The N-end rule relates the in vivo half-life of a protein to the identity of its N-terminal residue. In eukaryotes, the N-end rule pathway is a ubiquitin-dependent, proteasome-based system that targets and processively degrades proteins bearing certain N-terminal residues. Arg-DHFR, a modified dihydrofolate reductase bearing an N-terminal arginine (destabilizing residue in the N-end rule), is short lived in ATP-supplemented reticulocyte extract. It is shown here that methotrexate, which is a folic acid analog and high affinity ligand of DHFR, inhibits the degradation but not ubiquitination of Arg-DHFR by the N-end rule pathway. The degradation of other N-end rule substrates is not affected by methotrexate. We discuss implications of these results for the mechanism of proteasome-mediated protein degradation.

The surface of a protein molecule bears a number of peptide bonds, which are potential cleavage sites for proteases. Nonetheless, only some intracellular proteins are short-lived in vivo, indicating that most of these solvent-exposed bonds cannot be cleaved by intracellular proteases. Features of a protein that make it short-lived in vivo or in vitro are called degradation signals or degrons (Varshavsky, 1991). The resistance of a long-lived protein to proteases located in the same compartment is due in part to the sequence selectivity of proteases, which require the presence of a sequence motif or at least a specific residue in a substrate. In addition, the rate of peptide bond cleavage by even a relatively nonspecific protease depends on conformational flexibility of the motif that the protease recognizes. For example, only some of the potential cleavage sites on the surface of a globular protein are cleaved efficiently by the bacterial metalloendopeptidase thermolysin, and these preferred cleavage sites are located in exposed segments of the polypeptide chain which have the highest spatial mobility (Fontana et al., 1986).

Thus, even if a motif recognized by a protease is present on the surface of a protein, conformational rigidity of this potential degron may preclude its efficient utilization by the protease. Conversely, a conformationally destabilized protein may acquire degrons that are masked in an unperturbed version of the protein (Parsell and Sauer, 1989). This can happen not only through conformational relaxation of a previously rigid (and therefore cryptic) surface degron but also through exposure of degrons in previously buried regions of the protein. The mechanistic connection between segmental mobility of a polypeptide chain and its susceptibility to proteolysis stems from a scarcity of local chain conformations that can lend themselves to an optimal transition-state intermediate without an additional adjustment (Fontana et al., 1986; Creighton, 1992; Hubbard et al., 1994).

Dynamic aspects of a substrate's conformation are likely to play a major role in the functioning of intracellular proteolytic systems such as those that involve the multisubunit, multi-catalytic protease called the proteasome (for reviews, see Lupas et al., 1995; Rechsteiner et al., 1993; Goldberg and Rock, 1992; Orlowski, 1990). A salient feature of these ATP-dependent systems is their processivity: once the degradation of a protein begins, it proceeds to completion. Thus, a proteasome-mediated system should be able to perturb the conformation of a globular protein substrate before or during its processive degradation by the proteasome. The "conformational" problem to be solved by the proteasome is analogous to the problem faced by a protein translocation system: components of a transmembrane channel must unfold a protein before or during its "threading" across membrane, except that in this case the protein is transported rather than destroyed (Blobel, 1980; Eilers and Schatz, 1986; Sanders and Schekman, 1992; Arkowitz et al., 1992).

In the present work, we show that a proteasome-based proteolytic system called the N-end rule pathway is remarkably sensitive to alterations in the conformational stability of its substrates. The N-end rule relates the in vivo half-life of a protein to the identity of its N-terminal residue (for review see Varshavsky, 1992). Similar but distinct versions of the N-end rule operate in all organisms examined, from mammals to bacteria (Bachmair et al., 1986; Gonda et al., 1989; Tobias et al., 1991). In eukaryotes, the N-end rule-based degradation signal, called the N-degron, comprises two determinants: a destabilizing N-terminal residue and an internal lysine (or lysines) of a substrate (Bachmair and Varshavsky, 1989; Johnson et al., 1990; Hill et al., 1993). The Lys residue is the site of formation of a mult ubiquitin chain (Chau et al., 1989). Ubiquitin (Ub) is a protein whose covalent conjugation to other proteins (often in the form of a multi-Ub chain) plays a role in a number of processes, primarily through routes that involve protein degradation (for reviews, see Ciechanover and Schwartz, 1994; Parsell and Lindquist, 1993; Vierstra, 1993; Jentsch, 1992; Varshavsky, 1992; Finley, 1992; Hochstrasser, 1992).

The recognition of an N-end rule substrate is mediated by a
protein called N-recognin or E3. The binding of N-recognin to a substrate's destabilizing N-terminal residue is followed by the formation of a multi-Ub chain linked to an internal lysine of the substrate (the second determinant of its N-degron). A substrate-linked multi-Ub chain is required for the degradation of at least some N-end rule substrates (Chau et al., 1989; Doehmen et al., 1991). In both yeast and mammalian cells, the ~200 kDa N-recognin and a specific ~20-kDa Ub-conjugating (E2) enzyme (one of several such enzymes in a cell) are physically associated, forming a part of a larger targeting complex (Madura et al., 1993; Hershko and Ciechanover, 1992). A substrate bearing a multi-Ub chain is transferred (presumably while still bound by the targeting complex) to the 26S proteasome, a ~2,000-kDa multicatalytic protease that contains about 40 distinct subunits (Rechsteiner et al., 1993). The ensuing processing degradation of the substrate yields short (~10-residue) peptides and regenerates Ub from a multi-Ub chain. All Ub-dependent proteolytic systems, including the N-end rule pathway, apparently share many components of the 26S proteasome. Differences among these pathways include their distinct targeting complexes, whose recognins (associated with specific E2s) bind to degradation signals other than N-degrons (Varshavsky, 1992).

Previous studies (Gonda et al., 1989; Reiss et al., 1988) showed that the N-end rule pathway is active in ATP-supplemented reticulocyte extract. In the present work we used this in vitro system and methoxetate (MTX, a folic acid analog and inhibitor of the enzyme dihydrofolate reductase (DHFR)), to determine whether noncovalent conformational stabilization of a protein affects its degradation by the N-end rule pathway.

MATERIALS AND METHODS

DNA Constructs—The plasmids pEJ11-J and pEJ11-M expressed, respectively, Ub-Arg-eK-DHFRha and Ub-Met-eK-DHFRha (Fig. 1) from the T7 polymerase promoter in Escherichia coli and were constructed using a three-part ligation as follows. (i) The pT7-7 vector (Tabor and Richardson, 1985) was digested with NdeI and HindIII, and the large fragment was purified by agarose gel electrophoresis, yielding the first of the three fragments (the second one encoding either Arg or Met at the Ub-eK junction) yielding pEJ11-J and pEJ11-M, encoding, respectively, Ub-Arg-eK-DHFRha and Ub-Met-eK-DHFRha. The plasmids pFJJ1-R and pFJJ1-M, expressing, respectively, Ub-Arg-eK-DHFRha and Ub-Met-eK-DHFRha (Fig. 1), were produced from pEJ11-R and pEJ11-M by excising their BamHI-HindIII fragment (encoding eK·DHFRha) and replacing it with a ~600-base pair fragment of pPW17-R (Doehmen et al., 1994), which encoded Arg-Asp-Gly-Ser-Gly-Ile-Met-DHFRha.

E. coli JM101 and DH5α (Ausubel et al., 1992) were used as hosts in plasmid construction and in plasmid preparation for sequencing, respectively. The final constructs were verified by restriction mapping and nucleotide sequencing (Ausubel et al., 1992). E. coli BL21(DE3) (Studier and Moffat, 1986) was used for overexpression of DHFR-containing fusions.

Ub-X-DHFRha Test Proteins—Unless stated otherwise, all procedures were carried out at 4°C. 0.5 ml of an overnight culture of E. coli BL21(DE3) carrying one of the pT7-based plasmids was diluted into 50 ml of Luria broth containing ampicillin at 75 µg/ml, and the culture was grown at 37°C to an A600 of ~0.2; more ampicillin was then added, to a final concentration of 115 µg/ml, and the incubation was continued until an A600 of ~0.6. Isopropl-1-thio-ß-galactopyranoside was then added to a final concentration of 0.2 mM. The culture was grown for another 25 min at 30°C. The cells were harvested by centrifugation at 4,000 × g for 10 min; washed twice with M9 buffer supplemented with 0.22% glucose, rifampicin (0.2 mg/ml), ampicillin (75 µg/ml), and 0.1% methionine assay medium (Difco); and incubated with 0.5 µCi of Trans35S-label (ICN) for 10 min at 30°C. The cells were collected by centrifugation, washed once with Luria broth, twice with M9 buffer, and resuspended in 0.5 ml of 25% (v/v) sucrose, 50 mM Tris-HCl, pH 8.0. 0.1 ml of a lysozyme solution (10 mg/ml in 0.25 M Tris-HCl, pH 8.0) was then added. After 5 min at room temperature, 0.8 ml of the lysis buffer (1% Triton X-100, 37 mM Na-EDTA, 50 mM Tris-HCl, pH 8.0) was added, and the suspension was vortexed for 10 s twice, followed by a quick freezing in liquid N2 and thawing of the sample in a 37°C bath. The lysate was centrifuged at 40,000 × g for 25 min, and Ub-X-DHFRha (this term denotes both Ub-ß-gal-DHFRha and Ub-ß-de-DHFRha; Fig. 1) in the supernatant was bound to the monoclonal anti-ßha 12CA5 (Field et al., 1988; Johnson et al., 1992), added as an ascitic fluid,
Two equal samples were withdrawn from reaction tubes at the times required for rapid degradation of X-f3gal by the N-end rule. Findings with the same X-f3gals in the yeast cytoplasm extract, producing X-f3gal test proteins bearing different terminal residues (Bachmair and Varshavsky, 1989). Extracts after the last residue of Ub, making possible the production of these lysines was revealed by the N-end rule substrate such as Arg-eK-DHFRha in reticulocyte extract. In the constructs of Fig. 1, the C terminus of Ub-X-eK-DHFRha was extended with a 14-residue sequence containing ha, an N-terminal extension of DHFR, derived from X-f3gal test proteins (Fig. 1) (Bachmair and Varshavsky, 1989), is denoted as eK (extension (e) containing lysine (K)). In the present work, the strategy used by Gonda et al. (1989) with βgal-based substrates was employed to examine the degradation of similarly designed DHFR-based substrates by the N-end rule pathway in reticulocyte extract. The ~40-residue N-terminal extension of DHFR, derived from X-βgal test proteins (Fig. 1) (Bachmair and Varshavsky, 1989), is denoted below as eK (extension (e) containing lysine (K)) (Johnson et al., 1992). In the constructs of Fig. 1, the C terminus of Ub-X-eK-DHFRha was extended with a 14-residue sequence containing ha, an epitope tag derived from the influenza virus hemagglutinin, which could be recognized by a monoclonal antibody (Field et al., 1988; Johnson et al., 1992). Two fusion proteins, Ub-Met-eK-DHFRha and Ub-Arg-eK-DHFRha (Fig. 1), bore, respectively, Met and Arg, a stabilizing and a destabilizing residue at the junction between Ub and the rest of a fusion (Varshavsky, 1992). These proteins were overexpressed in E. coli, labeled in vivo with [35S]methionine, and purified by affinity chromatography, using anti-ha antibody (see “Materials and Methods”). Ub-Met-eK-DHFRha and Ub-Arg-eK-DHFRha were rapidly deubiquitinated upon addition to ATP-depleted reticulocyte extract, yielding, respectively, Met-eK-DHFRha and Arg-eK-DHFRha (Fig. 2, A and B, lanes a and b), analogous to the previously characterized deubiquitination of Ub-X-eK-βgal fusions under the same conditions (Gonda et al., 1989). Both Met-eK-DHFRha and Arg-eK-DHFRha remained metabolically stable in ATP-depleted extract (data not shown), but the addition of ATP resulted in a much faster degradation of Arg-eK-DHFRha than Met-eK-DHFRha, which remained long lived in ATP-supplemented extract (Fig. 2, A and B, lanes b–e, and Fig. 3A). The metabolic fates of DHFR-based test proteins were monitored by SDS-PAGE (Fig. 2) and also by measuring the amount of acid-soluble [35S] released during the incubation of an [35S]-labeled protein in ATP-supplemented extract (Fig. 3). As shown in Fig. 3A, 33% of the initially present [35S]-labeled Arg-eK-DHFRha was degraded to acid-soluble fragments in ATP-supplemented reticulocyte extract after a 30-min incubation. By contrast, only 6% of the otherwise identical Met-eK-DHFRha, bearing an N-terminal Met (stabilizing residue in the N-end rule), was degraded after a 30-min incubation (Fig. 3A). Analysis by SDS-PAGE showed a transient accumulation of multiply ubiquitinated Arg-eK-DHFRha derivatives and a decrease in intensity of the band of unmodified Arg-eK-DHFRha in the course of its incubation in ATP-supplemented extract (Fig. 2B). By contrast, Met-eK-DHFRha was neither ubiquitinated nor significantly degraded in ATP-supplemented extract (Fig. 2A), in agreement with the findings about similarly designed X-βgal substrates (Gonda et al., 1989).

**RESULTS**

Linear Ub fusions are rapidly cleaved in vivo or in cell-free extracts after the last residue of Ub, making possible the production of otherwise identical proteins bearing different N-terminal residues (Bachmair et al., 1986; Baker et al., 1992). In one application of this method, Gonda et al. (1989) incubated fusions of Ub to E. coli β-galactosidase (Ub-X-βgal) in reticulocyte extract, producing X-βgal test proteins bearing different N-terminal residues. Depending on the identity of a residue X, an X-βgal is either short-lived or metabolically stable in ATP-supplemented extract (Gonda et al., 1989), similar to the in vivo findings with the same X-βgals in the yeast Saccharomyces cerevisiae (Bachmair et al., 1986). Mutational analysis has shown that either one of two lysines (Lys-15 or Lys-17) in a non-βgal N-terminal region of an X-βgal test protein was also required for its degradation. Degradation by the N-end rule pathway (Fig. 1) (Bachmair and Varshavsky, 1989; Johnson et al., 1990). The function of these lysines was revealed by the finding that Lys-15 or, alternatively, Lys-17, is the site of formation of a multi-Ub chain (Chau et al., 1989). The non-βgal, ~40-residue extension at the N terminus of βgal was derived in part from an internal sequence of E. coli Lac repressor (Bachmair and Varshavsky, 1989).

In the present work, the strategy used by Gonda et al. (1989) with βgal-based substrates was employed to examine the degradation of similarly designed DHFR-based substrates by the N-end rule pathway in reticulocyte extract. The ~40-residue N-terminal extension of DHFR, derived from X-βgal test proteins (Fig. 1) (Bachmair and Varshavsky, 1989), is denoted below as eK (extension (e) containing lysine (K)) (Johnson et al., 1992). In the constructs of Fig. 1, the C terminus of Ub-X-eK-DHFRha was extended with a 14-residue sequence containing ha, an epitope tag derived from the influenza virus hemagglutinin, which could be recognized by a monoclonal antibody (Field et al., 1988; Johnson et al., 1992). Two fusion proteins, Ub-Met-eK-DHFRha and Ub-Arg-eK-DHFRha (Fig. 1), bore, respectively, Met and Arg, a stabilizing and a destabilizing residue at the junction between Ub and the rest of a fusion (Varshavsky, 1992). These proteins were overexpressed in E. coli, labeled in vivo with [35S]methionine, and purified by affinity chromatography, using anti-ha antibody (see “Materials and Methods”). Ub-Met-eK-DHFRha and Ub-Arg-eK-DHFRha were rapidly deubiquitinated upon addition to ATP-depleted reticulocyte extract, yielding, respectively, Met-eK-DHFRha and Arg-eK-DHFRha (Fig. 2, A and B, lanes a and b), analogous to the previously characterized deubiquitination of Ub-X-eK-βgal fusions under the same conditions (Gonda et al., 1989). Both Met-eK-DHFRha and Arg-eK-DHFRha remained metabolically stable in ATP-depleted extract (data not shown), but the addition of ATP resulted in a much faster degradation of Arg-eK-DHFRha than Met-eK-DHFRha, which remained long lived in ATP-supplemented extract (Fig. 2, A and B, lanes b–e, and Fig. 3A). The metabolic fates of DHFR-based test proteins were monitored by SDS-PAGE (Fig. 2) and also by measuring the amount of acid-soluble [35S] released during the incubation of an [35S]-labeled protein in ATP-supplemented extract (Fig. 3). As shown in Fig. 3A, 33% of the initially present [35S]-labeled Arg-eK-DHFRha was degraded to acid-soluble fragments in ATP-supplemented reticulocyte extract after a 30-min incubation. By contrast, only 6% of the otherwise identical Met-eK-DHFRha, bearing an N-terminal Met (stabilizing residue in the N-end rule), was degraded after a 30-min incubation (Fig. 3A). Analysis by SDS-PAGE showed a transient accumulation of multiply ubiquitinated Arg-eK-DHFRha derivatives and a decrease in intensity of the band of unmodified Arg-eK-DHFRha in the course of its incubation in ATP-supplemented extract (Fig. 2B). By contrast, Met-eK-DHFRha was neither ubiquitinated nor significantly degraded in ATP-supplemented extract (Fig. 2A), in agreement with the findings about similarly designed X-βgal substrates (Gonda et al., 1989).

Previous work has shown that an amino acid derivative such as a dipeptide that bears a destabilizing N-terminal residue can inhibit the degradation of a βgal-based N-end rule substrate either in vitro (reticulocyte extract) (Gonda et al., 1989) or in vivo (yeast cells) (Baker and Varshavsky, 1991). As shown in Figs. 2C and 3A, the degradation of a DHFR-based N-end rule substrate such as Arg-eK-DHFRha in reticulocyte extract was almost completely inhibited by the Arg-Ala dipeptide. Dipeptides bearing destabilizing N-terminal residues inhibit the N-end rule pathway by competing with N-end rule substrates for binding to N-recognin (Reiss et al., 1998; Gonda et al., 1989; Baker and Varshavsky, 1991). Arg-Ala not only precluded the degradation of Arg-eK-DHFRha (Fig. 3A) but also inhibited its ubiquitination (Fig. 2C), indicating that Arg-Ala blocks a step in the N-end rule pathway which precedes the ubiquitination step.

We asked whether the folate analog MTX, a high affinity DHFR ligand ($K_D \approx 10^{-11} \text{M}$) and a competitive inhibitor of DHFR (Matthews et al., 1985), would affect the degradation of
MTX Inhibits DHFR Degradation

FIG. 2. Effect of MTX on the degradation of DHFR-based N-end rule substrates in reticulocyte extract. Panel A: lane a, purified, 35S-labeled Ub-Met-eK-DHFRha (the amount of substrate added to this lane and analogous lanes in other panels was larger than the initial amounts of substrate in lane b and analogous lanes). Lane b, Ub-Met-eK-DHFRha was added to ATP-depleted reticulocyte extract and incubated for 10 min at 37 °C (time zero sample). Lanes c–e, same as lane a, but the samples were withdrawn and analyzed by SDS-PAGE 15, 30, and 60 min after the addition of ATP at time zero (see "Materials and Methods"). Panel B: same as panel A but with Ub-Arg-eK-DHFRha. Panel C: same as panel B, but the Arg-Ala dipeptide was added to reticulocyte extract (to the final concentration of 10 mM) together with Ub-Arg-eK-DHFRha. Panel D: lanes a–e, same as panel A, but the data are from another experiment. Lanes f–i, same as lanes b–e, but the assay was carried out in the presence of 20 µM MTX (see "Materials and Methods"). Panel E: lane a, purified, 35S-labeled Ub-Arg-Δe-DHFRha. Lanes b–d, same as lanes b–d in panel A, but the assay was carried out with Ub-Arg-Δe-DHFRha, and the incubation times were 0, 10, and 30 min (after the addition of ATP). Lanes e–h, same as lanes a–d, but the assay was carried out in the presence of 20 µM MTX. Arrowheads and arrows indicate, respectively, the bands of 36-kDa Ub-X-eK-DHFRha fusions and their 28-kDa deubiquitinated derivatives, X-eK-DHFRha (Ub-Arg-Δe-DHFRha and Arg-Δe-DHFRha in panel E). Asterisks indicate the band of 8-kDa Ub, produced by deubiquitination of 35S-labeled Ub-X-eK-DHFRha and Ub-Arg-Δe-DHFRha. Half-open square brackets denote the bands of multibiquitinated Arg-eK-DHFRha. The incubation times are indicated above the lanes.

FIG. 3. Degradation of DHFR-based N-end rule substrates in reticulocyte extract, measured by determining acid-soluble 35S. Panel A: □, Met-eK-DHFRha; ♦, Arg-eK-DHFRha; x, Arg-eK-DHFRha in the presence of 1 mM Arg-Ala dipeptide; ○, Arg-eK-DHFRha in the presence of 20 µM MTX. Panel B: □, same as in panel A but from another experiment with Met-eK-DHFRha; ♦, Arg-eK-DHFRha; ○, Arg-Δe-DHFRha; □, Arg-Δe-DHFRha in the presence of 20 µM MTX. Each decay curve was determined at least three times, in independent experiments, with the results differing by less than 15% for each of the time points. See "Materials and Methods" for the definition of a zero time point and other details.

A DHFR-based N-end rule substrate. Remarkably, the presence of MTX in ATP-supplemented reticulocyte extract resulted in a nearly complete inhibition of Arg-eK-DHFRha degradation (Fig. 3A). The inhibitory effect of MTX was confined to the actual proteolysis of Arg-eK-DHFRha: its multibiquitination was in fact enhanced by MTX, in contrast to the effect of Arg-Ala, which inhibited both the degradation and ubiquitination of Arg-eK-DHFRha (Fig. 2D; compare with Fig. 2C).

We also determined the effect of MTX on degradation of Arg-Δe-DHFRha (derived from Ub-Arg-Δe-DHFRha), which lacked most of the ~40-residue, lysine-containing eK extension of Arg-eK-DHFRha (Fig. 1). Previous work has shown that Arg-Δe-DHFRha is much longer lived than Arg-eK-DHFRha in the yeast S. cerevisiae at 30 °C (t1/2 of more than 4 h versus ~10 min, respectively); it has also been shown that a major reason for the increased metabolic stability of Arg-Δe-DHFRha is the...
absence of Lys-15 and Lys-17 residues: Arg-eK-DHFRha, which contains Arg instead of Lys at positions 15 and 17 of the otherwise unaltered eK extension, is nearly as long-lived in yeast as Arg-Δe-DHFRha (Bachmair and Varshavsky, 1989). In a qualitative agreement with these in vivo data, Arg-Δe-DHFRha was degraded more slowly than Arg-eK-DHFRha in ATP-supplemented reticulocyte extract (Fig. 3B). The degradation of Arg-Δe-DHFRha was mediated by the N-end pathway, inasmuch as Met-Δe-DHFRha, bearing a stabilizing N-terminal residue, was degraded at a much lower rate than Arg-Δe-DHFRha (data not shown).

Similarly to the findings with Arg-eK-DHFRha (Figs. 2D and 3A), the addition of MTX almost completely inhibited the degradation of Arg-Δe-DHFRha in ATP-supplemented reticulocyte extract (Fig. 3B). In contrast to the extensive ubiquitination of Arg-eK-DHFRha prior to its degradation (Fig. 2, B and D), the degradation of Arg-Δe-DHFRha was not accompanied by a significant accumulation of its mult ubiquitinated derivatives, and no enhancement of multiubiquitination of Arg-Δe-DHFRha could be detected in the presence of MTX as well (Fig. 2E).

The effect of MTX was confined to DHFR-based substrates: in parallel assays with βgal-based N-end rule substrates such as Arg-eK-βgal (Gonda et al., 1989), the addition of MTX did not alter the kinetics of Arg-eK-βgal degradation (data not shown).

**DISCUSSION**

The MTX-DHFR assay has been used previously to address the mechanistic and kinetic aspects of protein translocation across membranes (Eilers and Schatz, 1986, 1988; Vestweber and Schatz, 1988; Wienhues et al., 1991; Arkowitz et al., 1992). The present work extends the applications of MTX-DHFR to the problem of Ub-dependent protein degradation. We report the following results.

1) Arg-eK-DHFRha (Fig. 1) is degraded by the N-end rule pathway in ATP-supplemented reticulocyte extract in the absence but not in the presence of MTX, a folic acid analog and a competitive inhibitor of DHFR which binds to mammalian DHFRs with a K_0 of ~10^{-11} M (Fig. 3A).

2) The effect of MTX is confined to DHFR-based substrates, inasmuch as the degradation of other N-end rule substrates such as Arg-eK-βgal is not inhibited by MTX.

3) The degradation of Arg-eK-DHFRha (this work) and other N-end rule substrates (Gonda et al., 1989) in reticulocyte extract is highly processive: no degradation intermediates could be detected by SDS-PAGE in the course of proteolysis.

4) MTX inhibits the degradation but not ubiquitination of Arg-eK-DHFRha, whose multiubiquitinated derivatives become more abundant in the presence of MTX (Fig. 2D). Previous work (Chau et al., 1989; Bachmair and Varshavsky, 1989) has shown that the degradation of N-end rule substrates such as Arg-eK-βgal and Arg-eK-DHFRha requires (and is preceded by) the formation of a multi-Ub chain linked to one of two lysines (Lys-15 or Lys-17) in the ~40-residue extension (eK) at the N terminus of Arg-eK-DHFRha.

5) Arg-Δe-DHFRha, which lacks the eK extension, is also degraded (at a lower rate than Arg-eK-DHFRha) by the N-end rule pathway in reticulocyte extract, and this degradation is also inhibited by MTX (Fig. 3B). Only traces of multiubiquitinated Arg-Δe-DHFRha derivatives were observed during its degradation by the N-end rule pathway, and the relative content of these derivatives was further decreased in the presence of MTX (Fig. 2E and data not shown).

The extensive ubiquitination of Arg-eK-DHFRha prior to its degradation indicates that ubiquitination is not rate-limiting for the degradation of Arg-eK-DHFRha in reticulocyte extract. By contrast, the scarcity of ubiquitinated derivatives of Arg-Δe-DHFRha suggests that ubiquitination of Arg-Δe-DHFRha is among the slowest steps that precede its degradation by the N-end rule pathway. A likely explanation for these differences between Arg-eK-DHFRha and Arg-Δe-DHFRha is the difference in locations of multi-Ub chains attached to these substrates: in Arg-eK-DHFRha, a multi-Ub chain is linked to one of the sterically accessible lysines (Lys-15 or Lys-17) in a flexible N-terminal extension (eK) located outside of the folded DHFR globule (Fig. 1) (Bachmair and Varshavsky, 1989). By contrast, in the case of Arg-Δe-DHFRha a multi-Ub chain must form on one of the numerous but relatively immobile and (presumably) unfavorably located lysines of the folded DHFR globule (Dohmen et al., 1994), thus accounting for both the lower overall rate of Arg-Δe-DHFRha degradation and the low steady-state content of its ubiquitinated derivatives. This interpretation also accounts for the observed difference in the effect of MTX on the relative abundance of mult ubiquitinated Arg-eK-DHFRha and Arg-Δe-DHFRha. Indeed, a block to Arg-eK-DHFRha degradation due to a conformational stabilization of its DHFR moiety by MTX would not be expected to perturb the formation of a multi-Ub chain linked to the eK extension of DHFR, as observed. By contrast, a multi-Ub chain linked to Arg-Δe-DHFRha has to form on a lysine of the folded DHFR moiety. This is a slow step, whose rate is further decreased in the presence of MTX, which stabilizes the conformation of DHFR (Matthews et al., 1985; Eilers and Schatz, 1988). (It is assumed that the rate of formation of a multi-Ub chain linked to Arg-Δe-DHFRha is limited by the rate of chain initiation at one of the lysines of DHFR.)

A model that accounts for the findings of this work is consistent with other evidence is shown in Fig. 4. A major assumption is that the initiation of processive degradation of an N-end rule substrate such as Arg-eK-DHFRha requires at least a local conformational perturbation of the folded DHFR moiety which can be "utilized" by the proteasome. The probability of this perturbation would be decreased in the presence of MTX, which stabilizes the folded conformation of DHFR. Since MTX inhibits the degradation but not ubiquitination of Arg-eK-DHFRha, the relevant conformational perturbation must be a step that occurs after but not before the ubiquitination step (Fig. 4). It is not specified whether this "sufficient" perturbation of DHFR is a thermally driven fluctuation or is at least in part the result of DHFR interactions with components of the N-end rule pathway, including a DHFR-linked multi-Ub chain; the model is consistent with either possibility. Another assumption of the model is that an interaction between a ubiquitinated Arg-eK-DHFRha substrate and the proteasome-based proteolytic machine is reversible, in that a substrate-proteasome complex dissociates (or enters a state that cannot result in proteolysis; see below) after a stochastically determined time interval (Fig. 4).

Given these assumptions, the near cessation of degradation of DHFR-based N-end rule substrates in the presence of MTX is explained as follows. In the absence of MTX, the mean time interval between the formation of a substrate-proteasome complex and a relevant conformational perturbation of DHFR within the complex is either close to or shorter than the mean lifetime of the complex. In the presence of MTX, its binding to DHFR (and the resulting conformational stabilization of the DHFR globule) decreases the probability of a relevant perturbation of DHFR within the substrate-proteasome complex but does not influence the mean lifetime of the complex (Fig. 4). Specifically, the time interval before perturbation of the substrate is postulated to become significantly longer than the time interval before dissociation of the substrate-proteasome complex, resulting in abortive cycles of targeting and multiubiquitination but little net degradation of a DHFR-based
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Fig. 4. On the mechanism of MTX effect. Each of the four depicted transitions (I-IV) in the N-end rule pathway is actually a multistep reaction (Varshavsky, 1992). The shapes of structures are arbitrary. Among the protein-protein complexes shown in this diagram, the interactions between N-recognin (N-r) and the Ub-conjugating enzyme (E2), and between N-recognin and a DHFR-based N-end rule substrate were demonstrated directly (Bartel et al., 1990; Madura et al., 1993). By contrast, a direct interaction between N-recognin and proteasome is a conjecture that remains to be verified. The path of a multi-Ub chain (black ovals) in a complex between proteasome and a multiubiquitinated substrate is unknown. However, if the E2 enzyme that initiates the formation of a substrate-linked multi-Ub chain is also responsible for the chain elongation, the growing tip of the chain must be in proximity to this E2, as shown in the diagram. Superimposition of the substrate and proteasome denotes a complex (of unknown structure) between them. A DHFR-based N-end rule substrate such as Arg-Leu-DHFRha (Fig. 1), bearing a destabilizing N-terminal residue (d) and a mobile lysine residue (K) outside of the folded DHFR globule, is bound by a complex of N-recognin and E2 (step I). Formation of a lysine-linked multi-Ub chain (the chain's length is arbitrarily set at five Ub moieties) and the binding of a multiubiquitinated substrate by the proteasome take place at step II. In vitro, the multiubiquitination of an N-end rule substrate can occur in the absence of proteasome; however, it is possible that the binding of a targeted substrate by the proteasome accompanies or even precedes the substrate's multiubiquitination in vivo. At step III, a local or a global conformational perturbation of DHFR in a complex with the proteasome occurs, resulting in proteolysis (the irreversible step IV), which yields short fragments of DHFR and regenerates Ub from a multi-Ub chain. Formation of the MTX-DHFR complex stabilizes the folded DHFR conformation, decreasing the probability of a conformational perturbation of DHFR (step III) which can be utilized by the proteasome and thereby inhibiting the degradation of a DHFR-containing substrate. In the case of a protein such as Arg-Leu-DHFRha (Fig. 1), which lacks a targetable lysine residue outside of the DHFR globule, the binding of MTX and the resulting conformational stabilization of DHFR suppress both ubiquitination and degradation of Arg-Leu-DHFRha. See "Materials and Methods" for further details.

The understanding of proteasome mechanics is still rudimentary, and therefore the model of Fig. 4 is vague about details of the MTX effect. To cite just one example, the postulated "dissociation" of a substrate-proteasome complex may actually be less than a complete dissociation: for the model to be relevant, it is sufficient that the complex can enter a state in which the proteasome becomes unable to initiate the degradation of a DHFR-based substrate upon an otherwise sufficient perturbation of DHFR.

The degradation of DHFR-based N-end rule substrates in vivo (in S. cerevisiae cells) was found to be at most weakly inhibited by the addition of MTX to the growth medium. However, in contrast to the above in vitro assays (Figs. 2 and 3), in which the N-end rule pathway targets a previously synthesized, folded DHFR, in the in vivo assays the binding of MTX to a nascent DHFR-based substrate takes place in kinetic competition with the targeting of the same substrate by the N-end rule pathway. Moreover, while the binding of MTX requires the folded DHFR conformation, the targeting by the N-end rule pathway is expected to occur at any time after the (cotranslational) formation of a destabilizing residue at the N terminus of a substrate. A strategy in which the N-end rule pathway is repressed at first (in a specially constructed yeast strain) and then induced should eliminate the above kinetic competition and allow the effects of MTX to be tested in vivo under conditions in which a DHFR-based substrate is folded and MTX-bound before it is targeted by the N-end rule pathway. These experiments are in progress. Interestingly, the in vivo degradation of a DHFR-based N-end rule substrate whose DHFR moiety was fused to an unrelated protein such as S. cerevisiae Cdc28p (Dohmen et al., 1994) could be inhibited by MTX, in contrast to the degradation of an otherwise identical substrate lacking the Cdc28p domain. The role of a DHFR-linked protein in conferring the sensitivity to inhibition by MTX remains to be understood.

The ability to suppress the in vivo degradation of a short lived protein with its cell-penetrating, low M, ligand would have a number of applications, including the possibility of constructing new types of conditional mutants (Dohmen et al., 1994).

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