoforms were present in the oral apparatus fraction, and this pattern was similar to that of the purified 14-nm filament protein. Similar results were also obtained on immunoblot analysis with an anti-citrate synthase antibody instead of the anti-14-nm filament protein antibody (data not shown). These results indicate that the difference in the isoforms between the citrate synthase and the 14-nm filament protein was not caused by protein denaturation or an artifact during the purification.

NH_2-Terminal Amino Acid Sequences and Peptide Maps of the Isoforms of the Citrate Synthase and 14-nm Filament Protein—To compare all isoforms of the citrate synthase and the 14-nm filament protein, we determined

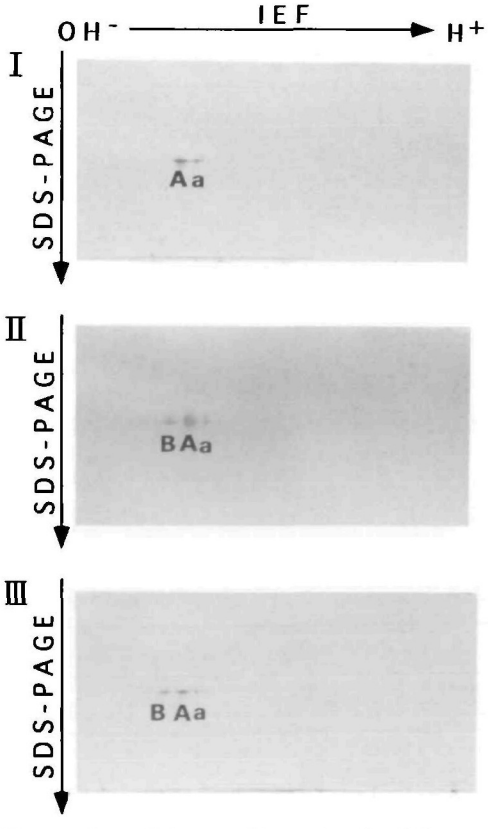

Fig. 1. **Comparison of the two-dimensional gel electrophoresis patterns of the citrate synthase (I), a mixture of the citrate synthase and the 14-nm filament protein (II), and the 14-nm filament protein (III).** The purified citrate synthase, the purified 14-nm filament protein and the mixture were subjected to two-dimensional electrophoresis with an Immobiline system, as described under "MATERIALS AND METHODS." First-dimension isoelectric focusing (horizontal) and second-dimension SDS-PAGE (vertical) were performed. The acid ends of the gels are to the right. The gels were silver stained. B, A, and a in the pictures denote the pI 8.4, 8.0, and 7.7 isoforms, respectively.

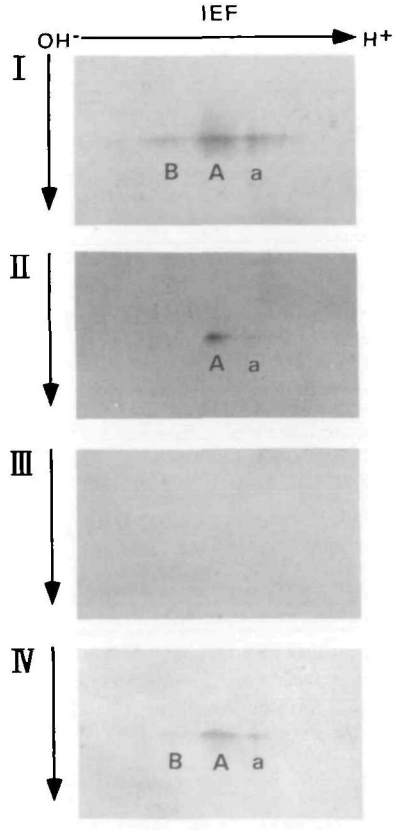

Fig. 2. **Identification of the isoforms of the citrate synthase and 14-nm filament protein recognized by the anti-14-nm filament protein antibody in the whole cell extract (I), mitochondrial fraction (II), cytoplasmic fraction (III), and oral apparatus fraction (IV).** Each subcellular fraction was prepared as described under "MATERIALS AND METHODS." The proteins were separated by two-dimensional gel electrophoresis, transferred to a PVDF filter, and then probed with the anti-14-nm filament protein antibody. Only the regions of the isoforms of the citrate synthase and 14-nm filament protein are shown. B, A, and a in the pictures denote the pI 8.4, 8.0, and 7.7 isoforms, respectively.

TABLE I. **Analysis of the NH_2-terminal amino acid sequences of the isoforms of the citrate synthase and 14-nm filament protein.** CS and 14-F denote the citrate synthase and 14-nm filament protein, respectively.

Protein	NH_2-terminal amino acid sequence
The deduced sequence of *T. pyriformis* 14-F	GQTNLKKVIA EIIPHKQAEL
pI 8.0 isoform of CS	- QTNLKKVIA EIIPHKQAEL
pI 7.7 isoform of CS	GQTNLKKVIA EIIPHKQAEL
pI 8.4 isoform of 14-F	GQTNLKKVIA EIIPHKQAEL
pI 8.0 isoform of 14-F	- QTNLKKVIA EIIPHKQAEL
pI 7.7 isoform of 14-F	GQTNLKKVIA EIIPHKQAEL

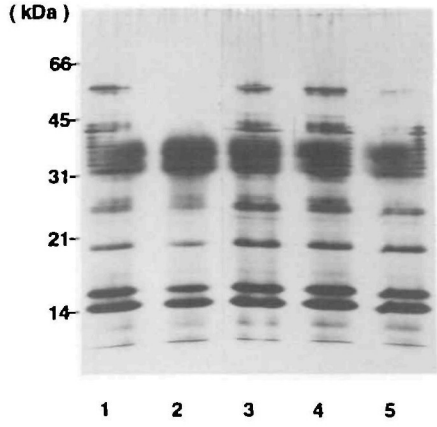

Fig. 3. **Analysis of the peptide maps of the isoforms of the citrate synthase and 14-nm filament protein.** The isoforms obtained on two-dimensional electrophoresis were excised from the gels, and then subjected the second SDS-PAGE, V8 protease being added. The gels were silver stained. Lane 1, pI 8.0 isoform of the citrate synthase; lane 2, pI 7.7 isoform of the citrate synthase; lane 3, pI 8.4 isoform of the 14-nm filament protein; lane 4, pI 8.0 isoform of the 14-nm filament protein; and lane 5, pI 7.7 isoform of the 14-nm filament protein.

their NH_2-terminal amino acid sequences. As shown in Table I, the NH_2-terminal amino acid sequences from the 2nd to 20th amino acid residues were completely identical in these isoforms. The NH_2-terminal 21-residues corresponding to the mitochondrial targeting sequence deduced from the 14-nm filament protein gene were absent. Although the first residues of the pI 8.0 isoform of the citrate synthase and the pI 8.0 isoform of the 14-nm filament protein were not determined, the first residues of the pI 7.7 isoform of the citrate synthase, and the pI 8.4 and 7.7 isoforms of the 14-nm filament protein were all glycine, which corresponded to the residue deduced from the 14-nm filament protein gene.

Further analysis of the isoforms was performed using peptide maps obtained by *S. aureus* V8 protease digestion. The peptide mapping patterns of all the isoforms were very similar, although some minor differences were seen in the lower bands (Fig. 3). Some of the upper bands are undigested or partially digested isoforms. Similar results were obtained with peptide maps obtained by CNBr digestion (data not shown). Since the citrate synthase and 14-nm filament protein are encoded by a single gene and translated from one species of mRNA, these isoforms may be derived from the same translational product, and the minor differ-

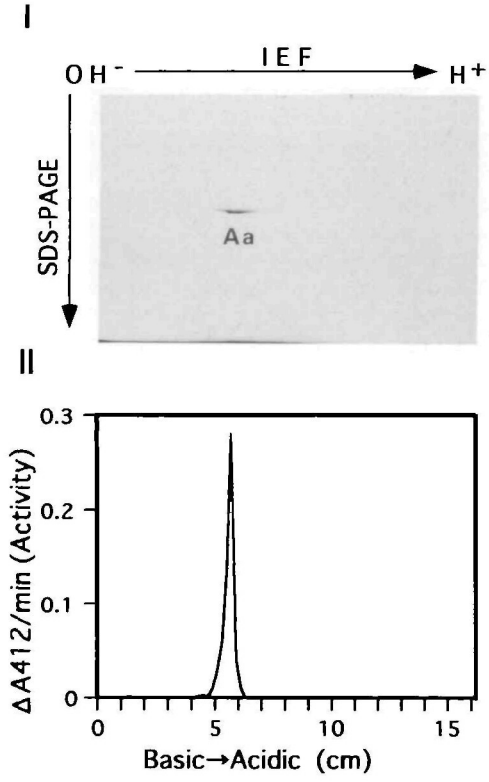

Fig. 4. **Non-urea isoelectric focusing and the enzymatic activity patterns of the purified citrate synthase.** (I) The purified citrate synthase was subjected to isoelectric focusing at 500 V for 16 h at 4°C, in the absence of urea. The gel was fixed with 10% trichloroacetic acid and 5% sulfosalicylic acid, and then subjected to SDS-PAGE in the second dimension. The gel was stained with Coomassie Brilliant Blue. A and a in the pictures denote the pI 8.0 and 7.7 isoforms, respectively. (II) After isoelectric focusing, the gel, which shrunk on focusing (160 mm long), was sliced into 2-mm sections, and then the citrate synthase activity of the protein extracted from each slice was measured.

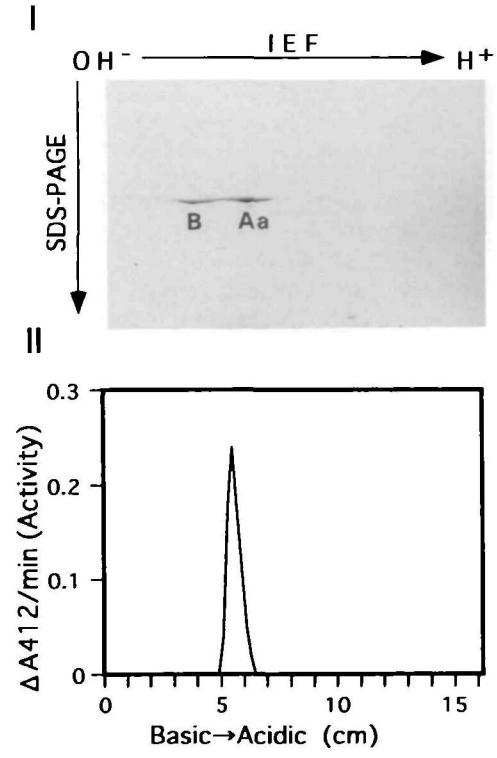

Fig. 5. **Non-urea isoelectric focusing and the enzymatic activity patterns of the purified 14-nm filament protein.** (I) The purified 14-nm filament protein was subjected to isoelectric focusing at 500 V for 16 h at 4°C, in the absence of urea. The gel was fixed with 10% trichloroacetic acid and 5% sulfosalicylic acid, and then subjected to SDS-PAGE in the second dimension. The gel was stained with Coomassie Brilliant Blue. B, A, and a in the pictures denote the pI 8.4, 8.0, and 7.7 isoforms, respectively. (II) After isoelectric focusing, the gel, which shrunk on focusing (160 mm long), was sliced into 2-mm sections, and then the citrate synthase activity of the protein extracted from each slice was measured.

ences observed in the peptide maps may be due to some modifications.

Relationship between the Isoforms and the Enzymatic Activity—To measure the citrate synthase activity of each isoform, the citrate synthase and 14-nm filament protein were subjected to isoelectric focusing under non-denaturing conditions. After the first-dimensional isoelectric focusing, the gels were sliced and then the citrate synthase activity of the protein extracted from each gel slice was measured. Figures 4I and 5I show the two-dimensional gel electrophoretic patterns (in the absence of urea) of the citrate synthase and 14-nm filament protein, respectively. Because of the non-denaturing conditions, this method gave slightly less separation than the Immobiline system, so the pI 8.0 and 7.7 isoforms could not be separated. As shown in Figs. 4II and 5II, the common isoforms, the pI 8.0 and/or 7.7 isoforms, possessed citrate synthase activity, but the pI 8.4 isoform only found for the 14-nm filament protein did not possess enzymatic activity. Since the amount of the pI 8.4 isoform is almost equal to those of both the pI 8.0 and 7.7 isoforms, some modification of the pI 8.4 isoform may cause loss of its enzymatic activity.

DISCUSSION

In this study, we demonstrated that a difference between the citrate synthase and the 14-nm filament protein exists in their isoforms. As shown in Fig. 1, the citrate synthase comprised pI 8.0 and 7.7 isoforms, while the 14-nm filament protein comprised pI 8.4, 8.0, and 7.7 isoforms. The subcellular distribution of these isoforms corresponded to the localization of the 14-nm filament protein and citrate synthase, that is to say, the pI 8.0 and 7.7 isoforms existed in the mitochondrial fraction, and the pI 8.4, 8.0, and 7.7 isoforms existed in the cytoskeletal fraction (Fig. 2). The difference in the isoforms between the citrate synthase and the 14-nm filament protein may reflect the differences in their functions and/or localization. The 14-nm filament protein is a cytoplasmic filamentous protein involved in gamete nuclear behavior during conjugation, and the purified 14-nm filament protein can polymerize to form the 14-nm filament. However, the citrate synthase is a mitochondrial TCA cycle enzyme and the purified citrate synthase cannot polymerize. Since the pI 8.4 isoform was only found for the 14-nm filament protein, it may be an indispensable factor for polymerization of the 14-nm filament protein.

All isoforms of the citrate synthase and the 14-nm filament protein had almost the same NH_2-terminal amino acid sequences (Table I), suggesting that there are no NH_2-terminal presequences in the cytoplasmic mature protein or the mitochondrial mature protein. In the previous study, Southern and Northern blot analysis revealed that the citrate synthase and 14-nm filament protein are encoded by a single gene and translated from a single species of mRNA (*7*). These results demonstrated that the genes, mRNAs, and NH_2-terminal amino acid sequences of the citrate synthase and 14-nm filament protein are identical, in other words, they are identical at least at the translational level. What causes the differences in the isoelectric points of the isoforms? We presume that some posttranslational modification may account for the polymorphism of the isoforms. For example, in *Chlamydomonas*, lysine residues in α-tubulin are acetylated only in the flagellar axoneme, *i.e.* not in the cytoplasm. It has also been shown that the acetylation and deacetylation of lysine residues in α-tubulin may be a control step in the assembly and disassembly of flagellar microtubules (*17-20*). Besides, it is well known that some modifications, including acetylation, phosphorylation, and glycosylation, lead to shifts in the isoelectric points of proteins. Therefore, we consider that the polymorphism of the isoforms of the citrate synthase and 14-nm filament protein may be due to some posttranslational modifications. The minor differences between the isoforms observed in the peptide maps (Fig. 3) may also support the possibility of posttranslational modifications.

The results of non-urea isoelectric focusing showed that the pI 8.4 isoform of the 14-nm filament protein did not possess citrate synthase activity (Fig. 5). It seems likely that the pI 8.4 isoform may be blocked at its catalytic domain or that its conformation is altered by some factors or modifications. For example, the phosphorylation of acetyl-CoA carboxylase and pyruvate dehydrogenase leads to the complete inhibition of their enzymatic activities (*21-24*). Similarly, some modifications of the pI 8.4 isoform possibly lead to inhibition of its activity. However, the 14-nm filament protein having a pI 8.4 isoform, can polymerize to form 14-nm filaments, but the citrate synthase cannot polymerize. Recently, it was found in our laboratory that the 14-nm filament protein expressed in *E. coli* can only form short filaments, and that the velocity of its polymerization is much slower than that of the purified 14-nm filament protein (*25*). In addition, the expressed 14-nm filament protein should not be posttranslationally modified. These results suggest that the pI 8.4 isoform arising at the posttranslational level is an important factor for polymerization of the 14-nm filament protein.

Although the separative capacity of non-urea isoelectric focusing is inferior, it could separate the pI 8.4 isoform from the pI 8.0 isoform, and could maintain the citrate synthase activity. The citrate synthase is a remarkably unstable enzyme in general, and its activity is very sensitive to pH, ionic strength, detergent treatment and so on. *Tetrahymena* citrate synthase forms a dimer complex and then expresses its enzymatic activity (*26*). In spite of the instability of the citrate synthase, we succeeded in measuring its enzymatic activity after isoelectric focusing under non-denaturing conditions. Therefore, we expect that non-urea isoelectric focusing can be applied to the separation of other proteins with various isoelectric points, their activities, conformations, and complexes being maintained.

Tetrahymena citrate synthase can be included in the multifunctional proteins, but it is a little different from most of the already reported multifunctional proteins. Crystallin is a typical example, which acts as a structural protein in the eye lens and also has various enzymatic activities (*27-30*). Other examples are protein disulfide isomerase/prolyl hydroxylase (*31*), the cation-independent mannose 6-phosphate receptor/the insulin-like growth factor (*32*), α-enolase/plasminogen receptor (*33*), and so on. Most multifunctional proteins are encoded by plural genes, so their functions are probably regulated at the gene level. Even if encoded by a single gene, their plural functions would be regulated at the transcriptional level through alternative splicing, or regulated at the transla-

tional level, by means of such as a multiple translational initiation site and a frame shift. Since *Tetrahymena* citrate synthase and the 14-nm filament protein are identical at the translational level, these modes of regulation do not apply in this case. Thus the dual functions and localization of *Tetrahymena* citrate synthase are possibly regulated by some posttranslational modifications.

Finally, elucidation of the posttranslational modifications of the citrate synthase and the 14-nm filament protein will provide significant information for understanding the regulatory mechanisms underlying the functions and localization of a multifunctional protein. To determine the posttranslational modifications of the citrate synthase and the 14-nm filament protein, we are currently conducting mass spectrometrical analysis of their isoforms.

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