Effect of pH on Diclofenac–Lysozyme Interaction: Structural and Functional Aspect

Mohd Basheeruddin, Sheeza Khan, Neesar Ahmed and Shazia Jamal*

School of Life Sciences, B. S. Abdur Rahman Crescent Institute of Science and Technology, Chennai, India

As a nonsteroidal antiinflammatory drug, diclofenac (DCF) is used in the treatment of a variety of human ailments. It has already been reported that the use of this class of drugs for a longer duration is associated with numerous side effects such as cardiovascular implications, reno-medullary complications, etc. In the present study, the effect of DCF on the structure, stability, and function of lysozyme was studied. The study was designed to examine the effect of DCF only at various pH values. Heat-induced denaturation of lysozyme was analyzed in the presence and absence of various molar concentrations of DCF at different pH values. The values of thermodynamic parameters, the midpoint of denaturation ($T_m$), enthalpy change at $T_m$ ($\Delta H_m$), constant pressure heat capacity change ($\Delta C_p$), and Gibbs energy change at 25°C ($\Delta G_D^{\circ}$), thus obtained under a given set of conditions (pH and molar concentration of DCF), demonstrated the following: 1) DCF destabilized lysozyme with respect of $T_m$ and $\Delta G_D^{\circ}$ at all the pH values, 2) the magnitude of protein destabilization is lesser at acidic pH than at physiological pH, 3) structural changes in lysozyme are less projecting at pH 2.0 than at pH 7.0, and 4) quenching is observed at both pH values. Furthermore, the process of protein destabilization in the presence of DCF is entropically driven.

Keywords: diclofenac sodium, lysozyme, thermal denaturation, protein stability, circula dichroism spectrum

INTRODUCTION

Protein–drug interaction studies are important and central in understanding biological processes. Such interactions may influence the transportation, absorption, metabolism, and excretion of drugs (Caldwell et al., 1995). Small ligands are known to intermingle with these molecules readily (Ajmal et al., 2017a; Zhang et al., 2020). Recently, such studies are hot spots of multidisciplinary research (Ajmal et al., 2017b; Zhang et al., 2020). Proteins are versatile molecules and perform many different functions in the human body. They are flexible molecules, and ligand binding can affect their hydrodynamics and function; these alterations can be harmful or useful (Babu et al., 2011; Elfaki et al., 2013; Ajmal et al., 2017a). Drug binding to transport proteins can significantly affect the metabolism of drug molecules. It becomes important to look at the different aspects of these interactions when designing the dosage of the drugs spatially in a multidrug therapy or treatment in comorbid conditions, where the picture can be more complicated; protein binding of drugs not only affects drug pharmacokinetics but can also affect its function.

Diclofenac (DCF) sodium and potassium salts have been used to treat a range of ailments including osteoarthritis, ankylosing spondylitis, rheumatoid arthritis, primary dysmenorrhea, and mild to moderate pain (Sharma et al., 2012; Tampucci et al., 2019). DCF is a nonsteroidal...
antiinflammatory drug that is a derivative of phenylacetic acid; that is, its chemical name is 2-(2,6-dichloroanilino) phenylacetic acid (Vane and Botting, 1996; Ibrahim et al., 2018; Boumya et al., 2021; Galisteo et al., 2021). It is an analgesic, antipyretic, and antirheumatic medicament. DCF use has also been implicated in defective cardiovascular function. Numerous studies exist implicating the role of DCF in cardiac, renal, and gastrointestinal complications (Gökçimen et al., 2000; Weir, 2002; Lewis et al., 2002; Baigent et al., 2013; Lundgren et al., 2017). Lysozyme is a small globular protein used as a model molecule to study the effect of external agents on its stability and functions (Ajmal et al., 2017b; Leone et al., 2019). Ever since its discovery, lysozyme has represented a prototype molecule for understanding the complexity of its structure and function (Saadati-Eskandari et al., 2019). Thus, the study on the interaction of drugs with lysozyme has important significance. Such studies are useful for providing information on the structural features of the molecule interaction with drugs and illuminating the therapeutic effectiveness of drugs (Ajmal et al., 2016; Karaman and Sippl, 2019). Interestingly, no study exists to date that could explain the pH dependence of DCF effects on the structural, functional, and stabilization properties of proteins. In this communication, we have analyzed the effect of DCF on the structure, stability, and function of lysozyme at different pH values by measuring \(\Delta G^o_{D}\) (Gibbs free energy change upon denaturation at 25°C) and enzyme kinetic parameters (\(K_m\) and \(k_{cat}\)) in the presence and absence of DCF.

**MATERIALS AND METHODS**

Lyophilized hen egg-white lysozyme and *M. luteus* cell wall were commercially available and purchased from Sigma. The ultrapure sample of guanidinium chloride (GdmCl), DCF, cacodylate, sodium acetate, and dialysis tubing was also procured from Sigma. KCl and glycine were obtained from SRL. All analytical grade chemicals were used without any further purification.

The stock solution of lysozyme was immensely dialyzed against 0.1 M KCl at pH 7.0. This solution was filtered with 0.45 μm millipore filter paper. Molar absorption coefficient (M\(^{-1}\) cm\(^{-1}\)) values of 39,000 at 280 nm for lysozyme were used to determine the concentration of protein (Sinha et al., 2000; Lindorff-Larsen, 2019). Refractive index measurements were used to find the concentration of GdmCl stock solution. All solutions were prepared in an appropriate buffer that contains 0.1 M KCl. In this study, 50 mM KCl–HCl buffer (pH2.0), 50 mM glycine–HCl buffer (pH3.0), 50 mM sodium acetate buffer (pH 4.0), and 50 mM cacodylic acid buffer (5.0–7.0) were used. The solutions of DCF were prepared in the respective buffers at different pH values. Heating or the addition of GdmCl may cause a change in pH; hence, the pH of the samples was measured before and after the experiment. There were no such changes observed at all pH values. All the solutions used were prepared fresh each time.

Heat-induced denaturation experimental studies were carried out in a spectrophotometer (Jasco Model: V-730 UV/VIS) with a temperature controller (peltier Model ETCS-761). At the rate of 1°C/min, the samples were heated, and this scan rate provides sufficient time for equilibration. All samples were thermally denatured in the temperature range of 20°C–85°C. An increase in temperature shows the variation in absorbance at 300 nm. Total data points were collected after thermally denaturing the samples. At a given wavelength, the absorbance values were converted to \(\Delta T_m\)\(^{1 cm}\), the difference molar absorption coefficient. All heat-induced transition curves were plotted as \(\Delta T_m\) versus temperature. \(T_m\) and \(\Delta H_m\) were determined from these plots using Eq. 1 (Santoro and Bolen, 1988; Sinha et al., 2000; Singh et al., 2007; Lin et al., 2020; Leite et al., 2021).

\[
y(T) = y_N(T) + y_D(T) \exp [-\Delta H_m / R(1/T - 1/T_m)] \frac{1 + \exp [-\Delta H_m / R(1/T - 1/T_m)]}{1 + \exp [-\Delta H_m / R(1/T - 1/T_m)]}
\]

(1)

The parabolic function (such as \(y_N(T)\) and \(y_D(T)\)) for the analysis of the transition curve explains the dependence of the optical properties of the folded and unfolded protein molecules (Islam, 2020; Parray et al., 2020; Parray et al., 2021). The value of temperature-independent \(\Delta C_p\) was determined by using the slope of the plot between \(\Delta H_m\) and \(T_m\) using Eq. 2 (Becktel and Schellman, 1987; Sinha et al., 2000; Rahman et al., 2015; Dragan et al., 2019).

\[
\Delta C_p = \frac{\partial \Delta H_m}{\partial T_m}
\]

(2)

With the values of \(T_m\), \(\Delta H_m\), \(\Delta C_p\), and \(\Delta G_D(T)\), the values of \(\Delta G_D\) were estimated at any temperature using the Gibbs–Helmholtz equation (Eq. 3) (Singh et al., 2005; Khan et al., 2013; Dragan et al., 2019; Naiyer et al., 2021).

\[
\Delta G_D(T) = \Delta H_m \left(\frac{T_m - T}{T_m}\right) - \Delta C_p \left[ (T_m - T) + T \ln \left(\frac{T}{T_m}\right) \right]
\]

(3)

The far-UV CD of lysozyme was measured in a Jasco spectropolarimeter (Model: J-810) having a temperature controller (peltier model-Jasco PTC-424S). The cuvette path length used for far UV was 1 mm. At each wavelength, the value of mean residue ellipticity (deg cm\(^2\) dmol\(^{-1}\)) was converted by the CD signal using Eq. 4.

\[
[\theta]_1 = \theta_M M_0 / 10lc
\]

(4)

Where the observed ellipticity in milli degrees is \(\theta\) at wavelength \(\lambda_{nm}\), \(M_0\) is the mean residue weight of the protein, \(c\) is the protein concentration in mg cm\(^{-1}\), and \(l\) is the path length (cm).

Fluorescence studies were carried out at different concentrations of DCF (2–20 μM) at two pH values (i.e., 2.0 and 7.0). Fluorescence quenching was monitored by measuring intrinsic fluorescence from the range of 315–500 nm with the excitation wavelength of 295 nm. The slits were set at 5 nm for the excitation and emission.

The *M. luteus* cell wall was used as a substrate for the lytic activity of lysozyme at pH 7.0 at 25°C. The effect of different concentrations of DCF on kinetic parameters (\(K_m\) and \(k_{cat}\)) was measured using the method of Maurel and Douzou (1976). The given concentrations of DCF were preincubated with the substrate and the enzyme. The change in absorbance on the addition of lysozyme to the substrate with constant stirring was recorded at 450 nm in a spectrophotometer (Model: Jasco V-660).
UV/Visible). The slope of the linear part (the first 30 s) was considered to find out the rate of lysis, as in this region 10–20% of the substrate was lysed. The value of apparent specific absorbance ($\varepsilon_{450}$) of the M. luteus cell wall was taken as 0.656 mg/L (Khan et al., 2013). The weight of cells lysed per second per mol of lysozyme is defined as the rate of lysis of lysozyme. The substrate was directly taken in a glass cell with a 1 cm path length with concentrations ranging from 10 to 200 mg m$^{-1}$. The final volume of solutions was made to 3 ml with buffer. Readings were taken in the spectrophotometer at 25°C ± 0.1°C. A constant amount of lysozyme (0.45 mM) was added to initiate the reaction in all samples. The kinetic parameters $K_m$ and $V_{max}$ were calculated from Michaelis–Menten plots (Eq. 5),

$$v = \frac{V_{max} [S]}{(K_m + [S])}$$

where, the initial velocity is $v$, and the concentrations of the substrate are [S]. The product of enzyme concentration and $V_{max}$ gives the value of $k_{cat}$.

RESULTS AND DISCUSSION

The effect of DCF on the stability of lysozyme was investigated by measuring the heat-induced denaturation of lysozyme in the presence of different concentrations of DCF (5–20 µM) at various pH values (i.e., 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0). Figure 1 explains the representative thermal denaturation curves of lysozyme.

The differences in molar absorption coefficient changes were observed in $\Delta \varepsilon_{300}$ as a function of temperature. The values for $T_m$ and $\Delta H_m$ were analyzed using Eq. 1. There was no complete transition in the range from 20°C to 80°C at pH 5.0, 6.0, and 7.0. Hence, 2.0 M GdmCl was added to bring down the denaturation curves in the range that can be measured, and GdmCl effects were corrected using the earlier published method (Becktel and Schellman, 1987; Singh et al., 2005; Khan et al., 2013; Shahid et al., 2019; Chowhan et al., 2021). Table 1 shows the values of $T_m$ and $\Delta H_m$ at pH 5.0, 6.0, and 7.0 were corrected for the contribution of GdmCl.

It can be seen that $T_m$ decreases with an increase in the concentration of DCF at all pH values. This can also be observed in the shift of denaturation curves in Figure 1 toward the left side. This decrease in the $T_m$ is less at pH 2.0. The values of $T_m$ and $\Delta H_m$ (both at a specific molar concentration of DCF) at different pH values were plotted in a graph, and the slope of the straight line of the $T_m$ and $\Delta H_m$ (as specific molar concentrations of DCF) at different pH values gives the value of $\Delta C_p$ [i.e., $\Delta C_p = (\delta \Delta H_m/\delta T_m)$]. The $\Delta C_p$ values obtained here and those obtained from DSC measurements are in agreement with an earlier report by Makhatadze and Privalov (1993; Eskew and Benight, 2021). However, $T_m$ is not a good measure of protein stability as the stability ($\Delta G^{\circ}$) depends not only on $T_m$ but also

FIGURE 1 | Representative thermal denaturation curves of lysozyme in the absence and presence of different concentrations of DCF at different pH values. Symbols in figures: (●), (●), (△), (○), and (∇) represent 0, 5, 10, 15, and 20 µM of DCF, respectively.
TABLE 1 | Thermodynamic parameters of lysozyme in the presence of different concentrations of DCF at different pH values.a,b

| pH  | [DCF], μM | T_m  | ΔH_m | ΔG°p |
|-----|----------|------|------|------|
| 2.0 | 0        | 58.0 | 82   | 5.63 |
| 5   | 58.6     | 84   | 5.35 |
| 10  | 54.9     | 81   | 5.14 |
| 15  | 54.0     | 78   | 5.05 |
| 20  | 52.2     | 77   | 4.99 |
| 3.0 | 0        | 77.3 | 98   | 8.04 |
| 5   | 76.5     | 96   | 7.89 |
| 10  | 75.9     | 97   | 7.74 |
| 15  | 75.0     | 94   | 7.60 |
| 20  | 74.2     | 95   | 7.45 |
| 4.0 | 0        | 79.0 | 102  | 8.64 |
| 5   | 78.0     | 100  | 8.49 |
| 10  | 77.2     | 98   | 8.34 |
| 15  | 76.3     | 101  | 8.19 |
| 20  | 75.1     | 99   | 7.89 |
| 5.0 | 0        | 80.0 | 118  | 11.05|
| 5   | 78.4     | 117  | 10.77|
| 10  | 77.1     | 113  | 10.42|
| 15  | 76.0     | 114  | 10.08|
| 20  | 74.5     | 112  | 9.73 |
| 6.0 | 0        | 84.0 | 128  | 12.77|
| 5   | 82.0     | 126  | 12.31|
| 10  | 80.3     | 122  | 11.77|
| 15  | 78.1     | 119  | 11.35|
| 20  | 76.7     | 119  | 11.15|
| 7.0 | 0        | 86.0 | 128  | 13.00|
| 5   | 83.9     | 125  | 12.54|
| 10  | 82.1     | 126  | 12.16|
| 15  | 80.5     | 124  | 11.78|
| 20  | 78.7     | 125  | 11.24|

From triplicate measurements, values of maximum errors from the means are 0.2–0.5, 2–5, and 3–5% in T_m, ΔH_m, and ΔG°p, respectively.

ΔT_m, ΔH_m, and ΔG°p are in °C, kcal mol⁻¹, and kcal mol⁻¹, respectively.

on ΔC_p and ΔH_m. Therefore, we determined the ΔG°p values at different experimental conditions using Eq. 3. Values of ΔG°p at 25°C (i.e., ΔG°p) were calculated at all pH values with the help of the T_m, ΔH_m, and ΔC_p values with Eq. 3. The values for ΔG°p given in Table 1 shows that an increase in the concentration of DCF decreases the values of ΔG°p at all pH values and also that the destabilizing effect of DCF is less at pH 2.0 than at pH 7.0.

Since structure determines stability, this decrement in instability should also be reflected in the structure of lysozyme; hence, structural studies on lysozyme were carried out. Figure 2 represents the absorption spectra of lysozyme in the absence and presence of DCF at pH 7.0 and pH 2.0. Observing the changes in the tertiary structure, it can be seen that pH 2.0 demonstrates no change, while significant change was observed at pH 7.0.

Further, to analyze the effect of DCF on the secondary structure of lysozyme, far-UV CD experiments were conducted in the absence and presence of 20 μM DCF. Monitoring the secondary structure probe (222 nm), a significant change can be observed at pH 7.0, but there were no significant changes at pH 2.0 (Figure 3). The changes in the absorption spectra depend on side chains of chromophores, tyrosine, and tryptophan (Wetlaufer, 1963; Pignataro et al., 2020), while far-UV CD demonstrates changes in the peptide backbone conformation. Therefore, it can be concluded that DCF induces loss of structure of lysozyme at pH 7.0, which is also reflected in protein stability in terms of thermodynamic parameters.

To shed some light on the interaction of DCF with lysozyme, intrinsic fluorescence spectroscopy was carried out at pH 7.0 and pH 2.0. DCF concentrations in the range of 2–20 μM were used. The fluorescence emission spectra were recorded in the range of 315–500 nm with an excitation wavelength of 295 nm. The residues Trp 62 and Trp108 are the most dominant fluorophores present in this protein (Saha et al., 2018). Quenching was observed at both pH values, but the magnitude of quenching was less at pH 2.0 (Figure 4A and insets).

Earlier intrinsic fluorescence studies on various proteins have demonstrated that quenching of fluorescence intensity is an indicator of destabilization (Bansal et al., 2018). Hence, we can conclusively say that lysozyme gets destabilized in the presence of DCF. Our finding gets further support from the study conducted by Kenawi and coworkers, who implicated DCF’s ability to form hydrogen bonds and intermolecular charge transfer complex with proteins to be responsible for its destabilization (Kenawi et al., 2005; Bielecka et al., 2019; Paul et al., 2021). This phenomenon of quenching shows a linear decrease with an increase in DCF concentration. To validate this statement, relative fluorescence
intensities (RFI) at 355 nm were plotted against DCF concentration at both pH values, which shows that there is a decrease in the RFI values with the addition of DCF (Figure 4B).

However, our finding leads us to speculate that the pI value of lysozyme and DCF are 11 and 4, respectively. Therefore, at pH 7.0, lysozyme exists as a positively charged structure, while DCF remains as a negative entity. This difference in like charge leads to the electrostatic attraction between both, thus bringing DCF in the close vicinity of our protein. This interaction of DCF with lysozyme leads to structural changes that cause a decrease in the stability of lysozyme. However, at pH 2.0, both lysozyme and DCF exist as positively charged entities. Hence, an electrostatic repulsive force exists between the two entities, which allows a small amount of DCF to bind to the lysozyme. This results in less
TABLE 2 | Stability parameters of lysozyme in the presence of DCF at two pH values.

| pH  | [DCF], μM | ΔH° | TΔS° |
|-----|-----------|-----|------|
| 2.0 | 0         | 30.9| 25.2 |
| 5   | 33.3      | 27.9|      |
| 10  | 35.7      | 30.5|      |
| 15  | 37.1      | 32.5|      |
| 20  | 38.6      | 34.2|      |
| 3.0 | 0         | 13.8| 5.8  |
| 5   | 17.7      | 9.6 |      |
| 10  | 20.7      | 12.4|      |
| 15  | 23.0      | 14.8|      |
| 20  | 25.1      | 16.9|      |
| 4.0 | 0         | 15.1| 6.5  |
| 5   | 19.4      | 10.6|      |
| 10  | 22.7      | 13.9|      |
| 15  | 25.1      | 18.3|      |
| 20  | 26.9      | 18.2|      |
| 5.0 | 0         | 29.5| 14.4|
| 5   | 33.6      | 22.7|      |
| 10  | 36.9      | 26.0|      |
| 15  | 38.6      | 27.8|      |
| 20  | 40.7      | 30.2|      |
| 6.0 | 0         | 33.0| 20.2|
| 5   | 37.2      | 24.6|      |
| 10  | 40.1      | 21.7|      |
| 15  | 42.5      | 30.6|      |
| 20  | 44.6      | 33.0|      |
| 7.0 | 0         | 30.7| 21.3|
| 5   | 35.0      | 25.8|      |
| 10  | 39.2      | 30.1|      |
| 15  | 42.0      | 34.6|      |
| 20  | 43.8      | 39.5|      |

*ΔH° is in kcal mol⁻¹ and TΔS° is in kcal mol⁻¹ K⁻¹.

The extent of destabilization of lysozyme at acidic pH than at neutral pH.

The other way to explain the process of protein destabilization is through the contribution of enthalpy and entropy components, which play an important role in the thermodynamic stability. Enthalpy and entropy contribute to its stabilization in terms of neutral pH.

Using these values, a graph of ΔH° versus TΔS° is plotted in the presence of different molar concentrations of DCF (Figure 5), which shows that there is no perfect enthalpy entropy compensation at both pH values. Rather, it can be seen that TΔS° > ΔH°, hence, the process of destabilization is entropically driven. The interaction between GdmCl and DCF was ruled out, as there is a linear trend found in a decrease in T in the presence of different concentrations of DCF with a fixed amount of GdmCl. Our results suggest that the entropic contribution to the protein destabilization outweighs the enthalpic contribution, which is further supported by the destabilizing effect of TMAO in RNase A, which also shows that this destabilizing effect is under entropic control. This finding is further supported by the destabilizing effect of RNase A in the presence of TMAO, which is also under entropically control (Singh et al., 2005).

The thermodynamic quantities are just the physical parameters that need to be validated with biological function. Henceforth, our observations were validated by measuring the kinetic parameters K and kcat of lysozyme in the absence and presence of DCF at pH 7.0 (Table 3).

It can be seen that with the addition of DCF, K and kcat of lysozyme increases, while kcat is decreased. In the absence of DCF, the values of the enzymatic parameters of lysozyme agree with the earlier reports (Wetlaufer, 1963; Weir, 2002; Jamal et al., 2009; Khan et al., 2013; Antosiewicz and Shugar, 2016; Beltrán and Franco, 2019; Costa et al., 2019; Nambiar, 2019), and we assure that all the values obtained from this study are authentic and accurate. DCF destabilizes the lysozyme by shifting the denaturation equilibrium (native state ↔ denatured state) toward the right side because it has the ability to bind the enzyme (Langman et al., 1994; Grosser et al., 2006; Decherchi and Cavalli, 2020; Migliore et al., 2021). The above observation can be explained in the light of the change in the functionally native conformation of lysozyme at pH 7.0, and the presence of DCF leads to a change in the conformation of enzymes, making it inefficient/slow to complete the reaction. The change in the enzyme active site may be the subtle reason for the observation of K and kcat values. This is in complete agreement with the previously published data on other proteins (Khan et al., 2013; Dragan et al., 2019). Since the overall catalytic activity of an enzyme cannot be defined by kcat alone, the ratio of kcat and K (kcat/Km) refers to the reaction of free enzyme and free substrate (Jamal et al., 2009; Nambiar, 2019), so the parameter kcat/Km was calculated (Table 4). It can be seen that in the presence of DCF, the overall catalytic efficiency of lysozyme decreases. This effect shows that DCF affects the association, either through solvation effects on the substrate or enzyme active sites or their thermodynamic activities.

In our study, we found the destabilizing effect of DCF predominant at physiological pH, and this could be a reason that patients put on prolonged use of DCF have serious defects such as kidney damage and cardiovascular disorder. However, there is no direct evidence or reference. The hypothesis needs to be tested by conducting studies on a protein isolated from the...
heart, kidney, and stomach to understand the mechanism involved in the damage of these organs due to the DCF prolonged usage.

CONCLUSION

Taken together, our outcomes suggest that DCF reduces the stability of protein at the physiological pH (pH 7.0). This decrease in the stability of the molecule is also reflected in the loss of structure at both the tertiary and secondary levels of its organization. The study on lysozyme also demonstrated a loss of function at the physiological pH in the presence of DCF. However, at a low pH, DCF exhibits no such effect on its structure, stability, and function.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

SJ contributed to the concept and objective design of the study; MB performed the experiments; SJ and NA analyzed the results; and SJ, SK, and NA wrote the manuscript.

FUNDING

This work was supported by the Grant from SERB Department of Science and Technology, Govt. of India, File No-SB/YS/LS-38/2014. PI Dr. Shazia Jamal

ACKNOWLEDGMENTS

The authors thank Dr. Athi N. Naganathan, IIT Madras, India, for helping with UV CD instrument. MB is a recipient of JRF from DST. SK, NA, and SJ are Assistant Professor at BSACIST.

REFERENCES

Ajmal, M. R., Nusrat, S., Alam, P., Zaidi, N., Khan, M. V., Zaman, M., et al. (2017a). Interaction of Anticancer Drug Clofarabine with Human Serum Albumin and Human α-1 Acid Glycoprotein. Spectroscopic and Molecular Docking Approach. *J. Pharm. Biomed. Analysis* 135, 106–115. doi:10.1016/j.jpba.2016.12.001

Antosiewicz, J. M., and Shugar, D. (2016). UV-vis Spectroscopy of Tyrosine Side-Groups in Studies of Protein Structure. Part 2: Selected Applications. *Biophys. Rev.* 8, 163–177. doi:10.1007/s12551-016-0197-7

Babu, M. M., van der Lee, R., de Groot, N. S., and Gsponer, J. (2011). Intrinsically Disordered Proteins: Regulation and Disease. *Curr. Opin. Struct. Biol.* 21, 432–440. doi:10.1016/j.sbi.2011.03.011

Baigent, C., Bhala, N., Emberson, J., Merhi, A., Abramson, S., Arber, N., et al. (2013). Vascular and Upper Gastrointestinal Effects of Non-steroidal Anti-

**TABLE 4** | Activity parameters of lysozyme in the absence and presence of DCF at pH 7.0 and 25°C.

| [DCF], μM | Km (μg ml⁻¹) | Kcat (mg ml⁻¹ s⁻¹ M⁻¹) | Kcat/Km |
|-----------|-------------|-----------------|--------|
| 0         | 77.8 ± 2    | 484.1 ± 29      | 6.22   |
| 5         | 86.7 ± 3    | 448.9 ± 21      | 5.16   |
| 10        | 92.5 ± 2    | 412.1 ± 24      | 4.45   |
| 15        | 95.3 ± 3    | 371.8 ± 26      | 3.91   |
| 20        | 104.8 ± 4   | 340.0 ± 27      | 3.22   |
