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Kinetic evidence that His-711 of neutral endopeptidase 24.11 is involved in stabilization of the transition state

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Neutral endopeptidase 24.11 (EC 3.4.24.11; NEP) is a membrane-bound Zn-metalloendopeptidase with a catalytic activity and a specificity very similar to that of thermolysin, a bacterial zinc-endoprotease. NEP can be inactivated by reaction with diethylpyrocarbonate, due to the modification of a histidine residue present in the active site of the enzyme. This histidine residue was proposed to be analogous to His^{231} in thermolysin, which is involved in the stabilization of the tetrahedral intermediate during the transition state. Using site-directed mutagenesis of the cDNA encoding rabbit NEP, we have created two mutants of NEP where His^{711} was replaced by either Gln or Phe (NEP-Gln^{711} and NEP-Phe^{711}). Determination of kinetic parameters showed that both mutants had K_m values very similar to that of the non-mutated enzyme but that their k_{cat} values were 25-fold lower. The calculated difference in free energy needed to form the transition state complex was increased by 2.2 kcal/mol for both mutants. These observations strongly suggest that His^{711} is involved in the stabilization of the transition state by forming an hydrogen bond with the oxyanion of the tetrahedral intermediate.

Neutral endopeptidase; Catalytic mechanism; Transition state

1. INTRODUCTION

Neutral endopeptidase (EC 3.4.24.11, neprilysin, NEP) is a membrane-bound zinc-metalloendopeptidase present at the plasma membrane of many cell types. The complete primary structure of NEP had been deduced from the sequences of cDNA clones from rabbit [1], rat [2], human [3] and, recently, mouse [4]. This 94 kDa ectoenzyme contains 749 amino acid residues. The protein has an NH_2-terminal cytoplasmic domain of 27 amino acid residues, an hydrophobic domain of 23 residues that anchors the protein in the plasma membrane, and finally, a large extracellular domain that contains the active site [5].

Although NEP is approximately twice the size of thermolysin (TLN), a bacterial zinc-endoprotease, both exhibit similar specificity with regard to substrates and inhibitors. Indeed, both enzymes prefer bulky P'1 residues, such as phenylalanine and leucine, in the sequence of their substrates, and both enzymes are inhibited by phosphoramidon. Furthermore, residues involved in zinc binding and in the acid/base catalysis are found in a similar sequence in both enzymes [6,7]. However, TLN and NEP react differently with the arginine-specific reagents, butanedione and phenylglyoxal, suggesting differences in the active site of these enzymes. Indeed, it has been shown recently that two arginine residues are involved in substrate binding in NEP [8,9] while X-ray diffraction studies showed only one arginine in the TLN active site [10].

The proposed catalytic mechanism for TLN involves an attack of a water molecule on the carbonyl of the scissile bond to form an oxyanion, a tetrahedral intermediate which subsequently breaks down into products [11,12]. The stabilization of this transition state by enzyme residues is crucial for catalysis. In TLN, X-ray diffraction studies indicated that the tetrahedral intermediate is stabilized by hydrogen bonding with His^{231} and Tyr^{157} [11]. In NEP an inactivation study with diethylpyrocarbonate (DEPC) suggested the presence in the active site of a histidine residue which is probably involved in the stabilization of the transition state [13,14]. Using photoaffinity labelling, Bateman et al. [15] have shown that His^{711} of NEP could be crosslinked to *N*-bromoacetyl-D-leucylglycine, an irreversible inhibitor of NEP which mimics the transition state of the catalytic reaction. Hence, they proposed that residue His^{711} of NEP was present at the active site and could explain the sensitivity of this enzyme to DEPC. However, mutations of this residue resulted in an inactive enzyme and no conclusion could be drawn as to its role in catalysis.

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Abbreviations: NEP, neutral endopeptidase 24.11, EC 3.4.24.11, neprilysin; TLN, thermolysin; DEPC, diethylpyrocarbonate; TBS, Tris-buffered saline; octylglucoside, 1-*O*-*n*-octyl-β-D-glucoside; MES, 2-(morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline; phosphoramidon, [*N*-(α-rhamnopyranosyloxy-hydroxyphosphinyl)-L-leucyl-L-tryptophan].

In the present study, we used site-directed mutagenesis of the cDNA encoding the NEP sequence and transfections of COS-1 cells to express mutated NEP enzymes in which the residue, His^{711}, was changed for either Gln or Phe (NEP-Gln^{711} and NEP-Phe^{711}). Changes observed in the kinetic parameters of the mutant enzymes are consistent with a role of His^{711} in the stabilization of the transition state during catalysis.

2. EXPERIMENTAL

2.1. *Site-directed mutagenesis and vector construction*

All DNA manipulations were performed using described procedures [16]. Oligonucleotide-directed mutagenesis was performed according to the method described by Taylor et al. [17], with an M13 subclone containing the proper fragment of NEP cDNA. Recombinant M13-NEP phages carrying the mutations were screened directly by DNA sequencing [18]. A DNA fragment containing the mutated region was isolated from the replicating form of the M13-NEP recombinant phage and substituted for the equivalent non-mutated fragment in pSVENK19, a previously described NEP expression vector [6]. The presence of the mutations in the expression vectors was confirmed by sequencing the mutated regions by the chain-termination method for double-stranded templates using T7 DNA polymerase [19].

2.2. *Transfection of COS-1 cells and binding of [^{125}I]2B12 monoclonal antibody*

COS-1 cells [20] were transfected using the standard calcium-phosphate co-precipitation procedure [21] as previously described [22]. Two days after transfection, cells were harvested by scraping in Tris-buffered saline (TBS; 10 mM Tris, pH 7.4, 150 mM NaCl) with a rubber spatula. The binding of [^{125}I]2B12 monoclonal antibody was performed as described previously [7], and was used to determine the relative amounts of NEP expressed at the cell surface.

2.3. *Enzyme assays and immunoblotting*

NEP from transfected COS-1 cells was solubilized in 50 mM MES-NaOH, pH 6.5, containing 1% octylglucoside and the enzymatic activity was measured essentially as described previously [23] using 50 nM of the tritiated substrate [tyrosyl-(3,5-^{3}H)](D-Ala_2)-Leu-Enkephalin (50 Ci/mmol) purchased from Commisariat à l'Énergie Atomique (Gif-Sur-Yvette, France), except that incubations were carried out in 50 mM MES-NaOH, pH 6.5, at 37°C. The K_m values were determined by the isotopic dilution method. Calculations were done using the program ENZFITER. In order to calculate the k_{cat} values, the amounts of enzyme were quantitated by immunoblot analysis using purified rabbit kidney NEP as standard [23]. Immunoblotting was performed as previously described using monoclonal antibody 18B5, which recognizes a linear epitope of the rabbit NEP sequence (Labonté and Crine, unpublished results).

2.4. *Inactivation of NEP with DEPC*

The enzymes were dissolved in 50 mM MES-NaOH, pH 6.5, containing 1% octylglucoside. DEPC previously diluted in 70% ethanol, was added to the final concentrations indicated. The reactions were allowed to proceed at 25°C for 30 min and the enzyme activity was determined as described in section 2.3.

3. RESULTS

3.1. *Mutagenesis of NEP cDNA and expression of the mutated enzymes*

Site-directed mutagenesis of the NEP cDNA was used to substitute Gln or Phe for His^{711}, and these mutated cDNAs were introduced in the expression vector pSVENK19 described previously [6]. Vectors, pSVENK19-Gln^{711} and pSVENK19-Phe^{711}, and the pSVENK19 carrying the non-mutated cDNA, were used to transfect COS-1 cells. Binding of Mab [^{125}I]2B12 as well as immunoblot analysis of proteins extracted from transfected COS-1 cell membranes showed similar levels of expression of mutated and non-mutated NEPs (results not shown).

3.2. *Enzymatic properties of mutated NEP*

It was hypothesized that since stabilization of the transition state is important for catalysis, mutation of one of the residues participating in this process would affect enzyme activity. To test the activity of mutated enzymes, we measured the rate of hydrolysis of the tritiated substrate (D-Ala_2)-Leu-Enkephalin. Mutated enzymes exhibited an important decrease in activity (Fig. 1). Under initial velocity conditions, the activity of mutated NEPs was approximately 20-fold lower than that of the non-mutated enzyme.

We next determined the kinetic parameters for non-mutated and mutated enzymes. The K_m values for non-mutated NEP was 86 ± 6 μM. Both mutants exhibited slightly higher K_m values, 144 ± 15 μM for NEP-Gln^{711} and 125 ± 16 μM for NEP-Phe^{711} (Table I). This increase may correspond to a variation in the interaction of the mutated enzymes with the substrate. On the other hand, the k_{cat} values for the mutants were 25-fold lower than that of the non-mutated NEP (Table I). Furthermore, the difference in free energy between the reaction catalyzed by NEP-Gln^{711} or NEP-Phe^{711} and non-mutated NEP ($\Delta\Delta G$) calculated from k_{cat}/K_m values [24], was found to be 2.2 kcal/mol for both mutated enzymes (Table I).

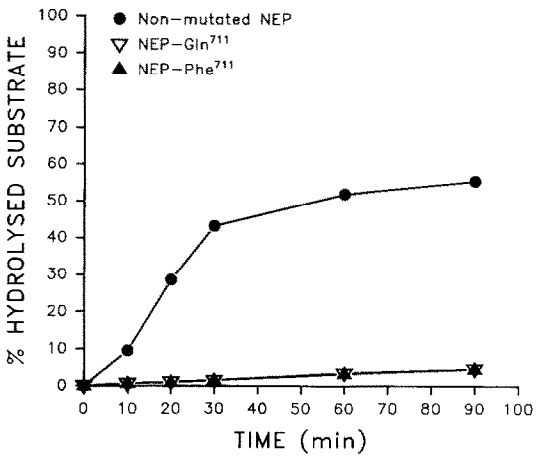

Fig. 1. Rate of hydrolysis of [tyrosyl-(3,5-^{3}H)](D-Ala_2)-Leu-Enkephalin. The activity of non-mutated (6 ng) and mutated (6 ng) NEPs was determined by measuring the rate of hydrolysis of [tyrosyl-(3,5-^{3}H)](D-Ala_2)-Leu-Enkephalin (50 nM). The amounts of enzymes in the reaction mixtures were determined by immunoblot using known quantities of purified kidney NEP as standards.

Table I

Kinetic parameters of non-mutated and mutated NEPs

	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\cdot\mu M^{-1}$)	$\Delta\Delta G$ (kcal·mol^{-1})
Non-mutated NEP	86 ± 6	802 ± 25	9.3	-
NEP-Gln^{711}	144 ± 15	34 ± 2	0.24	2.25
NEP-Phe^{711}	125 ± 16	31 ± 2	0.25	2.23

K_m and k_{cat} values are the mean ± S.D. of at least three separate determinations performed as described in section 2. Differences in free energy ($\Delta\Delta G$) for mutated enzymes were calculated using the relation: $\Delta\Delta G = -RT \cdot \ln((k_{cat}/K_m)_{mutant}/(k_{cat}/K_m)_{non\text{-}mutant})$ [24].

3.3. *Chemical modification by DEPC*

It has been shown that NEP is sensitive to modification by DEPC, a compound reacting with histidine, tyrosine, arginine and lysine residues. From the kinetics of inhibition, it was postulated that a histidine residue in the active site of NEP was the target of DEPC [13,14]. In order to verify that His^{711} corresponds to the residue sensitive to DEPC, we proceeded to the modification of the non-mutated and mutated NEPs with this reagent. Thus, if His^{711} is the unique target of DEPC in NEP active site, we would expect that the residual activity of mutants NEP-Gln^{711} and NEP-Phe^{711} will be insensitive to modification by this reagent. Surprisingly, mutants NEP-Gln^{711} and NEP-Phe^{711} were still sensitive to the action of DEPC (Fig. 2), albeit at a much reduced level. The IC_{50} values determined for non-mutated NEP, NEP-Gln^{711} and NEP-Phe^{711} were 50 ± 7, 156 ± 12 and 226 ± 24 μM, respectively.

4. DISCUSSION

Photoaffinity labelling has shown that His^{711} of NEP could be crosslinked to *N*-bromoacetyl-D-leucylglycine, an irreversible inhibitor of NEP which mimics the transition state of the catalytic reaction [14]. This suggested that His^{711} of NEP has a role similar to that of the His^{231} of TLN. Interestingly, His^{711} is located 65 residues from the zinc-coordinating residue, Glu^{646}, in the NEP sequence, and the same distance is observed between His^{231} and Glu^{166} in the TLN sequence. Furthermore, Kim et al. [25] recently mutated all the histidine residues in the NEP sequence, except those involved in zinc ligation, and observed that only the mutation of His^{711} abolished catalytic activity of the enzyme. However, since the enzymatic parameters for the mutated enzyme were not determined, it was impossible to evaluate the role of His^{711} in catalysis.

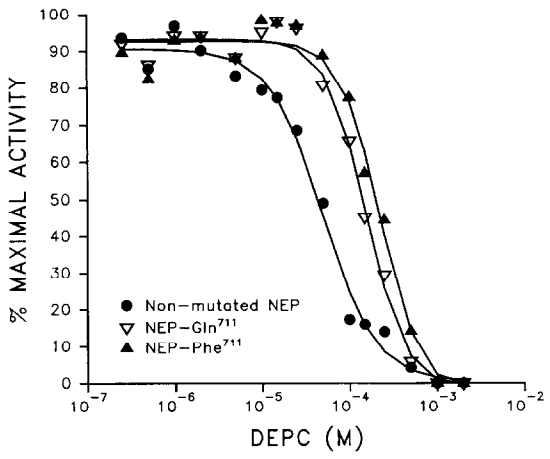

Fig. 2. Inactivation with diethylpyrocarbonate. Activity of non-mutated and mutated enzymes was determined by measuring the hydrolysis of [tyrosyl-(3,5-^{3}H)](D-Ala$_2$)-Leu-Enkephalin (50 nM) in the presence of different concentrations of DEPC.

In the present study, we have used site-directed mutagenesis to evaluate the role of His^{711} of NEP in catalysis. Either Gln or Phe were substituted for His^{711} of NEP and the kinetic parameters of the mutated enzymes were determined for both mutants. When measured under initial velocity conditions, both NEP-Gln^{711} and NEP-Phe^{711} showed 5% of the activity of non-mutated NEP, suggesting an important role for His^{711} in enzyme activity. However, this residual activity of mutant NEP-Gln^{711} and NEP-Phe^{711} was still sensitive to modification by DEPC. This result suggested either that His^{711} is not the residue sensitive to DEPC modification, or that a second residue in or near the active site of NEP can also be modified by this reagent.

It can be argued that replacing His^{711} by either Gln or Phe disrupted the integrity of the active site of NEP, thus resulting in the lower activity observed. We believe this possibility to be improbable for the following reasons. Firstly, the K_m values measured for the mutated enzymes are very similar to that determined for the non-mutated enzyme, thus suggesting that the substrate-binding subsites have been preserved. Secondly, the mutated and non-mutated enzymes showed similar IC_{50} for inhibition by the chelating agent, 1-10 phenanthroline (results not shown). This result indicates that the zinc atom binds with similar affinity to the mutated and non-mutated enzymes and suggests that the geometry of the ligands has not been grossly perturbed.

Unlike the K_m values, the k_{cat} values determined for the mutated enzymes were greatly reduced resulting in a calculated loss in binding energy ($\Delta\Delta G$) of 2.2 kcal/mol. This value is consistent with the loss of one hydrogen bond in the tetrahedral intermediate [26]. The decrease in activity observed for mutant NEPs is similar to that observed in *Bacillus subtilis* neutral protease, a protease homologous to TLN, bearing a His^{228}-to-Leu mutation [27]. It was proposed that this decrease in activity was due to the loss of a hydrogen bond in the tetrahedral intermediate. Thus, these observations strongly suggest that His^{711} of NEP participates in the catalytic mechanism by forming a hydrogen bond with the oxyanion formed during the transition state, as does

His^{231} in TLN [11], and further confirm the similarity between the catalytic mechanisms of NEP and TLN.

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