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Molecular Pharmacological Interaction of Phenylbutazone to Human Neutrophil Elastase

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Human neutrophil elastase (HNElastase, EC 3.4.21.37), a causative factor of inflammatory diseases, was purified by Ultrogel AcA54 gel filtration and CM-Sephadex ion exchange chromatography. HNElastase was inhibited by phenylbutazone in a concentration dependent manner up to 0.4 mM, but as the concentration increased, the inhibitory effect gradually diminished. Binding of phenylbutazone to the human neutrophil elastase caused strong Raman shifts at 200, 440, and 1194 cm^{-1}. The peak at 1194 cm^{-1} might be evidence of the presence of -N=N-Φ radical. The core area of the elastase, according to the visual molecular model of human neutrophil elastase, was structurally stable. A deeply situated active center was at the core area surrounded by hydrophobic amino acids. Directly neighboring the active site was one positively charged atom and two atoms carrying a negative charge, which enabled the enzyme and the drug to form a strong interaction. Phenylbutazone may form a binding, similar to a key & lock system to the atoms carrying opposite charges near the active site of the enzyme molecule. Furthermore, the hydrophobicity of the surrounding amino acid near the active site seemed to enhance the binding strength of phenylbutazone. Binding of phenylbutazone near the active site may cause masking of the active site, preventing the substrate from approaching the active site and inhibiting elastase activity.

Key Words: Neutrophil elastase, Molecular modeling, Phenylbutazone, Inhibition of elastase

INTRODUCTION

Human neutrophil elastase(HNE, EC 3.4.21.37), a causative factor in inflammatory diseases such as emphysema and rheumatoid arthritis (Janoff, 1972, Starkey, 1980, Oholson & Olsson, 1977, Fritz et al, 1986, Abramsom & Weissman, 1989), is regulated by plasma proteinase inhibitors, alphal-proteinase inhibitor, and alpha$_2$-macroglobulin (Salvesen et al, 1980, Cohen, 1975). Under certain pathological conditions, however, the released enzymes or the abnormal function of inhibitors may cause various inflammatory diseases (Janoff, 1972, Starkey, 1980, Oholson & Olsson, 1977, Fritz et al, 1986). Aspirin-like drugs (NSAIDs) are known as clinically effective agents for the treatment of inflammatory diseases such as rheumatoid arthritis(Lentin et al, 1987, Vane et al, 1987 and 1995, Insel, 1996). However, It is difficult to ascribe the antirheumatoid effects of aspirin-like drugs solely to the inhibition of prostaglandin synthesis (Insel, 1996). We already reported that some NSAIDs such as oxyphenbutazone and phenylbutazone inhibited human neutrophil elastase, but some other drugs did not inhibit elastase under specific condition (Kang et al, 1996). There may be a specific relationship between the structure of an individual drug and its inhibitory effect on neutrophil elastase. To investigate the molecular mechanism of action, one has to investigate the tertiary and quaternary structure of the enzyme in a reaction medium similar to the in vivo condition, such as in an aqueous solution. Raman spectroscopy is especially useful in that the interference from water, the main biological solvent, is only minimal (Keller et al, 1994). A question that always arises in crystallography is whether the conformational structure of protein in the neutral

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state is identical to the structure deduced by X-ray diffraction method. This paper, will describe the pharmacological characteristics of the inhibition of human neutrophil elastase by phenylbutazone, the method for visualization of the molecular model from PDB data, and the molecular pharmacological mechanism of the inhibition by analyzing Raman spectroscopic data and the molecular model of human neutrophil elastase.

METHODS

N-Succinyl-Ala-Ala-Pro-Phe-p-Nitroanilide (SAPN A), N-Succinyl-Ala-Ala-Ala-p-Nitroanilide (SANA), and phenylbutazone were Sigma products. Tris, glycine, and sodium dodecyl sulfate were acquired from Bio-Rad. Spectro pore 2 dialysis membrane was from Spectrum Medical Industries INC., L.A., U.S.A., Ultrafiltration membrane was the product of Amicon Co.. All other chemicals were of the highest quality obtainable.

Purification of human neutrophil elastases

Human neutrophils were separated from whole blood (acquired from the blood bank of Kosin Medical Center) by Ficoll-hypaque step gradient centrifugation at 200×g for 40 minutes and washed three times with 50 mM Tris-Cl buffer, pH 7.3 (Jeung et al, 1987). The method used to purify human neutrophil elastase was a modification of the methods used by Baugh & Travis and Kang (Baugh et al, 1976, Kang, 1985). In brief, separated neutrophils were suspended in 0.5 M Tris-Cl buffer, pH 7.3, containing 1.5 M NaCl and 0.05 M $CaCl_2$, they were then homogenized with Polytron at 4°C and centrifuged at 30,000×g for 60 minutes. The supernatant was then collected and kept at 4°C until used. The extract was chromatographed through an AcA54 gel filtration column equilibrated with 50 mM of Tris-Cl buffer containing 150 mM of NaCl. Elastase-rich fractions were pooled, concentrated, and dialyzed against 50 mM of NaAc, pH 4.5, containing 150 mM NaCl. Ultrogel purified elastase was chromatographed again with an CM- Sephadex ion exchange column equilibrated with 50 mM NaAc buffer, pH 4.5, containing 150 mM of NaCl. Bound protein was eluted by a linear salt gradient (0.15~0.7 M) in the same buffer. Elastase- rich fractions were pooled, concentrated, measured for the protein content and elastase activity, and kept at −20°C until use. Elastase purified by ion exchange chromatography showed four bands of isozyme molecules in the SDS-PAGE. These isozymes were chromatographed by HPLC with TSK 250 column and the least molecular weighted E4 elastase was used in most of this study(Baugh & Travis, 1976, Kang, 1985).

Elastase assay

Elastase assay was carried out in 200 ul of reaction medium (in a 96 well plate) containing 60~150 mM NaCl, 50 mM $CaCl_2$, pH 7.3 with or without inhibitor, depending upon the design of the experiment (Kang, 1985). After the substrate, SANA, was added to the reaction medium, it was incubated at 37°C for an apropropriate period (usually 10~40 minutes depending on the nature of the experiment). Elastase activity was measured spectrophotometrically at 410 nm by monitoring the concentration of liberated p-nitroaniline, using a Titertek Multiskan Spectrophotometer (MCC/340, Flow laboratories, Switzerland). Percent of inhibition was calculated by $100*[1-(V_{\text{inhibitor present}}/V_{\text{inhibitor absent or control}})]$.

Preparation of phenylbutazone bound elastase

362 μg of purified elastase was placed in 200 μl of 50 mM Tris buffer, pH 7.3, containing 5 mM $CaCl_2$, 150 mM of NaCl, and 20% of dimethylsulfoxide (DMSO). After 24 μl of 0.5 mM phenylbutazone was added to the reaction medium,it was incubated at room temperature for 30 minutes. The mixture of drug and elastase sample was placed in Spectro pore 2 membrane, then dialyzed three times against one liter of 50 mM Tris-Cl buffer, pH 7.3. After the final dialysis, sample was frozen and kept at −20°C until used.

Raman experiments

Ramen spectra were recorded in melting point capillaries using Laser Raman Spectrophotometer System (Sepex 1403, 0.85 m double spectrometer equipped with RCA-C 31034 phototube and DM 3000 software). All spectra were recorded using the 514.5-nm laser wavelengths for excitation. Data were collected at 1 cm^{-1} intervals with 0.3 $^{-S}$ integration time and 4 cm^{-1} spectral slit width.

Table 1. Crystallographic informations

HYDROLASE(SERINE PROTEINASE), HUMAN NEUTROPHIL ELASTASE (E.C.3.4.21.37) (ALSO REFERRED TO AS HUMAN LEUCOCYTE ELASTASE, COMPLEX WITH METHOXYSUCCINYL-ALA-ALA-PRO-ALA CHLOROMETHYL KETONE, SOURCE: HUMAN (HOMO SAPIENS) NEUTROPHILS ISOLATED FROM PURULENT SPUTUM. AUTHOR: M.A.NAVIA, B.M.McKEEVER, J.P.SPRINGER, T.-Y.LIN, H.R.WILLIAMS, E.M. FLUDER, C.P.DORN, K.HOOGSTEEN. REVDAT: 15-OCT-89.
JOURNAL: AUTHORS: M.A.NAVIA, B.M.McKEEVER, J.P.SPRINGER, T.-Y.LIN, H.R.WILLIAMS, E.M.FLUDER, C.P.DORN, K.HOOGSTEEN. TITL: STRUCTURE OF HUMAN NEUTROPHIL ELASTASE IN COMPLEX WITH A PEPTIDE CHLOROMETHYL KETONE INHIBITOR AT 1.84-ANGSTROMS RESOLUTION. PROC.NAT.ACAD. SCI.U.S.A. 86: 7~11, (1989) <SEQUENCE>aILE VAL GLY GLY ARG ARG ALA ARG PRO HIS ALA TRP PRO PHE MET VAL SER LEU GLN LEU ARG GLY GLY HIS PHE CYS GLY ALA THR LEU ILE ALA PRO ASN PHE VAL MET SER ALA ALA HIS CYS VAL ALA ASN VAL ASN VAL ARG ALA VAL ARG VAL VAL LEU GLY ALA HIS ASN LEU SER ARG ARG GLU PRO THR ARG GLN VAL PHE ALA VAL GLN ARG ILE PHE GLU ASP GLY TYR ASP PRO VAL ASN LEU LEU ASN ASP ILE VAL ILE LEU GLN LEU ASN GLY SER ALA THR ILE ASN ALA ASN VAL GLN VAL ALA GLN LEU PRO ALA GLN GLY ARG ARG LEU GLY ASN GLY VAL GLN CYS LEU ALA MET GLY TRP GLY LEU LEU GLY ARG ASN ARG GLY ILE ALA SER VAL LEU GLN GLU LEU ASN VAL THR VAL VAL THR SER LEU CYS ARG ARG SER ASN VAL CYS THR LEU VAL ARG GLY ARG GLN ALA GLY VAL CYS PHE GLY ASP SER GLY SER PRO LEU VAL CYS ASN GLY LEU ILE HIS GLY ILE ALA SER PHE VAL ARG GLY GLY CYS ALA 218 SER GLY LEU TYR PRO ASP ALA PHE ALA PRO VAL ALA GLN 218 PHE VAL ASN TRP ILE ASP SER ILE ILE GLN

Crystallographic and sequence information

Two available crystallographic data files loaded from different laboratories were available from the Protein Data Bank (PDB) in Brookhaven National Laboratory, New York (also available from Tokyo and Cambridge). The PDB contains atomic co-ordinates, structure factors, and phases from different studies of macromolecules. It is essentially a depository of data, made into a computer-readable form. The PDB contains atomic co-ordinate entries for 47 macromolecules, and 13 sets of structure factors and phases. The atomic co-ordinate entries, which include descriptive texts and partial bond connectivities, conform to a uniform format. Atomic coordinate entries consist of records each of 80 characters. Columns 1 to 6 contain a record type identifier, and columns 7 to 70 contain data (Bernstein et al, 1977). The crystallographic information (Protein Data Bank) (Bode et al, 1986; Navia et al, 1989) of human neutrophil elastase was transferred from Brookhaven National Laboratory (e-mail: pdb@bnlchm.bitnet or pdb@chm.chm.bnl.gov) via FTP. This description, Navia's PDB datum instead of Bode's since Navia's PDB crystallographic datum was obtained from the elastase crystal crystallized after the activity was inhibited by an inhibitor with a smaller molecular weight. The following description is a summary of the crystallographic informations. Sina's datum was used as a reference for the sequence (Sina et al, 1987).

Molecular modeling

The molecular model was constructed by a molecular visualization program (Roger Sayler Biomolecular Structure, Glaxo Research and Development, Greenford, Middlesex, UK) using Navia's PDB datum of crystallographic information on human neutrophil elastase. The specific details in drawing each model will be described in the legend of the individual figure.

RESULTS

The elastase which was purified by ion exchange chromatography showed four bands of isozyme molecules in the SDS-PAGE. These four bands of isozyme are known as same elastase with different molecular weights due to the modification by different carbohydrates. The least molecular weighted enzyme, E4 elastase, that was obtained by HPLC chromatography with TSK 250 column was used in most of this studies(Baugh & Travis, 1976, Kang, 1985). N-terminal amino acid sequence of this ela-

stase was identical to those of previous studies (Sina et al, 1987).

Inhibition of HNElastase by phenylbutazone and Raman spectral observation

Phenylbutazone inhibited human neutrophil elastase concentration dependently up to 0.4 mM, but with the increase in concentration the inhibitory effect gradually diminished (Fig. 1). The binding of phenylbutazone to the human neutrophil elastase molecules was so strong that it did not dissociate during dialysis of the sample mixture to remove the unbound phenylbutazone from the elastase-phenylbutazone complex. Raman spectra of human neutrophil elastase with and without binding of phenylbutazone are in Fig. 2, Fig. 2A, B, and C are the Raman spectra for control elastase, phenylbutazone bound elastase, and medium only, respectively. Raman spectra of reaction medium containing 20% of dimethylsulfoxide showed several intense bands (676, 716 cm^{-1}) and weaker bands such as 302, 342, 390, 950, 1018, 1318, 1418 cm^{-1}(Fig. 2C). Comparison of the Raman spectrum of elastase without drug binding (Fig. 2A) with phenylbutazone-elastase complex (Fig. 2B) showed both spectral similarities and some differences between 200 and 1800 cm^{-1}. The new bands at 200, 440, and 1194 cm^{-1} appeared in the phenylbutazone-elastase complex(Fig. 2B), but not in the control.

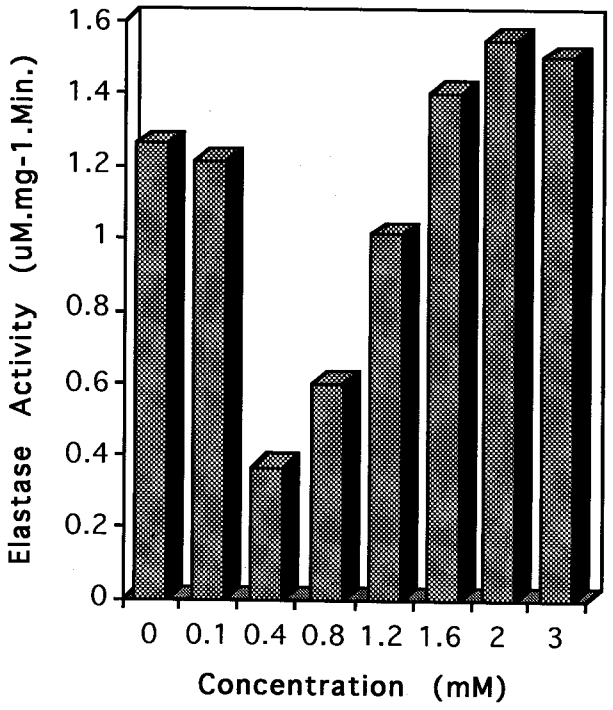


Fig. 1. Inhibition of human neutrophil elastase by different concentrations of phenylbutazone: Experimental details are in the Methods.

Molecular modeling of human neutrophil elastase by a molecular visualization system

Molecular structure could be visualized by several different models such as wireframe, stick, backbone, spacefill, ball & stick, ribbon, and strand. Each display of molecular structure could also be expressed in different color and visualized by using unique molecular structural characteristics such as atoms, rigidity of the structure (highly mobile atoms are colored red, while stable atoms are colored blue), or

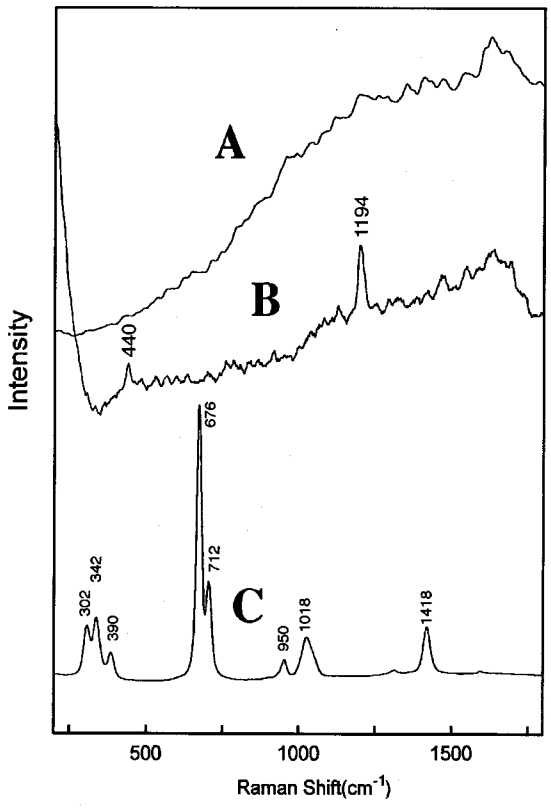


Fig. 2. Raman spectra: Experimental details of preparation of the samples were described in the Methods. Sample A and B were dialyzed against 50 mM Tris-Cl, pH 7.3. A: control elastase, B: human neutrophil elastase-phenylbutazone complex, C: reaction medium containing dimethylsulfoxide (while elastase reacted with phenylbutazone, 20% of dimethylsulfoxide was added in the reaction medium for prevention of precipitation).

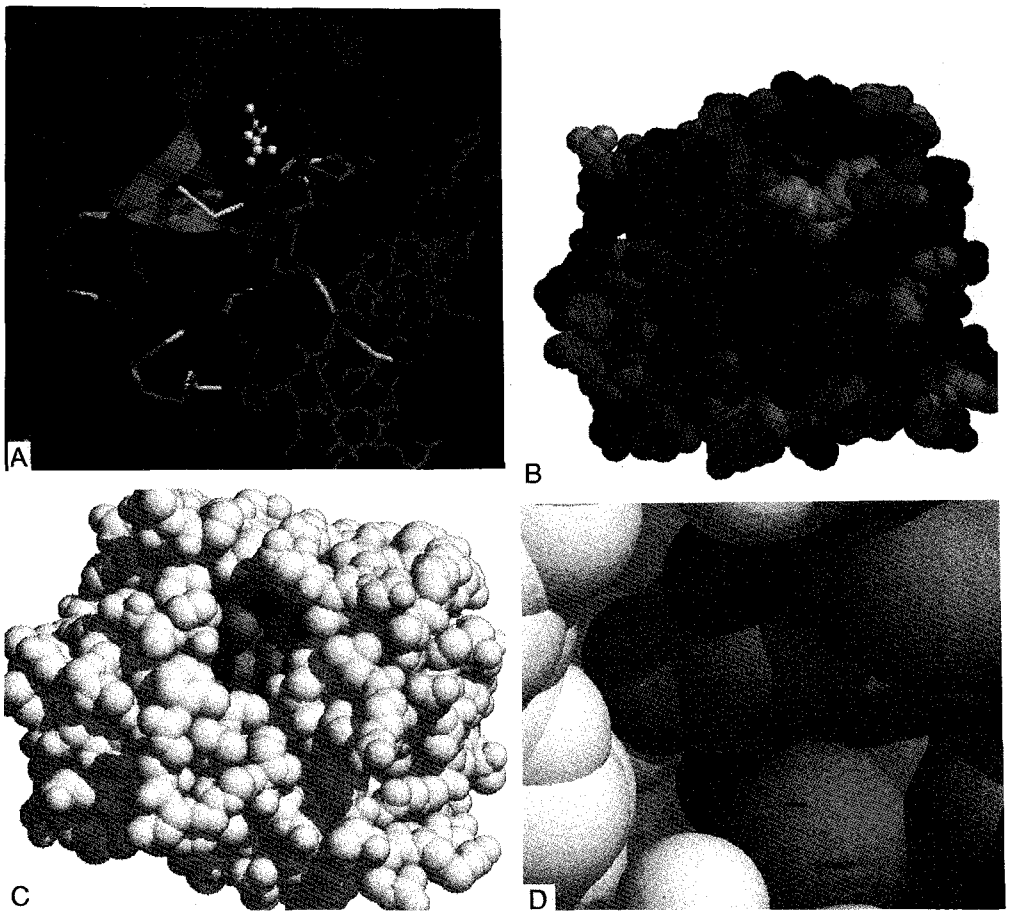


Fig. 3. Molecular structures of human neutrophil elastase visualized by various styles: Technical details for constructing molecular models of human neutrophil elastase were described in the Methods. A: the model was visualized by stick, backbone, spacefill, ball & stick, ribbon, and strand. The amono acids expressed with ball & stick are those amino acids involved in <charge-relay system>a and colored by the secondary structure; Ser^{173} (active site, red color=alpha helix), His^{41} (blue color), and Asp^{88} (white color). C-terminal colored with red and gradually changed to yellow, white, gray, green, blue, and dark blue color toward the N-terminal. Asp^{88} (83~93) area was colored with green, Red-yellow colored area is Ser^{173} (168~178), green to blue colored area is His^{41} (36~46). Red color portions are alpha helices. B: Molecular model visualized by spacefill. Colors of the peptide molecule were expressed in order of the structural stability. Dark blue colored area is the most stable & rigid portion, and the color was gradually changed toward red (least stable, the most variable area). The amino acids expressed by ball & stick are the amino acids of active site (Ser^{173}) and charge-relay system (His^{41}, Asp^{88}). C: Monochromal visualization of human neutrophil elastase: Whole molecule was colored with white except the active site (CPK color) and the neighboring disulfide bond (yellow color). The active site (CPK color) located in the deep pocket. D: Magnefied structure of active site. Ser^{173}, His^{41}, and Cys^{26} & Cys^{42} forming -S-S- bond. Red, violet, gray, and yellow colored atoms are oxygen, nitrogen, carbon, and sulfur atoms, respectively. Two shlfur atom are charged negatively and one nitrogen atom of histidine was charged positively.

secondary structure (alpha helices are colored magenta, betha sheets are colored yellow, and turns are colored pale blue). A analysis of the visual molecular model of human neutrophil elastase molecule(Fig. 3) indicates the following: 1) the core area of elastase molecule was structurally stable; 2) the active site was at the core of the molecule; 3) the active center was in a deep pocket; 4) the rim of the pocket was surrounded by hydrophobic amino acids such as Phe^{170}, Gly^{171}, Phe^{25}, Leu^{20}, Va^{146}, Tyr^{80}, Pro^{82}, and Leu^{85}; 5) at the direct neighboring area of active site, there was one positive charged atom (nitrogen atom of His^{41}) and two negatively charged atoms (sulfur atoms of Cys^{26} and Cys^{42}).

DISCUSSION

The inhibitory characteristic of phenylbutazone on human neutrophil elastase (V-shape curve of elastase activity v.s concentration, Fig. 1), was increased in a concentration-dependent manner and peaked at the concentration of 0.4 mM. Afterwards the effects diminished even when the dose of phenylbutazone was raised. This characteristic may come from the intermolecular interaction at high concentrations of phenylbutazone, and that may cause loss or decrease in binding property. The weak electrostaticly charged portions (-N- or -C=O parts) of the drug molecule will form intermolecular complexes between drug molecules carrying opposite ionic charges (positive or negative) at neutral pH. As a result the total number of drug molecules that can bind to the active sites of the elastase will be reduced. As the drug concentration increases, the active site of the enzyme will not be occupied by the phenylbutazone molecule, and more likely be exposed to the substrate, leading to a gradual recovery of enzyme activity.

Raman frequencies of human neutrophil elastase also undergo drastic changes upon binding of phenyl-

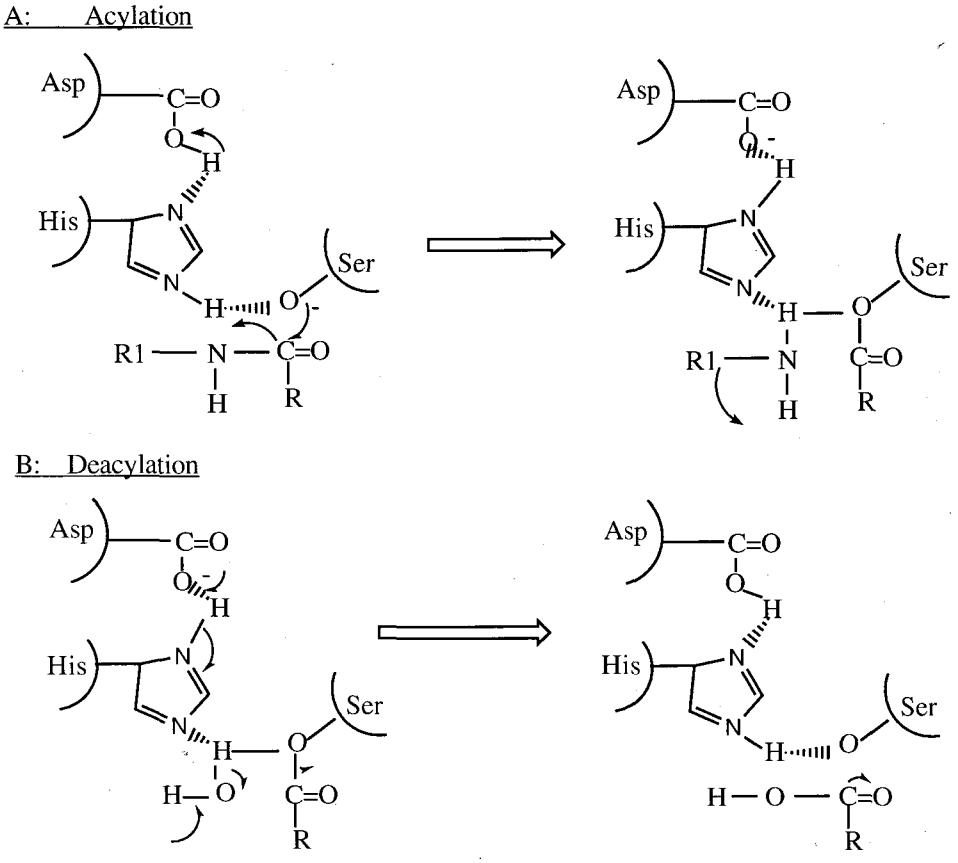
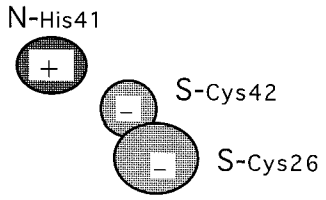
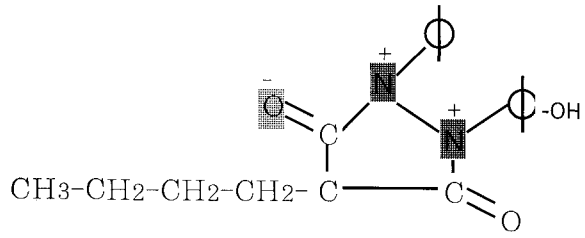


Fig. 4. "Charge-relay System", Blow's hypothesis of machanism of action of serine protease.



Charged atoms at near the active site



Phenylbutazone

Fig. 5. Charged atoms at the active site of elastase and phenylbutazone.

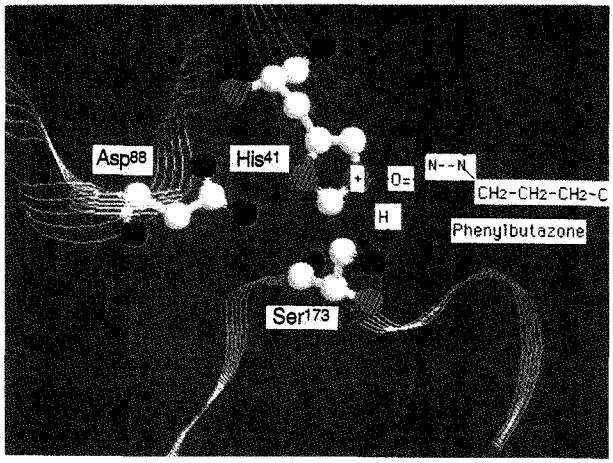


Fig. 6. Model of the binding of phenylbutazone to the human neutrophil elaastase: Electrostatically negative charged oxygen of the phenylbutazone molecule bound to the nitrogen atom (histidine ring, positive charged) at the active site of the elastase.

butazone. The differences between Fig. 2A and 2B reflects the effects of the enzyme complex formation with phenylbutazone. In the elastase-phenylbutazone complex, the Raman shift at 1194 cm^{-1} could be the evidence of the two -N-Φ radicals of the phenylbutazone molecule (Scheule et al, 1977 and 1979). The active site of human neutrophil elastase is Ser^{173} therefore, the positive charge will be relayed from hydrogen atom of Asp^{88} through His^{41} as in the "Charge-relay" hypothesis regarding the mechanism of action of serine protease (Blow et al, 1969)(Fig. 4). The inhibitory effects of human neutrophil elastase must be related to the property of the drug binding to the active site of the elastase resulting in complete or partial masking of the active site. In phenylbutazone inhibition, both -C=O portions and nitrogen atoms of the pyrazole ring in the phenylbutazone molecule might be important factors for the binding of phenylbutazone molecule to the active site of the human neutrophil elastase molecule (Fig. 5). The negatively charged oxygen atoms in the $--C=O^-$ portion of pyrazole ring may interact with the nitrogen atoms carrying a weak positive charge of the histidine ring of the active site. As one can see in the molecular models (Fig. 3, A.B.C.D) constructed by the chrystallographic PDB data, active sites of human neutrophil elastase showed important molecular structural factors; 1) the vicinity of the active site of human neutrophil elastase is surrounded by hydrophobic amino acids such as Phe, Gly, Leu, Val, Pro, and Leu, 2) one positively charged atom (nitrogen atom of His) and two negatively charged atoms (sulfur atoms of Cys^{26} and Cys^{42}) near the active site formed rigid comformation (Fig. 5). Therefore, three charged atoms might be bound electrostatically to the corresponding opposite charge of the phenylbutazone molecule, since it has the charges that are opposite to the enzyme molecule, i.e., one of the two negatively charged C=O and two positively charged $-N^+$ in the pyrazole ring of the phenylbutazone molecule (Fig. 6) lead to a strong interaction between the drug and enzyme. Phenylbutazone may form key & lock like binding to the atoms carrying opposite charges near the active site of enzyme molecule. Furthermore, the surrounding hydrophobic amino acid molecules of enzyme will also interact very effectively with the hydrophobic parts of the phenylbutazone such as phenyl rings and aliphatic carbon chain. Binding of phenylbutazone to an area near the active site of human neutrophil elastase may cause masking of the active site since drugs prevent the substrate from approaching the active site, possibly inhibiting the elastase activity. The technique that this study applied using computer aided constructing of the molecular model enables one to visualize the details of the molecular structure. This is an important method for the investigation of the mechanism of drug actions. Furthermore, this technology can be applied to the development and

design of new drugs. There may be two different classes of NSAIDs, elastase sensitive and insensititive drugs. If that is the case, it will be an important factor for the treatment of tissue destructive inflammatory disease and for the development of new drugs.

In conclusion, the inhibitory effects of phenylbutazone on elastase, besides the inhibition of cyclooxygenase, could be another important mechanism of action of in the treatment of inflammatory diseases. The mechanism of the elastase inhibition may be related to the specific molecular structure of elastase and phenylbutazone. Those structural features must be playing an important role in the drug binding to the active site of human neutrophil elastase. Furthermore, visualization of the molecular model by using PDB data is an important technique for the investigation of the mechanism of drug action and the development of new drugs.

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