The Structural Basis for the Prevention of Nonsteroidal Antiinflammatory Drug-Induced Gastrointestinal Tract Damage by the C-Lobe of Bovine Colostrum Lactoferrin

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ABSTRACT Nonsteroidal antiinflammatory drugs (NSAIDs), due to their good efficacy in the treatment of pain, inflammation, and fever, are among the most prescribed class of medicines in the world. The main drawback of NSAIDs is that they induce gastric complications such as peptic ulceration and injury to the intestine. Four NSAIDs, indomethacin, diclofenac, aspirin, and ibuprofen were selected to induce gastropathy in mouse models. It was found that the addition of C-terminal half of bovine lactoferrin (C-lobe) reversed the NSAID-induced injuries to the extent of 47–70% whereas the coadministration of C-lobe prevented it significantly. The C-lobe was prepared proteolytically using serine proteases. The binding studies of C-lobe with NSAIDs showed that these compounds bind to C-lobe with affinities ranging from 2.6 to 4.8 $\times$ 10^{-4} M. The complexes of C-lobe were prepared with the above four NSAIDs. All four complexes were crystallized and their detailed three-dimensional structures were determined using x-ray crystallographic method. The structures showed that all the four NSAID molecules bound to C-lobe at the newly identified ligand binding site in C-lobe that is formed involving two α-helices, α10 and α11. The ligand binding site is separated from the well known iron binding site by the longest and the most stable β-strand, βj, in the structure. Similar results were also obtained with the full length lactoferrin molecule. This novel, to our knowledge, binding site in C-lobe of lactoferrin shows a good complementarity for the acidic and lipophilic compounds such as NSAIDs. We believe this indicates that C-lobe of lactoferrin can be exploited for the prevention of NSAID-induced gastropathy.

INTRODUCTION

Nonsteroidal antiinflammatory drugs (NSAIDs) are prescribed extensively all over the world and their current consumption is among the highest for any drug. The analgesic and antipyretic properties of NSAIDs are very useful but their long term administration causes a severe damage in gastroduodenal mucosa resulting in gastric and intestinal injuries that include peptic ulcer and formation of strictures in the small and large intestines (1,2). The adverse effects of NSAIDs to the mucosal cell wall represent a range of pathologies that may be asymptotic but some are certainly life threatening (2). It has been indicated that the unbound NSAID molecules present in the mucosal region of the gut tend to damage the mucosal lining causing severe ulcers as a result of prolonged administration (3,4). The existing methods used for preventing the NSAID-induced gastropathy include co-medication of acid suppressants like proton pump inhibitors and prostaglandin analogs (5,6). However, these methods also show side effects and have only limited efficacy. Hence, to make the applications of NSAIDs safer, there is an urgent need to develop novel therapeutic agents that could prevent gastropathy by eliminating the unbound molecules of NSAIDs. Some reports have shown that bovine colostrum produces observable effects against NSAID-induced gastric ulcers (7,8). There are preliminary reports that the recombinant human lactoferrin also has the ability to prevent NSAID-induced intestinal injuries in rats and mice (9,10) indicating that lactoferrin is a main component of colostrum that is responsible for the useful effects against NSAID-induced gastropathy. It has been shown recently that NSAID-like molecules bind to bovine lactoferrin (11,12). Lactoferrin is an 80-kDa bilobal protein with two similarly sized N- and C-terminal halves (N- and C-lobes). It has been shown that lactoferrin can be cleaved by limited proteolysis into N- and C-lobes (13). It was further observed that longer incubations of lactoferrin with proteases results in the complete digestion of N-lobe whereas C-lobe survives such an enzymatic degradation (13). We show that the C-lobe of bovine lactoferrin protects the gastrointestinal tract of mouse from NSAID-induced gastropathy. The binding studies of four NSAIDs, 1-(4-chlorobenzoyl)-5-methoxy-2-methyl-1-H-indole-3-acetic acid (indomethacin), [2-(2,6-dichlorophenylamino) phenyl]acetic acid (diclofenac), 2-(acetyloxy) benzoic acid (aspirin), and α-methyl-4-(2-methylpropyl) benzene acetic acid (ibuprofen) carried out with C-lobe have shown that these NSAIDs bind to C-lobe at affinities of ~10^{-4} M. To identify the site of binding in the C-lobe and for characterizing the nature of interactions between C-lobe and NSAIDs, the crystal structures of four complexes of C-lobe with indomethacin, diclofenac, aspirin and ibuprofen have been determined. The structure analyses of these complexes have shown the presence of what we believe to be a novel ligand binding site in C-lobe that is located at the back of the well known iron binding cleft in

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the C-lobe (14). All four NSAID molecules occupy an identical region in the new ligand binding site although their orientations differ slightly. Several residues of the C-lobe are involved in the interactions with NSAIDs. Such a promiscuous mode of binding by C-lobe signifies the importance of its widely useful sequestering property.

EXPERIMENTAL PROCEDURES

Preparation of C-lobe

Lactoferrin was isolated from bovine colostrum and purified to homogeneity using the procedure described earlier (15). The freshly purified and lyophilized samples of lactoferrin were dissolved in 50 mM Tris-HCl, pH 8.0 that are 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 (Sigma-Aldrich, St. Louis, MO) 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 fraction corresponding to the 40 kDa molecular mass was pooled and 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 261K. It was confirmed using sodium dodecyl sulfate polyacrylamide gel electrophoresis and N-terminal sequence determination of the first 20 amino residues from the N-terminus that the eluted protein was a C-lobe of bovine lactoferrin (13).

Binding studies

The binding studies of NSAIDs with C-lobe were carried out using fluorescence spectroscopic technique with spectrofluorometer, FP-6200 (Shimadzu, Koyoto, Japan). C-lobe (2.5 mL 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 μL, 40 μL, 60 μL, and 80 μL of NSAIDs (indomethacin, aspirin, and ibuprofen) from their stock solutions 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 (Fig. 1, *a–c*). The fluorescence spectra with diclofenac could not be recorded because of its very high intrinsic fluorescence. The spectral changes of the protein were recorded with different NSAIDs at various concentrations.

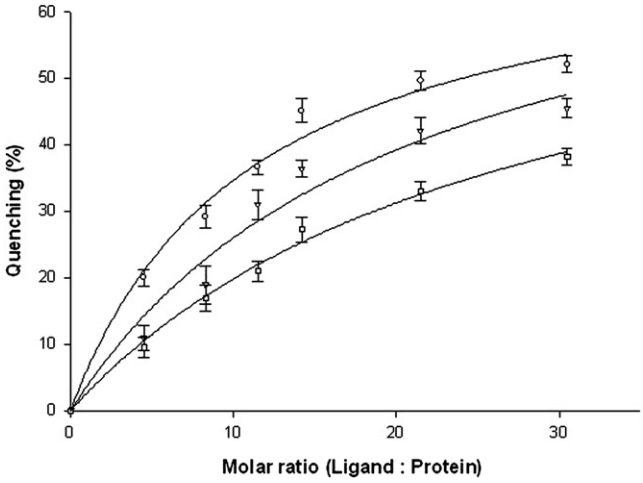

FIGURE 1 Binding curves, ○ (indomethacin), △ (aspirin), and □ (ibuprofen) showing the change in fluorescence intensities ($\Delta F/Fo$) at 280 nm as the NSAIDs/C-lobe molar ratio was increased. Indomethacin exhibited slightly large increase in fluorescence as compared to others while observed. The errors on the experimental points have been indicated.

Animal studies

The ability of C-lobe to prevent the gastric damage caused by NSAIDs was established by using mouse animal model. All the mice samples were procured from the experimental animal house facility of All India Institute of Medical Sciences, New Delhi. All animal experiments were conducted in accordance with the guidelines for the care and use of laboratory animals by CPCSEA (Ministry of Social Justice, government of India) and adopted by Ethics Committee on Animal Experimentation by the All India Institute of Medical Sciences. The experiments were carried out on female Swiss albino mice (age, 6–8 weeks; weight, ~25 g) that were fasted 12 h with free access to water before the experiment. The doses of C-lobe and NSAIDs used in these experiments were 200 mg/kg and 10 mg/kg weight of mice, respectively. All the preparations were made in phosphate buffer saline (PBS) containing 50 mM potassium phosphate; 150 mM NaCl; pH 7.2. The animals were randomized in three groups designated as control, NSAID, and the test group with C-lobe + NSAID. At zero time animals of the test and NSAID groups were treated orally with NSAIDs (10 mg/kg weight of mice) whereas PBS was given to the animals of control group. After 1 h, 200 mg/kg of C-lobe was administered orally to the test group and PBS was given to the control and NSAID groups; 24 h later, all animals were euthanized and the whole gut was removed for the analysis of bleeding and inflammation index. The analysis of intragastric bleeding was carried out by measuring the concentration of hemoglobin. The distal end of the small intestine was excised and flushed with 2 mL of distilled water for the hemoglobin assay. In addition, ileal tissues from small intestine were excised and processed for myeloperoxidase (MPO) assay that is known to act as an index of inflammation (16).

Hemoglobin assay

The hemoglobin concentration that is an indicator of intragastric bleeding was estimated using the modified method of Crosby and Furth (17); ~25 μL of the test sample was added to 1 mL reaction mixture containing 1% benzidine/HCl in acetic acid + 1% H_2O_2 and incubated it for 20 min. This was followed by the addition of 5 mL diluent consisting of 10% acetic acid incubated for 10 min. The absorbance was measured spectrophotometrically using a spectrophotometer Cary 100 Bio (Varian, Palo Alto, CA) at a wavelength of 515 nm.

Myeloperoxidase assay

The indicator of tissue neutrophil accumulation of MPO activity was determined using the modified method of Brandley et al. (16). The equally weighed ileal tissues of three experimental groups were homogenized in 50 mM phosphate buffer, pH 6.0 containing 0.5% hexadecyltrimethylammonium bromide. It was sonicated and freeze-thawed, and followed by centrifugation for 40 min at 12000 rpm. An aliquot (0.1 mL) of the supernatant was allowed to react with 2.9 mL of buffer containing O-dianisidine (0.167 mg/mL) and 0.0005% H_2O_2. The rate of change in absorbance was measured spectrophotometrically, using a spectrophotometer Cary 100 Bio (Varian), at 460 nm. The results were expressed as units of MPO per mg of protein. The protein was estimated using bicinchoninic acid method using bovine albumin as a standard.

Statistical analysis

All values are statistically derived and presented as mean ± SE. The comparison among the groups was made using one-way analysis of variance. The differences with p-values < 0.05 were considered significant.

Crystallization of C-lobe complexes with NSAIDs

The C-lobe was cocrystallized with four NSAIDs (indomethacin, diclofenac, aspirin, ibuprofen) using identical molar ratios (1:20) of protein to NSAIDs. A protein solution (80 mg/mL) was prepared in the buffer containing 20% ethanol and 50 mM MES at pH 6.5. The samples of NSAIDs were added to this solution and allowed to stay for 24 h at 298 K. The incubated protein solutions were mixed in a 1:1 ratio with crystallization buffer containing 0.1 M MES pH 6.5, 25% (v/v) polyethylene glycol monomethyl ether 550, and 0.01 M zinc sulfate heptahydrate, and allowed to equilibrate via vapor diffusion over 1-mL well solution consisting of above crystallization buffer at 298 K. The irregular-shaped and dark brown-colored crystals of the complexes appeared after two days.

Detection of indomethacin, diclofenac, aspirin, and ibuprofen in crystals

To confirm the presence of indomethacin, diclofenac, aspirin, and ibuprofen in the crystals of C-lobe complexes, the crystals of four complexes were used for the identifications of indomethacin, diclofenac, aspirin, and ibuprofen. Initially, the crystals were washed with distilled water and dissolved in the buffer containing 50 mM Tris-HCl, pH 8.0 separately. These crystal solutions were ultrafiltered in the same buffer containing 1 M NaCl using a membrane with a cutoff of 1 kDa.

The presence of indomethacin in the filtrate was observed by adding ammonium oxalate and concentrated sulphuric acid followed by heating the contents in the water bath. Indomethacin reacts with oxalate ions in acidic solution to form a violet-colored complex and the absorbance of this complex was measured at wavelength of 578 nm using the spectrophotometer Lamda 25 (PerkinElmer, Boston, MA).

The presence of diclofenac in the filtrate was observed by adding Fe^{3+} ions and 2,2′-bipyridine. Diclofenac reduces Fe^{3+} to Fe^{2+} on heating in aqueous solution and the ferrous ions produced reacts with 2,2′-bipyridine to form a complex and its absorbance was measured at wavelength of 520 nm using the spectrophotometer Lamda 25 (PerkinElmer).

The presence of aspirin in the filtrate was observed by adding Fe^{3+} ions. When treated with basic solution the acetylsalicylic acid hydrolyzes quickly to salicylic acid. The salicylate ions form an intensely purple-colored complex with the ferric ion in acidic solution and the absorption of this complex was measured at wavelength of 530 nm using the spectrophotometer Lamda 25 (PerkinElmer).

X-ray intensity data collection and processing

The freshly grown crystals of all four complexes of C-lobe with NSAIDs were used for x-ray crystallographic studies. The intensity data were collected at 287 K using a 345 mm diameter MAR Research Imaging plate scanner (Marresearch, Norderstedt, Germany) mounted on a RU-300 Rigaku rotating anode x-ray generator (Rigaku, Tokyo, Japan) operating at 50 kV and 100 mA. The Osmic Blue confocal optics (Rigaku) was used for focusing Cu Kα radiation. The data were processed with DENZO and SCALEPACK from HKL package (18). The crystals of four complexes are isomorphous and belong to monoclinic space group $P2_1$. The summary of data collection statistics for all the four complexes is given in Table 1.

Structure determination and refinement

The structures of all four complexes were determined with molecular replacement method using AMoRe (19). The coordinates of native C-lobe (14) (Protein Data Bank code: 1NKX) were used as the search model. The rotation and translation search functions were calculated with data between the resolution range of 12.0–4.0 Å yielding clear solutions with distinct peaks for the four structures. The transformed coordinates using AMoRe (19) were initially subjected to 40 cycles of rigid body refinements. Density modifications and crystallographic refinement procedures were carried out using ARP/wARP (20) and REFMAC (21) respectively. The model buildings were carried out using program O (22). The difference Fourier $|Fo - Fc|$ at 3σ and Fourier $|2Fo - Fc|$ maps at 1σ before inclusion of ligands were calculated at R_{cryst} factors of ≤ 0.24 in all the cases. These showed reasonably characteristic electron densities at the ligand binding site in the C-lobe. The ligands were modeled into the respective electron densities (Fig. 2, *a–d*) and their coordinates were included in the subsequent cycles of refinements. Note that the fitting of aspirin in the electron density is slightly ambiguous as it can be adjusted in two orientations in which either carboxylic group or acetyl group could be placed in the proximity of the side chain of Thr^{663}. However, chemical considerations such as the possibility of hydrogen bond formation favor the present mode of binding. The position of metal ions, Fe^{3+} and Zn^{2+}, anions CO_3^{2-}, and SO_4^{2-}, atoms of sugar residues from three glycan chains and water oxygen atoms were also determined. All these atoms were included in the subsequent refinement cycles. The refinements converged with R_{cryst}/R_{free} factors of 0.209/0.244, 0.193/0.219, 0.182/0.228, and 0.165/0.220 for the complexes with indomethacin, diclofenac, aspirin, and ibuprofen, respectively. The refinement statistics of these structures are summarized in Table 1.

RESULTS

Analysis of binding studies

To exploit the fluorescence properties of protein molecule, the binding studies were carried out as described in Experimental Procedures. The intrinsic fluorescence of C-lobe when excited at 280 nm is due to the presence of at least four aromatic residues, which are located in the proximity of the binding pocket. The fluorescence spectra were recorded in the presence of increasing amounts of NSAIDs. The fluorescence intensities of C-lobe decreased gradually when the concentrations of NSAIDs were enhanced although the positions of emission maxima as well as the shape of the peaks remained unaltered. This suggested that NSAIDs bound to C-lobe without altering the environment in the vicinity of aromatic residues. The observed fluorescence data were used for calculating the fluorescence quenching ($Q = (Fo - F)/Fo$, where F is the measured fluorescence and Fo is the fluorescence in the absence of NSAIDs). Plots of graph of Q (%) against the molar ratios of ligand/protein were prepared. As seen from Fig. 1, it gave rise to three smoothly rising curves for the binding of indomethacin, aspirin, and ibuprofen. The least-squares fit of the fluorescence intensity changes for the NSAID-C-lobe binding curves were obtained by Sigma Plot 8.0 (23). The R^2 values for the fit curves of the binding of indomethacin, aspirin, and ibuprofen to C-lobe are 0.98, 0.96, and 0.99, respectively. The error bars on the experimental points were estimated from the average of values that were obtained by repeating each experiment six times. The binding constants were calculated using the binding equation described by Scatchard (11,12). From these curves binding constants for indomethacin, aspirin, and ibuprofen were obtained. The approximate values of binding constants were found to be 2.6×10^{-4} M, 3.3×10^{-4} M, and 4.8×10^{-4} M for indomethacin, aspirin, and ibuprofen, respectively indicating that all the compounds were attached to C-lobe at an approximate affinity of 10^{-4} M.

TABLE 1 Summary of data collection and refinement statistics

Parameters	(a) C-lobe indomethacin	(b) C-lobe diclofenac	(c) C-lobe aspirin	(d) C-lobe ibuprofen
Data collection				
Space group	P21	P21	P21	P21
Unit cell dimensions (Å)				
a	63.8	61.4	63.3	63.3
b	50.6	49.9	50.5	50.5
c	66.1	66.1	65.9	66.1
β (°)	107.6	107.1	107.7	107.7
Molecules in the unit cell (n)	2	2	2	2
Vm (Å^3/Da)	2.5	2.4	2.5	2.7
Solvent content	~51.0	~48.0	~51.0	~54.3
Resolution range (Å)	20.0–2.2 (2.25–2.20)	20.0–1.4 (1.43–1.40)	20.0–2.0 (2.07–2.00)	20.0–2.3 (2.35–2.30)
Total measured reflections (n)	113,121	613,858	153,732	156,288
Unique reflections (n)	20,430	74,524	27,067	15,841
Redundancy	6.5	5.6	7.4	6.4
Overall completeness of data	91 (93)	99.7 (98.5)	96.1 (90.2)	90.0 (90.2)
Overall R_{sym}	12.4 (48.0)	6.2 (45.0)	7.1 (40.3)	12.5 (47.7)
Overall $I/\sigma(I)$	9.8 (2.2)	17.5 (2.0)	13.2 (2.5)	6.4 (2.0)
Refined model				
Protein Data Bank code	3IB1	3IB0	3IAZ	3IB2
R_{cryst} (%)	20.9	19.3	18.2	16.5
R_{free} (%)	24.4	21.9	22.8	22.0
Protein atoms	2605	2605	2605	2605
Fe^{3+} ion	1	1	1	1
Zn_2^{+} ions	2	2	2	2
CO_3^{2-} ion	1	1	1	1
SO_4^{2-} ion	1	1	1	1
Sugar residues (N-linked)	11	13	13	7
Atoms of ligand	25	19	13	15
Water molecules	247	509	260	143
RMSD* in bond length	0.02	0.01	0.01	0.01
RMSD in bond angles	2.2	1.7	1.7	1.7
RMSD in torsion angles	20.4	16.9	17.9	18.3
Overall G factor	−0.04	0.04	0.07	−0.1
Mean B-factor (Å^2)				
Main chain atoms	34.6	17.7	27.9	29.4
Side chain atoms	36.8	20.2	30.7	31.7
Water molecules	52.2	38.9	46.3	41.7
All atoms	39.0	23.5	33.7	32.5
Ramachandran plot residues (%)				
Residues in the most favored regions	85.4	88.0	89.0	83.7
Residues in the additionally allowed regions	14.3	11.7	10.6	15.0
Residues in the generously allowed regions	0.0	0.0	0.0	1.0
Residue in the disallowed regions (Leu^{640})	0.3	0.3	0.3	0.3

Values in parentheses are for the highest resolution shell.
*RMSD, root mean-square deviation.

Analysis of studies in vivo

Gastric bleeding

The examination of gastric hemoglobin concentration gives an index of gastric bleeding. The administration of NSAIDs in mice increased the hemoglobin concentration due to induced gastric injury ($p < 0.05$; Fig. 3 *i*, *A*). The orally administered C-lobe in mice after the administration of NSAIDs showed reduction in hemoglobin concentration considerably ($p < 0.05$; Fig. 3 *i*, *B*). When compared with the concentration of hemoglobin in NSAIDs treated mice, it corresponded to a recovery to the extent of 60% in indomethacin-induced injury, 52% in diclofenac-induced injury, 51% in aspirin-induced injury, and 47% in ibuprofen-induced injury. The values after recovery in the cases of indomethacin and diclofenac were slightly higher than those of control samples ($p < 0.05$; Fig. 3 *i*, *C*) whereas in the cases of aspirin and ibuprofen, the hemoglobin concentrations after the administration of C-lobe were comparable to those observed in the control samples ($p < 0.05$; Fig. 3 *i*, *C*). This indicates that the effect of C-lobe was significant in reducing the gastric bleeding in mice treated with NSAIDs. In a separate experiment when C-lobe was coadministered with NSAIDs, it prevented any injuries completely.

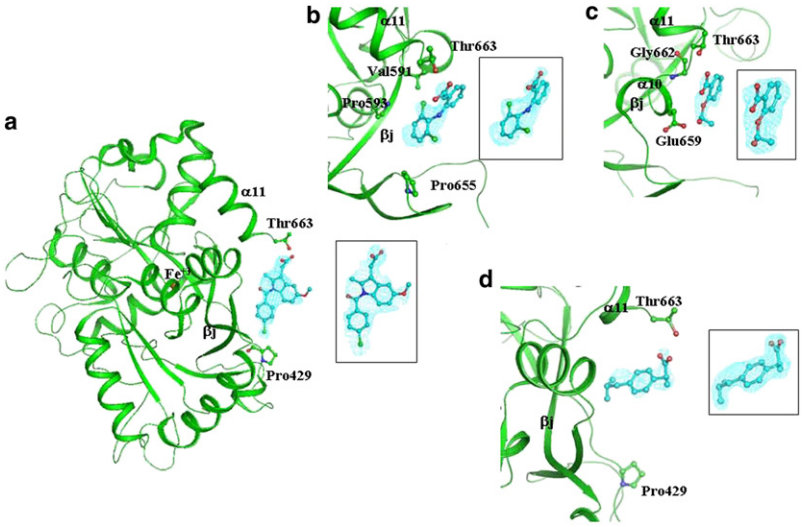

FIGURE 2 Initial $|Fo - Fc|$ difference Fourier maps calculated at $R_{cryst} = 0.24$ before inclusion of ligands showing electron densities at 3σ cutoff (along side in the box $|2Fo - Fc|$ maps at 1σ cutoff) for the ligands (*a*) indomethacin, (*b*) diclofenac, (*c*) aspirin, and (*d*) ibuprofen.

MPO activity

The infiltration of neutrophils during gastric injury, as an index of inflammation, was measured by the activity of marker enzyme MPO. The enhancement in neutrophil migration was observed as an increase in the MPO activity ($p < 0.05$; Fig. 3 *ii*, *A*) in ileum tissue after NSAIDs were administered to the mice that were significantly higher as compared to control ($p < 0.05$; Fig. 3 *ii*, *C*). When the C-lobe was orally administered to mice after giving the dosage of NSAIDs, it reduced the activity of MPO considerably and the recoveries occurred to the extent of 70%, 69%, 60%, and 68% from the injuries induced by indomethacin, diclofenac, aspirin, and ibuprofen, respectively ($p < 0.05$; Fig. 3 *ii*, *B*) as compared to only NSAIDs treated mice. These values were comparable to the values obtained for control samples ($p < 0.05$, Fig. 3 *ii*, *C*). The lowering of MPO activity of samples clearly indicates the repairing role played by the C-lobe. As in the case of gastric bleeding, when C-lobe was coadministered with NSAIDs, the MPO activity in the ileum tissue was found to be significantly low.

Structural analysis

The final models of the complexes of C-lobe with indomethacin, diclofenac, aspirin, and ibuprofen have been summarized in Table 1 (a–d, respectively). The structure of the proteolytically prepared C-lobe of bovine lactoferrin consists of residues from 342 to 676. The structural organization of C-lobe is comprised of 12 α-helices, and 11 β-strands. The structure of C-lobe is divided into two similarly sized domains, C-1 and C-2. The C-1 domain is comprised of six α-helices and six β-strands, whereas the domain C-2 is made up of six α-helices and five β-strands. The deep iron binding cleft is formed between domains C-1 and C-2. The base of the iron binding cleft is closed by the longest β-strand in the structure. The structures of four complexes of C-lobe with indomethacin (Fig. 4 *a*), diclofenac (Fig. 4 *b*), aspirin (Fig. 4 *c*), and ibuprofen (Fig. 4 *d*) show a common binding site for all the four NSAID molecules. All the four ligands are located at the site in the back of the iron binding site. This site is structured into a shallow cleft. It is formed with an α-helix, α10, a β-strand, β_j, and including two loops, one between helices α10 and α11 and the other

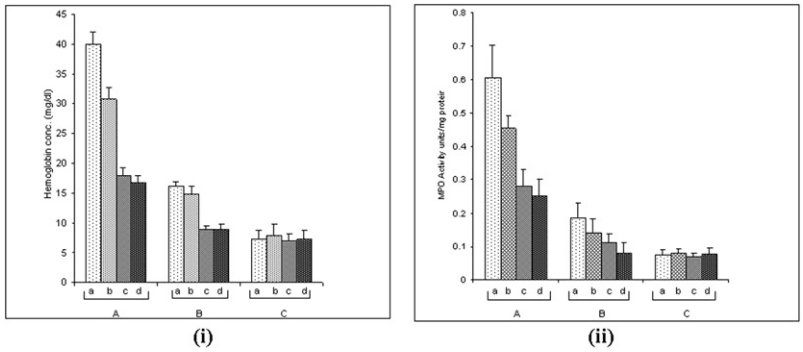

FIGURE 3 Effect of C-lobe on acute NSAID-induced intestinal injury in mice. The mice were treated as described in the methods. (*i*) Rinses of the distal small intestinal lumen were analyzed for hemoglobin: (*A*) samples treated with (*a*) indomethacin, (*b*) diclofenac, (*c*) aspirin, and (*d*) ibuprofen; (*B*) C-lobe was administered orally to the mice after the treated with (*a*) indomethacin, (*b*) diclofenac, (*c*) aspirin, and (*d*) ibuprofen; and (*C*) control samples treated with PBS. The results are presented as mean ± SE, $n = 15$, $p < 0.05$. (*ii*) The ileal tissues were analyzed for MPO activity: (*A*) samples treated with (*a*) indomethacin, (*b*) diclofenac, (*c*) aspirin, and (*d*) ibuprofen; (*B*) on adding C-lobe after the NSAIDs were added (*a*) indomethacin, (*b*) diclofenac, (*c*) aspirin, and (*d*) ibuprofen; and (*C*) in control samples. Results are presented as mean ± SE, $n = 15$, $p < 0.05$.

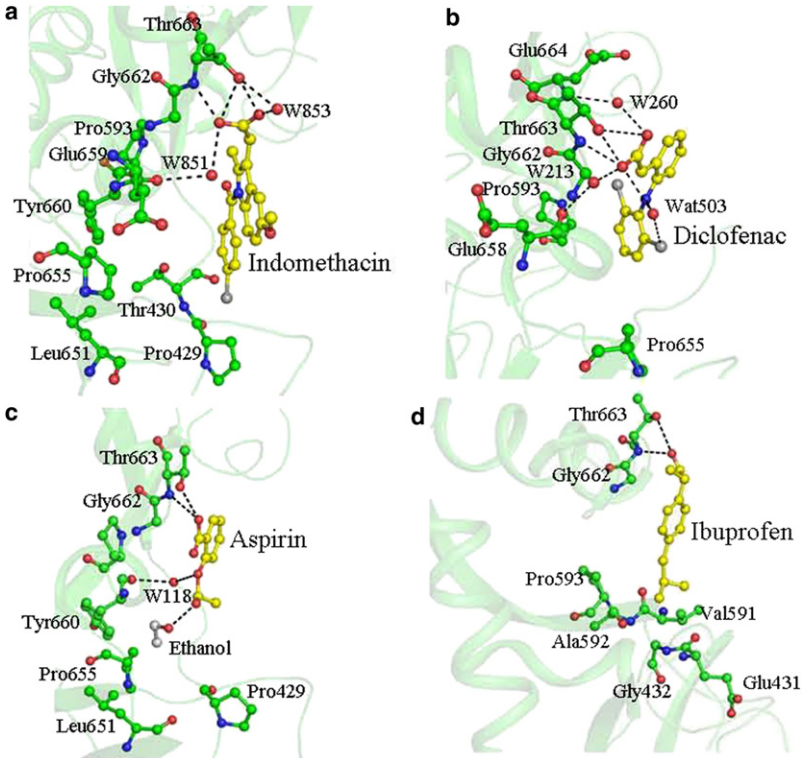

FIGURE 4 Binding of NSAIDs in the ligand-binding site of C-lobe. Protein residues are shown in green and NSAID molecules are yellow: (*a*) indomethacin, (*b*) diclofenac, (*c*) aspirin and (*d*) ibuprofen. Dashed lines indicate hydrogen bonds. Residues are labeled.

between β-strands βd and βe. This binding site has an opening to the surface of the protein. The NSAID binding site consists of hydrogen bond forming features involving Thr^{663} and the backbone N atoms on one side and hydrophobic residues Pro^{429}, Thr^{430}, Val^{591}, Pro^{593}, Leu^{651}, and Pro^{655} on the opposite side. It has dimensions of the order of 16 Å in length and 8 Å deep from the surface and seems to be specific to acidic and lipophilic ligands such as NSAID molecules.

Binding of NSAIDs to C-lobe

Structure of the complex of C-lobe with indomethacin

The structure of the complex of C-lobe with indomethacin has been determined at 2.2 Å resolution. The crystals were obtained by cocrystallization using protein to indomethacin molar ratio of 1:10. The unit cell dimensions were found to be $a = 63.8$ Å, $b = 50.6$ Å, $c = 66.1$ Å, and $\beta = 107.6°$. As illustrated in Fig. 4 *a*, indomethacin is held parallel to the surface in the NSAID binding site with its carboxylic group forming several hydrogen bonds with Thr^{663} and a water molecule. The hydrophobic interactions are provided by Gly^{662}, Thr^{430}, and Pro^{429}. Indomethacin is bound firmly at two ends. On one side it forms hydrogen bonds whereas the second side is held by van der Waals forces indicating a typical model for the compounds having both hydrophilic and hydrophobic moieties as in NSAIDs. Indomethacin covers ~26% volume of the ligand binding site (Fig. 5 *a*).

Structure of C-lobe complex with diclofenac

The structure of the complex formed between C-lobe and diclofenac was determined at 1.4 Å resolution. The crystals of the complex formed between C-lobe and diclofenac were obtained by cocrystallizing the C-lobe with diclofenac at 1:10 molar ratio. The unit cell dimensions were determined to be $a = 61.4$ Å, $b = 49.9$ Å, $c = 66.1$ Å, and $\beta = 107.1°$. As seen from Fig. 4 *b*, Thr^{663} forms three direct hydrogen bonds and one through solvent water molecule with the carboxylic group of diclofenac. The residues Gly^{662}, Pro^{593}, and Val^{591} provide van der Waals contacts at the other end. The diclofenac molecule is fitted into the newly identified ligand binding site of C-lobe and occupies ~21% volume of the ligand binding site (Fig. 5 *b*).

Structure of C-lobe complex with aspirin

The structure of the complex formed between C-lobe and aspirin was determined at pH 6.5 as similar conditions were used for the spectroscopic studies of this complex. The crystals of the complex were obtained by cocrystallization using the mixture of C-lobe with aspirin at 1:10 molar ratio. The cell dimensions were $a = 63.3$ Å, $b = 50.5$ Å, $c = 65.9$ Å, and $\beta = 107.7°$. As seen from Fig. 4 *c*, aspirin binds to C-lobe at the NSAID binding site and forms two direct hydrogen bonds involving one of the carboxyl group oxygen atoms of aspirin with Thr^{663} NH (Thr^{663} NH•••O1 aspirin = 2.9 Å) and $O^{\gamma}H$ (Thr^{663} $O^{\gamma}H$•••O1 aspirin = 2.8 Å). There are three other hydrogen bonds involving Glu^{659} O Tyr^{660} O with O3 of aspirin through solvent water

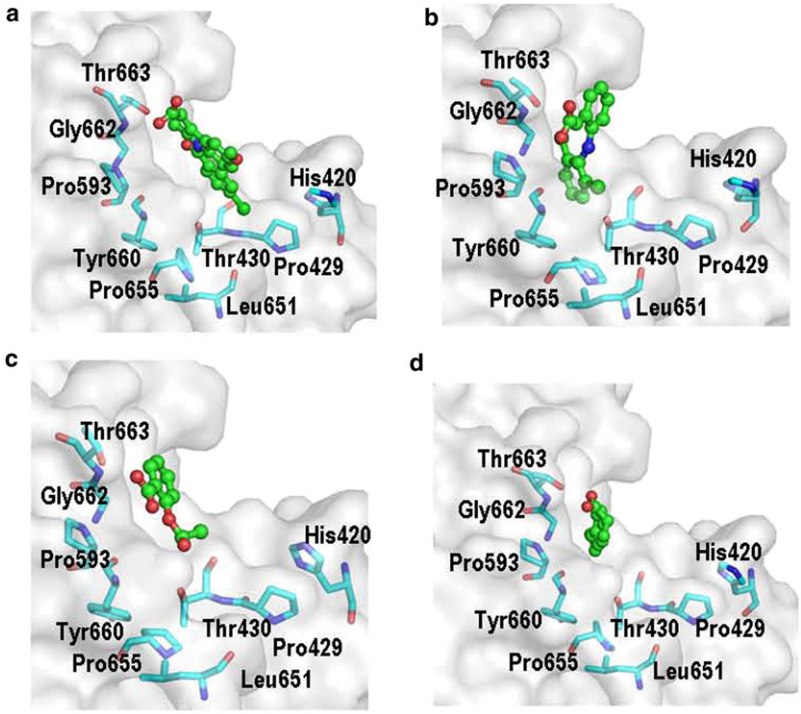

FIGURE 5 Placement of NSAID molecules in the ligand-binding site. The molecules of (*a*) indomethacin, (*b*) diclofenac, (*c*) aspirin, and (*d*) ibuprofen are shown well fitted in the figure (drawn using grasp) (26).

molecule W118 (aspirin O3•••W118 = 3.3 Å, W118•••O Glu^{659} = 3.1 Å, and W118•••O Tyr^{660} = 3.0 Å). Another acetyl group oxygen atom O4 forms a hydrogen bond with ethanol OH at a distance of 2.7 Å. The methyl group of ethanol, on the other hand, forms strong van der Waals contacts with Pro^{655}, Glu^{659}, and Gly^{662}. The position of aspirin molecule indicates that it occupies a 14% volume of the ligand binding site in the proximity of Thr^{663} (Fig. 5 *c*).

Structure of the complex of C-lobe with ibuprofen

The crystals of the complex of C-lobe with ibuprofen were obtained using cocrystallization by mixing C-lobe and ibuprofen at 1:10 molar ratio. The cell dimensions were found to be $a = 63.3$ Å, $b = 50.5$ Å, $c = 66.1$ Å, and $\beta = 107.7°$. Ibuprofen binds to C-lobe at the same site that is occupied by indomethacin, diclofenac, and aspirin. The interactions between C-lobe and ibuprofen involve two hydrogen bonds and three notable van der Waals contacts (Fig. 4 *d*). As seen in Fig. 5 *d*, it is fitted into the cleft covering ~17% volume of the ligand binding site.

The structures of the complexes of C-lobe with four NSAIDs indicate that a new independent ligand binding site is present in the C-lobe of lactoferrin. This site is particularly suitable for the binding of NSAIDs and it is highly accessible from the surface. This is also independent from the other role of C-lobe of carrying out the iron binding and release. Therefore this can act as a sequesterer in both iron saturated and apo form of C-lobe. This NSAID binding property of C-lobe seems to be responsible for its effects against gastric ulcers.

DISCUSSION

The effect of sequestration of NSAIDs by C-lobe does not hamper the efficiency of their binding to the target proteins because the binding affinities of NSAIDs to PLA_2 and COX-2 enzymes are significantly higher (10^{-6} to 10^{-8} M) than their affinities toward C-lobe ($\approx 10^{-4}$ M). On the other hand, the human serum albumin (HSA) also has been shown to sequester NSAIDs through more than one binding site with affinities of the order of 10^{-6} to 10^{-7}M (24,25). These affinities are comparable to those of NSAIDs with PLA_2 and COX-2 target proteins. As a result, HSA is unsuitable for coadministration for sequestering the unbound NSAIDs as it impairs the availability of drug molecules due to their high affinities for HSA (25). We believe the experiments carried out to study the side effects of NSAIDs on mouse models using indomethacin, diclofenac, aspirin, and ibuprofen show clearly that the significant damages occur to the gastric mucosal lining. The observed concentrations of gastric hemoglobin after the administration of NSAIDs show that the levels of hemoglobin increase considerably. The concentrations of the marker proteins indicate that the lesions caused by NSAIDs on gastric mucosal lining are maximum with indomethacin whereas diclofenac produces 80% of the damage produced by indomethacin whereas both aspirin and ibuprofen cause significantly lower damages than that caused by indomethacin (Fig. 3 *i*, *A*). However, when C-lobe was administered, the levels of hemoglobin dropped considerably (Fig. 3 *i*, *B*). Similar results were obtained when the activity of lower intestinal myeloperoxidase (MPO) was estimated (Fig. 3 *ii*, *A*). As seen from Fig. 3 *ii*, *A*, the activity of MPO

increased nearly 3–5 times when NSAIDs were administered. Again the maximum damage was caused by indomethacin. When C-lobe was administered, the recorded activity of MPO dropped nearly to the levels of values obtained for the controlled cases indicating a complete reversal of NSAID-induced gastropathy. In other experiments with mouse models, when C-lobe was coadministered with NSAIDs, the gastric lesions were not observed. The binding studies for NSAIDs with C-lobe show that indomethacin, diclofenac, aspirin, and ibuprofen bind to C-lobe with affinities ranging from 2.6 to 4.8 $\times$ 10^{-4} M that seem to be good enough to sequester the unbound molecules in the gut without affecting the efficacy of the drugs. The structural studies have shown the presence of a single promiscuous binding site that is suitable for compounds with acidic and lipophilic nature such as those of NSAIDs. This is a shallow cleft on the surface of the protein that is situated at the back of the well formed deep hydrophilic iron-binding cleft in the C-lobe. It is well separated from the iron binding site by the longest β-strand β_j (residues, 590–602) in the structure of C-lobe. The new ligand binding site is comprised of protein segments, 429–432 (loop, β_d–β_e), a part of β-strand β_j (residues, 570–593), apart of α-helix, α10 (residues, 658–666), and a loop between α-helices α10 and α11 (residues 661–664). On one side, the site is rich in hydrophobic environment whereas at the opposite end, it has favorable hydrogen bond formers. As a result, this binding site in C-lobe attracts the ligands with lipophilic and acidic characters. The site is nearly 16 Å long and ~8 Å deep. The sizes of NSAID molecules are within the range of 12 Å in length and 6 Å width. Thus the dimensions of the binding site in C-lobe can accommodate various NSAIDs. In comparison, the HSA has two large binding cavities with dimensions of 16 Å $\times$ 15 Å. The drug binding site in HSA consists of apolar residues and basic residues with a complete absence of acidic residues providing a clear definition of specificity of the binding. This supports the observed high affinity of $\approx 10^{-7}$M for NSAIDs (25). All compounds studied are reported to be positioned overlappingly to make contacts either through hydrogen bonds or salt bridges in addition to extensive hydrophobic interactions. In the case of C-lobe, all four molecules occupy same regions of the binding site although their orientations differ slightly (Fig. 5). On binding of NSAIDs, the residues of the ligand binding site, Asn^{414}, Thr^{430}, Glu^{431}, Pro^{655}, Tyr^{660}, Thr^{663}, and Glu^{664} undergo minor conformational adjustments. The root mean-square shifts for the side chains of above residues in the complexes of C-lobe with indomethacin, diclofenac, aspirin, and ibuprofen when superimposed on the same residues of the native structure (14) were found to be 0.7 Å, 1.0 Å, 0.9 Å, and 0.8 Å, respectively.

CONCLUSION

These structural and binding studies of C-lobe complexes with NSAIDs show that NSAIDs bind to C-lobe at a common site indicating the presence of a new ligand binding site in C-lobe of lactoferrin. The binding affinities of C-lobe for indomethacin, diclofenac, aspirin, and ibuprofen are of the order of 10^{-4} M. Therefore, C-lobe is capable of sequestering the unbound NSAIDs in the gut resulting in the reversal of NSAID-induced gastropathy. Because C-lobe is resistant to enzymatic degradation, its sequestering property has potentially useful therapeutic applications. The bindings of NSAIDs to C-lobe unlike to that of human serum albumin does not hamper the availability of drugs because the binding constant of NSAIDs with C-lobe is considerably lower than that reported for NSAIDs with targets, COX-2, and PLA_2. In this respect, human serum albumin is unsuitable because it has a high binding constant for NSAIDs (10^{-7} M) that is comparable to that of actual drug targets (10^{-6}–10^{-8} M). Therefore, coadministration of C-lobe with NSAIDs offers a great advantage for the prevention of NSAID-induced gastropathy. We believe the animal studies support these conclusions unambiguously.

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