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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.