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Tubulin Dimer Dissociation and Proteolytic Accessibility

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ABSTRACT: The α and β subunits of the tubulin dimer each possess a distal C-terminal subtilisin cleavage site which, when cleaved, releases an acidic, small peptide. In addition, each possesses an internal site, cleaved by trypsin in α and chymotrypsin in β, which connects the amino and carboxyl structural domains. A model of the dimer is presented which suggests that the β C-terminal subtilisin site may be more accessible in the monomer than in the dimer. Kinetics of cleavage at this site on the dimer yield straight-line plots of log (undigested fraction) versus time, from which pseudo-first-order rate constants are obtained. Temperature effects on the rate constant are due to changes in the activity of subtilisin, not to temperature-induced unfolding around this site. The rate constant is proportional to the subtilisin/tubulin ratio, whether this is varied by changing the concentration of subtilisin or of tubulin. However, if the rate constant increases due to decreasing tubulin concentration, the extrapolated zero time intercept decreases. The decrease in zero time intercept is interpreted as being due to the appearance of a rapidly digested fraction upon dilution of tubulin. The increase observed in this fast fraction with dilution of tubulin is fully reversible upon reconcentration. It is suggested that this fast fraction represents monomeric β-tubulin and the concentration dependence of this fast fraction indicates a dissociation constant of about 1.5×10^{-7} M.

Tubulin is a heterodimer composed of two quite similar but nonidentical subunits, α- and β-tubulin. These are each composed of an amino-terminal domain and a carboxy-terminal domain whose extreme end is highly charged. The linker region connecting the two domains of α-tubulin is split by trypsin (after Arg-339) and that connecting the two domains of β-tubulin by chymotrypsin (after Tyr-281) (Kirchner & Mandelkow, 1985; Sackett & Wolff, 1986). These cleavage sites are not accessible in microtubules or zinc sheet polymers. This, combined with the geometry of the polymers, indicates that these sites are both contained within the long axis of the polymer protofilament and thus likely exposed on opposite sides

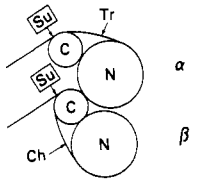

FIGURE 1: Proposed structure of the tubulin dimer. Each subunit is shown composed of an amino-terminal domain, a carboxy domain, an exposed linker region connecting these, and an exposed carboxy-terminal tail. The amino domain is larger than the carboxy domain in both subunits, and the two domains of each subunit are shown in contact. The intradimer α–β contact is shown between the β C-domain and the α N-domain. Sites of enzyme cleavage are indicated.

of the free dimer [see Figure 1 and Sackett and Wolff (1986)].

Limited subtilisin treatment cleaves the small carboxy-terminal tail from both subunits, resulting in a derivative of substantially altered assembly properties (Serrano et al., 1984; Bhattacharyya et al., 1985; Sackett et al., 1985). With extended incubation, subtilisin can also cleave between the two domains of both subunits (Sackett et al., 1986).

Cross-linking combined with proteolysis has indicated that the *intra*dimer contact region between the α and β subunits is between the α N-domain and the β C-domain (Kirchner & Mandelkow, 1985; Serrano & Avila, 1985). The *inter*dimer contact in protofilaments of microtubules is between the α C-domain and the β N-domain (Kirchner & Mandelkow, 1985). It is presumably the formation of this *inter*dimer, α C-domain–β N-domain contact which results in the loss of accessibility of the trypsin and chymotrypsin sites mentioned above. Electron microscopy and image processing of tubulin polymers indicate that the dimer unit cell is rhomboid (Baker & Amos, 1978; Amos, 1982). Combining all these features suggests that the overall structure of the dimer may be similar to that shown in Figure 1 [see also Sackett and Wolff (1986)]. Examination of the model suggests that, in addition to the limitations on access to trypsin and chymotrypsin sites imposed by formation of *inter*dimer contacts, formation of the *intra*dimer contact might limit access to the β C-terminal subtilisin site simply due to the presence of the α subunit.

Protease digestion can be used to probe changes in conformation of a protein as well as the average native structure. As an extreme example, many proteins are protease resistant in the native state but quite easily digested when denatured. Regions of a native protein which are relatively unfolded are more protease sensitive than compact domains. The ability of proteases to cleave the region between the amino- and carboxy-terminal domains of tubulin presumably reflects this (Kirchner & Mandelkow, 1985; Sackett & Wolff, 1986).

Conformational changes more subtle than frank denaturation can also be probed by proteolysis. Local melting of the helical structure of myosin has been examined in this manner (Ueno & Harrington, 1984) as well as structural flexibility in tropomyosin (Ueno, 1984). Transitions in protein folding as well as changes in the state of association of oligomeric proteins have been determined by proteolytic susceptibility (Girg et al., 1981; Rudolph, 1985; Optiz et al., 1987). Any change in a protein's conformation that enhances the accessibility of a region to proteolytic attack should, in principle, be detectable, either as a change in the digestion pattern or as a change in the kinetics of cleavage (Neurath, 1980; Ueno & Harrington, 1984).

In this paper, subtilisin digestion is used to probe conformational changes in tubulin. This study provides an example of changes in the apparent accessibility of the subtilisin cleavage site resulting from the interaction between the α and β subunits.

Materials and Methods

Tubulin was prepared by phosphocellulose chromatography of microtubule protein purified from rat brain by two cycles of temperature-dependent polymerization in Mes assembly buffer [0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes),[1] 1 mM $MgCl_2$, and 1 mM EGTA, pH 6.9] with 1 mM GTP (Sloboda & Rosenbaum, 1982). Protein was stored at −70 °C or in liquid nitrogen, following drop freezing in liquid nitrogen.

Subtilisin BPN and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated α-chymotrypsin were from Sigma. They were dissolved in water at 1 mg/mL and stored in aliquots at −70 °C. Aliquots were thawed only once.

The intrinsic activity of subtilisin was monitored by using *p*-nitrophenyl acetate (PNA). A stock solution of 0.25 M PNA in dimethyl sulfoxide was diluted to a final concentration of 1.5 mM in assembly buffer. After equilibration to the desired temperature, enzyme was added to 0.01 mg/mL and the increase in absorbance at 400 nm monitored continually with a Cary 219 double-beam spectrophotometer. In a similar way, chymotrypsin activity was monitored using *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTNA).

Digestion of tubulin was at the indicated temperature in Mes assembly buffer with 1 mM GTP except as noted. At the appropriate time points, aliquots were removed from the reaction mixture, and the reaction was stopped by the addition of phenylmethanesulfonyl fluoride in dimethyl sulfoxide to a concentration of 0.2 mM. Experiments with PNA and BTNA confirmed that this stopped both subtilisin and chymotrypsin hydrolysis essentially instantaneously. Samples were placed on ice until prepared for electrophoresis. Aliquots from dilute solution were precipitated with 80% ethanol (−20 °C, 1 h) and centrifuged so that equal amounts were loaded on the gels.

Electrophoresis of samples was performed in vertical sodium dodecyl sulfate–polyacrylamide slab gels, using a modification of the method of Laemmli (1970). Gels contained 9% acrylamide and 0.6% Acrylamide (FMC) and were cast using Gelbond PAG (FMC). The lower gel buffer pH was 9.2, and the SDS in the electrode buffer (0.1%) was Sigma "Lauryl Sulfate" or equivalent (i.e., containing significant tetradecyl sulfate), in order to increase separation of α- and β-tubulins (Best et al., 1981). Samples were prepared for electrophoresis by removing from ice, mixing with SDS loading solution (1% w/v SDS, 10% v/v glycerol, 5% v/v mercaptoethanol, and 0.01 M Tris-HCl, pH 6.8, with bromophenol blue as tracking dye), and boiling immediately for 1 min. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250, diffussion destained, soaked in 5% glycerol, and air-dried. When dried, gels were covered with a piece of clear plastic which was pressed in place on the gel and served to preserve the surface and prevent curling. Molecular weights were estimated by using the following standards (Bio-Rad Corp., molecular weights × 10^{-3}): phosphorylase B (94), bovine serum albumin (66), ovalbumin (46), carbonic anhydrase (29), and soybean trypsin inhibitor (21.5).

Quantitation of gels was performed on dried gels, using an LKB 2202 laser densitometer equipped with a Model 2220 recording integrator. The intensity of each band was normalized to the total absorbance in each scan in order to eliminate variations due to loading volume or staining (Ueno

[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid; PNA, *p*-nitrophenyl acetate; BTNA, *N*-benzoyl-L-tyrosine-*p*-nitroanilide; SDS, sodium dodecyl sulfate.

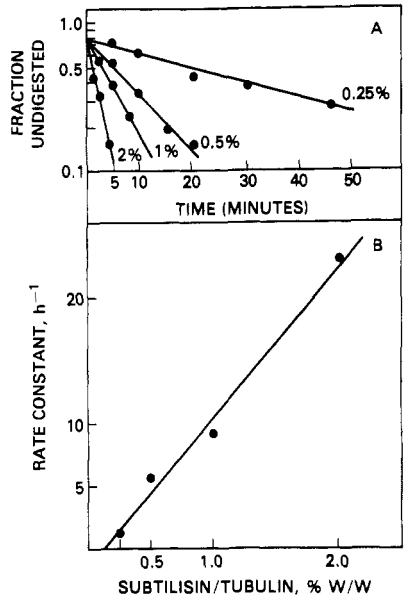

FIGURE 2: Variation of subtilisin concentration at constant tubulin concentration. Tubulin, at 1 mg/mL, was digested with subtilisin at 2%, 1%, 0.5%, and 0.25% w/w to tubulin at 30 °C. (A) Semilog plots of digestion data were linear at all subtilisin concentrations. (B) Reaction rate constant [from (A)] increases linearly with subtilisin concentration.

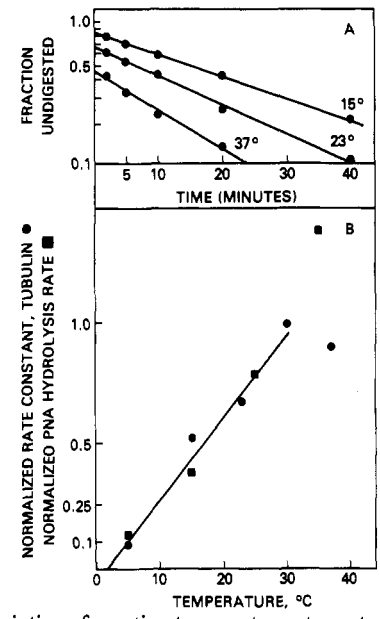

FIGURE 3: Variation of reaction temperature at constant tubulin and subtilisin concentrations. (A) Tubulin was preequilibrated to the desired temperature at 1 mg/mL and digested with 0.01 mg/mL subtilisin. (B) Rate constants for tubulin digestion were normalized to the value at 30 °C set equal to 1 (●). Hydrolysis rates for subtilisin cleavage of PNA preequilibrated to the desired temperatures were also normalized to the value at 30 °C set equal to 1 (■).

& Harrington, 1984). The fraction undigested was calculated for each time point, and data were plotted as log (undigested fraction) versus time of digestion. Lines were chosen by linear regression. The half-times for digestion were obtained graphically, and the apparent pseudo-first-order rate constant was calculated as $k = 0.693/t_{1/2}$.

Results

Examination of the time course of digestion of tubulin by subtilisin reveals that the β subunit is cleaved more rapidly than the α subunit (Bhattacharyya et al., 1985; Sackett et al., 1986). Semilog plots of band density versus time yield straight lines for digestion of both subunits, and the apparent pseudo-first-order rate constant derived from these plots is typically 3–4-fold higher for β than for α digestion (Sackett et al., 1986). Quantitation of digestion of the α C-terminus is technically more difficult than for β. This is due to the longer digestion time periods required by the lower apparent rate constant for α, incomplete electrophoretic separation of α from cleaved α, and reasons discussed below. Therefore, the rest of these studies refer to digestion of the β C-terminus.

Variation of the concentration of subtilisin yields the data in Figure 2. As expected, increasing subtilisin concentration at constant tubulin concentration causes a linear increase in the apparent rate constant (Figure 2B). At all rates, linear semilog plots are obtained (Figure 2A), and the pattern of digestion on the gel is not changed by altering the subtilisin concentration.

When the temperature is varied at contstant tubulin and subtilisin concentrations, the apparent rate constant increases with increasing temperature as expected (Figure 3). In order to determine if the temperature change caused a change in structure around the β C-terminal cleavage site, the apparent rate constant for β cleavage was compared to the rate for PNA hydrolysis (Figure 3B). A change in the protein which altered this region would be expected to change the apparent rate constant for β hydrolysis relative to the rate of PNA hydrolysis (Ueno & Harrington, 1984). The close agreement suggests that there is no major structural change around the β C-terminal site in this temperature range and that the rate of change primarily reflects the properties of the enzyme. Note, however, that while the data yield straight lines on semilog plots (Figure 3A), the zero time intercept is no longer constant but decreases with increasing temperature (see below).

It has previously been observed that internal cleavage by subtilisin (cleavage of the linker between the N- and C-domains) occurs with prolonged time (~1 h) at higher temperature (Sackett et al., 1986). For this reason, and to minimize the displacement of the zero time extrapolation, further studies were done at low temperatures (5 °C) and shorter times. Cleavage of the α C-terminus is suppressed disproportionately at low temperature (Bhattacharyya et al., 1985). The basis of this is not understood, and this reinforced the reasons given above for limiting the analysis to the β C-terminus.

Figure 4 presents data from experiments in which the tubulin concentration was varied at constant subtilisin concentration. The digestion pattern (Figure 4A) is not changed by dilution of the tubulin. Some digestion of α becomes evident at low concentration, but there are no new bands nor is there any evidence of internal cleavage. Quantitation of the gels reveals (Figure 4B) that the apparent rate constant increases with decreasing tubulin concentration and that the zero time displacement from 1.0 increases with decreasing tubulin concentration. The reaction rate constant is actually inversely proportional to tubulin concentration (Figure 4C).

The apparent rate constant for β hydrolysis is proportional to the subtilisin/tubulin ratio whether the ratio is increased by increasing subtilisin concentration (Figure 2) or by decreasing tubulin concentration (Figure 4). There is a difference between the two cases, however. If the apparent rate constant is increased by increasing subtilisin concentration, the zero time intercept is unaffected. By contrast, when the apparent rate constant increases due to decreasing tubulin concentration, however, the zero time intercept does not remain the same but decreases with decreasing tubulin concentration (Figure 4B).

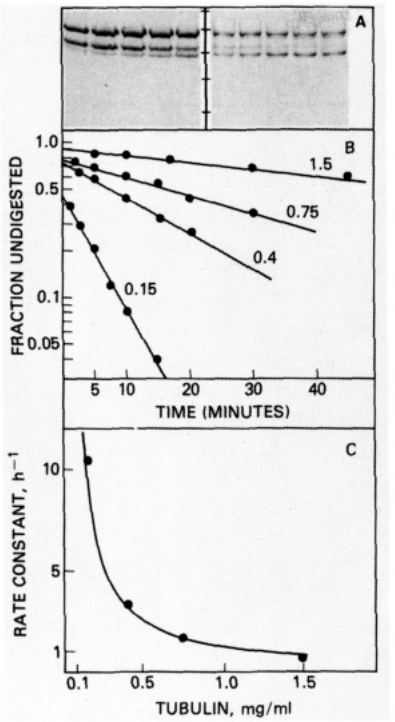

FIGURE 4: Variation of tubulin concentration at constant subtilisin concentration and temperature. (A) Time course of digestion of tubulin at 1.5 mg/mL (left) and 0.15 mg/mL (right), with 0.01 mg/mL subtilisin at 5 °C. Aliquots were removed at 5, 10, 17, 30, and 45 min from the 1.5 mg/mL sample and at 1, 2.5, 5, 7.5, and 10 min from the 0.15 mg/mL sample. Time increases left to right in both panels. Aliquots were processed for electrophoresis as described under Materials and Methods. Approximate molecular weights ($\times 10^{-3}$) are indicated by the tic marks on the center axis and are (top to bottom) 70, 60, 50, 40, and 30. (B) Quantitation of digestion time courses at different tubulin concentrations. Semilog plots yielded straight lines at all tubulin concentrations. The decrease in the zero time extrapolation with decreasing tubulin concentration suggests the existence of a rapidly digested fraction (see text). (C) The reaction rate constant is plotted versus tubulin concentration. The solid line is that calculated for the case that the rate constant is inversely proportional to tubulin concentration.

While more complex explanations might be constructed, the simplest interpretation of the downward displacement of the zero time intercept is that there are two fractions: one digested very fast and one digested more slowly. At low concentrations of tubulin, the fast fraction accounts for more than half of the total, while at higher concentrations the slow fraction is predominant. As tubulin concentration is steadily decreased, the "fast" fraction steadily increases, as indicated by the decrease in the zero time value (notice in Figure 4A that significant hydrolysis of β is evident even at very short time periods). The relation between the fast fraction and tubulin concentration is not the same as that between the apparent rate constant and tubulin concentration (see below).

The experiments presented in Figure 5 demonstrate that the increase in the fast fraction with decreasing tubulin concentration is reversible and not due to dilution-induced denaturation. Tubulin was diluted to 0.1 mg/mL and incubated at 5 °C for 30 min. The solution was then concentrated by ultrafiltration and adjusted to 1 mg/mL. The time course of subtilisin digestion for this solution (Figure 5A, gel b; Figure 5B, curve b) was compared with that for freshly prepared solutions of tubulin at 1 mg/mL (Figure 5A, gel a; Figure 5B, curve a) and 0.1 mg/mL (Figure 5A, gel c; Figure 5B, curve c). Clearly the appearance of the fast fraction is reversible. Thus, the percent fast fraction detected is a function of the concentration of tubulin and not the history of the solution.

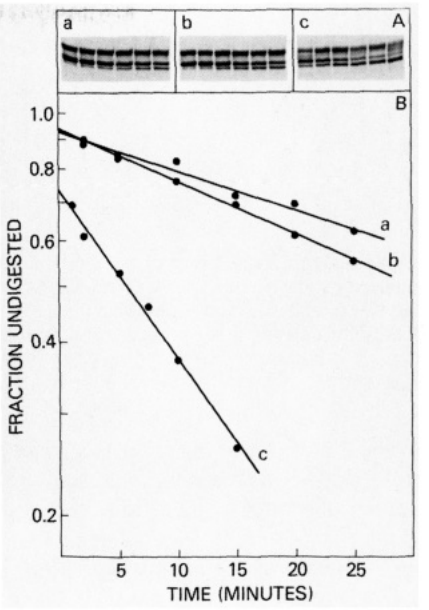

FIGURE 5: Reversibility of the fast fraction. Tubulin was diluted to 0.1 mg/mL and incubated at 5 °C for 30 min. The solution was concentrated to 1 mg/mL by centrifugation at about 5 °C in an Amicon Centricon-30 (89% recovery). The reconcentrated solution, a freshly prepared 1 mg/mL solution, and a freshly prepared 0.1 mg/mL solution of tubulin were equilibrated at 5 °C for 15 min after which subtilisin was added (0.01 mg/mL) and samples were removed at indicated times and processed for electrophoresis. Gel patterns are shown in panel A, quantitation in panel B. In both panels, a = 1 mg/mL, b = 0.1 mg/mL reconcentrated to 1 mg/mL, and c = 0.1 mg/mL. Time points for the gel samples are (left to right) 2, 5, 10, 15, 20, and 25 min for curves a and b and 1, 2.5, 5, 7.5, 10, and 15 min for curve c.

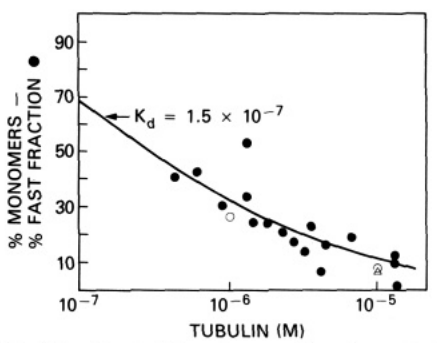

FIGURE 6: Rapidly digested fraction as a function of tubulin concentration. Data from a number of experiments such as Figure 5 were combined. The percent fast fraction, corresponding to the displacement of the zero time extrapolation from 1, is plotted versus tubulin concentration. The open symbols are from the experiment of Figure 5; the open triangle represents the diluted and reconcentrated sample. The line shows the relation between percent monomers and tubulin concentration for a dimer dissociating with K_d of 1.5×10^{-7} M.

It is known (Detrich & Williams, 1978; Detrich et al., 1982) that at low concentrations, tubulin dimers reversibly dissociate into monomers. Thus, the possibility occurred to us that the rapid rate of β hydrolysis might represent hydrolysis of the β as monomer whereas the slow rate would represent β hydrolysis in the dimer. Figure 6 shows the concentration dependence of the fast fraction. The line in this figure shows the percent of monomers versus concentration for a monomer–dimer system with a K_d of 1.5×10^{-7} M. The apparent fit of the data suggests that the existence of the fast fraction may indeed be due to the dissociation of the tubulin dimer into monomers, with the monomers being digested much more rapidly than the dimer.

As discussed above (Figure 1), the β C-terminal subtilisin site might be expected to become more accessible in the mo-

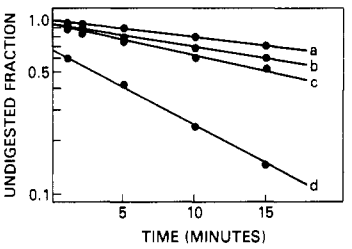

FIGURE 7: Chymotryptic digestion does not show a fast fraction at low tubulin concentrations. Time course of digestion of β C-terminus by subtilisin at two tubulin concentrations is compared to chymotryptic digestion of the internal linker region of β-tubulin at the same two concentrations of tubulin. (a, b) Chymotryptic digestion; (c, d) subtilisin digestion; (a, c) 0.45 mg/mL tubulin; (b, d) 0.15 mg/mL tubulin.

nomer than the dimer, while the other three sites would not. Despite the difficulties in obtaining reliable quantitative data on α C-terminal digestion by subtilisin mentioned above, it is clear that dilution of tubulin does not generate a substantial fast fraction for α cleavage (compare short times of cleavage of α versus β in Figure 4A, right panel). This point can be examined better by using a site whose cleavage is not suppressed at these temperatures and whose products are better resolved, such as the chymotrypsin site on the β subunit. This has the advantage of being on the same subunit as the subtilisin site examined above.

As shown in Figure 7, chymotrypsin cleaves β-tubulin, yielding the N- and C-terminal domains as previously shown (Sackett & Wolff, 1986), but no fast fraction is observed upon lowering the tubulin concentration. This is in sharp contrast to the response to subtilisin (line d in Figure 7).

Discussion

In this study, we have used proteolytic probes of different regions of the protein to examine changes in tubulin structure associated with temperature and concentration changes. The β subunit is the focus of these studies with subtilisin. At high concentrations of tubulin, the hydrolysis appears simple, yielding a straight-line plot of log (uncleaved β) versus time which extrapolates to 1 at zero time. As tubulin concentration is decreased, the gel pattern of digestion is not altered. No novel bands appear, and internal cleavage is not observed. However, the reaction becomes markedly biphasic. A fraction of the protein is still digested similarly to that seen at higher concentrations, yielding straight semilog plots and apparent rate constants proportional to the subtilisin/tubulin ratio. Furthermore, the apparent rate constant for this cleavage shows a temperature dependence that is essentially the same as that for subtilisin cleavage of NPA, indicating that there is no temperature-induced structural change to this site in this fraction.

As tubulin concentration is decreased, the data no longer extrapolate to 1 at zero time, suggesting the existence of a rapidly digested fraction. Since the gel pattern is unaltered at low tubulin concentrations, this fast fraction is not due to major unfolding of the protein. The cleavage products appear to be the same in the fast and slow fractions. The existence of the fast fraction is not due simply to the increase in rate constant of the slow fraction with decreasing tubulin concentration, since the rate constant can be increased by increasing subtilisin concentration at constant tubulin concentration (Figure 2); no dependence of the zero time value on subtilisin concentration (and hence rate constant) is seen. In other words, increases in the rate of the slow fraction cannot account for the creation of a second component, the fast fraction. Moreover, dilution-induced denaturation is not likely to be the explanation for the fast fraction as shown by the essentially complete reversibility of dilution-induced increases in the fast fraction (Figure 5).

Equilibrium ultracentrifugation studies of bovine brain tubulin have indicated that tubulin dimer exists in equilibrium with dissociated monomers (Detrich & Williams, 1978; Detrich et al., 1982). At high tubulin concentration, essentially all is present as dimer, with progressively more monomer at lower concentrations. The concentration dependence of the fast fraction observed here (Figure 6) suggests that dimer dissociation may explain the appearance of the fast fraction. The β monomer may be a better substrate for the enzyme than β in the $\alpha\beta$ dimer simply because the α subunit is not there.

If the rapidly digesting fraction seen with subtilisin treatment does correspond to monomeric β-tubulin, these studies indicate that the dissociation constant for the tubulin dimer is about 1.5×10^{-7} M (Figure 7). This result is lower than that obtained in centrifuge studies, $(8–10) \times 10^{-7}$ M (Detrich & Williams, 1978; Detrich et al., 1982). Although the data in Figure 3 indicate that increased temperature results in an increase in the fast fraction,[2] this is not likely to be the cause of the discrepancy, since both the centrifuge data and the data in Figure 6 were obtained at 5 °C. The complete explanation for the discrepancy is not clear, but contributing factors may include the tubulin source (rat brain here versus bovine brain), the buffer used (Mes buffer here versus Pipes), and the length of the experiment (30–45 min here versus 15–30 h) as well as the considerable difference in the techniques involved. Further study is needed to resolve this point.

The increased susceptibility of the monomer shown here is similar to that of lactate dehydrogenase, a "dimer of dimers". The tetrameric form of the enzyme is resistant to thermolysin while the dimer is cleaved quite rapidly, even though the dimer does not differ from the tetramer in the backbone structure of its subunits (i.e., the dimer is not unfolded relative to the tetramer) (Girg et al., 1981; Rudolph, 1985; Optiz et al., 1987). Apparently, a single dimer is a better substrate just because another dimer is not associated.

In the model of the tubulin dimer (Figure 1), the cleavage site for the β terminus is the only one of the four cleavage sites which might be expected to be more exposed in the monomer. The internal cleavage site for chymotrypsin is exposed on the dimer and might not be expected to be any more exposed in the monomer unless the protein unfolded upon dissociation. Unfolding does not seem to occur in the monomer within the time scale of these experiments (no new bands occur), and no rapidly digesting fraction is observed in chymotryptic digestion upon dilution of tubulin (Figure 7). Rather, dilution causes a change which is preferentially seen at the subtilisin β C-terminal site. Dissociation of the tubulin dimer could easily make this site more accessible (Figure 1), and thus the increase in the fast fraction with dilution is a monitor of the concentration dependence of dimer dissociation.

Acknowledgments

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[2] A combination of the temperature effect at 30 °C (Figure 3) and the concentration effect at 1 mg/mL (Figure 6) probably accounts for the low intercept in Figure 2A.

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Genetic Engineering of *Eco*RI Mutants with Altered Amino Acid Residues in the DNA Binding Site: Physicochemical Investigations Give Evidence for an Altered Monomer/Dimer Equilibrium for the Gln144Lys145 and Gln144Lys145Lys200 Mutants†

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ABSTRACT: We have genetically engineered the Arg200 → Lys mutant, the Glu144Arg145 → GlnLys double mutant, and the Glu144Arg145Arg200 → GlnLysLys triple mutant of the *Eco*RI endonuclease in extension of previously published work on site-directed mutagenesis of the *Eco*RI endonuclease in which Glu144 had been exchanged for Gln and Arg145 for Lys [Wolfes et al. (1986) *Nucleic Acids Res. 14*, 9063]. All these mutants carry modifications in the DNA binding site. Mutant *Eco*RI proteins were purified to homogeneity and characterized by physicochemical techniques. All mutants have a very similar secondary structure composition. However, whereas the Lys200 mutant is not impaired in its capacity to form a dimer, the Gln144Lys145 and Gln144Lys145Lys200 mutants have a very much decreased propensity to form a dimer or tetramer depending on concentration as shown by gel filtration and analytical ultracentrifugation. This finding may explain the results of isoelectric focusing experiments which show that these two mutants have a considerably more basic p*I* than expected for a protein in which an acidic amino acid was replaced by a neutral one. Furthermore, while wild-type *Eco*RI and the Lys200 mutant are denatured in an irreversible manner upon heating to 60 °C, the thermal denaturation process as shown by circular dichroism spectroscopy is fully reversible with the Gln144Lys145 double mutant and the Gln144Lys145Lys200 triple mutant. All *Eco*RI endonuclease mutants described here have a residual enzymatic activity with wild-type specificity, since *Escherichia coli* cells overexpressing the mutant proteins can only survive in the presence of *Eco*RI methylase. The detailed analysis of the enzymatic activity and specificity of the purified mutant proteins is the subject of the accompanying paper [Alves et al. (1989) *Biochemistry* (following paper in this issue)].

It is believed that class II restriction endonucleases like repressors recognize their palindromic target size as dimers composed of identical subunits by forming a symmetrical complex in which both protein subunits are engaged in the same set of interactions with their substrate (Smith, 1979). While considerable progress has been made in elucidating the structural basis for the high specificity of repressors toward their operator sites (Anderson et al., 1981, 1987; Pabo & Lewis, 1982; Schevitz et al., 1985; Lehming et al., 1987), similar detailed knowledge is only beginning to emerge for restriction enzymes, in particular for the *Eco*RI endonuclease (McClarin et al., 1986). The target sites of restriction endonucleases are small, typically four to eight base pairs long, hence considerably smaller than those of repressors (Modrich & Roberts, 1982). Nevertheless, restriction endonucleases

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