ELSEVIER

Biophysical Chemistry 59 (1996) 289–297

Biophysical Chemistry

A bifunctional fusion protein containing the maltose-binding polypeptide and the catalytic chain of aspartate transcarbamoylase: assembly, oligomers, and domains

Ying R. Yang, H.K. Schachman *

Department of Molecular and Cell Biology, Wendell M. Stanley Hall, University of California, Berkeley, CA 94720-3206, USA

Abstract

The in vivo synthesis of many target proteins or polypeptides has been enhanced dramatically and their purification facilitated through the use of gene fusion techniques which lead to the expression of fusion proteins. This approach was used to characterize the product formed in *Escherichia coli* encoded by a DNA construct comprising *malE*, the gene encoding maltose binding protein, linked to a small 30 nucleotide region which, in turn, was linked to *pyrB*, the gene encoding the catalytic (c) chains of aspartate transcarbamoylase (ATCase). The resulting fusion protein, MBP-C, was produced in excellent yield and readily purified in two steps because of its binding to an amylose column and displacement by maltose. The complex was studied by both sedimentation velocity and sedimentation equilibrium and shown to be a trimer of c chains with one MBP linked covalently to each chain. Treatment of the fusion protein with factor Xa cleaved each chain at the tetrapeptide encoded by the linker region yielding purified MBP with a minor modification at the C-terminus and the catalytic (C) trimer of ATCase. The MBP-C complex was fully active as an enzyme and could be reversibly denatured in 6 M urea. Scanning calorimetry studies on the fusion protein demonstrated that the MBP domain melted at the same temperature as did the purified protein. Similarly, the T_m for the C trimer in the complex was identical to the value for C trimer isolated from ATCase. Moreover, the thermal stability of the C trimer in the MBP-C complex was greatly enhanced by the addition of the bisubstrate ligand, *N*-(phosphonacetyl)-L-aspartate (PALA), just as observed with purified C trimer. Analogous denaturation experiments with varying concentrations of guanidine-HCl indicated that the fusion protein was denatured at much lower concentration of denaturant than observed for C trimer. These experiments demonstrate that the linker between the two structural genes encodes a polypeptide of sufficient length to permit independent folding and assembly of each protein and permit the subsequent specific cleavage at the factor Xa recognition site, thereby yielding both active proteins.

Keywords: Gene fusion; Covalent linkage of different polypeptide chains; Bifunctional protein

Abbreviations: MBP, Maltose binding protein; ATCase, Aspartate transcarbamoylase; c, Catalytic polypeptide chain; C, Catalytic subunit or trimer; MBP-C complex, A complex containing MBP and C trimer of ATCase; R, Regulatory polypeptide chain; R, Regulatory subunit; PALA, *N*-(phosphonacetyl)-L-aspartate; Mops, Morpholinopropanesulfonic acid

* Corresponding author.

Elsevier Science B.V.
PII S0301-4622(96)00018-X

1. Introduction

Among the many applications of molecular biological techniques is the linkage of different genes for the expression of complex molecules containing different proteins linked covalently on a single polypeptide chain. In most cases the linkage of genes encoding glutathione S-transferase, maltose binding protein, ubiquitin, or protein A, for example, to a second gene encoding a target protein or polypeptide of interest has been used to enhance expression of the desired protein or fragment or to facilitate its purification based on the properties of the protein to which it is bound [1–7]. Fusion proteins have also been used as bifunctional ligands which serve in therapeutic or diagnostic purposes [8]. After the fusion protein is purified, cleavage of the polypeptide chain at the site of the fusion is employed to yield the desired protein or polypeptide. For protein chemists interested in the folding of polypeptide chains and the role of domains in the folding process, the isolation of fragments of the chain is crucial. Since the in vivo expression of these fragments is frequently very low, and they are often subject to proteolysis as well as being difficult to purify, we like others increasingly are turning to fusion proteins. Our interest in the assembly of the *Escherichia coli* regulatory enzyme aspartate transcarbamoylase (ATCase, carbamoylphosphate: L-aspartate carbamoyl transferase, E.C. 2.1.3.2) from its constituent catalytic (C) and regulatory (R) subunits, coupled with the recent demonstration [9–12] of the effect of a 70-amino acid zinc-containing polypeptide from the regulatory (r) chain on the properties of the C subunit, encouraged us to explore the possibility of using fusion with the maltose binding protein to isolate smaller fragments.

In initiating these studies we were concerned about the availability of the N-terminal region of the c chain of ATCase or its fragments for coupling to the C-terminus of the maltose binding protein, the folding of the maltose binding chains to give the proper tertiary structure, the formation of the two domains of the polypeptide chains in ATCase, and the assembly of the complex into an oligomer with the appropriate properties. Would the complex chain fold and form the required oligomer so as to yield a bifunctional protein? How many amino acid residues in the linker between the C-terminus of the maltose binding protein and the N-terminus of the target protein are required? Where in the linker peptide should the cleavage site be located? Would the two constituent proteins have the desired activities or would one or both exhibit diminished activity? Would the proteins in the complex be less stable as a result of the covalent coupling through the linker region? In order to answer some of these questions, we conducted model studies aimed at determining whether a stable, active C trimer of ATCase can be formed while linked covalently to the maltose binding protein. The C trimer of ATCase is a particularly interesting model because the active sites of the enzyme are located at the interfaces between adjacent chains and catalysis is dependent upon the participation of amino acid residues from each of the adjoining chains [13,14]. As shown below, such a fusion protein is formed containing three active maltose binding proteins per active C trimer of ATCase. The thermal and guanidine–HCl denaturation properties and cleavage of this complex are presented in order to provide tentative answers to some of the questions posed above.

This study involves considerations of the folding of polypeptide chains, the role of domains and the assembly of oligomers. All of these areas of protein science were of primary interest to Bill Harrington throughout his entire scientific career starting with his work on tobacco mosaic virus, continuing with his research on the folding of polypeptide chains and the assembly of collagen, and culminating with his perceptive, insightful long-term investigations of myosin and muscle contraction. He would be pleased to know that we are still using the ultracentrifuge as well.

2. Experimental

2.1. Plasmid construction

A DNA fragment containing the *pyrB* gene which encodes the c chain of ATCase was amplified by the PCR technique and subcloned into the pMAL-c vector obtained from New England BioLabs. The cloned gene was inserted downstream from the *malE* gene,

which encodes the maltose binding protein (MBP) excluding the last four residues; it also contains a sequence that encodes 10 additional amino acids including at its terminus the tetrapeptide (ILE–GLU–GLY–ARG) recognition site for the protease factor Xa. This region of the DNA is linked to the 5′ end of the *pyrB* gene. Synthesis of the fusion protein, comprising the maltose binding protein linked to the c chain of ATCase, MBP-C, is under the control of the *tac* promoter and the *mal*E translation initiation signal to produce a high level of expression.

2.2. Expression and purification

The cloned plasmid was transformed into an *E. coli* strain, HB101-4442 [15], which lacks *pyrB* as well as the *pyrI* gene encoding the r chain of ATCase [16]. Cells were grown at 37°C in 1 l of LB medium containing 50 mg/l ampicillin. Expression of the fusion protein was induced with 0.2 mM IPTG at mid-log phase. Cells were harvested by centrifugation 3 h after induction, and the cells were ruptured by sonication. The cell extract was dialyzed overnight against 500 ml of 50 mM Tris–HCl at pH 8.0 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA before loading onto a 100 ml Q-Sepharose column. Proteins were eluted by a linear gradient of solution formed from 300 ml each of 0.1–0.5 M KCl in the same buffer. Fractions containing the fusion protein were pooled and dialyzed against a column buffer (10 mM sodium phosphate at pH 7.2 containing 30 mM NaCl, 2 mM 2-mercaptoethanol, 1 mM EDTA and 1 mM sodium azide) and loaded onto a 100 ml amylose column. Unbound proteins were eluted with 200 ml of column buffer, and MBP-C was collected by elution of the bound protein with column buffer containing 10 mM maltose. This two-step purification procedure yielded 70 mg of MBP-C at a purification of approximately 95%. Following purification, 10 mg of MBP-C were dialyzed overnight against 500 ml of 20 mM Tris–HCl at pH 8, containing 0.1 M NaCl and 0.2 mM $CaCl_2$. The fusion protein at 2 mg/ml was subjected to proteolytic digestion at room temperature by adding 0.1 mg of factor Xa and incubating for periods varying from 2 h to 5 days. SDS-PAGE was used to monitor the extent of digestion. After completion of the cleavage of MBP-C, the protein mixture was dialyzed against the column buffer and the two components were separated on the amylose column in the absence and presence of maltose as described above. Assays for ATCase enzyme activity were performed at 30°C with [^{14}C]carbamoyl phosphate as described by Davies et al. [17] in a 50 mM Mops buffer at pH 7 containing 0.2 mM EDTA and 2 mM 2-mercaptoethanol.

2.3. Physical chemical studies

Sedimentation velocity and equilibrium experiments were performed at 20°C with a Beckman XL-A analytical ultracentrifuge equipped with an absorption optical system. Protein solutions were dialyzed against 40 mM potassium borate buffer at pH 9, containing 5 mM 2-mercaptoethanol and 0.2 mM EDTA. Measurements of the distribution of protein in the ultracentrifuge cell were made with ultraviolet light at wavelengths of either 280 nm or 235 nm. For sedimentation equilibrium experiments the protein concentrations were between 50 μg/ml and 1 mg/ml. Data processing was accomplished with the computer program Origin 2.01 provided by Beckman Instruments.

Differential scanning microcalorimetry was performed with a Microcal MC-2 instrument interfaced with an IBM-XT computer for data analysis. A scan rate of 45°C/h was used over the temperature range from 20–90°C. Protein solutions at 2–4 mg/ml were dialyzed against 40 mM potassium borate at pH 9, containing 5 mM 2-mercaptoethanol and 0.2 mM EDTA [18]. For measurements of the effect of binding of the bisubstrate analog *N*-(phosphonacetyl)-L-aspartate (PALA) on the temperature of denaturation the concentration of PALA was 20 moles PALA per mole of active site.

The denaturation of MBP-C and C trimers by guanidine–HCl was monitored by ultraviolet absorption spectrophotometry in a Cary 118 instrument. Samples containing different concentrations of denaturant were incubated overnight at 0°C to ensure completion of the reaction, and measurements in 20 mM Tris–Cl at pH 7.5 were made at 8°C.

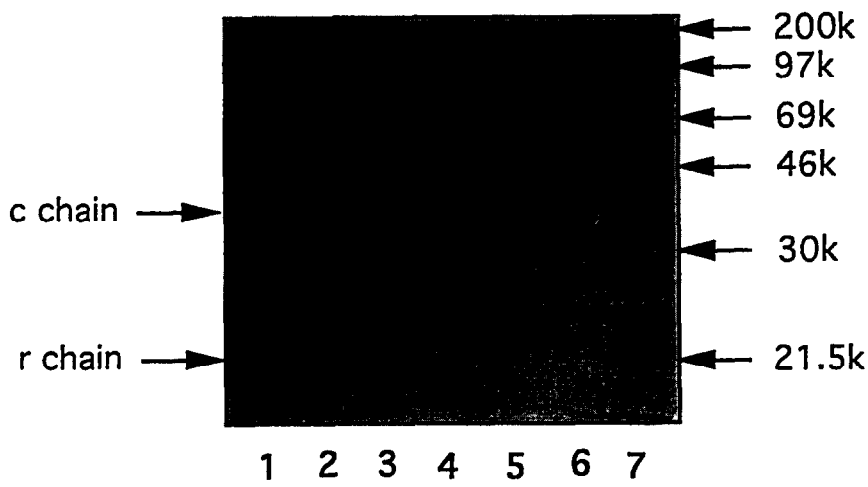

Fig. 1. Expression and purification of MBP-C. Detection of polypeptide chains and determination of their approximate molecular weights were performed by electrophoresis in polyacrylamide gels containing SDS according to the procedure of Laemmli [19]. The samples in the various lanes were: (1) c and r chains from wild-type ATCase holoenzyme; (2) crude extract from cells containing the fusion protein MBP-C; (3) purified fusion protein, MBP-C, from amylose column; (4) cleavage products formed by incubating MBP-C and factor Xa at room temperature for 2 days; (5) MBP isolated from amylose column after cleavage of MBP-C; (6) purified c chains from C trimer isolated from complex after digestion with factor Xa; (7) molecular weight markers.

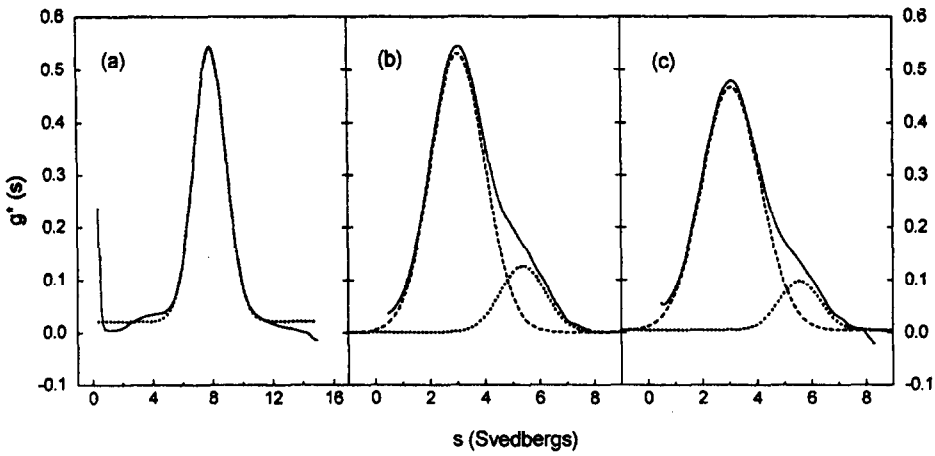

Fig. 2. Apparent sedimentation coefficient distributions of MBP-C complex and the individual proteins formed by cleavage of the fusion protein. Sedimentation velocity experiments were performed in a Beckman XL-A ultracentrifuge equipped with absorption optics. The curves of $g^*(s)$ vs. s were calculated from the time derivative of the sedimentation velocity concentration profile according to the method of Stafford [20]. Experiments were performed at 20°C with a rotor speed of 50,000 rpm. The protein concentration was 1.1 mg/ml in 40 mM potassium borate at pH 9.0 containing 5 mM 2-mercaptoethanol and 0.2 mM EDTA. Data were fit to either a single Gaussian or two Gaussians. (a) Distribution curve for MBP-C fit with a single Gaussian; (b) distribution curve (—) for MBP-C after digestion with factor Xa. This pattern was deconvoluted into two Gaussian curves (- - -) with s values of 3.0S and 5.4S; (c) distribution curve (—) for a mixture of equal molar amounts of purified MBP and C trimer. The s values from the deconvolution (- - -) were 3.0S and 5.6S, respectively.

3. Results

3.1. Purification of MBP-C and its cleavage

Purification of MBP-C was monitored by subjecting it to electrophoresis in polyacrylamide gels containing SDS [19]. As seen in Fig. 1, the dominant polypeptide in the crude extract (lane 2) had a mobility corresponding to a molecular weight of about 80,000. After purification most of the other polypeptides were removed yielding one major component (lane 3). Incubation of the MBP-C complex with factor Xa for 2 days at room temperature caused virtually complete cleavage at the linker between MBP and the c chains of ATCase as seen in the gel pattern (lane 4). One component had a mobility matching precisely that for c chains from ATCase (lane 1), and the second band corresponded to that of purified MBP produced by the cleavage reaction and isolation from the amylose column (lane 5). The pattern for the c chains obtained from the C trimer after the cleavage reaction is shown in lane 6. Its mobility is equal to that of c chain obtained directly from purified ATCase (lane 1).

These patterns from SDS-PAGE experiments demonstrate the ease and effectiveness of the purification and the proper size of the fused polypeptide chain comprising the maltose binding protein and the c chain of ATCase. They show that cleavage of the intact MBP-C complex is complete yielding the two proteins which when subjected to electrophoresis in SDS gels exhibit polypeptide chains with the correct molecular weights. Thus factor Xa is effective and specific and the linkage is available for the proper cleavage.

3.2. Physical properties of MBP-C complex and cleavage products

Although the electrophoresis patterns in Fig. 1 demonstrate that the genetic construct leads to a single polypeptide chain comprising both the region corresponding to MBP and that representing a c chain of ATCase, those experiments provide no evidence as to the molecular weight and stability of the complex under nondenaturing conditions. Hence both sedimentation velocity and sedimentation equilibrium experiments were conducted on the purified MBP-C complex and the products resulting from the digestion by factor Xa. Sedimentation velocity experiments yielded a single sharp boundary indicative of a single species with a sedimentation coefficient (s) of 8.0S. Fig. 2a shows the apparent sedimentation coefficient distribution computed from the time derivative of the sedimentation velocity concentration profile according to the method of Stafford [20]. This value of 8.0S is consistent with that expected for a relatively compact complex consisting of a C trimer and three bound MBP molecules. A direct molecular weight determination by sedimentation equilibrium yielded 2.03×10^5 as compared to the value, 2.28×10^5, calculated from the sum of the values for one C trimer and three MBP molecules. These results show clearly that the complete polypeptide chain representing the MBP-C complex forms stable trimers like wild-type c chains and that the segments of the chains corresponding to MBP fold into stable, functional domains with one such molecule linked covalently to each folded c chain in the trimer.

Treatment of the MBP-C complex with factor Xa, as described above, leads to the cleavage of each of the chains in the trimer yielding a mixture of MBP and C trimers. This result is indicated by sedimentation coefficient distribution shown in Fig. 2b. Unlike the symmetrical $g^*(s)$ vs. s curve observed for the intact MBP-C complex (Fig. 2a) the distribution curve for the cleavage products shows pronounced asymmetry which can be accounted for readily by two Gaussian curves with s values of 3.0S and 5.4S. The former is the expected value for MBP with a molecular weight of 4.2×10^4 and the latter corresponds to the known value for the C trimer [21]. It should be noted that that extinction coefficient per mg of protein is much higher for MBP (about 1.7 per mg/ml at 280 nm) than that of C trimer (0.7 per mg/ml) thus leading to a much larger apparent amount of MBP relative to C trimer. In a control experiment, equal molar amounts of purified MBP and C trimer were mixed and examined in the ultracentrifuge. The resulting distribution curve, $g^*(s)$ vs. s, shown in Fig. 2c, is very similar to that resulting directly from the incubation of the MBP-C complex with factor Xa.

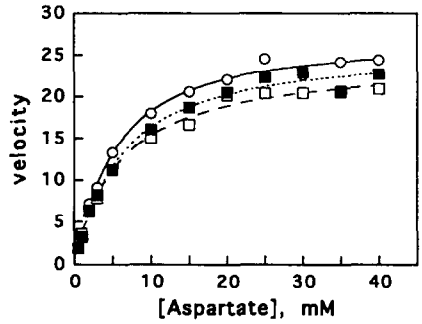

Fig. 3. Enzyme activity of MBP-C and C trimer before and after denaturation by urea. Assays were performed at 30°C in 50 mM Mops buffer at pH 7.0 containing 2 mM 2-mercaptoethanol and 0.2 mM EDTA using [^{14}C]carbamoyl phosphate according to the procedure of Davies et al. [17]. Enzyme activity as a function of the concentration of aspartate is expressed as velocity in micromoles of carbamoylaspartate formed per microgram of C trimer per hour. □, MBP-C; ○, purified C trimer from wild-type ATCase; ■, MBP-C formed after incubating the complex at 0°C for 45 min in a solution containing 6 M urea followed by a 10-fold dilution at 0°C and dialysis overnight at 0°C in the assay buffer.

3.3. Enzyme activity of MBP-C complex and reversibility of denaturation by urea

Assays for ATCase activity were conducted on the MBP-C complex to determine whether the linkage of the MBP chains at the N-terminus of each c chain caused any distortion of the C trimer sufficient to affect the active sites at the interface between the adjacent chains. As seen in Fig. 3 the saturation curves for enzyme activity as a function of aspartate concentration for the complex and pure C trimer are very similar with almost identical values of V_{max} and K_m. Moreover, as had been shown previously with pure C trimers [22], denaturation of the complex in 6 M urea, which leads to single, unfolded, inactive chains, followed by a 10-fold dilution and dialysis against a dilute buffer at pH 7.0 resulted in almost complete recovery of enzyme activity. The saturation curve for the renatured complex shown in Fig. 3 is very similar to those observed for the MBP-C complex and C trimers.

3.4. MBP and C trimer melt independently in thermal denaturation of the MBP-C complex

Differential scanning microcalorimetry experiments on the MBP-C complex showed two distinct endotherms (Fig. 4a) with the sharp, symmetrical peak at 53°C corresponding to the heat denaturation of MBP and the broad, asymmetric curve at about 63°C representing the thermal denaturation of the C trimers. The endotherm for the C trimers in the partially melted complex is very similar to that observed previously [12,18] for the wild-type C trimers under the same experimental conditions.

Fig. 4a also shows that the addition of the bisubstrate ligand *N*-(phosphonacetyl)-L-aspartate (PALA), which is bound very tightly at the active sites of ATCase [23], had no effect on the melting of

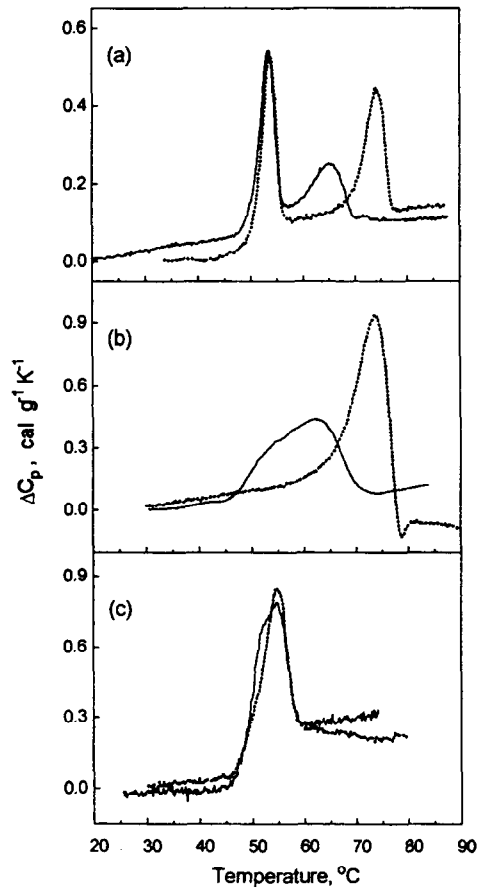

Fig. 4. Differential scanning calorimetry of fusion protein and the products formed by cleavage of MBP-C by digestion with factor Xa. Plots of the observed increase in heat capacity (ΔC_p) vs. temperature are shown by the solid line and the dotted line represents the data obtained for the solutions containing the bisubstrate ligand, PALA, at a concentration of 20 moles of PALA per mole of active sites. (a) fusion protein MBP-C; (b) C trimer from wild-type ATCase; (c) MBP.

the MBP in the complex. In contrast, both the shape of the endotherm and the T_m were markedly altered for the C trimer in the fusion protein upon the addition of PALA. This marked increase in the T_m of the PALA-liganded C trimer in the partially melted MBP-C complex, as compared to the unliganded C trimer in the complex, is in agreement with previous experiments with pure C trimer in the absence and presence of PALA [24,25].

Additional evidence supporting the view that the two proteins melt independently despite being linked covalently is shown in Fig. 4b and 4c. Purified C trimer exhibits the same asymmetrical thermal transition as observed in the fusion protein. Not only is the T_m similar but also, in the presence of PALA, the transition is much sharper and the T_m is increased from 63°C to 75°C. In contrast, as shown in Fig. 4c, PALA has no effect on the melting of MBP. As observed in the low melting component of the MBP-C, which is ascribed to the MBP with a T_m of 53°C, pure MBP exhibits a similar endotherm with a T_m also at 53°C.

3.5. Denaturation of the MBP-C complex by guanidine–HCl

Although little is known about the unfolding of the MBP chain upon treatment of the protein with denaturants like guanidine–HCl, it seemed of interest to study the denaturation of the complex to determine whether the individual domains "melt" independently as demonstrated above in the differential scanning microcalorimetry experiments. Accordingly the perturbation of the ultraviolet absorption at 287 nm was measured at different concentrations of guanidine–HCl. Fig. 5 shows the resulting titration curve for both the MBP-C complex and pure C trimer. The data demonstrate that a substantial change in absorbance occurs with the complex at guanidine–HCl concentrations which have little impact on the quaternary and tertiary structure of the C trimer.

No attempt was made to deconvolute the titration curve for the MBP-C complex in Fig. 5, but the data are consistent with the conclusion from the thermal denaturation experiment shown in Fig. 4a that MBP is denatured before the C trimer. It should be noted that the spectral changes in the MBP-C complex at guanidine–HCl concentrations below 1 M are heavily weighted toward the effect on the MBP part of the complex because its molar absorbance is more than two times that of C trimer. Without quantitative data for the denaturation of pure MBP, the inference that the two proteins in the complex are denatured independently must be considered tentative.

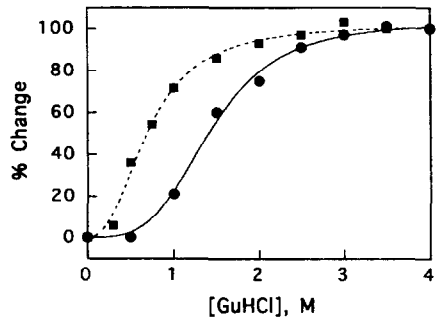

Fig. 5. Denaturation of MBP-C and C trimer by guanidine–HCl. The disruption of the quaternary and tertiary structures was measured by changes in the absorption spectra. Experiments were performed with tandem cells in a Cary 118 spectrophotometer and the values at 287 nm from the complete difference spectra are recorded as a function of the concentration of guanidine–HCl. After the contents of one of the tandem cells were mixed, that cell along with the other tandem cell were maintained at 0°C overnight in order to be certain that the denaturation was complete. Results for MBP-C are designated by ■ and for C trimer from ATCase by ●.

4. Discussion

There have been innumerable studies describing the use of fusion proteins and the requirements for their successful application in the expression and purification of target proteins and polypeptides. However, few of these studies involved physical chemical investigations of both the fusion protein and the individual components after the cleavage reaction. Moreover, it was of special interest to examine the use of gene fusion for a system in which the product of the target gene must form an oligomer to generate the shared active sites essential for enzyme activity. Although different proteins have been used in fusions for the isolation of target proteins, we used MBP because the vector leading to its expression is readily available and the fusion complex can be purified in good yield due to the binding of the MBP domain to an amylose column and its release upon the addition of maltose. Moreover, the unique

sensitive site for cleavage by factor Xa is already in the vector.

The gene fusion involving *mal*E and *pyrB* described above led to the expression of polypeptide chains encompassing the amino acid sequences of both MBP and the c chains of ATCase, as well as the 10 amino acid peptide linker between them. Both polypeptides in the complex folded to form their active, stable tertiary structures and, moreover, the chains did associate efficiently to give trimers with the same specific activity as the C trimers isolated from ATCase. The molecular weight data and the sedimentation coefficients demonstrated that the presence of MBP attached to the N-terminus of each c chain did not interfere with oligomer formation. Nor did it have any significant effect on the stability of the trimers. Conversely, the trimer formation did not have any effect on the active sites of MBP as judged by the binding and elution properties of MBP-C to the amylose column.

The two proteins were readily separated after cleavage of the complex by factor Xa indicating that the N-terminal region of each c chain in the trimer was sufficiently flexible and available for efficient rupture at the ILE–GLU–GLY–ARG recognition site. For some systems this is not feasible because the N-terminal region of the target protein is partially buried and the cleavage by factor Xa can be achieved on only the denatured fusion protein. Preliminary studies indicate that attempts to construct fusion proteins in which both components can fold successfully and oligomers can form is a useful way to assess the flexibility and availability of the N-terminal region of the target protein. We have succeeded, for example, in constructing a fusion between MBP and a C trimer in which the c chains are circularly permuted, i.e., the wild-type N- and C-terminal regions are linked covalently and new N- and C-termini are introduced elsewhere in the molecule ([26], Zhang and Schachman, submitted for publication). One such fusion protein involved circularly permuted c chains with the N-terminus at residue 236. This region of the chain is known from the crystallographic studies [27] to exist in the form of a flexible loop; hence the N- and C-termini formed in that region would be expected to be exposed and available for linkage to other proteins. It will be of interest to determine whether fusions can occur as well with circularly permuted chains having their new N- and C-termini in much less accessible regions of the structure.

Even though the linker between the globular MBP [28,29] and the folded c chain in the trimer is only 10 amino acids, the two constituents have little impact on each other. This is shown most clearly by thermal transitions in the differential scanning calorimetry experiments. MBP in the complex melts precisely at the same T_m as observed for the purified protein (Fig. 4). Similarly, the C trimer in the fusion protein has the same thermal transition as the purified C trimer. Moreover, the addition of PALA has the same impact on the melting curve of C trimer in the fusion protein as it does on the isolated C trimer. Hence these two proteins act independently despite being linked covalently through a relatively short peptide. Comparable heat denaturation experiments with other fusions and especially with different linkers will be useful in determining the extent to which the different domains are coupled energetically. Fusion proteins are likely to prove of value to protein chemists not only because of the increased expression and ease in isolating the target protein or polypeptide but also because they serve as excellent models to study folding of chains, assembly of oligomers, and the extent of domain-domain interactions in the bifunctional complexes.

Acknowledgements

This research was supported by National Institute of General Medical Sciences Grant GM 12156 and by National Science Foundation Grant DMB 9020605. The technical assistance of Mrs. Reiko Terauchi is greatly appreciated.

References

[1] M. Uhlén and T. Moks, Methods Enzymol., 185 (1990) 129.
[2] C.V. Maina, P.D. Riggs, A.G. Grandea III, B.E. Slatko and L.S. Moran, Gene, 74 (1988) 365.
[3] R.T. Baker, S.A. Smith, R. Marano, J. McKee and P.G. Board, J. Biol. Chem., 269 (1994) 25381.
[4] K. Nagai and H.C. Thøgersen, Nature, 309 (1984) 810.
[5] T.D. Parks, K.K. Leuther, E.D. Howard, S.A. Johnston and W.G. Dougherty, Anal. Biochem., 216 (1994) 413.
[6] J. Germino and D. Bastia, Proc. Natl. Acad. Sci. USA, 81 (1984) 4692.

[7] D.B. Smith and K.S. Johnson, Gene, 67 (1988) 31.
[8] R.J. Kreitman, R.K. Puri and I. Pastan, Proc. Natl. Acad. Sci. USA, 91 (1994) 6889.
[9] D.W. Markby, B.-B. Zhou and H.K. Schachman, Proc. Natl. Acad. Sci. USA, 88 (1991) 10568.
[10] B.-B. Zhou and H.K. Schachman, Protein Sci., 2 (1993) 103.
[11] B.-B. Zhou, G.L. Waldrop, L. Lum and H.K. Schachman, Protein Sci., 3 (1994) 967.
[12] C.B. Peterson, B.B. Zhou, D. Hsieh, A.N. Creager and H.K. Schachman, Protein Sci., 3 (1994) 960.
[13] E.A. Robey and H.K. Schachman, Proc. Natl. Acad. Sci. USA, 82 (1985) 361.
[14] S.R. Wente and H.K. Schachman, Proc. Natl. Acad. Sci. USA, 84 (1987) 31.
[15] K.F. Foltermann, M.S. Shanley and J.R. Wild, J. Bacteriol., 157 (1984) 891.
[16] H.K. Schachman, C.D. Pauza, M. Navre, M.J. Karels and L. Wu, Proc. Natl. Acad. Sci. USA, 81 (1984) 115.
[17] G.E. Davies, T.C. Vanaman and G.R. Stark, J. Biol. Chem., 245 (1970) 1175.
[18] C.B. Peterson and H.K. Schachman, Proc. Natl. Acad. Sci. USA, 88 (1991) 458.
[19] U.K. Laemmli, Nature, 227 (1970) 680.
[20] W.F. Stafford III, Anal. Biochem., 203 (1992) 295.
[21] J.C. Gerhart and H.K. Schachman, Biochemistry, 4 (1965) 1054.
[22] D.L. Burns and H.K. Schachman, J. Biol. Chem., 257 (1982) 8648.
[23] K.D. Collins and G.R. Stark, J. Biol. Chem., 246 (1971) 6599.
[24] L.P. Vickers, J.W. Donovan and H.K. Schachman, J. Biol. Chem., 253 (1978) 8493.
[25] V. Edge, N.M. Allewell, and J.M. Sturtevant, Biochemistry, 24 (1985) 5899.
[26] Y.R. Yang and H.K. Schachman, Proc. Natl. Acad. Sci. USA, 90 (1993) 11980.
[27] W.N. Lipscomb, Adv. Enzymol., 68 (1994) 67.
[28] J.C. Spurlino, G.-Y. Lu and F.A. Quiocho, J. Biol. Chem., 266 (1991) 5202.
[29] A.J. Sharff, L.E. Rodseth, and F.A. Quiocho, Biochemistry, 32 (1993) 10553.