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Peroxynitrite-mediated oxidation of the C85S/C152E mutant of dihydrofolate reductase from *Escherichia coli*: functional and structural effects

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Abstract

Peroxynitrite is a potent reactive oxygen species that is believed to mediate deleterious protein modifications in a wide variety of neurodegenerative disorders. In this study, we have analysed the effects of oxidative damage induced by peroxynitrite on a cysteine-free mutant of dihydrofolate reductase (SE-DHFR), from a functional and a structural point of view. The peroxynitrite-mediated oxidation results in the inhibition, concentration-dependent, of the catalytic activity. This effect is strongly influenced by the HCO_3^-/CO_2 buffering system, that we observed to significantly affect the yield of protein oxidation by modulating the peroxynitrite-induced modification of aromatic residues. Because of this effect, in presence of bicarbonate system, we have observed a protection of enzymatic activity of SE-DHFR with regard to peroxynitrite. The thermodynamic stability of the oxidized protein has been studied in comparison with the non-oxidized protein by differential scanning calorimetry. The thermodynamic parameters obtained showed a decrease of stability of SE-DHFR upon oxidation, evaluated in terms of Gibbs free energy of about 1.25 kcal/mol at 25 °C, with respect to the non-oxidized protein. Together, these data indicate that structural and functional alterations induced by peroxynitrite may play a direct role in compromising DHFR function in multiple pathological conditions.

Keywords: Dihydrofolate reductase; Peroxynitrite; Oxidative damage; Differential scanning calorimetry

Protein oxidation plays a key role in many disorders and diseases (like Alzheimer's and Parkinson's diseases) as indicated by the higher level of oxidized proteins found in tissues of ill subjects with respect to healthy subjects [1]. In addition to the other forms of reactive oxygen species (ROS), proteins are highly susceptible to modification by peroxynitrite, which is produced endogenously by the rapid reaction of nitric oxide ($^{\bullet}NO$) with superoxide anion ($O_2^{\bullet-}$) [2]. The main end-product of this reaction is the peroxynitrite anion ($ONOO^-$), which, under physiological conditions, is in equilibrium with the peroxynitrous acid (ONOOH), and rapidly decays to nitrate through the formation of a very active secondary species (ONOOH*).

The peroxynitrite system ($ONOO^-$, ONOOH, and ONOOH*) leads to a covalent modification of several amino acid residues in proteins, such as cysteine, methionine, tryptophan, and tyrosine residues [3,4]. The peroxynitrite anion ($ONOO^-$) reacts rapidly with CO_2 [5,6] to form a short-lived intermediate identified as the nitrosoperoxycarbonate adduct ($ONO_2CO_2^-$). This species is more reactive in tyrosine oxidation than $ONOO^-$ itself and produces 3-nitrotyrosine and 3,3-dityrosine. The reaction of peroxynitrite with CO_2 generally is of dominating importance since bicarbonate is

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the main buffering system in tissues, and aerobic life forms depend on the CO_2/O_2 exchange. Enzymes are known targets of ROS, with ROS modifications associated with the accrual of enzymes with impaired function. These alterations often lead to the formation of catalytically less active enzymes that are more sensitive to heat inactivation and proteolytic degradation. In addition, altered forms of proteins can aggregate and thus become more resistant to proteolysis, resulting in their potential accumulation [7]. Oxidative modification of an enzyme induces structural and functional changes with possible relationships between defects in the catalytic function and stability of proteins.

Dihydrofolate reductase (DHFR)[1] is a ubiquitous enzyme for normal cellular metabolism in both eukaryotic and prokaryotic cells. DHFR catalyzes the NADPH-dependent reduction of dihydrofolate or folate to tetrahydrofolate, a precursor of cofactors required for the biosynthesis of purines, pyrimidines, and several amino acids. Recent studies indicate that low blood levels of folate are associated with Alzheimer's disease [8,9], suggesting that defects in the metabolism of folic acid could cause an increment of the DNA damage and make the neurons more sensitive to the β-amyloid neurotoxicity. New studies point to folic acid deficiency (with associated high homocysteine levels) as a risk factor for another age-related neurodegenerative disorder, Parkinsons's disease, because key dopaminergic neurons become sensitized to environmental toxins causing their death or degeneration [10].

DHFR from *Escherichia coli* is a monomeric α/β protein containing two non-essential cysteine residues among a total of 159 amino acids. At high temperatures cysteine residues can form disulphide bonds (intra- and intermolecular), which can cause proteins to become cross-linked and negatively impact protein folding and unfolding. This is especially true if the cross-links occur as the result of modified disulphide bonds [11]. When conditions are returned to the native state, there is a great impairment for the proper non-covalent interactions to occur, and as a result the protein is unable to assume its correct native configuration. Together, these events can ultimately compromise the overall thermodynamic properties by preventing the innate reversibility in the thermal unfolding process. To circumvent this problem a double mutant was created, in which Cys 85 was replaced by Ser and Cys 152 by Glu (C85S/C152E, SE-DHFR) [11]. The two mutations do not appear to have any effect on the mechanism of catalysis; in fact at 15 °C the kinetic parameters of the enzymatic reaction of SE-DHFR are unchanged with respect to the wild-type [11–13].

In this work, we report the effects of peroxynitrite on function and stability of SE-DHFR. The thermal stability of SE-DHFR has been investigated by differential scanning calorimetry (DSC), analysing the heat capacity function of the non-oxidized and oxidized enzyme [14,15]. The characterization of peroxynitrite-induced modifications was also conducted in the present report using fluorescence measurements, Western blotting analysis, and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) titration. Lastly, we have analysed the effect of peroxynitrite on wild-type DHFR and SE-DHFR enzymatic activity.

Materials and methods

Materials

SE-DHFR was overexpressed and purified in the laboratory of Dr. C. R. Matthews (The Pensylvania State University, Pensylvania, USA), who kindly supplied us, as previously described [12]. The specific activity of SE-DHFR was 48.87 $U\,mg^{-1}$ at 20 °C and pH 7.0. Wild-type DHFR was isolated in our laboratory from *E. coli* strain AG-1 (Stratagene) containing the plasmid pWT1-3, kindly supplied by Dr. C. R. Matthews, as previously described [12]. The specific activity of wt-DHFR was 5.5 $U\,mg^{-1}$ at 20 °C and pH 7.0. SE-DHFR and wt-DHFR, stocked in ammonium sulphate, before each experiment, were dialyzed against 4 L of 50 mM phosphate buffer, 0.2 mM EDTA at pH 7.0 overnight. The concentration of SE-DHFR and wt-DHFR was determined spectrophotometrically using a Cary 1 (VARIAN) dual-beam spectrophotometer, at 280 nm using a molar extinction coefficient of 31,100 $M^{-1}cm^{-1}$ [13]. The anti-nitrotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY, USA).

Determination of enzymatic activity

Enzymatic activity of dihydrofolate reductase was followed spectrophotometrically [16]. The reaction mixture contained 1440 μl of 50 mM potassium phosphate buffer, 0.2 mM EDTA, pH 7.0, 30 μl of 3.3 mg/ml NADPH, and 30 μl of 0.0035 M H_2F. The solution was thermostated for 5 min at 20 °C and 20 μl of 5 μM SE-DHFR or wt-DHFR was added to start the reaction. The decrease of the absorbance at 340 nm, due to the lowering of NADPH concentration in the time, was registered and related to the enzymatic activity. The experimental error in the measure of the enzymatic activity has been determined by performing repeated assays at each 10 min in the 24 h time span. From these measurements the mean values and standard deviation were calculated.

[1] *Abbreviations used:* H_2F, dihydrofolic acid; SE-DHFR, C85S/C152E double mutant of dihydrofolate reductase from *Escherichia coli*; DHFR, dihydrofolate reductase; wt-DHFR, wild-type dihydrofolate reductase.

Effect of peroxynitrite on enzymatic activity

Peroxynitrite was synthesized according to the protocol reported by M. Uppu et al., and stocked at −80 °C [6]. The concentration of peroxynitrite was determined at 302 nm ($\varepsilon = 1670\ M^{-1}cm^{-1}$) [17]. The peroxynitrite stock solution was diluted in 0.1 M KOH before use. The effect of peroxynitrite on enzyme activity was studied by assaying the enzymatic activity of SE-DHFR and wt-DHFR after incubation of the enzyme with peroxynitrite. The solution of 5 μM SE-DHFR or wt-DHFR was thermostated at 20 °C for 4 min and then peroxynitrite was added to the solution, under stirring. After 2 min of incubation at 20 °C, aliquots were added to the assay solution and the enzymatic activity monitored at 340 nm as described above. Moreover, in order to evaluate the possible effects of basic pH and byproducts of peroxynitrite on the enzymatic activity, a control activity assay has been performed after addition of SE-DHFR and wt-DHFR to buffer solution containing decayed peroxynitrite. In practice peroxynitrite was added to buffer solution, at the pH used for the enzyme assay (pH 7), then after 5 min of incubation (during which the peroxynitrite decomposed to the byproducts) the enzyme was added and the solution was incubated for 2 min. Aliquots were added to the assay mixture and the activity recorded. To evaluate the effect of the HCO_3^-/CO_2 system on the peroxynitrite oxidation of the protein, SE-DHFR, and wt-DHFR were oxidized in presence of $NaHCO_3$ concentrations ranging between 0.7 and 20 mM.

Polyacrylamide gel electrophoresis and Western blotting

The presence of nitrotyrosines was proved by immunoblotting analysis. The peroxynitrite-exposed enzyme samples, in presence and in absence of 20 mM $NaHCO_3$, were previously dialyzed against 20 mM phosphate buffer, at pH 7.0. Immunoblotting detection of nitrotyrosine groups was performed using anti-nitrotyrosine antibodies. The immunoblotting experiment was performed by electroblotting 2 μg of the enzyme samples, previously separated on a 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) [18], onto PVDF membranes (Millipore, Bedford, MA) according to Towbin et al. [19]. The immunoblot detection was carried out with ECL (Enhanced Chemiluminescence) Western blotting analysis system (Amersham–Pharmacia, Biotech) using peroxidase-conjugated anti-rabbit secondary antibodies. Relative amounts of 3-nitrotyrosine groups on oxidized proteins were quantitated by densitometric analysis using Scion Image software (Scion, Frederick, MD) and a MATLAB R12 program. Measurement of bands intensities from uniform area with accurate background subtraction was performed.

Cysteine titration

The sulphydryl content of non-oxidized and oxidized 5 μM wt-DHFR, in absence and in presence of $NaHCO_3$, was determined by Ellman's method [20,21] using DTNB 500 μM. Sulphydryl groups titration was performed in presence of 8 M urea to determine the total sulphydryl content. The increase in absorbance at 412 nm due to the release of 5-thio-nitrobenzoate was monitored.

Spectrofluorimetric studies

An ISS model GREG-200 spectrofluorometer was used for fluorescence measurements. Samples of 10 μM SE-DHFR and wt-DHFR non-oxidized and oxidized by peroxynitrite in presence and in absence of $NaHCO_3$ (from 0.7 to 20 mM in the reaction mixture) were analysed and the fluorescence emission spectra ($\lambda_{exc} = 280$ nm, $\lambda_{em} = 310$–450 nm) were collected.

Separation of the SE-DHFR oxidized forms by reversed phase chromatography by HPLC

The samples, previously dialyzed against 10 mM phosphate buffer, pH 7.0, of 140 μM SE-DHFR non-oxidized and oxidized by treatment with peroxynitrite (from 2.72 to 5 mM in the mixture reaction) in presence and in absence of 20 mM $NaHCO_3$ were injected into an HPLC column (C-4 HAMILTON, reversed phase, PRP3, 4.1 × 150 mm). Elution was carried out with H_2O + 0.1% TFA (A) and CH_3CN + 0.1% TFA (B) with the following gradient: from 25 to 40% of B in 2 min, from 40 to 45% of B in 15 min, and from 45 to 80% of B in 5 min; B remains at 80% for 5 min and returns to 25% in 5 min. The elution pattern was monitored with a UV–vis detector at the wavelength of 254 nm (flow rate 1 ml/min).

Separation of the SE-DHFR oxidized oligomeric forms by gel filtration by FPLC

Samples of 60 μM SE-DHFR non-oxidized and oxidized by treatment with peroxynitrite (from 0.2 to 5 mM in the mixture reaction) in absence and in presence of 20 mM $NaHCO_3$ were injected onto an FPLC gel filtration column (SUPERDEX 75 HR 10/30). Elution was carried out with 50 mM potassium phosphate buffer, 0.2 mM EDTA, pH 7.0. The elution pattern was monitored with a UV–vis detector at the wavelength of 254 nm (flow rate 1 ml/min).

Differential scanning calorimetry measurements

The calorimetric studies were carried out with a N-DSC II calorimeter (MODEL 6100, Calorimetry

Sciences, USA) in 0.3268 ml cells at scanning rate of 1°C/min. Data reduction and analysis were performed with the software preinstalled in the instrument (Applied Thermodynamics). Samples of 140 μM SE-DHFR non-oxidized and oxidized by treatment with peroxynitrite (from 2.72 to 5 mM in the mixture reaction), in presence and in absence of 20 mM $NaHCO_3$, were diluted to 50 μM and analysed. The peroxynitrite-exposed enzymes were previously dialyzed against 10 mM potassium phosphate buffer, pH 7.0. Protein samples were degassed before loading in the calorimetric cells.

Analysis of the heat capacity function and structural thermodynamics

Deconvolution of the heat capacity function has been performed with software developed by Applied Thermodynamics. The basic thermodynamics used for data analysis and interpretation has been extensively described in the literature [22]. Several laboratories have shown that the heat capacity change associated with the unfolding of the native state of a protein can be expressed as a linear combination of the change in polar (ΔASA_{pol}) and apolar (ΔASA_{ap}) solvent-accessible surface areas between those states [23]. All our accessible surface area calculations of protein structures were analysed using the implementation of Lee and Richards's algorithm [24] in the program ACCESS (S.R. Presnell, University of California, San Francisco), with a probe radius of 1.4 Å and a slice of 0.25 Å.

Results

Effect of peroxynitrite on enzymatic activity

The effect of peroxynitrite on the enzymatic activity of SE-DHFR is reported in Fig. 1. Peroxynitrite causes a concentration-dependent reduction of the enzymatic activity, with an Ec_{50} value of about 0.1 mM (corresponding to a ratio $[ONOO^-]/[SE\text{-}DHFR] = 20$) and the maximal effect observed is a residual activity of 10% at a ratio $[ONOO^-]/[SE\text{-}DHFR] = 670$. The effect of the HCO_3^-/CO_2 system on the protein oxidation by peroxynitrite is displayed in Fig. 2. As shown the effect of peroxynitrite on the enzymatic activity of SE-DHFR decreases by increasing the concentration of $NaHCO_3$ (ranging from 0.71 to 20 mM) in the reaction mixture, and the values of Ec_{50} calculated are reported in legend of Fig. 2. The effect of decomposition products of peroxynitrite [25], and the effect of $NaHCO_3$ on SE-DHFR enzymatic activity was tested. From these experiments a 1.97% decrease of the reaction initial rate resulted, a value lower than the calculated experimental error (4.6%). These data consist with the hypothesis that only the $ONOO^-$ form is the cause of the inhibitory effect of peroxynitrite system on SE-DHFR activity.

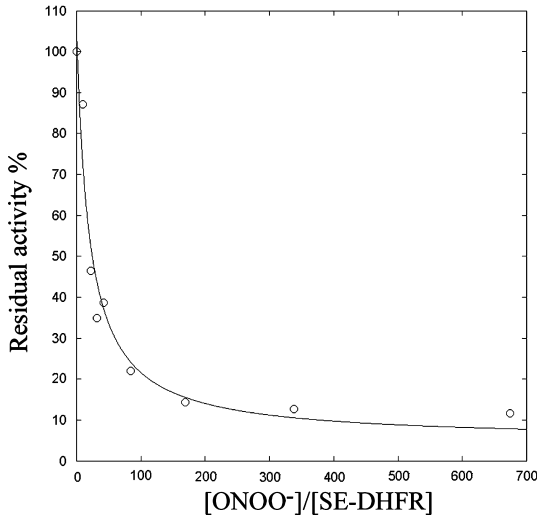

Fig. 1. Dependence of residual activity percentage of SE-DHFR on the ratio $[ONOO^-]/[SE\text{-}DHFR]$. The value of Ec_{50} obtained was 0.100 mM ± 0.027 with $\chi^2 = 407.04$. Ec_{50} is the concentration of peroxynitrite giving a residual activity of 50% with respect to the total inhibition of enzymatic activity.

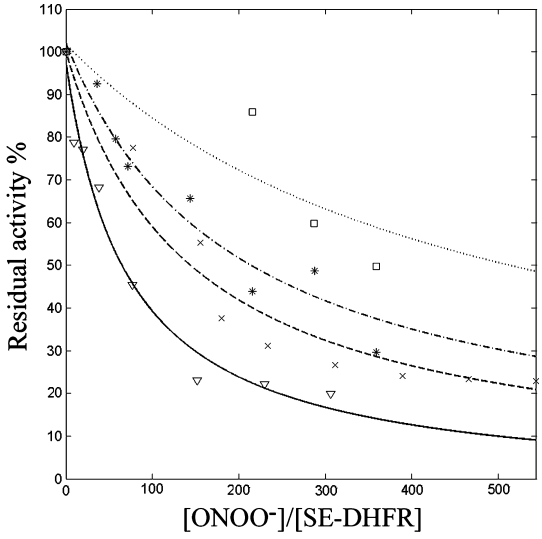

Fig. 2. Effect of the HCO_3^-/CO_2 system on the oxidation of SE-DHFR with peroxynitrite. Curve (—): $[HCO_3^-] = 0.71$ mM, $Ec_{50} = 0.359$ mM ± 0.084. Curve (---): $[HCO_3^-] = 1.72$ mM, $Ec_{50} = 0.720$ mM ± 0.231. Curve (– · –): $[HCO_3^-] = 5$ mM, $Ec_{50} = 0.933$ mM ± 0.259. Curve (· · · · ·): $[HCO_3^-] = 20$ mM, $Ec_{50} = 2.39$ mM ± 0.64.

The effect of peroxynitrite on the enzymatic activity of wt-DHFR, both in presence and in absence of $NaHCO_3$, caused a 10% inhibition of the enzymatic activity at a ratio $[ONOO^-]/[SE\text{-}DHFR] = 600$. The peroxynitrite concentration producing a 50% inhibition of SE-DHFR activity (0.1 mM) induces an inactivation on wt-DHFR enzymatic activity that is of the order of magnitude of the experimental error.

Effect of peroxynitrite on tryptophan oxidation

The reaction of SE-DHFR with peroxynitrite resulted in a decrease of protein tryptophan fluorescence (Fig. 3). A residual tryptophan fluorescence of 37.5%, with respect to the non-oxidized protein, was observed from the fluorescence spectrum of SE-DHFR oxidized at a ratio $[ONOO^-]/[SE\text{-}DHFR] = 10$. Since the fluorescence of tryptophan is dependent on the integrity of the indole ring, the loss of fluorescence probably results from the disruption of the ring by oxidation, or N-nitration. Fig. 3 also shows the effect of the system HCO_3^-/CO_2 on the oxidative modification of tryptophan residues by peroxynitrite. The effect of the oxidant did not seem to be influenced by the presence of low concentrations of $NaHCO_3$, while a restoration of the tryptophan fluorescence was obtained at high concentrations of $NaHCO_3$ (the residual fluorescence of the oxidized protein was of 89% in presence of 20 mM $NaHCO_3$ with respect to the non-oxidized protein). The enzymatic activity of SE-DHFR samples analysed by fluorescence measurements was assayed to investigate a possible correlation between residual tryptophan fluorescence and residual enzymatic activity. The data displayed in Table 1 show that the system HCO_3^-/CO_2 can modulate the oxidative modification of the tryptophan residues by peroxynitrite, allowing at the same time a restoration of the enzymatic activity of SE-DHFR, under these experimental conditions (i.e., $[ONOO^-]/[SE\text{-}DHFR] = 10$). It should be pointed out that at these protein and peroxynitrite concentrations the residual enzymatic activity is around 73% while the residual Trp fluorescence is around 37%, suggesting that even if tryptophan residues play a role in catalytic activity their modification does not strongly affect the function of the enzyme. By measuring the fluorescence intensities and specific activities of the SE-DHFR oxidized with different amounts of peroxynitrite concentrations giving a ratio $[ONOO^-]/[SE\text{-}DHFR]$ ranging from 5 to 150 in absence of bicarbonate (data not shown) it emerged that at peroxynitrite concentrations higher than 0.1 mM ($[ONOO^-]/[SE\text{-}DHFR] > 10$) the residual activity decreased as already shown in Fig. 1 but the Trp fluorescence remained constant (37.5% of residual tryptophan fluorescence).

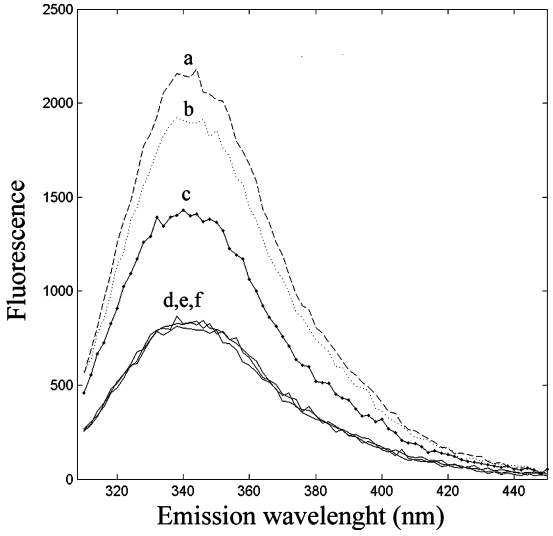

Fig. 3. Fluorescence emission spectra of non-oxidized and oxidized SE-DHFR oxidized at a ratio $[ONOO^-]/[SE\text{-}DHFR] = 10$ in presence and in absence of HCO_3^-/CO_2 system, as described under Materials and methods. Excitation wavelength was 280 nm. Curve a (---) non-oxidized enzyme in presence of products of degradation of peroxynitrite; Curve b (·····) enzyme oxidized in presence of 20 mM HCO_3^-; Curve c (–·–·–) enzyme oxidized in presence of 5 mM HCO_3^-; and Curves d–f (—) enzyme oxidized in absence of HCO_3^- (d), in presence of 0.71 mM (e), and 1.72 mM HCO_3^- (f).

Fluorescence emission spectra have been recorded also for the wt-DHFR oxidized by peroxynitrite in absence and in presence of $NaHCO_3$. The reaction of wt-DHFR with peroxynitrite resulted in a decrease of protein tryptophan fluorescence of about 15% at a ratio $[ONOO^-]/[SE\text{-}DHFR] = 10$ (Fig. 4). The protection of tryptophan fluorescence against peroxynitrite in absence and in presence of 0.7–5 mM HCO_3^- (see Table 1B) on wild-type enzyme suggests that free thiol targets in wt-DHFR decrease the reactivity of $ONOO^-$ and the $ONO_2CO_2^-$ against tryptophan residues; it can be noted from the data shown in Table 1B that the in absence of $NaHCO_3$ the presence of cysteines determines a residual tryptophan fluorescence comparable with the value in

Table 1
Comparison between residual tryptophan fluorescence of oxidized SE-DHFR (A) and wt-DHFR (B) by peroxynitrite and residual enzymatic activity, in presence and in absence of $NaHCO_3$

	In absence of $NaHCO_3$ (%)	0.71 mM $NaHCO_3$ (%)	5 mM $NaHCO_3$ (%)	20 mM $NaHCO_3$ (%)
(A) SE-DHFR				
Residual tryptophan fluorescence at 340 nm	37.5	38.3	66.5	88.9
Residual enzymatic activity	74.33	73.64	87.32	94.06
(B) wt-DHFR				
Residual tryptophan fluorescence at 340 nm	85	77.4	79.1	88
Residual enzymatic activity	98.72	88.83	98	99.1

In all experiments the ratio $[ONOO^-]/[SE\text{-}DHFR] = 10$.

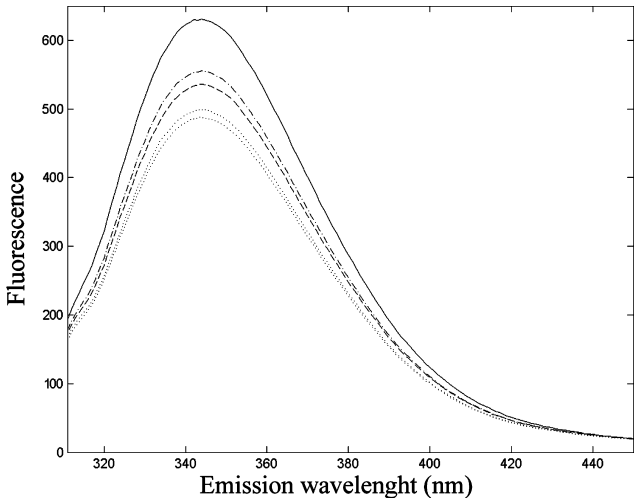

Fig. 4. Fluorescence emission spectra of non-oxidized and oxidized wt-DHFR oxidized at a ratio $[ONOO^-]/[SE\text{-}DHFR] = 10$ in presence and in absence of HCO_3^-/CO_2 system, as described under Materials and methods. Excitation wavelength was 280 nm. Curve (—) non-oxidized enzyme in presence of products of degradation of peroxynitrite; Curve (– · –) enzyme oxidized in presence of 20 mM HCO_3^-; Curve (---) enzyme oxidized in absence of HCO_3^-; Curves (· · · · · ·) enzyme oxidized in presence of 0.71 and 5 mM HCO_3^-.

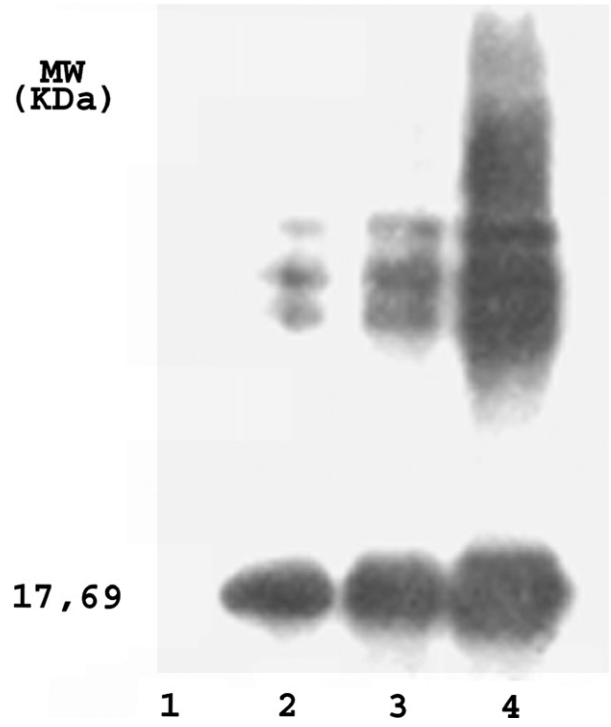

Fig. 5. Immunoblot detection of 3-nitrotyrosine groups. One hundred and forty micromolar of SE-DHFR was treated with increasing amounts of peroxynitrite. The ratios $[ONOO^-]/[SE\text{-}DHFR]$ are reported (lane 1, 0; lane 2, 20; lane 3, 35; and lane 4, 35, in presence of 20 mM $NaHCO_3$:), separated on 12% SDS–PAGE, and then electroblotted onto a PVDF membrane. The ratios between the intensity of the band of oxidized SE-DHFR in presence of 20 mM HCO_3^- and the band intensity of oxidized SE-DHFR by 2.72 and 5 mM peroxynitrite were, respectively, 1.62 and 1.30.

presence of 20 mM $NaHCO_3$. This behaviour can be explained basing on the order of reactivity of amino acid residues with regards to the two oxidant species $ONOO^-$ and $ONO_2CO_2^-$: the $ONOO^-$ anion is more reactive toward cysteines than toward aromatic residues [26], in contrast the $ONO_2CO_2^-$ adduct preferentially oxidizes aromatic residues with respect to cysteines, in the order Tyr > Trp ≫ Cys.

Immunoblot detection of 3-nitrotyrosine groups

The nitrated tyrosine is a specific biomarker of the attack of reactive nitrogen species, such as peroxynitrite, over proteins [27,28]. Immunoblot detection of 3-nitrotyrosine groups on SE-DHFR was performed in order to evaluate the susceptibilities of the enzyme to nitration under the conditions used in calorimetric measurements. The results obtained (Fig. 5) clearly indicate that increasing amounts of peroxynitrite induce a gradual increase of 3-nitrotyrosine groups on SE-DHFR. More specifically the presence of 20 mM $NaHCO_3$ in the mixture reaction causes a relevant nitration of tyrosine residues (Fig. 5, lane 4). The control experiments revealed a high specificity of the anti-nitrotyrosine antibodies; in fact no bands were detected after reduction of the blotted proteins with dithionite [29,30] (data not shown). In Fig. 5 in both of the lanes of the Western blotting several high molecular weight bands were observed suggesting the formation of cross-linked polypeptides or aggregated species resistant to SDS. The presence of high molecular weight species in presence of bicarbonate has been confirmed by gel filtration analysis in FPLC (Fig. 6), to assess the formation of oligomeric species in absence of heat treatment of the oxidized protein (procedure used for the sample denaturation in SDS–PAGE).

The presence of nitrotyrosines and cross-linked species on oxidized SE-DHFR and wt-DHFR at low protein concentration (5 μM) and peroxynitrite amounts ranging from 0.1 to 100 μM, both in presence and in absence of $NaHCO_3$, has been verified by Western blotting analysis, using the experimental conditions utilized in fluorimetric and enzymatic assays (Fig. 7). In the wild-type enzyme we observed an increase in the modification of tyrosines with increasing peroxynitrite concentrations, both in presence and in absence of $NaHCO_3$. With the mutant SE-DHFR, in presence of $NaHCO_3$, we determined that there was an increase of 3-nitrotyrosines relative to the monomeric band. In contrast, with the wt-DHFR 3-nitrotyrosines were concentrated within the high molecular weight bands, with the absence of bicarbonate enhancing this effect. This behaviour may be due to the fact that the nitrosoperoxycarbonate adduct is less reactive toward thiol targets with respect to peroxynitrite [31]. In wt-DHFR the cysteine residues are responsible for the formation of disulphide bridges

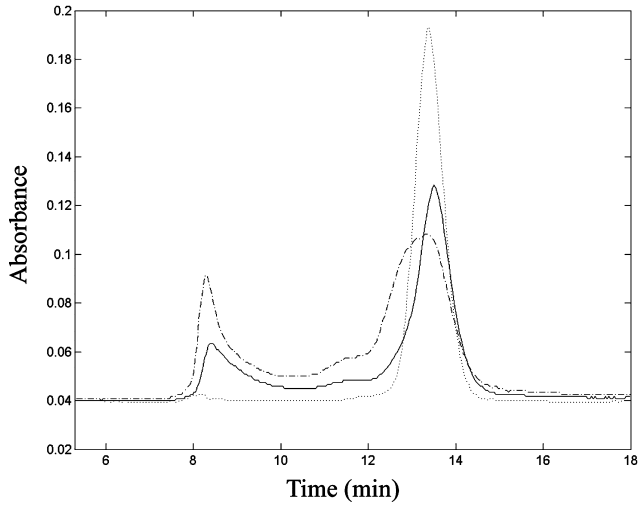

Fig. 6. FPLC gel filtration chromatograms of non-oxidized and oxidized SE-DHFR by treatment with peroxynitrite in presence of 20 mM HCO_3^-. The ratios $[ONOO^-]/[SE\text{-}DHFR]$ are reported: (· · · · · ·) 0; (—) 60; and (– · –) 80. Molecular weight standards were injected under the same conditions, and the retention time for each was recorded: (Tyroglobulin MW = 670 kDa: 7.9 min; IgG MW = 158 kDa: 9.3 min; ovalbumin MW = 44 kDa: 11.4 min; myoglobin MW = 17 kDa: 13.5 min; and Vit.B_{12} MW = 1.35 kDa: 19.1 min).

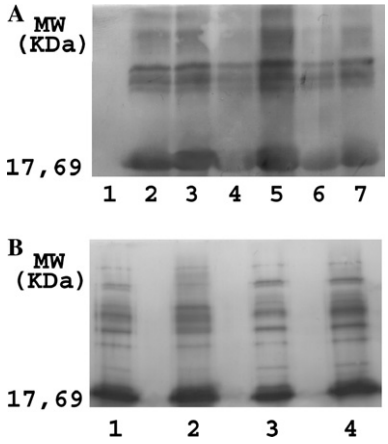

Fig. 7. Immunoblot detection of 3-nitrotyrosine groups. (A) 5 μM SE-DHFR was treated with increasing amounts of peroxynitrite. The ratios $[ONOO^-]/[SE\text{-}DHFR]$ are reported (lane 1, 0.02; lane 2, 20; lane 3, 10; lane 4, 0.2; lane 5, 20 in presence of 20 mM $NaHCO_3$; lane 6, 20 in presence of 1.7 mM $NaHCO_3$; and lane 7, 20 in presence of 0.7 mM $NaHCO_3$). (B) 5 μM wt-DHFR was treated with increasing amounts of peroxynitrite. The ratios $[ONOO^-]/[SE\text{-}DHFR]$ are reported (lane 1, 20 in presence of 20 mM $NaHCO_3$; lane 2, 330 in presence of 20 mM $NaHCO_3$; lane 3, 20; and lane 4, 330).

that partially account for the presence of cross-linked species as revealed by Western blotting (Fig. 7B). It is emerging from these results that in presence of $NaHCO_3$ the disulphide bridges are formed at lower extent than in absence of $NaHCO_3$ [26], especially at high peroxynitrite concentration. These data are explained also tacking into account the results of cysteine titration after peroxynitrite modification in absence and in presence of NaHCO3 as shown below.

Cysteine titration of wt-DHFR

The wild-type DHFR contains two cysteine residues, which are good substrates for oxidation by peroxynitrite [32]. The sulphydryl groups oxidized by increasing concentrations of peroxynitrite in absence of $NaHCO_3$ are summarized in Fig. 8. In absence of $NaHCO_3$ about 50% of total sulphydryl groups was rapidly oxidized at a ratio $[ONOO^-]/[SE\text{-}DHFR] = 10$, while the total content of cysteine residues was oxidized at a ratio $[ONOO^-]/[SE\text{-}DHFR] = 400$. In presence of 20 mM $NaHCO_3$ the percentage of sulphydryl groups oxidized was of about 55% in all the conditions tested, as shown in Fig. 6.

Separation of SE-DHFR oxidized forms by reversed-phase chromatography by HPLC

Samples of non-oxidized and oxidized protein by increasing concentrations of peroxynitrite in presence and in absence of 20 mM of $NaHCO_3$ were analysed by HPLC as described under Materials and methods. The results are summarized in Fig. 9. In absence of $NaHCO_3$, the oxidized protein shows a shorter retention time with respect to the non-oxidized protein, as a result of the introduction of polar groups (nitro groups, carboxyl groups, sulphoxide groups, etc.) by oxidation [3,26,32–34]. In presence of 20 mM $NaHCO_3$ the product of the oxidation includes several chemical species, probably because the oxidation yield is not complete. This is confirmed by the fact that the peak of the non-oxidized form is still present (retention time

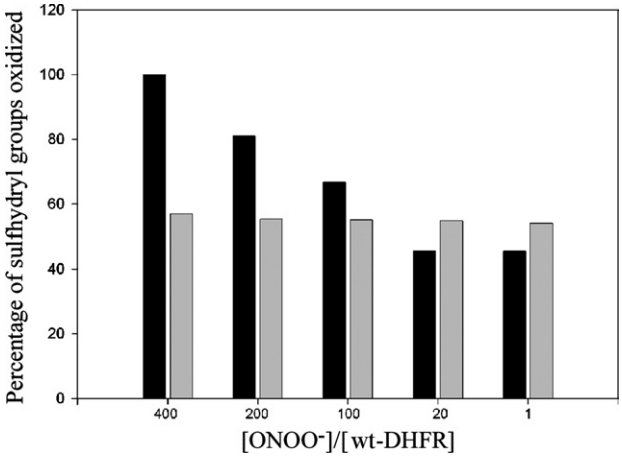

Fig. 8. Determination of the cysteine residues oxidized by increasing concentrations of peroxynitrite in absence (black) and in presence (grey) of 20 mM $NaHCO_3$ on wt-DHFR (5 μM). The enzyme was denaturated by 8 M urea after the oxidation as described in Material and methods.

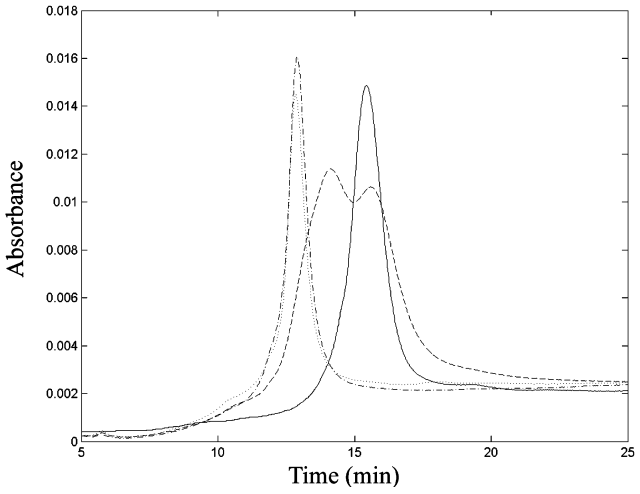

Fig. 9. HPLC reversed phase chromatograms of non-oxidized and oxidized SE-DHFR by treatment with peroxynitrite in presence and in absence of 20 mM HCO_3^-. The ratios $[ONOO^-]/[SE\text{-}DHFR]$ are reported: (—) 0; (– · –) 20; (· · · · · ·) 35; and (- - -) 35 in presence of 20 mM HCO_3^-.

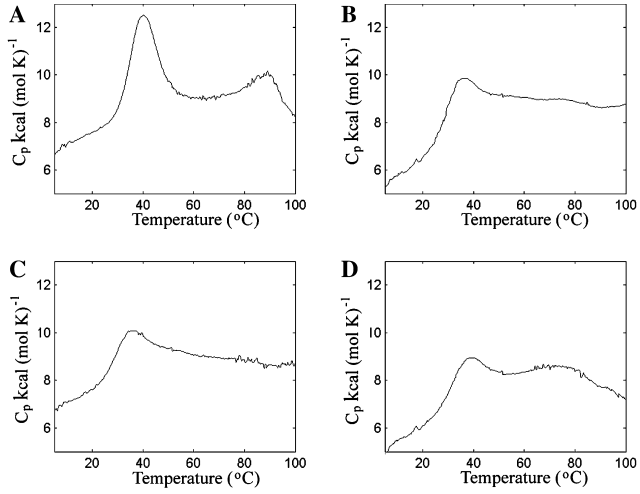

Fig. 10. Partial molar heat capacity of non-oxidized and oxidized SE-DHFR at pH 7.0 as function of temperature. (A) non-oxidized SE-DHFR. (B) $[ONOO^-]/[SE\text{-}DHFR] = 20$. (C) $[ONOO^-]/[SE\text{-}DHFR] = 35$. (D) $[ONOO^-]/[SE\text{-}DHFR] = 35$ in presence of 20 mM $NaHCO_3$. The buffer used was 10 mM potassium phosphate, pH 7.0. The thermodynamic parameters obtained by the fit are shown in Table 3.

15.44 min); a complete oxidation is instead obtained in absence of $NaHCO_3$. This kind of analysis allowed us to verify the nature of the samples analysed by differential scanning calorimetry.

Peroxynitrite effect on SE-DHFR folding

Calorimetric measurements of the effect of heating solution of non-oxidized and oxidized SE-DHFR are shown in Fig. 10A–D. The quantity measured was the energy required to raise the temperature of the entire solution, which is its heat capacity at constant pressure (3 atm). Fig. 10 shows the partial molar heat capacity function of non-oxidized SE-DHFR at a concentration of 50 μM in 10 mM potassium phosphate, pH 7.0 (Fig. 10A). Under these experimental conditions, the protein shows two peaks: the first one occurring at 39.19 °C corresponds to the transition from the native to the folding intermediate; the second one, which occurs at 88.32 °C, accounts for the transition from the folding intermediate to the unfolded state. The same calorimetric measurements were performed with the oxidized enzymes, in presence and in absence of $NaHCO_3$ as described in Materials and methods. The oxidized enzymes in absence of $NaHCO_3$ do not show the second transition (Figs. 10B and C); in presence of $NaHCO_3$ the second transition is restored at a lower temperature with respect to the non-oxidized protein (75.16 °C) (Fig. 10D).

The thermodynamic parameters of non-oxidized and oxidized SE-DHFR are summarized in Table 2. The results show that the protein is destabilized by oxidative modification. The T_m and ΔH of oxidized proteins are lower than the value obtained for the non-oxidized protein, and at high concentration of peroxynitrite the values of T_m and ΔH tend to decrease. The T_m and ΔH of oxidized proteins in presence of $NaHCO_3$ are intermediate between that of non-oxidized protein and oxidized proteins in absence of $NaHCO_3$. Furthermore, the ΔC_p of oxidized proteins are greater than that of the non-oxidized protein, and at high concentration of peroxynitrite the value of ΔC_p tends to increase. The oxidizing effect of peroxynitrite on thermal unfolding is destabilizing, in fact the T_m of enzymes treated with per-

Table 2
Summary of the non-linear deconvolution analysis of differential scanning calorimetry data of non-oxidized and oxidized SE-DHFR

	First transition T_m (°C)	$\Delta H(T_m)$ (kcal/mol)	$\Delta S(T_m)$ (cal/molK)	$\Delta C_p(T_m)$ (kcal/molK)	Second transition T_m (°C)
Non-oxidized SE-DHFR	39.19 ± 0.05	55.90 ± 0.67	178.97 ± 2.14	0.829 ± 0.010	88.32 ± 0.05
$[ONOO^-]/[SE\text{-}DHFR] = 20$	32.62 ± 0.05	42.97 ± 0.49	140.50 ± 1.60	0.967 ± 0.011	Absent
$[ONOO^-]/[SE\text{-}DHFR] = 35$	31.79 ± 0.05	37.80 ± 0.31	123.96 ± 1.02	1.106 ± 0.009	Absent
$[ONOO^-]/[SE\text{-}DHFR] = 35 + 20$ mM HCO3^{-*}	35.47 ± 0.05	42.20 ± 0.42	136.73 ± 1.36	1.001 ± 0.010	75.16 ± 0.05

T_m is the temperature at which $\Delta G = 0$ for the first transition. $\Delta H(T_m)$ an $\Delta C_p(T_m)$ are the values of ΔH and ΔC_p at T_m. The errors in the parameters have been derived from the χ^2 obtained by non-linear least-square analysis, using the error propagation analysis. The thermodynamic parameters (ΔH, ΔC_p, and T_m) were obtained for a two state fit. In the present analysis, the heat capacities are considered to be temperature-dependent.

* The calculated thermodynamic parameters are not accurate because of the heterogeneity of the protein system as explained in the text.

Table 3
Accessible surface area changes, after the first thermal transition, in presence and in absence of oxidative agents

	ΔASA_{ap} (Å^2)	ΔASA_{pol} (Å^2)
Non-oxidized SE-DHFR	3876.90	3372.01
$[ONOO^-]/[SE\text{-}DHFR] = 20$	4167.02	3332.14
$[ONOO^-]/[SE\text{-}DHFR] = 35$	4532.56	3416.06

oxynitrite decrease of about 8 °C, and an increase of ΔC_p is simultaneously observed. These results show that the oxidized proteins have undergone an advanced unfolding process respect to the non-oxidized protein, exposing a larger surface area to the solvent. The structural thermodynamics analysis has been performed after having calculated the enthalpy change, relative to the first transition, at the reference temperature (i.e., 60 °C see Materials and methods). The results for the change in accessible surface areas apolar and polar upon the transition have been reported in Table 3 for SE-DHFR non-oxidized and oxidized in absence of $NaHCO_3$. For SE-DHFR, the complete unfolding of the native state is accompanied by an increase in ΔASA_{ap} of 9175.74 Å^2 and an increase in ΔASA_{pol} of 5615.91 Å^2 as calculated by ACCESS program (see Materials and methods). By comparing the results summarized in Table 3 it appears that the partly folded state populated by the non-oxidized protein retains 57% of the apolar surface area buried from the solvent while the polar surface area exposed is the 60%. This behaviour is characteristic of compact intermediates. The effect of peroxynitrite oxidation is an increase in the amount of apolar surface area exposed to the solvent after the transition while no big variation is observed about the polar surface area exposure to the solvent.

Discussion

In this work we have investigated the effects of peroxynitrite-induced oxidative modifications on enzymatic activity of a cysteine-free mutant of DHFR from *E. coli*, in comparison with the effects on its structural stability. Peroxynitrite significantly affects the catalytic activity of SE-DHFR both in absence and in presence of HCO_3^-/CO_2; at the same time the modulating effect of $NaHCO_3$ on the inactivation of the enzyme by peroxynitrite is remarkable (the value of Ec_{50} increased from 0.1 to 2.39 mM in presence of 20 mM $NaHCO_3$, see legend of Figs. 1 and 2). All the experimental data obtained regarding the different protein modification induced by peroxynitrite in absence and in presence of $NaHCO_3$ can be described according to the different oxidizing system arising from the presence of the buffer HCO_3^-/CO_2. The speed of the reaction of peroxynitrite with CO_2, in biological systems, is faster than the uncatalysed decomposition of peroxynitrite. Thus, many of the reactions of peroxynitrite are more likely to be mediated by the reactive intermediates derived from the reaction of $ONOO^-$ with CO_2 than by peroxynitrite itself [6]. The production of the short-lived $ONO_2CO_2^-$ adduct in presence of bicarbonate first results in a decrease of the yield of oxidation (as revealed by HPLC analysis Fig. 9) and second produces a more efficient tyrosine nitration with respect to tryptophan modification (see Western blotting and fluorimetric data, Figs. 5 and 3). These data are in direct agreement with the literature [35–37].

The lower catalytic inactivation in presence of $NaHCO_3$, can be due either to the decreased yield of oxidation, either, but only in part, to the lower tryptophan modification induced by the system peroxynitrite/bicarbonate.

The first effect could be explained based on the fact that in presence of CO^2/HCO_3^- the reaction of peroxynitrite with CO_2 might represent the rate-limiting step in the nitration process. The second effect suggests that the modification of the tryptophan residue may affect the enzyme/substrate orientation. In particular the Trp22, which is placed in the binding site of the protein, has been shown to be involved in the hydrogen-bonding network stabilizing the binding of the substrate [38–40]. The enzyme DHFR contains five tryptophan residues (Trp22, Trp30, Trp47, Trp74, and Trp133). The literature reports mainly the importance of Trp22 on catalysis by DHFR with respect to other tryptophan residues [40,41]. At $[ONOO^-]/[SE\text{-}DHFR] = 10$ at increasing $NaHCO_3$ concentrations we observed an increasing both of residual Trp fluorescence and residual enzymatic activity, suggesting a correlation between tryptophan integrity and the recovery of 20% residual activity, as shown in Table 1A. From our data it emerged that tryptophan residues have a partial role in the enzymatic function and their modification is closely related to the reduced activity of the enzyme; but the oxidation/nitration of Trp 22 definitively is not the only process involved in the loss of function. This hypothesis is based on the observation that at high peroxynitrite concentrations (in absence of bicarbonate) the residual enzymatic activity decreases without any variation of fluorescence intensity (assessed at a value of 37.5% as reported in Results). Many aspects of SE-DHFR modification by peroxynitrite in absence and in presence of $NaHCO_3$ remain to be elucidated, especially tacking into account the characteristics of high flexibility of this protein where several residues placed in crucial positions in the molecular structure (like the loop Met 20, and the loop 117–131 [42]) exert the modulation of ligand binding properties, that reflect on the enzymatic activity. Studies on peroxynitrite-mediated aromatic modification have been reported in other proteins (Cu,Zn-SOD, [43]) where Trp nitration produces a partial inactivation of the enzyme activity without perturbating the active site integrity.

Because bicarbonate is the most important biological buffer and its plasmatic concentration is significant [31,44] (its limit concentration of 20 mM HCO_3^- is maintained in these experiments), it can cover a physiological role in the modulation of peroxynitrite oxidation of aromatic residues. The enzyme DHFR has four tyrosine residues, among which Tyr100 mediates hydrophobic contacts between the nicotinamide ring of the NADPH cofactor and the enzyme [40,41]. In peroxynitrite modification the accessibility of protein residues to the solvent is a crucial factor. The solvent accessibility of tyrosine residues of SE-DHFR has been analysed using the programme ACCESS (see Materials and methods). The accessible surface areas observed for tyrosine residues (Tyr100, Tyr111, Tyr128, and Tyr151) have revealed that Tyr100 is buried to the solvent with respect to the other tyrosines. Based on these calculations we can assume that Tyr100 is less susceptible toward peroxynitrite nitration. This might explain the discrepancy between the increase of nitrotyrosines in presence of bicarbonate and the increase of residual enzymatic activity when $NaHCO_3$ is added to peroxynitrite; the $ONO_2CO_2^-$ adduct is more reactive toward tyrosines modification in comparison with tryptophans, and as consequence the tryptophan fluorescence is recovered with parallel restoration of 20% catalytic activity, see Table 1A.

We have compared the mutant to the wild-type enzyme in their behaviour on peroxynitrite-mediated inhibition of enzymatic activity. The immunoblot data and the determination of oxidized sulphydryl groups (see Figs. 7B and 8) respectively, show that both in presence and in absence of $NaHCO_3$, the production of 3-nitrotyrosines and the oxidation of cysteines in the wild-type enzyme oxidized by peroxynitrite can be observed. As shown the increase of cysteine oxidation (Fig. 8) and also the increase of 3-nitrotyrosines (Fig. 7B) has no macroscopic effect on wt-DHFR enzymatic activity (see the effect of peroxynitrite on wild-type activity in Results). The presence of two cysteine residues in the wild-type enzyme seems to exert a protection of the enzymatic activity against peroxynitrite inactivation probably by acting as sacrificial targets. The fluorescence spectra of Fig. 4 and the data of Table 1 show that peroxynitrite preferentially oxidizes cysteines, as reported in other studies [26], producing in the wt-DHFR an increase of the residual fluorescence of about 50% with respect to the SE-DHFR; the same effect is observed for the residual activity. When the $NaHCO_3$ is introduced to the oxidizing system the order of reactivity of amino acid targets is changed resulting in a different modulation of Trp fluorescence and enzymatic activity either in the wt-DHFR and in SE-DHFR, as shown in Table 1A and B and discussed above.

In this work, in order to characterize the structural changes of the enzyme associated with the oxidation by peroxynitrite, the thermal stability of non-oxidized and oxidized SE-DHFR was studied by high-sensitivity differential scanning calorimetry. Under the conditions reported in legends of Fig. 10, the thermal transition of oxidized SE-DHFR is characterized by a decrease of T_m (~8 °C) and ΔH (~18 kcal/mol) with respect to the non-oxidized protein. The enthalpy decrease is related to a reduction of a significant number of non-covalent interactions that stabilize the native state with respect to the unfolded state. It is clear that the oxidized proteins are destabilized by peroxynitrite and that the second transition becomes absent in presence of this oxidative agent. The destabilizing effect of peroxynitrite can be evaluated in terms of transition free energy decrease ($\Delta\Delta G$) relative to the non-oxidized protein, calculated according to the fundamental equation as reported in literature [23]. ΔG decreases by 1.25 kcal/mol for SE-DHFR oxidized at a ratio $[ONOO^-]/[\text{SE-DHFR}] = 20$ at 25 °C, compared to the non-oxidized protein. For SE-DHFR oxidized at a ratio $[ONOO^-]/[\text{SE-DHFR}] = 35$ the ΔG decreases of about 1.46 kcal/mol. Since the oxidation yield is not complete and the protein is found to be heterogeneous (see HPLC results Fig. 9) the destabilizing effect exerted by peroxynitrite in presence of HCO_3^-/CO_2 buffer cannot be described with a rigorous thermodynamic analysis. In this case the thermodynamic parameters can only offer the most general account of the protein behaviour induced by heat. The calorimetric approaches for the study of thermal unfolding of oxidized protein, is interesting and innovative where the two-state model is applicable, while occurrence of heterogeneous species in the oxidation process is a big impairment to a rigorous thermodynamic analysis. Furthermore, the presence of oligomeric forms, produced during protein modification (like cross-linked species) is also a limitation of calorimetric application to oxidized protein systems. We have to take into account the presence of these forms evidenced by Western blotting experiments; however, the FPLC performed for the gel filtration separation of oxidized forms of SE-DHFR and Western blotting has revealed the low percentage of high molecular weight polymers in absence of bicarbonate, and a high percentage of cross-linked species in presence of HCO_3^- (Fig. 6). The HPLC analysis and the data above show that only in absence of bicarbonate we have a statistically predominant species and the two-state model for the study of thermal unfolding is applicable.

Calorimetric studies have allowed us to know the effect of oxidation by peroxynitrite on SE-DHFR thermodynamic stability, oxidized in absence of bicarbonate, basing on the fact that the oxidation produces an homogeneous species (see HPLC results Fig. 9). From the thermodynamic parameters obtained we have also observed that peroxynitrite modification produces a decrease of packing interactions, as it results from the

lower ΔH values relative to the first transition showed by the oxidized proteins. Therefore, from the calculations of the changes in accessible surface areas upon thermal transition, we have found that peroxynitrite oxidation causes an increase in the ΔASA_{ap} with respect to the non-oxidized protein, with the consequent loss of the second transition. This leads to conclude that the folding intermediate populated by the oxidized proteins exposes a larger portion of hydrophobic residues with respect to the non-oxidized protein that makes the partly folded state more prone to form aggregated species, rather than undergoing the transition to the completely denaturated state.

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