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# 20S proteasome mediated degradation of DHFR: implications in neurodegenerative disorders

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**Abstract**

The 20S proteasome is responsible for the degradation of protein substrates implicated in the onset and progression of neurodegenerative disorders, such as α-synuclein and tau protein. Here we show that the 20S proteasome isolated from bovine brain directly hydrolyzes, in vitro, the dihydrofolate reductase (DHFR), demonstrated to be involved in the pathogenesis of neurodegenerative diseases. Furthermore, the DHFR susceptibility to proteolysis is enhanced by oxidative conditions induced by peroxynitrite, mimicking the oxidative environment typical of these disorders. The results obtained suggest that the folate metabolism may be impaired by an increased degradation of DHFR, mediated by the 20S proteasome.


*Keywords:* 20S proteasome; Dihydrofolate reductase; Neurodegenerative pathologies; Oxidation

The 20S proteasome or multicatalytic proteinase complex is 700 KDa particle which, in a barrel-shape, assembles 28 subunits organized in four stacked seven-membered rings. The two outer rings contain different but related α subunits, whereas the inner ones are composed by similar β subunits, which harbor the catalytic sites of the complex [1]. Even though only three β subunits are catalytically active, the importance of subunit pairs for the proteolytic activity has been demonstrated with yeast 20S proteasome mutants [2]. In fact, besides the three major catalytic components: chymotrysin-like (ChT-L),[1] trypsin-like (T-L), and peptidyl-glutamyl peptide hydrolyzing (PGPH) each related to a specific β subunit [3–5] the 20S proteasome expresses other activities such as branched chain amino acid preferring (BrAAP) and small neutral amino acid preferring (SNAAP) [6].

The 20S proteasome, binding PA700 regulatory particles on each α ring, forms a larger complex, the 26S proteasome which is part of the ATP, ubiquitin-dependent degradation pathway, but it can also degrades non-ubiquitinated protein substrates [7].

In the cells, the free 20S proteasome is the predominant form of the complex and more and more proteins are demonstrated to be directly degraded by the complex [7]. Some of them are implicated in the onset and progression of neurodegenerative disorders [8,9], such as the microtubule-associated protein tau which accumulates in the neurofibrillary tangles present in the cerebral cortex of patients with Alzheimer's disease (AD) [10] and α-synuclein, the main component of the Lewy body's filaments, two mutations of whose gene have been identified in Parkinson's disease (PD), as well as in other forms of dementia [11,12].

Recent studies suggest that low blood levels of folate can be associated with AD; furthermore it has been reported that a folate deficiency causes the increase of homocysteine plasma concentration, demonstrated to be directly toxic to neurons through DNA damage, having, therefore, a relevant role in the pathogenesis of PD and AD [13,14].

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[1] *Abbreviations used:* AD, Alzheimer's disease; SNAAP, small neutral amino acid preferring; PD, Parkinson's disease; BrAAP, branched chain amino acid preferring; DHFR, dihydrofolate reductase; ChT-L, chymotrysin-like; T-L, trypsin-like; MTX, methotrexate; TMP, trimethoprim.

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Folate, in vivo, can be reduced to tetrahydrofolate in a double step reaction catalyzed by the enzyme dihydrofolate reductase (DHFR); the product of that reaction is implicated, as a donor of methyl groups, in the DNA synthesis and in other important biochemical reactions. Folic acid analogs and DHFR inhibitors are used either as antitumor drugs, e.g., methotrexate (MTX), or antibacterial agents, e.g., trimethoprim (TMP) [15–18].

It is well documented that poly-ubiquitinated DHFR is efficiently degraded by the 26S proteasome [19,20], however, no evidences about a ubiquitin independent and 20S proteasome mediated degradation of DHFR are available.

In the present study, using the 20S proteasome isolated from bovine brain, in an in vitro assay, we have demonstrated that DHFR is directly hydrolyzed by the 20S complex, without any previous ubiquitination and that the degradation is increased under oxidative conditions.

## Materials and methods

### *Materials*

Bovine liver DHFR, dihydrofolate ($H_2F$), NADPH, and substrates for assaying the ChT-L and PGPH proteolytic activities (Z-GGL-pNA, Z-LLR-2NA) were purchased from Sigma (USA). The Z-GPALG-pAB substrate was the kind gift of Prof. M. Orlowski (Mount Sinai School of Medicine, NY). The C85S/C152E double mutant of dihydrofolate reductase (SE-DHFR) from *Escherichia coli* was a kind gift of Dr. C.R. Matthews, Department of Chemistry, University of Pennsylvania. Precast gels 4–20% linear gradient were obtained from Bio-Rad Laboratories S.r.l.

### *Isolation and purification*

Isolation and purification of 20S proteasome from bovine brain were carried out as previously reported [21,22]. A higher degree of purification was obtained by a hydrophobic interaction chromatography step which improves the separation of proteasome from the copurifying chaperonine Hsp90.

### *Determination of proteolytic activities*

The ChT-L, PGPH, and BrAAP activities of the brain 20S proteasome were determined as reported previously [21,23,24], using Z-GGL-pNA, Z-LLR-2NA, and Z-GPALG-pAB respectively, as substrates. Aminopeptidase N (EC 3.4.11.2), used for coupled assay utilized for detecting the BrAAP activity [24], was purified from pig kidney, as reported elsewhere [25,26]. Proteolytic activity assays were performed in the presence of increasing amounts of MTX (from 0 to 300 mM): MTX did not affect the assayed activities (data not shown).

### *Preparation of DHFR proteins*

The bovine liver DHFR and the SE-DHFR were obtained as suspensions in 3.6 M ammonium sulfate solution, pH 7.0. Protein samples were dialyzed over night against phosphate buffer 20 mM, pH 7.5, and protein concentrations were determined spectrophotometrically ($\varepsilon_{280} = 27,280\,\mathrm{cm^{-1}\,M^{-1}}$ for the bovine DHFR [27], $\varepsilon_{280} = 31,100\,\mathrm{cm^{-1}\,M^{-1}}$ for the SE-DHFR [28]).

### *Exposure of DHFRs to peroxynitrite*

Peroxynitrite was synthesized according to the protocol reported by Uppu et al. [29] and stocked at −80 °C. The concentration was determined spectrophotometrically at $\lambda = 302$ nm ($\varepsilon = 1670\,\mathrm{M^{-1}\,cm^{-1}}$). The stock solution was diluted in KOH 0.1 M before use. Phosphate buffer solutions of the bovine liver DHFR and the SE-DHFR (22.84 μM) were incubated with peroxynitrite (final concentrations 0, 50, and 100 μM) for 15 min at room temperature. Peroxynitrite decomposition in phosphate buffer was followed spectrophotometrically ($\lambda = 302$ nm): within 1 min the absorbance went down to the baseline. Control experiments on the 20S proteasome activities were performed in order to check the effect of peroxynitrite decomposition side products.

### *Polyacrylamide gel electrophoresis*

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [30,31] was done in 4–20% linear gradient precast gels for DHFR proteins treated with increasing concentrations of peroxynitrite (0, 10, 40, 80, and 100 μM).

### *Determination of SE-DHFR degradation by HPLC*

Incubation of SE-DHFR, 30.7 μM, with the 20S proteasome, 0.34 μM, was carried out in phosphate buffer 20 mM, pH 7.5, $MgCl_2$ 5 mM (incubation buffer), at 37 °C. Twenty microliters of aliquots were withdrawn at different times (from 0 to 120 min), acidified with 2 ml of TCA 10%, and subjected to HPLC on a Hamilton PRP-3 column (4.1 × 150 mm). Elution was carried out at a flow rate of 0.7 ml/min with the buffers: 0.1% trifluoroacetic acid in water (A) and acetonitrile containing 0.1% trifluoroacetic acid (B). Samples were loaded in 25% buffer B and eluted according to the following gradient: step 1, from 25 to 40% buffer B in 5 min; step 2, from 40 to 45% buffer B in 15 min; step 3, from 45 to 80% buffer B in 5 min; and step 4, 80% buffer B for 5 min. The rate of DHFR degradation was determined measuring the peak height of the protein ($\lambda = 210$ nm).

Determinations of SE-DHFR degradation by 20S proteasome were executed after incubation either with 2 mM MTX or with peroxynitrite (50 and 100 μM). Effect of peroxynitrite decomposition side products were analyzed by diluting the peroxynitrite into the buffer and allowed to decompose for 5 min before adding the enzyme: no effect was observed (data not shown).

Each experimental set was repeated three times and relative mean values and standard errors were calculated.

*DHFR activity assay*

The activity of DHFRs was determined spectrophotometrically following the decrease in absorbance at 340 nm of NADPH. The reaction mixture containing 50 mM potassium phosphate buffer, 0.2 mM EDTA, pH 7.0, 130 μM NADPH and 35 μM $H_2F$ in a final volume of 1500 μl, was thermostated for 5 min at 20 °C, then 50 μl of DHFR solution were added [32].

The peroxynitrite oxidative effect was checked preincubating the DHFRs with 0, 10, 50, 100, 200, and 400 μM peroxynitrite for 5 min at room temperature, then the activity was performed as described above. The peroxynitrite side products effect was tested as reported in the 2.7. paragraph. Activity assays were also performed with bovine DHFR pre-incubated or not with the 20S proteasome at 37 °C in a time range from 0 to 120 min. Control experiments were carried out in order to evaluate the effect of the protein pre-incubation at 37 °C.

*Enzyme assays using fluorescamine*

Degradation of DHFR and SE-DHFR was also monitored spectrofluorimetrically utilizing the fluorescamine (4-phenylspiro[furan-2(3H),1′-phathalan]-3,3′-dione) assay. Fluorescamine is a non fluorescent reagent, at alkaline pH, reacts rapidly with primary amines forming a fluorescent conjugate. Therefore, the fluorescent assay is a high sensitive and reproducible method which provides a measure of the number of peptide bonds cleaved [33,34]. The 20S proteasome and DHFR were pre-incubated in incubation buffer (see above), at 37 °C. Aliquots were withdrawn at different times (from 0 to 120 min) and transferred to a fluorometer cuvette containing 600 μl borate buffer (0.1 M, pH 9.0), followed by 300 μl of fluorescamine solution (0.1 mg/ml in acetone) with immediate mixing. Fluorescence emission was measured using a Hitachi model F-4500 spectrofluorometer ($\lambda_{exc} = 390$ nm, $\lambda_{em} = 475$ nm). Control experiments were performed pre-incubating the DHFR at 37 °C in the absence of the 20S proteasome. The assays were executed with the bovine liver DHFR and the SE-DHFR treated or not with peroxynitrite (0, 5, 10, and 50 μM); the same experiments were carried out pre-incubating the bovine-DHFR with 150 μM MTX.

## Results

*Degradation of SE-DHFR by isolated 20S proteasome*

Preliminary experiments of proteolysis by the 20S proteasome, isolated from bovine brain, were performed using a cysteine-free mutant (C85S/C152E) of dihydrofolate reductase (SE-DHFR) from *E. coli*.

*HPLC analysis*

The degradation of SE-DHFR was monitored by RP-HPLC measuring the protein peak height after increasing incubation times with the 20S proteasome at 37 °C. The elution profile of SE-DHFR, both in the absence and presence of 2 mM MTX, is characterized by a defined peak with a retention time of 15.35 min. After incubation with the 20S proteasome the chromatograms presented a decrease of the peak height and the appearance of peaks at lower retention times attributable to degradation products. Treatment of SE-DHFR with 2 mM MTX completely inhibited the degradative action of 20S proteasome, even at 120 min of incubation.

The results obtained are summarized in Fig. 1, which reports the % residual area of the SE-DHFR peak at different incubation times with 20S proteasome, in the presence and absence of MTX.

*Fluorescamine assays*

Fluorescamine assays were performed either to confirm the HPLC data obtained using the SE-DHFR (see

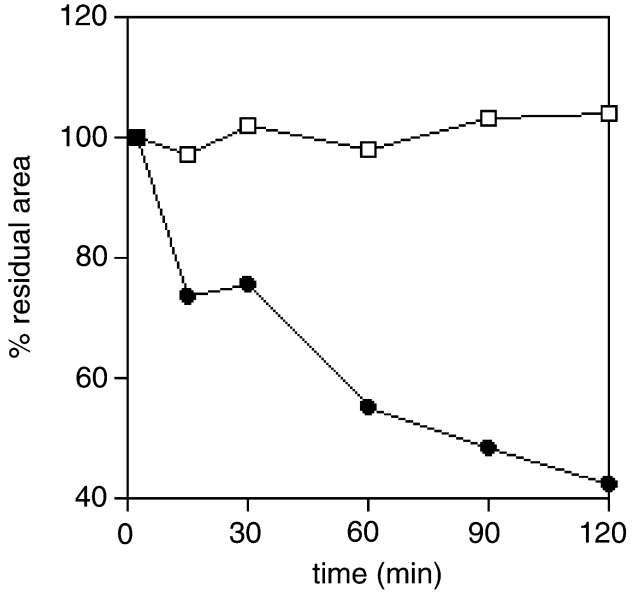


Fig. 1. Degradation of SE-DHFR by the 20S proteasome. HPLC analysis of the effect of methotrexate (control (●), and 2 mM (□)) on the SE-DHFR degradation by the 20S proteasome. Each data point is the mean value ± 4% SE and comes from three separate determinations. Error bars are not shown because they correspond to the size of the symbol.

above) or for its higher sensitivity which allowed us to circumvent several experimental difficulties connected with the application of RP-HPLC to the bovine DHFR (most likely the enzyme specifically interacts with several matrices and is present in very limited amount in the reaction mixture for the degradation by the 20S proteasome). The results obtained, summarized in Table 1, clearly show that the fluorescamine fluorescence emission increases as a function of the incubation times of SE-DHFR with the proteasome indicating the proteolytic degradation of the enzyme.

### *Degradation of bovine liver DHFR by isolated 20S proteasome*

#### *Effect of 20S proteasome on the bovine DHFR activity*

To test the 20S proteasome effect on the bovine DHFR functionality, activity assays were performed after incubation of bovine liver DHFR with the proteasomal complex at 37 °C. Aliquots were withdrawn after 2, 30, 60, 90, and 120 min and then added to a thermostated solution at 20 °C containing NADPH and $H_2F$. Table 2 reports the % activity remaining of DHFR after incubation with 20S proteasome at 37 °C. Control experiments without proteasome showed no effect of exposure at 37 °C on the DHFR functionality.

The incubation of bovine DHFR with the 20S proteasome induces a gradual decrease of DHFR activity; this effect could be ascribed both to the DHFR degradation and to the two macromolecules interaction making changes in protein structure and functionality.

#### *Fluorescamine assays*

The proteolytic effect of the 20S proteasome on the bovine liver DHFR was analyzed measuring the fluorescence emission of fluorescamine. Table 3 shows the fluorescence emission values, as percent of control, after incubation at 37 °C with the proteasome.

The gradual increase of fluorescence emission confirms the DHFR proteolysis by the 20S proteasome.

### *Degradation of oxidized DHFR proteins by 20S proteasome*

The proteolytic action of the bovine brain 20S proteasome was tested on SE-DHFR and bovine DHFR exposed to various amounts of peroxynitrite, from 0 to 100 μM.

To verify if peroxynitrite exposure induced fragmentation of the DHFR proteins a 4–20% linear gradient SDS–PAGE was performed: a single band was detectable after silver staining (data not shown).

Based on the observation that oxidants produces modifications to proteins leading to loss of function [35], the oxidative effect of increasing peroxynitrite concentrations on the DHFRs was checked looking at the remaining activity after peroxynitrite exposure.

In Table 4, data from these experiments are shown.

Table 1
Degradation of SE-DHFR by the 20S proteasome

| Incubation time (min) | 0 | 30 | 60 | 90 | 120 |
|---|---|---|---|---|---|
| % emission | 100 | 102 | 104 | 116 | 116 |

The 20S proteasome and SE-DHFR were pre-incubated in incubation buffer, at 37 °C. Aliquots were withdrawn at different times and added to 600 μl borate buffer (0.1 M, pH 9.0), followed by 300 μl of fluorescamine solution (0.1 mg/ml in acetone) with immediate mixing. Fluorescence emission was measured, $\lambda_{exc} = 390$ nm and $\lambda_{em} = 475$ nm and the associated standard deviation was always lower than 4%.

Table 2
Effect of the 20S proteasome on the bovine liver DHFR activity

| | % bovine DHFR activity remaining | | | | |
|---|---|---|---|---|---|
| Incubation time (min) | 0 | 30 | 60 | 90 | 120 |
| Control | 100 | 98.09 | 101.88 | 102.00 | 99.62 |
| After incubation with the 20S proteasome | 100 | 77.02 | 76.20 | 57.11 | 50.77 |

The activity of bovine DHFR was determined by monitoring spectrophotometrically the decrease in absorbance at 340 nm of NADPH. The reaction mixture (50 mM potassium phosphate buffer, 0.2 mM EDTA, pH 7.0, 130 μM NADPH, and 35 μM $H_2F$ in a final volume of 1500 μl) was thermostated for 5 min at 20 °C, then 50 μl of DHFR solution were added.

Table 3
Degradation of the bovine DHFR by the 20S proteasome

| Incubation time (min) | 0 | 30 | 60 | 90 | 120 |
|---|---|---|---|---|---|
| % emission | 100.0 | 103.3 | 113.7 | 124.7 | 126.0 |

The 20S proteasome and the bovine DHFR were pre-incubated in incubation buffer, at 37 °C. Aliquots were withdrawn at different times and added to 600 μl borate buffer (0.1 M, pH 9.0), followed by 300 μl of fluorescamine solution (0.1 mg/ml in acetone) with immediate mixing. Fluorescence emission was measured, $\lambda_{exc} = 390$ nm and $\lambda_{em} = 475$ nm.

Table 4
Peroxynitrite effect on the DHFRs activity

| Peroxynitrite concentrations (μM) | % SE-DHFR activity remaining | % bovine DHFR activity remaining |
|---|---|---|
| 0 | 100.00 | 100.00 |
| 10 | 95.62 | 97.13 |
| 50 | 87.11 | 91.25 |
| 100 | 46.50 | 58.30 |
| 200 | 38.70 | 41.55 |
| 400 | 22.05 | 27.22 |

The activity of DHFRs was determined by monitoring spectrophotometrically the decrease in absorbance at 340 nm of NADPH. The reaction mixture (50 mM potassium phosphate buffer, 0.2 mM EDTA, pH 7.0, 130 μM NADPH, and 35 μM $H_2F$ in a final volume of 1500 μl) was thermostated for 5 min at 20 °C, then 50 μl of DHFR solution, previously treated with peroxynitrite (0, 10, 50, 100, and 200 μM) were added. The peroxynitrite side products action was tested: no effect was detected.

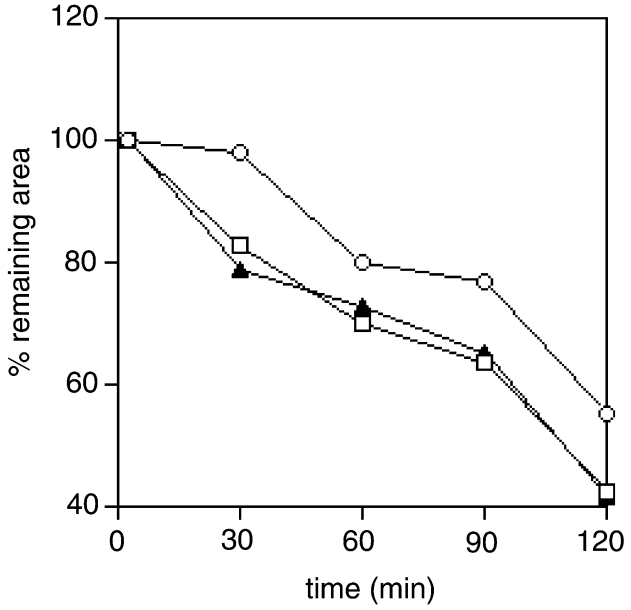


Fig. 2. HPLC analysis of the effect of peroxynitrite (control (○), 50 μM (□), and 100 μM (▲)) on the SE-DHFR degradation by the 20S proteasome. Data are reported as % residual area with respect to the control. Each data point is the mean value ± 4% SE and comes from three separate determinations. Error bars are not shown because they correspond to the size of the symbol.

*HPLC analysis*

The degradation of SE-DHFR treated with 0, 50, and 100 μM peroxynitrite was performed by HPLC analysis.

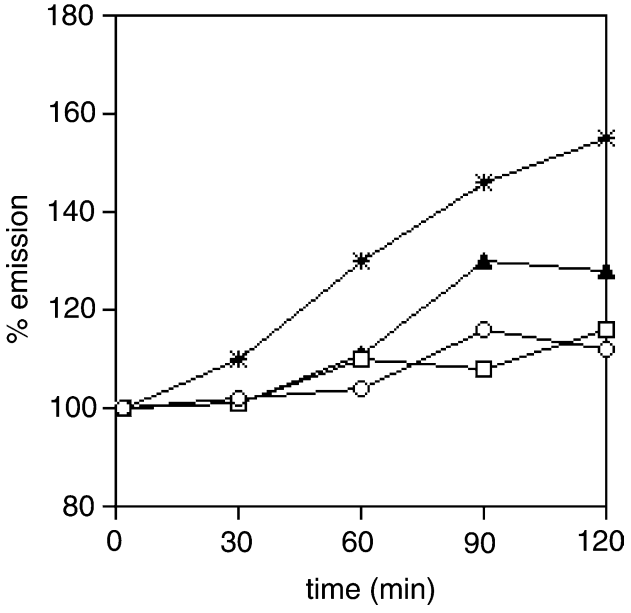


Fig. 3. Fluorescence emission related to the effect of peroxynitrite (control (○), 5 μM (□), 10 μM (▲), and 50 μM (*)) on the SE-DHFR degradation by the 20S proteasome, analyzed with fluorescamine. Each data point is the mean value ± 4% SE and comes from four separate determinations. Error bars are not shown because they correspond to the size of the symbol.

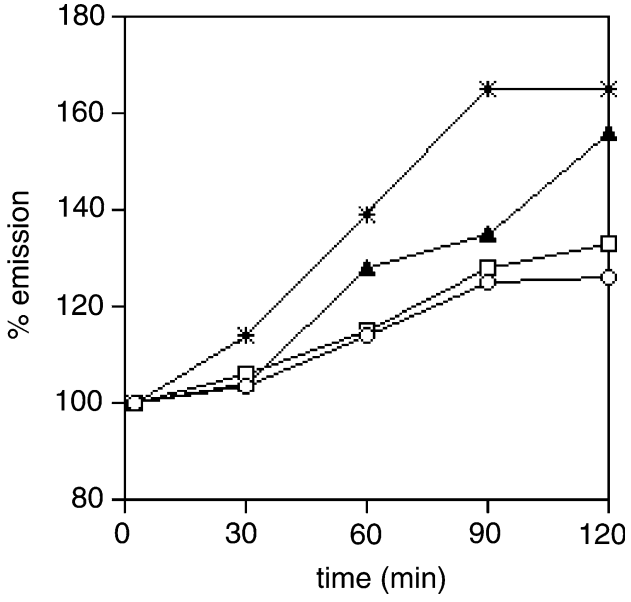


Fig. 4. Fluorescence emission related to the effect of peroxynitrite (control (○), 5 μM (□), 10 μM (▲), and 50 μM (*)) on the bovine DHFR degradation by the 20S proteasome, analyzed with fluorescamine. Each data point is the mean value ± 4% SE and comes from four separate determinations. Error bars are not shown because they correspond to the size of the symbol.

The elution profiles show a main peak, at the retention time of native SE-DHFR, characterized by a poorly resolved shoulder attributable to the oxidized form.

Treatment with peroxynitrite induced an increase of the SE-DHFR degradative rate with respect to the control, even if no differences were detectable between 50 and 100 μM peroxynitrite (Fig. 2).

*Fluorescamine assays*

Figs. 3 and 4 report the results of the fluorescamine assays for the 20S proteasome digestion of SE-DHFR and bovine DHFR treated with 0, 5, 10, and 50 μM peroxynitrite, respectively.

The 20S proteasome was able to recognize and to degrade both DHFR proteins: the degradation rate and the proteolyzed substrate amounts increased as a function of peroxynitrite concentration. Besides, the bovine DHFR, oxidized or not, was faster degraded by the 20S proteasome than the SE-DHFR.

## Discussion

In the present study we have demonstrated that the enzyme DHFR is directly proteolyzed by the 20S proteasome, with no previous ubiquitination step. To the best of our knowledge it is the first time that DHFR is recognized as a substrate for the 20S proteasome, in fact in earlier works a multi-ubiquitined DHFR, also in the presence of its inhibitor MTX, has been used as a model

either to determine how the N-end rule pathway is related to the protein conformational stabilization [20] or to analyze the substrate recognition by the 26S proteasome disclosing the dependence on the length of the polyubiquitin chains for efficient targeting [19].

Furthermore, taking into account the DHFR implication in the onset and progression of several neurodegenerative disorders, a 20S proteasome isolated from bovine brain has been used. In order to have a homogeneous model, a bovine DHFR was utilized as substrate for the 20S complex. The DHFR is a folded protein, in detail it is a $\alpha/\beta$ protein characterized by a high conformational flexibility [36], on the contrary of other proteins known to be substrates for the 20S proteasome and involved in neuropathologies, like $\alpha$-synuclein [9] and tau protein [8], which are native unfolded proteins.

In this context, it should be noticed that the two DHFRs, considered in this study, in spite of a relatively low sequence homology [37] show a similar three-dimensional structure.

Our data clearly indicate that both DHFRs are degraded by the 20S proteasome although the bovine enzyme is more rapidly hydrolyzed than the mutant one.

This evidence could be explained by the different conformation exhibited by the two proteins at the assay temperature of 37 °C. In fact the bovine DHFR, which shows a thermal transition around 50 °C, at 37 °C is in its native conformation, whereas, at the same temperature, the SE-DHFR is in a stable intermediate state resembling a molten globule which is more resistant to the proteolytic degradation [38,39].

The interaction between the bovine DHFR and the 20S proteasome has been also approached looking at the DHFR enzymatic activity: it came out that in the presence of the proteasome the DHFR showed a decreased activity. The qualitative data obtained from the fluorescamine assays suggest that the decrease of DHFR enzymatic activity resulted from its degradation by the 20S proteasome.

Several recent works report an increased oxidative stress in the brain of subjects affected by neurodegenerative disorders [40–42]. Since, as Davies et al. have demonstrated, the 20S proteasome plays a key role in the removal of mildly oxidized proteins [43], degradation experiments with the two DHFRs exposed to relatively low peroxynitrite concentrations were performed. Our data are in good agreement with Davies' observations in fact an increase in the rate of DHFR proteolysis was evident. Even after oxidation, the difference in the proteolysis rate has been maintained, being the bovine nitrated DHFR more rapidly degraded than its *E. coli* counterpart.

It is well known that neuropathologies are characterized by both an increase in oxidative conditions and an impairment of the proteasome functionality [40–42,44,45], which could be the cause of the accumulation of ubiquitinated and not-ubiquitinated proteins, among them $\alpha$-synuclein and tau protein, and of oxidized proteins.

Furthermore, clinical studies suggest a relationship between folate deficiency and correlated increased homocystein levels and neurological disorders, including AD and PD. It has been shown, in cultured neurons, that folate deprivation induces an increase in cytosolic calcium, in reactive oxygen species (also through a decrease of the reduced form of glutathione), in phosphorylated-tau, and in apoptosis, providing evidences that folate is directly involved in the neuronal regulatory mechanisms [46].

Our findings demonstrate the role of 20S proteasome in the removal of DHFR which became more efficient in an oxidative environment. Although data from the present study refer to in vitro conditions, the effect of 20S proteasome on the DHFR remains informative. In fact, being the DHFR directly degraded by the 20S proteasome, under oxidative conditions which induce a change in the proteasomal complex functionality, the DHFR turnover and the correlated folate levels might be altered, thus, contributing to the onset and progression of neurodegenerative disorders. In future studies, it will be important to elucidate the role of the 20S proteasome on the DHFR degradation on in vivo systems in order to verify the results shown in this study.

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## References

[1] W. Baumeister, J. Walz, F. Zuhl, E. Seemuller, Cell 92 (1998) 367–380.
[2] T.P. Dick, A.K. Nussbaum, M. Deeg, W. Heinemeyer, M. Groll, M. Schirle, W. Keilholz, S. Stevanovic, D.H. Wolf, R. Huber, H.G. Rammensee, H. Schild, J. Biol. Chem. 273 (1998) 25637–25646.
[3] M. Groll, L. Ditzel, J. Lowe, D. Stock, M. Bochtler, H.D. Bartunik, R. Huber, Nature 386 (1997) 463–471.
[4] W. Heinemeyer, M. Fischer, T. Krimmer, U. Stachon, D.H. Wolf, J. Biol. Chem. 272 (1997) 25200–25209.
[5] C.S. Arendt, M. Hochstrasser, Proc. Natl. Acad. Sci. USA 94 (1997) 7156–7161.
[6] M. Orlowski, C. Cardozo, C. Michaud, Biochemistry 32 (1993) 1563–1572.
[7] M. Orlowski, S. Wilk, Arch. Biochem. Biophys. 415 (2003) 1–5.
[8] C. Cardozo, C. Michaud, Arch. Biochem. Biophys. 408 (2002) 103–110.
[9] G.K. Tofaris, R. Layfield, M.G. Spillantini, Febs Lett. 509 (2001) 22–26.

[10] L. Gasparini, M. Racchi, G. Binetti, M. Trabucchi, S.B. Solerte, D. Alkon, R. Etcheberrigaray, G. Gibson, J. Blass, R. Paoletti, S. Govoni, FASEB J. 12 (1998) 17–34.
[11] M.H. Polymeropouos, C. Lavendan, E. Leroy, S.E. Ide, A. Dehejia, A. Dutra, B. Pike, H. Root, J. Rubestein, R. Boyer, E.S. Stenroos, S. Chandrasekharappa, A. Athanasiaddou, T. Papapetropoulos, W.G. Johnson, A.M. Lazzarini, R.C. Duvoisin, G. Di Iorio, L.I. Golbe, R.L. Nussbaum, Science 276 (1997) 2045–2047.
[12] R. Kruger, W. Kuhn, T. Muller, D. Woitalla, M. Graeber, S. Kosel, H. Przuntek, J.T. Epplen, L. Schos, O. Riess, Nat. Genet. 18 (1998) 106–108.
[13] W. Duan, B. Ladenheim, R.G. Cutler, I.I. Kruman, J.L. Cadet, M. Mattson, J. Neurochem. 80 (2002) 101–110.
[14] I.I. Kruman, T.S. Kumaravel, A. Lohani, W.A. Pedersen, R.G. Cutler, Y. Kruman, N. Haughey, J. Lee, M. Evans, M.P. Mattson, J. Neurosci. 22 (2002) 1752–1762.
[15] R.L. Blakley, in: The Biochemistry of Folic Acid and Related Pteridines, North-Holland Publishing, Amsterdam, 1969, pp. 139–187.
[16] R.L. Blakley, in: A.C. Sartorelli, J.S. Lazo, J.R. Bertino (Eds.), Molecular Actions and Targets for Cancer Chemotherapeutic Agents, Academic Press, New York, 1981, pp. 303–332.
[17] G.H. Hitchings, B. Roth, in: M. Sandler (Ed.), Enzyme Inhibitors as Drugs, Macmillan, London, 1980.
[18] G.H. Hitchings, S.L. Smith, Adv. Enzyme Regul. 18 (1980) 349–371.
[19] J.S. Thrower, L. Hoffman, M. Rechsteiner, C.M. Pickart, EMBO J. 19 (2000) 94–102.
[20] J.A. Johnston, E.S. Johnson, P.R.H. Waller, A. Varshavsky, J. Biol. Chem. 270 (1995) 8172–8178.
[21] A.M. Eleuteri, M. Angeletti, G. Lupidi, R. Tacconi, L. Bini, E. Fioretti, Protein Expr. Purif. 18 (2000) 160–168.
[22] M. Orlowski, C. Michaud, Biochemistry 28 (1989) 9270–9278.
[23] S. Wilk, M. Orlowski, J. Neurochem. 40 (1983) 842–849.
[24] M. Orlowski, C. Cardozo, C. Michaud, J. Biochem. 32 (1993) 1563–1572.
[25] G. Pfleiderer, Methods Enzymol. 19 (1970) 514–521.
[26] J. Almenoff, M. Orlowski, Biochemistry 22 (1983) 590–599.
[27] B.T. Kaufman, V.F. Kemerer, Arch. Biochem. Biophys. 172 (1976) 289–300.
[28] J. Lou, M. Iwakura, C.R. Matthews, Biochemistry 34 (1995) 10669–10675.
[29] R.M. Uppu, G.L. Squadrito, R. Cueto, W.A. Pryor, Methods Enzymol. 269 (1996) 311–321.
[30] U.K. Laemmli, Nature 277 (1970) 680–685.
[31] W. Wray, T. Bonlikas, W.P. Wray, R. Hancock, Anal. Biochem. 118 (1981) 197.
[32] B.L. Hillcoat, P.F. Nixon, R.L. Blakley, Anal. Biochem. 21 (1967) 178–189.
[33] S. Stein, P. Böhlen, J. Stone, W. Dairman, S. Udenfriend, Arch. Biochem. Biophys. 155 (1973) 202–212.
[34] C.H. Evans, J.D. Ridella, Anal. Biochem. 142 (1984) 411–420.
[35] T. Grune, K. Merker, G. Sandig, K.J. Davies, Biochem. Biophys. Res. Commun. 305 (2003) 709–718.
[36] G.P. Miller, S.J. Benkovic, Chem. Biol. 5 (1998) R105–R113.
[37] P. Lai, Y.E. Pan, J.M. Gleisner, D.L. Petrson, K.R. Williams, R.L. Blakley, Biochemistry 21 (1982) 3284–3294.
[38] J. Luo, M. Iwakura, C.R. Matthews, Biochemistry 34 (1995) 10669–10675.
[39] S. Sasso, I. Protasevich, R. Gilli, A. Makarov, C. Briand, J. Biomol. Struct. Dyn. 12 (1995) 1023–1032.
[40] B.S. Berlett, E.R. Stadtman, J. Biol. Chem. 272 (1997) 20313–20316.
[41] G. Carrard, A.L. Bulteau, I. Petropoulos, B. Friguet, Int. J. Biochem. Cell Biol. 34 (2002) 1461–1474.
[42] M.A. Smith, C.A. Rottkamp, A. Nunomura, A.K. Raina, G. Perry, Biochim. Biophys. Acta 1502 (2000) 139–144.
[43] T. Grune, K. Merker, G. Sandig, K.J.A. Davies, Biochem. Biophys. Res. Commun. 305 (2003) 709–718.
[44] J.N. Keller, K.B. Hanni, W.R. Markesbery, J. Neurochem. 75 (2000) 436–439.
[45] P.A. Szweda, B. Friguet, L.I. Szweda, Free Radic. Biol. Med. 33 (2002) 29–36.
[46] P.I. Ho, D. Ashline, S. Dhitavat, D. Ortiz, S.C. Collins, T.B. Shea, E. Rogers, Neurobiol. Dis. 14 (2003) 32–43.