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Structural and binding studies of C-terminal half (C-lobe) of lactoferrin protein with COX-2-specific non-steroidal anti-inflammatory drugs (NSAIDs)

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ABSTRACT

Three COX-2-specific non-steroidal anti-inflammatory drugs (NSAIDs), etoricoxib, parecoxib, and nimesulide are widely prescribed against inflammatory conditions. However, their long term administration leads to severe conditions of cardiovascular complications and gastric ulceration. In order to minimize these side effects, C-terminal half (C-lobe) of colostrum protein lactoferrin has been indicated to be useful if co-administered with NSAIDs. Lactoferrin is an 80 kDa glycoprotein with two similar halves designated as N- and C-lobes. Since NSAID-binding site is located in the C-terminal half of lactoferrin, C-lobe was prepared from lactoferrin by limited proteolysis using proteinase K. The incubation of lactoferrin with serine proteases for extended periods showed that N-lobe was completely digested but C-lobe was resistant for more than 72 h indicating its long half life in the animal gut. The solution studies have shown that COX-2-specific NSAIDs bind to C-lobe with binding constants ranging from 10^{-4} to 10^{-5} M showing significant affinities for sequestering these compounds. In order to understand the mode of binding and sequestering properties, the complexes of C-lobe with all these three compounds, etoricoxib, parecoxib, and nimesulide were prepared and the structures of their complexes with C-lobe were determined at 2.2, 2.9, and 2.7 Å resolutions, respectively. The analysis of the structures of complexes of C-lobe with NSAIDs clearly show that all the three compounds bind firmly at the same ligand-binding site in the C-lobe revealing the details of the interactions between C-lobe and NSAIDs. The mode of binding of COX-2-specific NSAIDs to C-lobe is similar to that of the binding of COX-2 non-specific NSAIDs to C-lobe.

Introduction

The severity of diseases and dosages of drugs administered to patients are usually determined empirically [1]. In order to ascertain that the sufficient quantity of drug is received by the patient, it often occurs that more than the required quantity of drug is administered over a long period. As a result of it, all the drug molecules are not utilized by the intended targets. The unbound molecules tend to bind non-specifically at other low affinity sites causing damage to various other tissues. This is particularly true about the non-steroidal anti-inflammatory drugs (NSAIDs).[1] It may be mentioned here that NSAIDs include both non-specific and cyclooxygenase-2 (COX-2) specific agents [2]. The therapeutic usefulness of NSAIDs against various inflammatory disorders is well known. As a result, these are among the most prescribed drugs in the world. However, the long term administration of NSAIDs causes gastric ulceration [3] while the more potent COX-2-specific NSAIDs, etoricoxib, parecoxib, and nimesulide in addition to producing moderate gastric ulcers [4] also cause cardiovascular complications due to thrombosis [5–7]. It has already been shown that gastric ulcers can be reduced by administering lactoferrin [8,9] and C-lobe [10] with NSAIDs. It has also been reported that application of lactoferrin reduces platelet aggregation [11]. In order to continue to exploit the useful therapeutic properties of NSAIDs, it is important to examine their side effects which may partly be addressed by regulating the supply of drugs to the system. It may be noted that the basis of dosage calculation is still based on empirical considerations and the dosage is generally arbitrarily increased or decreased based on the severity of the disease. The more scientific way to achieve the supply of an accurate dosage may be by non-covalently associating the excess drug molecules with such compounds that have no adverse effects of their own so that the release of drugs occurs gradually for the binding to their macromolecular targets. This is possible if the drug sequestering molecules bind to the compounds with lower affinities than that of their actual targets. Recently, it has been shown that C-lobe of lactoferrin possesses a well defined binding site for ligands having an aromatic moiety together with a hydrophilic carboxyl or hydroxyl groups [10]. It has already been demonstrated that C-lobe binds

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[1] *Abbreviations used:* COX-2, cyclooxygenase-2; NSAIDs, non-steroidal anti-inflammatory drugs; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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to non-specific NSAIDs [10] and sugars [12] and has been shown to reduce their concentrations in the gut [10]. In order to examine the role of C-lobe in minimizing the side effects of COX-2-specific NSAIDs, we have carried out binding studies of etoricoxib (5-chloro-6′-methyl-3-[4-(methylsulfonyl)phenyl]-2,3′-bipyridine), parecoxib (*N*-{[4-(5-methyl-3-phenylisoxazol-4yl)phenyl]sulfonyl}propanamide) and nimesulide (*N*-(4-nitro-2-phenoxyphenyl)methanesulfonamide) with C-lobe and crystallized the complexes of C-lobe with all the three compounds. It is pertinent to note here that COX-2-specific NSAIDs cause only moderate gastropathy [4] but produce a more serious cardiovascular complication [6,7]. In this regard also, it is reasonable to allow the release of drugs gradually so that the intensity of side effects is not increased suddenly. We report here the binding studies of etoricoxib, parecoxib and nimesulide with C-lobe as well as the structures of the complexes of C-lobe with all the three drugs. The results of these studies show that the hydrophobic moieties of these compounds are attached to the hydrophobic pocket while hydrophilic portions form several interactions with the side chains of Thr663 and Glu659 at the two ends of the binding pocket in the C-lobe. The binding studies revealed the modest binding affinities of NSAIDs towards C-lobe which are of the order of 10^{-4}–10^{-5} M. Since these affinities are relatively lower than the affinities of these drugs to COX-2 enzyme ($\approx 10^{-8}$ M) [13], these compounds are always available for the binding to target enzymes.

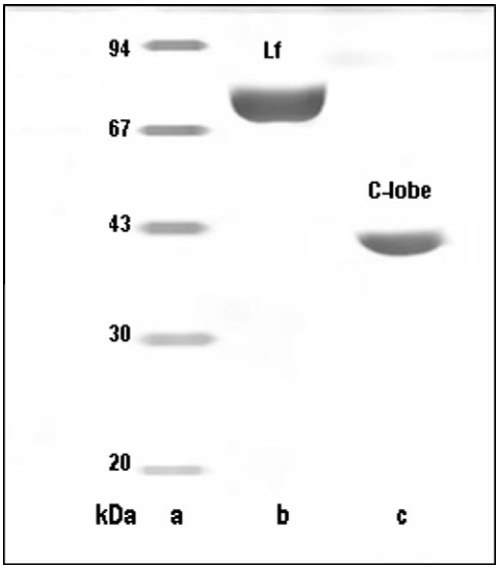

Fig. 1. SDS–PAGE indicating the preparation of 40 kDa N- and C-lobes. Lane (a) molecular weight markers (phosphorylase (b) 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa) (lane b) intact lactoferrin and (lane c) C-lobe.

Materials and methods

Preparation and purification of C-lobe

Lactoferrin was isolated and purified from bovine colostrum to homogenity using the procedure described earlier [14]. The freshly purified and lyophilized samples of lactoferrin were dissolved in 50 mM Tris–HCl, pH 8.0 which were incubated with proteinase K at a lactoferrin: proteinase K molar ratio of 25:1 for 30 min at room temperature. The hydrolyzed product of protein was passed through a cation exchanger CM-Sephadex C-50 column (150 mm × 15 mm) using a salt gradient of 0.04–0.5 M NaCl in 0.05 M Tris–HCl, pH 8.0. The fractions corresponding to the protein of molecular weight of 40 kDa were pooled and then passed through a gel filtration column (100 mm × 10 mm) of Sephadex G-50 in 0.05 M Tris–HCl, pH 8.0. The eluted protein was dialyzed against deionized water, lyophilized and stored at 261 K. It was confirmed as the C-lobe using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 1) and by N-terminal sequence determination of the first 20 amino residues from the N-terminus that the eluted protein was a C-lobe of lactoferrin [15].

Binding studies

The binding studies of NSAIDs with C-lobe were carried out using fluorescence spectroscopic technique with spectrofluorometer, FP-6200 (Shimadzu, Koyoto, Japan). About 2.5 mL of C-lobe at a concentration of 1×10^{-5} mol/L was loaded in 1 cm quartz cuvette. The concentrations of NSAIDs were varied by adding increasing volumes of 20, 40, 60, and 80 μL of NSAIDs, (a) etoricoxib (b) parecoxib, and (c) nimesulide from their stock solutions prepared at concentrations of 5×10^{-4} mol/L. The fluorescence experiments were conducted under the conditions of both entrance and exit slit widths at 5 nm and scanning speed of 240 nm/min. The fluorescence emission spectra of the protein were recorded in the range of 300–550 nm at an excitation wavelength of 280 nm at 298 K. The spectral changes of the protein were recorded with different NSAIDs at various concentrations (Fig. 2).

Co-crystallization of C-lobe complexes with NSAIDs

The freshly purified and lyophilized samples of C-lobe were dissolved in 50 mM MES at pH 6.5 to a concentration of 80 mg/mL. All the three NSAID molecules (etoricoxib, parecoxib, and nimesulide) were dissolved in 20% ethanol and 50 mM MES at pH 6.5 at a concentration of 1 mg/mL. The solutions of NSAIDs were mixed with protein solution in 1:1 (v/v) ratio, giving protein: NSAIDs molar ratios of approximately 1:1 and were allowed to stay for 24 h at 298 K. A reservoir solution containing 0.1 M MES pH 6.5, 25% (v/v) polyethylene glycol monomethyl ether 550 and 0.01 M zinc sulfate heptahydrate was prepared and mixed with the incubated protein solutions in 1:1 ratio for hanging drop vapor diffusion method at 298 K. The irregular-shaped and dark brown colored crystals of the complexes appeared after a week.

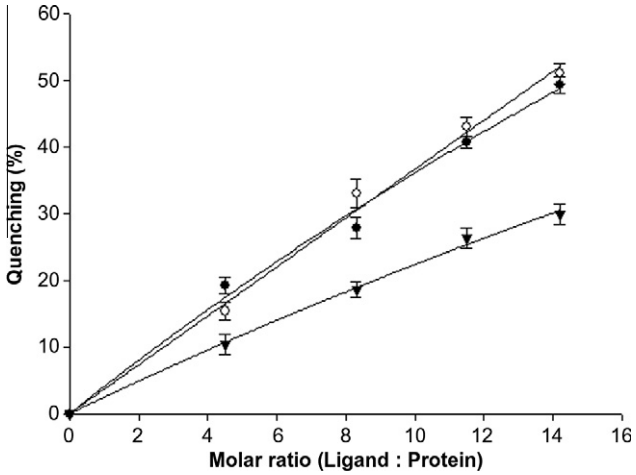

Fig. 2. Binding curves, ○ (nimesulide), ● (etoricoxib) and ▼ (parecoxib) showing the change in fluorescence intensities (ΔF/Fo) at 280 nm when the NSAIDs to C-lobe molar ratio were increased. The errors on the experimental points have been indicated.