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Original Article

# Glutaredoxin proteins from *E. coli* isoforms were compared in terms of energy frustration

## Proteínas glutaredoxinas de isoformas de *E. coli* foram comparadas em termos de frustração de energia

A. Patel[a], K. Tiwari[a,b], P. Asrani[b], H. Alothaid[c], A. F. A. Alahmari[d], R. Mirdad[e], M. R. Ajmal[f] and M. Tarique[g*]

[a]King Khalid University, College of Medicine, Department of Clinical Biochemistry, Abha, Kingdom of Saudi Arabia
[b]Amity University, Amity Institute of Microbial Biotechnology, Noida, UP, India
[c]Al Baha University, Faculty of Applied Medical Sciences, Department of Basic Medical Sciences, Al Baha, Al Baha Province, Saudi Arabia
[d]King Khalid University, College of Medicine, Department of Clinical Biochemistry, Abha, Saudi Arabia
[e]King Khalid University, Department of Surgery, Abha, Saudi Arabia
[f]University of Tabuk, Faculty of Science, Biochemistry Department, Physical Biochemistry Research Laboratory, Tabuk, Saudi Arabia
[g]Almanac Life Science India Private Limited, New Delhi, India

**Abstract**

Glutaredoxin (GRXs) protein plays a vital role inside the cell, including redox control of transcription to the cell's antioxidant defense, apoptosis, and cellular differentiation regulation. In this study, we have investigated the energy landscape and characterized the pattern of local frustration in different forms and states of the GRX protein of *E. coli.* Analysis was done on the conformational alterations, significant changes in the frustration pattern, and different GRXs such as GRX-II, GRX-III, GRX-II-GSH, and GRX-III-GSH complex. We have found the practice of frustration, and structure was quite similar in the same isoform having different states of protein; however, a significant difference was observed between different isoforms. Moreover, oxidation of GRX-I introduced an extra α-helix increasing the destabilizing interactions within the protein. The study of frustrated contacts on oxidized and reduced GRX and with bound and unbound Glutathione indicates its potential application in activating and regulating the behavior of GRXs.

**Keywords:** glutaredoxins, frustration index, protein stability, structure-function relationships, bioinformatics, structural biology.

**Resumo**

A proteína glutaredoxina (GRXs) desempenha um papel vital dentro da célula, incluindo o controle redox da transcrição para a defesa antioxidante da célula, apoptose e regulação da diferenciação celular. Neste estudo, investigamos a paisagem energética e caracterizamos o padrão de frustração local em diferentes formas e estados da proteína GRX de *E. coli*. A análise feita foi sobre as alterações conformacionais, mudanças significativas no padrão de frustração e diferentes GRXs, como GRX-II, GRX-III, GRX-II-GSH e complexo GRX-III-GSH. Encontramos a prática da frustração, e a estrutura era bastante semelhante na mesma isoforma com diferentes estados de proteína; no entanto, uma diferença significativa foi observada entre diferentes isoformas. Além disso, a oxidação de GRX-I introduziu uma α-hélice extra, aumentando as interações desestabilizadoras dentro da proteína. O estudo de contatos frustrados em GRX oxidado e reduzido e com glutationa ligada e não ligada indica sua potencial aplicação na ativação e regulação do comportamento de GRXs.

**Palavras-chave:** glutaredoxinas, índice de frustração, estabilidade da proteína, relações estrutura-função, bioinformática, biologia estrutural.

## 1. Introduction

Disulfide bond plays a vital role in determining the structure and function of proteins (Doig and Williams, 1991); (Creighton, 1992). These bonds are rare to form in cytoplasmic proteins because of the reducing environment (Schulz and Schirmer, 1979). Glutaredoxin (GRXs) is one such enzyme that is responsible for reduction of such bonds in the cytoplasm. They are heat stable in nature and are smaller in size with a low molecular weight of around 9-15 kDa. Multiple isoforms of GRXs are present inside the cell having glutathione (GSH) system and belonging to a superfamily of thiol-disulfide exchange catalyst (Berndt et al., 2008). The sequence of the protein

*e-mail: tariqueaiims@gmail.com
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contains signal sequence that allows them to attain a unique conformation. These interactions enable the isoforms of the proteins to obtain different structure but identical energetic conformations (Husain et al., 2018).

*E. coli* possess reduced form of both thioredoxins (TRX) and GRX in its cytoplasm to reduce the disulfide bonds. TRX pathway consists of TRX and thioredoxin reductase whereas GRX pathways have glutathione, glutathione reducatase, GRX-I, GRX-II and GRX-III (Holmgren, 1985). Within the superfamily of TRX, sequence resemblance is low but a common structural motif called TRX fold is present. This TRX fold comprises of three flanking α-helices, four strands of β-sheets and an active site which is analogous and essential for their redox activities (Martin, 1995).

GRXs plays multiple role inside the cell such as reduction of protein disulfides by means of GSH, providing reduction of equivalents for ribonucleotide reductase, control of cellular redox state, antioxidant defense, the redox control of transcription (Bandyopadhyay et al., 1998; Nakamura et al., 1999; Hirota et al., 2000), signal transduction (Herrero and de la Torre-Ruiz, 2007; Lillig et al., 2008), dehydro ascorbate reduction (Wells et al., 1990), apoptosis (Chrestensen et al., 2000; Daily et al., 2001), regulation of cellular differentiation (Takashima et al., 1999), and represent the main enzyme class responsible for deglutathionylation reactions (Ströher and Millar, 2012). GRXs also functions as electron donors and regulators of cellular function in response to oxidative stress such as in sulfur assimilation (Lillig et al., 1999; Berndt et al., 2004). Human GRXs are involved in many diseases (Lillig and Holmgren, 2007; Berndt et al., 2007). A typical mechanism of Glutaredoxin involves the reduction of oxidized Glutaredoxin by glutathione which in turn is reduced by glutathione reductase.

Protein folding is a complex phenomenon that involves the movement and coordination of different amino acid residues and atoms to obtain a native three-dimensional (3-D) structure (Clementi, 2008; Best et al., 2013). Theory of energy landscape states that a protein molecule tends to organize itself in number of intermediate structures with a flow of energy rather than directly assembling into a native conformation (Bryngelson and Wolynes, 1987). Within these intermediate structures, amino acid residues tend to interact with each other through minimally and highly frustrated contacts. Frustration refers to the tendency of amino acid residues for exhibiting the non-native interactions (Huynh et al., 2017). The protein folding mechanisms are guided by the native contacts (minimally frustrated and highly frustrated) among the amino acid residues (Clementi et al., 2000). In these studies, we calculate and compare the energy contribution of particular amino acid residues of a native protein to that of extra energy contributed by different set of amino acid residues in the same native environment (mutational frustration index) or by placing the residues in different local environment along with an interacting pair (configurational frustration index) (Parra et al., 2016). If this extra energy is sufficiently stabilizing in nature and normalizes with the energy fluctuation for formation of a native 3-D structure of a protein, then the local environment is referred to as minimally frustrated however; if this interaction is destabilizing in nature, then the local environment is referred to as highly frustrated (Ferreiro et al., 2014). Thus, amino acid residue tends to adopt those alternative intermediate structures in attaining the native conformation of a protein where minimum frustration contacts are present (Kluber et al., 2018). Previous studies on frustration of redundant protein reveals that 40% of native interactions in protein domain are minimally frustrated, 10% are highly frustrated interactions clustered on the protein surface. Remaining 50% represent neutral contacts which are randomly distributed in the protein structure (Ferreiro et al., 2007).

Different work propensity of various forms of GRXs and its interaction with GSH makes it a growing interest using computational studies to understand the role of local frustration on its energy landscape and functionality (Hasanuzzaman et al., 2017; Aravind and Prasad, 2005; Bashandy et al., 2010). In this paper, we tried to know the similarity and differences between various isoforms of GRXs, their states comparison (oxidized/reduced) and their interaction with GSH in context to energy landscape theory. To investigate the role of frustration and conformation changes we will use the algorithm to compute local frustration in all residues of protein (Hazlett, 2003). This study will focus on the theoretical mechanism of the effect of GRXs interaction with GSH as well as energy landscape of different isoforms of GRXs. The proteins under study despite having different lengths belongs to a similar domain of thioredoxin like CLAN family. Our comparisons are restricted up to the first 100 amino acid residues because the CLAN similarity exists up to these residues only.

## 2. Methods

### *2.1. Structure analysis*

We examined the structure of different forms of GRXs and their interaction with GSH from *E.coli*. The different structures that we took into the consideration are, oxidized GRX-I (PDB ID: 1GRX), reduced GRX-I (PDB ID: 1EGR), oxidized GRX-III (PDB ID: 1FOV), reduced GRX-II (PDB ID: 1G7O), oxidized GRX-III complex with GSH (GRX-III-GSH) (PDB ID: 3GRX) and GRX-II complex with GSH (GRX-II-GSH) (PDB ID: 4KX4). Atomic coordinates for all these proteins were also taken from PDB. Before taking the atomic coordinates, structure was extensively analyzed to check missing residues. In addition, we have minimized the energy using SPDBv tool to get quality structure for frustration analysis. The bond angle and distance were calculated using PyMol software. The calculation of bond distance is used to describe intramolecular hydrogen-bonded interactions.

### *2.2. Residual local frustration analysis*

We employed the algorithm to calculate the residual frustration in different GRXs structure. This frustratometer server is based on algorithm which analyzes and quantifies the location of frustration inside the protein molecules. We computed the 'single residual frustration index' and 'configurational frustration index' using the frustratometer server.

The frustration index determines the favorability of a particular contact relative to the set of all possible contacts in a particular normalized location by using the variance of that distribution (Shea et al., 2000). This server works by estimating the energy of a protein structure and comparing it to the energies of a set of 'decoy' states. i was defined as the residual frustration index for the contact between amino acids, whereas j was defined as a Z-score of the energy of the native pair when compared to N decoys. A native contact (or residue) was considered as minimally frustrated when its Z-score was > (+1.0). Eventually a native contact (or residue) was defined as highly frustrated when its Z-score was < (-1.0). However, if the native energy lies between these two limits given above then the native contact (or residue) was defined as neutral.

## 3. Results and discussion

### *3.1. Comparison of oxidized GRX-I with reduced GRX-I*

We examined the local frustration and characterized the network of highly and minimally frustrated interactions responsible for structural stability and activation to investigate the role of local frustration in conformation transitions between oxidized GRX-I (Figure 1A, B) (inactive) and reduced GRX-I (Figure 1C, D) (active). Configurational frustration index categorized every individual native contact presents in the protein structure according to their frustration level and it was used to measure relative stability of a particular native contact corresponding to all possible contacts in that particular site.

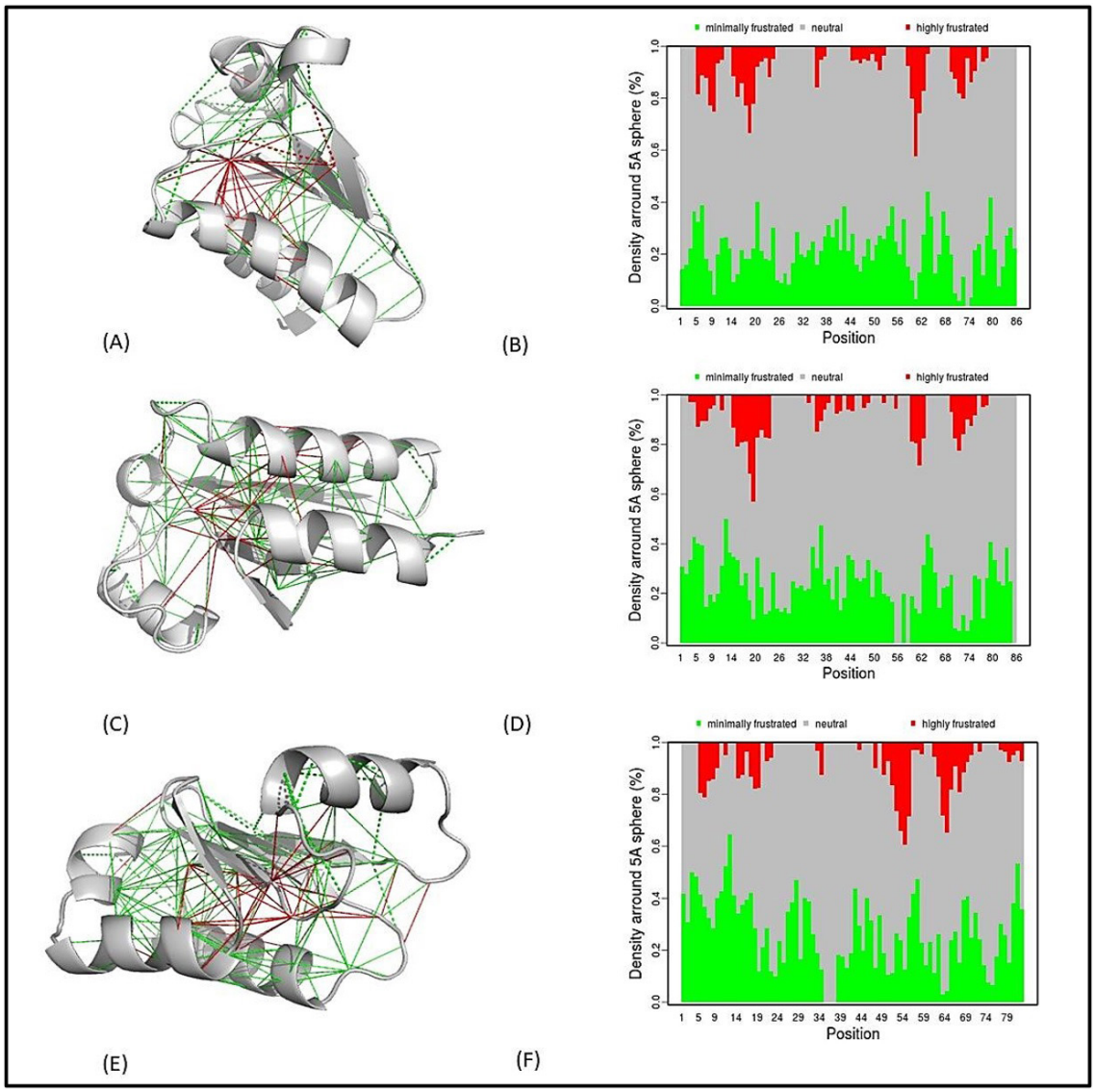


**Figure 1.** Frustration in oxidized GRX-I, reduced GRX-I and oxidized GRX-III. Localized frustration network (A), highly frustrated and minimally frustrated residues of oxidized GRX-I (PDB id: 1GRX) (B). Localized frustration network (C), highly frustrated and minimally frustrated residues of reduced GRX-I (PDB id: 1EGR) (D). Localized frustration network (E), highly frustrated and minimally frustrated residues of Oxidized GRX-III (PDB id: 1FOV) (F). Both minimally and highly frustrated interactions are shown in green and red, respectively.

Considering the previous estimated cutoff value of frustration indices, we came to know that oxidized GRX-I protein is less minimally frustrated i.e. 17% than the typical nonredundant globular protein which is about 40% minimally frustrated (Table 1 and Table S1). We also observed various highly frustrated contacts in Oxidized GRX-I which is about 8% of the total frustrated residues. Reduced GRX-I showed 19% minimally frustrated contacts whereas 7% contacts in the protein was highly frustrated. Unlike most globular protein, highly frustrated contacts in oxidized GRX-I and reduced GRX-I do forms a cluster spanning core of the protein whereas minimally frustrated contacts cover the surface of the protein (Figure 1).

The source of generation of highly frustrated contacts in oxidized GRX-I is clearly visible in the Figure 1. Residues from 14 to 21 which are component of α1-helixhave highly frustrated contacts with Pro 60, which is also the center of origin of these contacts. On the other hand, in reduced GRX-I the center of the origin of highly frustrated contacts was at the same residue Pro 60 but lies at the junction of loop and β-strand. The calculated frustrated value in oxidized GRX-I ranges between -4 to +3 whereas in reduced GRX-I it was from -3 to +2. The distribution of frustration intensity and its range value is visible in the frustration chart. The frustration value is the z-score of a particular minimally or highly frustrated contact (Tzul et al., 2017) however; frustration intensity is the peak of minimally or highly frustrated value obtained at a particular amino acid residue when observed around 5 Å sphere (Ferreiro et al., 2014). The frustration intensity is much higher at position 5-13 and 57-64 in chart depicting oxidized GRX-1 as compared to the chart describing reduced GRX-I (Figure 1). Moreover, residue from 4 to 15 was abundant of minimally frustrated contacts in both oxidized and reduced GRX-I and in both proteins, these residues accounted for some part of β-strand as well as α-helix.

The single residual frustration indices value of oxidized and reduced GRX-I at the level of amino acid has been outlaid (Table S1). Considering the frustration cutoff value, we can observe changes in frustration indices between some of residues such as from 28 to 35 of both oxidized and reduced GRX-I differ from each other. These residues lie in the bend and β2-strand. In oxidized GRX-I at position 29 and 35 which is aspartic acid and tyrosine, respectively, showed highly frustrated contacts whereas its counterpart reduced GRX-I showed no frustration (neutral) at both positions. In contrast to the highly frustrated contacts, residue no. 31 of oxidized GRX-I which is phenylalanine showed minimally frustrated contacts and reduced GRX-I had no frustration at that position. Apart from this, residues from 55 to 57 (loop) showed differences in frustration. In reduced GRX-I residue no. 55 and 57 which is proline and glutamic acid showed highly and minimally frustration respectively but their counterpart oxidized GRX-I was neutral and rather have minimally frustration residue at position 56 (Valine) which is neutral in reduced GRX-I.

### *3.2. Local frustration across the contact maps of GRX-I*

At the residue-residue contact level, we further calculated the configurational frustration indices of GRX-I (Figure 2). Frustratometer server was used to visualize the effect of oxidation by constructing the residue-residue contact maps of oxidized and reduced GRX-I. In both states (oxidized-reduced), frustration distribution was similar which were dominated by minimally frustrated interaction. Considerable amount of overlap of contacts between both states of GRX-I was displayed in the contact maps network and oxidation causes a minor difference in contact networks. When oxidized and reduced GRX-I were compared with each other, we came to know that oxidation minimizes the highly frustrated contacts in α1-helix (residues 12-20) significantly as well as in residues 50-62.

One of the major changes in the structure of GRX-I is that oxidation induces an extra α-helix at the residues number 38-41 which was earlier a turn. The main feature after the formation of an extra α-helix in the context of frustration contacts is that it increases the propensity of highly frustrated contacts at Glu41. The introduction of new α-helix in oxidized form is thought to be responsible for structural stability to perform reduction of disulfide bond as well as ribonucleotide reductase.

### *3.3. The energy landscape and local frustration*

In this comparison, both GRX-I (PDB ID: 1EGR) and GRX-II (PDB ID: 1G7O) are in their active form which means we have compared the energy landscape of two isoform of same class of protein which is present in the same state. Standard cut-off value of frustration index was taken into the consideration to evaluate the configurational frustration present in the each of the native contact of protein.

**Table 1.** Frustration distribution according to configurational frustration index in different GRXs of *E. coli*.

| PDB ID | Protein | No. of amino acids residues | No. of frustrated contacts | Highly frustrated contacts (H) % | Minimally frustrated contacts (M) % |
|---|---|---|---|---|---|
| 1GRX | Oxidized GRX-I | 85 | 465 | 8 | 17 |
| 1EGR | Reduced GRX-I | 85 | 470 | 7 | 19 |
| 1G7O | Reduced GRX-II | 215 | 1383 | 11 | 23 |
| 4KX4 | GRX-II-GSH | 217 | 1342 | 11 | 23 |
| 1FOV | GRX-III | 82 | 478 | 9 | 25 |
| 3GRX | GRX-III-GSH | 82 | 474 | 12 | 20 |

The percentage of the contacts showing highly and minimally frustrated interactions in; oxidized GRX-I (1GRX), reduced GRX-I (1EGR), reduced GRX-II (1G7O), oxidized GRX-III (1FOV), GRX-III-GSH (3GRX) and GRX-II-GSH (4KX4).

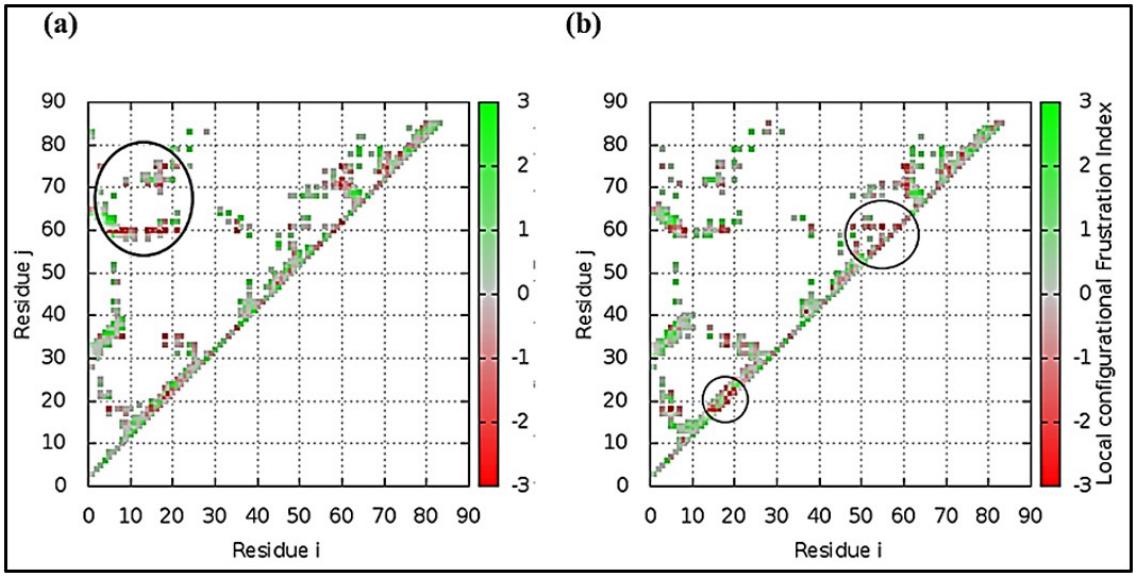


**Figure 2.** Contact maps of GRX-I in (a) oxidized state and (b) reduced state. Minimally frustrated and highly frustrated contacts are shown in green and red respectively. The distinct contacts in the oxidized GRX-I compared with reduced GRX-I are circled black.

The cutoff value index to categorize frustration was < -1 for highly frustrated contacts whereas for minimally frustrated contacts it was > +1. According to this cutoff value of frustration indices, reduced GRX-I has 7% highly frustrated region as compared to the reduced GRX-II which has around 11% of highly frustrated contacts in total protein. Both reduced GRX-I and reduced GRX-II have 19% and 23% minimally frustrated contacts respectively (Table 1).

In reduced GRX-I (Figure 3A, B), highly frustrated contacts do form a cluster in the core of protein structure and minimally frustrated contacts surrounded the surface of protein structure however; in reduced GRX-II (Figure 3C, D), the frustration distribution was random where most of the surface and core of protein structure was occupied by minimally frustrated contacts and highly frustrated contacts covers a little area both in core and on the surface. While in reduced GRX-I the center of minimally frustrated contacts was at joint of loop and β-strand at Pro6o, it was difficult in reduced GRX-II to point out one center of origin because of the complex matrix. Thus, majority of frustrated contacts were originated at two points, one in the loop at Lys21 and another one in the α3-helix at Lys 81. Frustration value of reduced GRX-I was in range between -3 to +2 whereas for reduced GRX-II the range was -4 to +3. Because of the huge difference in the size of both proteins, we measured the chart of frustration distribution differently. We only considered same number of residues in both protein and then pointed the differences. Intensity of highly frustrated contacts in reduced GRX-I at position from 24 to 26 was much higher when it was compared to its counterpart. In contrast, there was a humongous peak of minimally frustrated contacts at position 50-60 in reduced GRX-II (Figure 3).

Observing the single residual frustration indices of reduced GRX-I at amino acid level (Table S1), N-terminal β1-strand (2-7), β2-strand (32-37), and α1-helix (14-27) of protein is found to be occupied predominantly by minimally frustrated and neutral residues. In case of reduced GRX-II, N-terminal region shows minimally frustrated residues while C-terminal mainly has highly frustrated residues. Moreover, middle portion of protein is abundant by highly frustrated residues. In α 4-helix (84-107) a region (99-103) have minimally frustrated residues which is flanked by highly frustrated residues. In contrast to this, α5-helix (111-113) has highly frustrated residues flanked by minimally frustrated residues.

### *3.4. Local frustration across the contact maps of reduced GRX-I & II*

Calculating the frustration indices using frustratometer server at the level of residue-residue contact, we came to know that frustration distribution in both the proteins are lot different. Reduced GRX-II has much greater number of residues (more than 200) than the reduced GRX-I and this is the reason which increases the difference between the two a bit higher. The contact maps of reduced GRX-I show residues from 18-23 having highly frustrated contacts with residues 13-20 (Figure 4). Similar kind of highly frustrated contacts was found between the residues from 50-75 also. The comparison in contacts maps was visualized only through the residue no. 100 in both proteins as the length of reduced GRX-I was smaller. The frustration distribution in the contact maps of reduced GRX-II was symmetrical along with the map. The majority of contacts showed in the map till 100 residues were minimally frustrated contacts. If we proceed for the further mapping, we would be able to see some highly frustrated contacts.

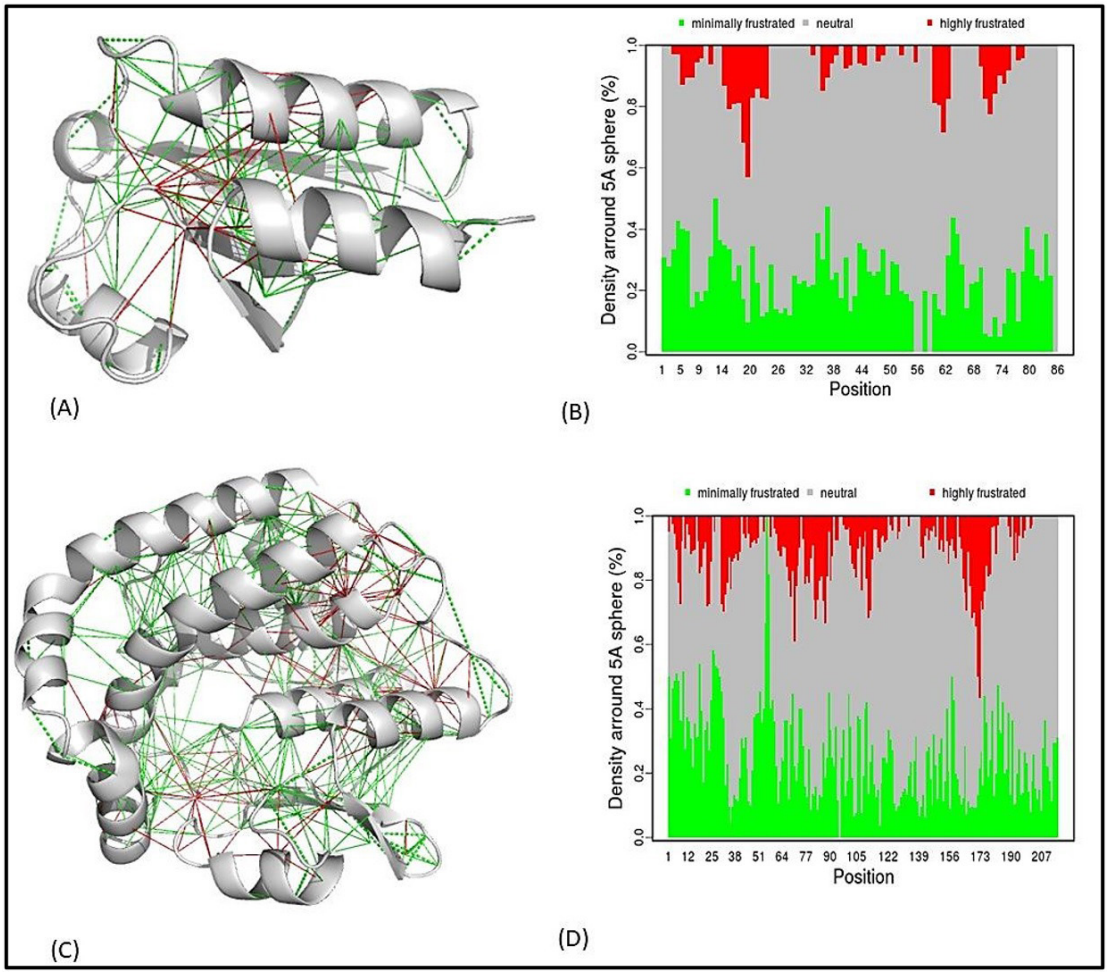


**Figure 3.** Frustration in reduced GRX-I and reduced GRX-II. Localized frustration network (A), Highly frustrated and minimally frustrated residues of GRX-I (PDB id: 1EGR) (B). Localized frustration network (C), highly frustrated and minimally frustrated residues of reduced GRX-II (PDB id: 1G7O) (D). Both minimally and highly frustrated interactions are shown in green and red, respectively.

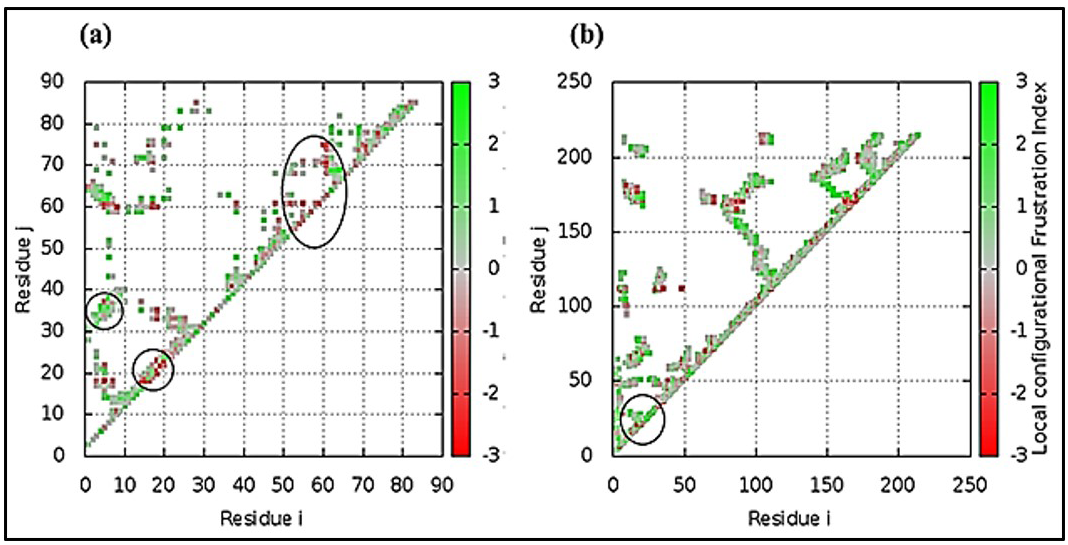


**Figure 4.** Contact maps of GRX-I in (a) reduced state and (b) GRX-II reduced state. Minimally frustrated and highly frustrated contacts are shown in green and red respectively. The distinct contacts in the oxidized GRX-I compared with reduced GRX-I are circled black.

### *3.5. Oxidized GRX-I with oxidized GRX-III*

#### *3.5.1. The energy landscape and local frustration*

The result of this comparison is discussed in the context of energy landscape theory similar to the previous one. We characterized the highly and minimally frustrated interactions in these two different isoforms of the same protein to find out the role of local frustration in localizing similarity and differences in the conformation of both inactive proteins i.e. Oxidized GRX-I (PDB ID: 1GRX) with Oxidized GRX-III (PDB ID: 1FOV). Accounting the used cutoff value of frustration index, we calculated that oxidized GRX-I and Oxidized GRX-III had 17% and 25% minimally frustrated contacts respectively. Highly frustrated contacts accounted for 8% for oxidized GRX-I and 12% for oxidized GRX-III (Table 1).

Highly frustrated contacts of Oxidized GRX-I protein (Figure 1A, B) do forms clusters in the core of the protein structure and minimally frustrated contacts extended over the surface of protein structure whereas in the oxidized GRX-III (Figure 1E, F), one side of protein structure is dominant by minimally frustrated contacts from core to one-side surface and another side highly frustrated contacts resides from core to the surface. Center of origin of highly frustrated contacts and distribution of frustrated contacts of oxidized GRX-I have already been discussed. In oxidized GRX-III the origin of minimally frustrated contacts is at Val 3 which is component of β1-strand. Gln 54 of β3-strand is the center for the origin of majority of highly frustrated contacts. The frustration values in Oxidized GRX-I range between -4 to +3 and for Oxidized GRX-III, range was -3 to +3. In the frustration distribution chart, the difference in intensity of frustration in oxidized GRX-I & III revealed that contacts at position from 63-69 is very high in oxidized GRX-III when compared to the oxidized GRX-I (Figure 1B). In contrast, in oxidized GRX-III the intensity of minimally frustrated contacts at position 9-14 is much higher than the oxidized GRX-I (Figure 1F).

When we examined single residual frustration of protein at amino acid level, we came to know that N-terminal of oxidized GRX-I is dominated by minimally frustrated residues whereas highly frustrated residues randomly distributed in middle portion and C-terminal of protein (Table S1). Residues 4, 5 and 6 (β1-strand) have multiple minimally frustrated contacts with different amino acids. Distribution of minimally and highly frustrated residues in oxidized GRX-III was random throughout in both N-terminal and C-terminal but the middle portion of protein (33-57) is mainly composed of neutral and some of minimally frustrated residues. Ser14 and Lys18 have multiple highly frustrated contacts throughout with different amino acids.

#### *3.5.2. Local frustration across the contact maps of oxidized GRX-I & GRX-III*

When we further calculated the frustration indices at the residue-residue level of contact maps using frustratometer server, we came to know that frustration distribution of both proteins is quite similar which is dominated by minimally frustration contacts however distribution of highly frustrated contacts were a little different in both the proteins. In the contact maps of oxidized GRX-I, it was displayed that residue no. 60 has highly frustrated contacts with residues from no. 5-22. Similar kind of contacts was visible in the oxidized GRX-III. In this case residue no. 52 and 53 had highly frustrated contacts with residues from 7-19. Contact pattern of Oxidized GRX-III which were totally different from Oxidized GRX-I was the highly frustrated region from 45-50 residues. The contact maps are highlighted and the difference of frustration at residue-residue level has been shown in the Figure 5.

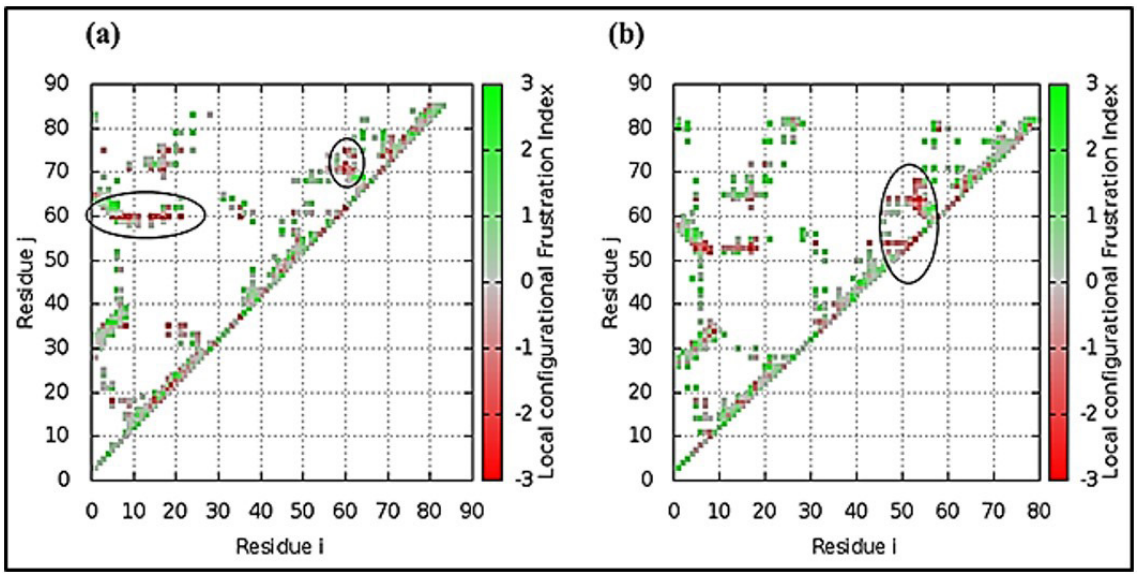


**Figure 5.** Contact maps of (a) GRX-I in oxidized state and (b) GRX-III in oxidized state. Minimally frustrated and highly frustrated contacts are shown in green and red respectively. The distinct contacts in the oxidized GRX-I compared with reduced GRX-I are circled black.

*3.5.3. Oxidized GRX-III with GRX-III-GSH complex*

Glutathione (GSH) is responsible for the reduction and activation of GRX using NADPH as a reducing agent. Hence the binding of GSH with GRX can provide different frustration pattern than the unbound GRX. In this part we will look up to the frustration pattern in oxidized GRX-III unbound (inactive) and in GRX-III-GSH (bound) complex.

*3.5.4. The energy landscape and local frustration*

Characterizing the highly and minimally energy frustration interactions in oxidized GRX-III (PDB ID:1FOV) and GRX-III-GSH complex (PDB ID: 3GRX) using frustratometer server measuring the data in energy landscape theory leads to the information that frustration pattern in both the proteins are quite similar. Considering the frustration cutoff value, we came to know that oxidized GRX-III had 25% of total contacts as a minimally frustrated whereas the value of highly frustrated contacts was 9% which is a bit lower than the typical globular protein. Making complex with Glutathione (GRX-III-GSH) decreases the minimally frustrated contacts. In GRX-III-GSH complex, the number of minimally frustrated contacts is 20% and highly frustrated contacts are 12% a bit higher than the globular protein (Table 1). This data indicates that binding of GSH decreases the number of minimally frustrated contacts and in contrast increases the number of highly frustrated contacts.

The distribution of energy frustration in the structure of both the proteins is throughout similar except the difference in number of contacts. In both oxidized GRX-III (Figure 6E, F) and GRX-III-GSH complex (Figure 6C, D),

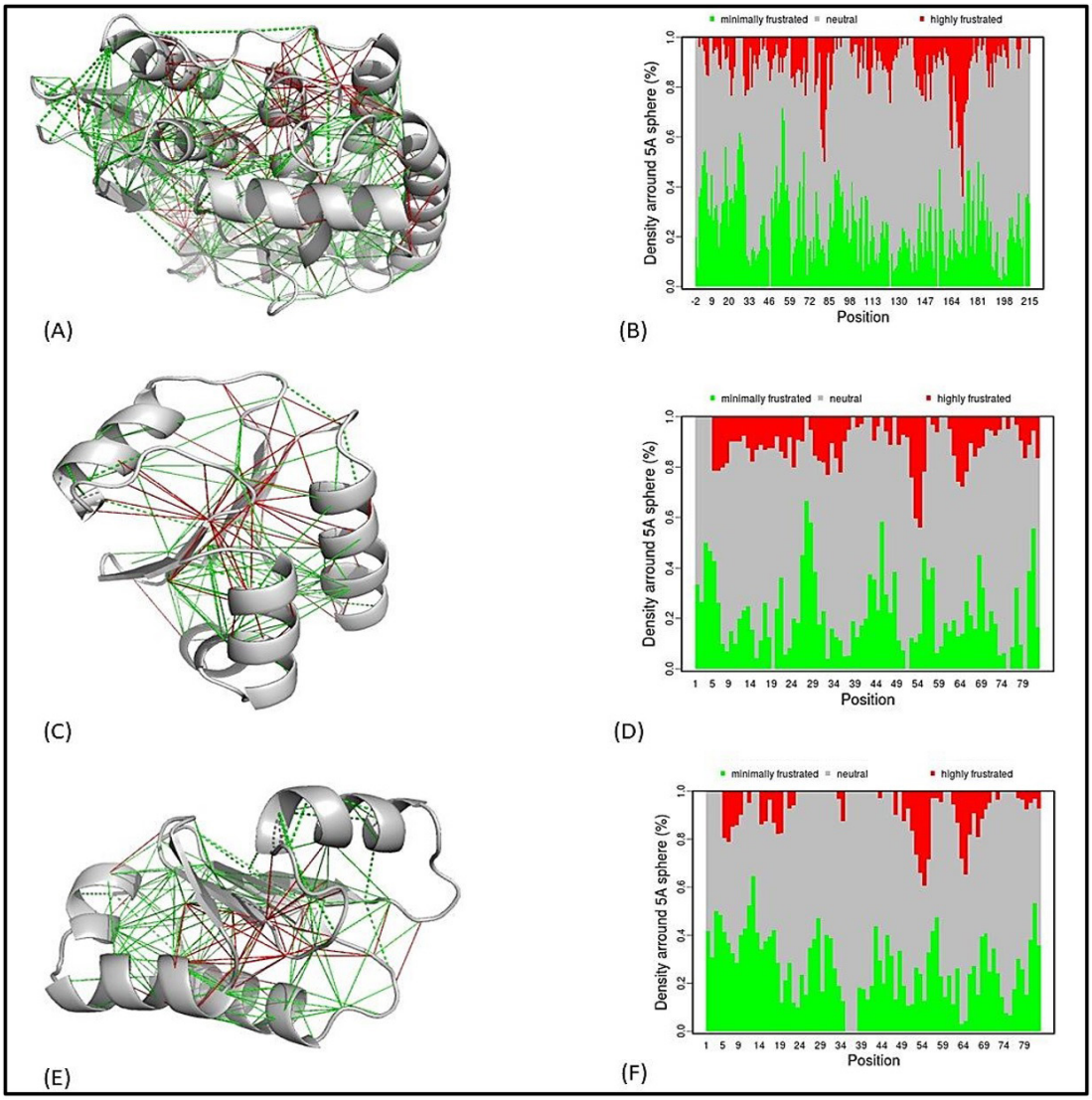


**Figure 6.** Frustration in GRX-II-GSH complex, GRX-III-GSH complex and oxidized GRX-III. Localized frustration network (A), highly frustrated and minimally frustrated residues of GRX-II-GSH (PDB id: 4KX4) (B). Localized frustration network (C), highly frustrated and minimally frustrated residues of GRX-III-GSH (PDB id: 1EGR) (D). Localized frustration network (E), highly frustrated and minimally frustrated residues of Oxidized GRX-III (PDB id: 1FOV) (F). Both minimally and highly frustrated interactions are shown in green and red, respectively.

the distribution of minimally frustrated contacts started from core of the protein and expands over one side of protein structure. A similarly highly frustrated contacts form cluster in the core of protein covers another side of the surface of protein. The origin of minimally and highly frustrated contacts in oxidized GRX-III has already been discussed in the previous result. In this discussion, we will be looking for the pattern and origin of distribution in GRX-III-GSH complex. Multiple origins were found for minimally frustrated contacts in GRX-III-GSH complex but Val3 which lies in the β1-strand is main because of majority of contacts. For highly frustrated contacts, we found it similar to oxidized GRX-III which is Gln54.

Frustration indices for both protein ranges between approx. -3 to +3 for oxidized GRX-III and GRX-III-GSH. In the chart of the energy distribution of GRX-III and GRX-III-GSH, we saw that distribution of minimally frustrated contacts and highly frustrated contacts were different. In oxidized GRX-III at position from 5-50 the highly frustrated contacts were widespread whereas in GRX-III-GSH it was quite absent. Similarly, minimally frustrated contacts at position 9-14 in oxidized GRX-III were at peak as compared to GRX-III-GSH (Figure 7).

Comparison of single residual frustration between Oxidized GRX-III and GRX-III-GSH complex at amino acid level has been shown (Table S1). Highly frustrated residues at the C-terminal of oxidized GRX-III (Asp 78 and Pro 79) which contributed in α4-helix loses its frustration upon complex with glutathione. Pro 32 and Met 43 (α2-helix) which are highly and minimally frustrated residues respectively, loses its frustration and become neutral after binding of glutathione. Ser14 (α1-helix) and Ala37 (loop) were the only residues where frustration was gained (minimally and highly frustrated respectively) after formation of complex.

*3.5.5. Local frustration across the contact maps of oxidized GRX-III and GRX-III-GSH complex*

Further visualization of contact maps at residue-residue level using frustratometer server was done and it was a bit difficult to find out the difference in the two contacts maps. Distribution of both minimally and highly frustrated contacts in the maps was highly similar. Although at some position the propensity of frustration was a bit higher, such as highly frustrated contact of residue 54 with residues 43-50 in GRX-III-GSH (Figure 7b). In addition, distribution of highly frustrated contacts at position 20, 25, 29, 31 and 35 in GRX-III-GSH accounted for the higher number of contacts than the oxidized GRX-III.

*3.5.6. GRX-III-GSH with GRX-II-GSH*

In this part we will discuss and compare about two different isoforms of same protein in a same form of state, their differences in interaction with glutathione and furthermore. The two proteins are GRX-III-GSH (PDB ID: 3GRX) and GRX-II-GSH (PDB ID: 4KX4). However, because of the large structure of GRX-II protein, the comparison would be made on only equal number of residues.

*3.5.7. The energy landscape and local frustration*

Frustratometer server provided the information required to calculate the energy frustration of these two proteins. The cutoff value to calculate the frustration value was same as for the previous ones. Upon using the frustratometer server we calculated that GRX-III-GSH has 20% minimally frustrated contacts and 12% highly frustrated contacts as discussed earlier. The number of minimally frustrated contacts in the GRX-II-GSH is higher i.e. 23% compare to the other one and highly frustrated contacts accounted for 11% (Table 1).

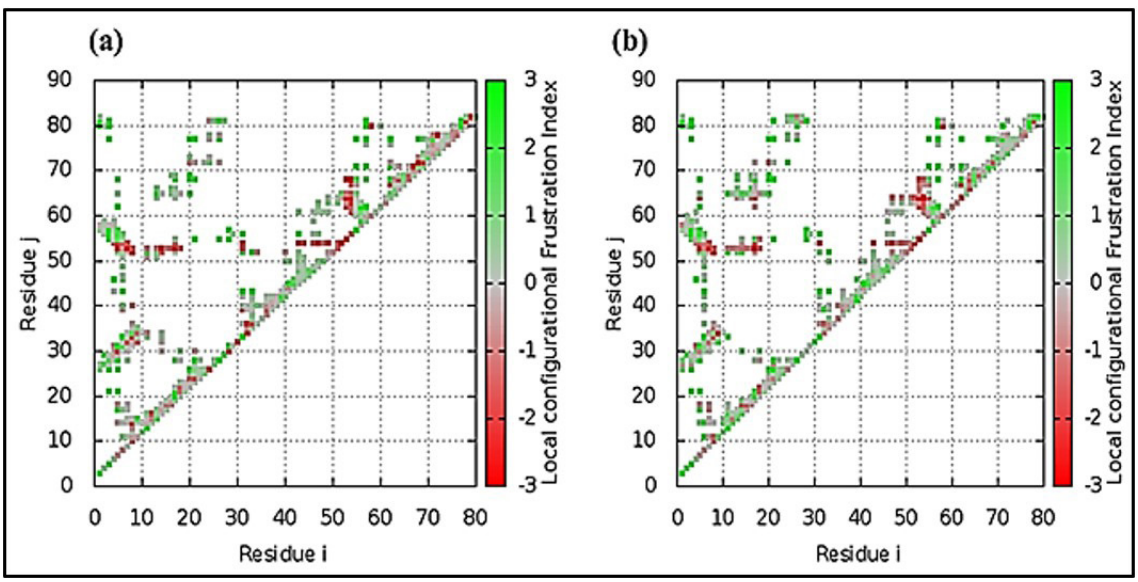


**Figure 7.** Contact maps of frustration in (a) GRX-III & (b) GRX-III-GSH complex. Minimally frustrated and highly frustrated contacts are shown in green and red respectively. The distinct contacts in the oxidized GRX-I compared with reduced GRX-I are circled black.

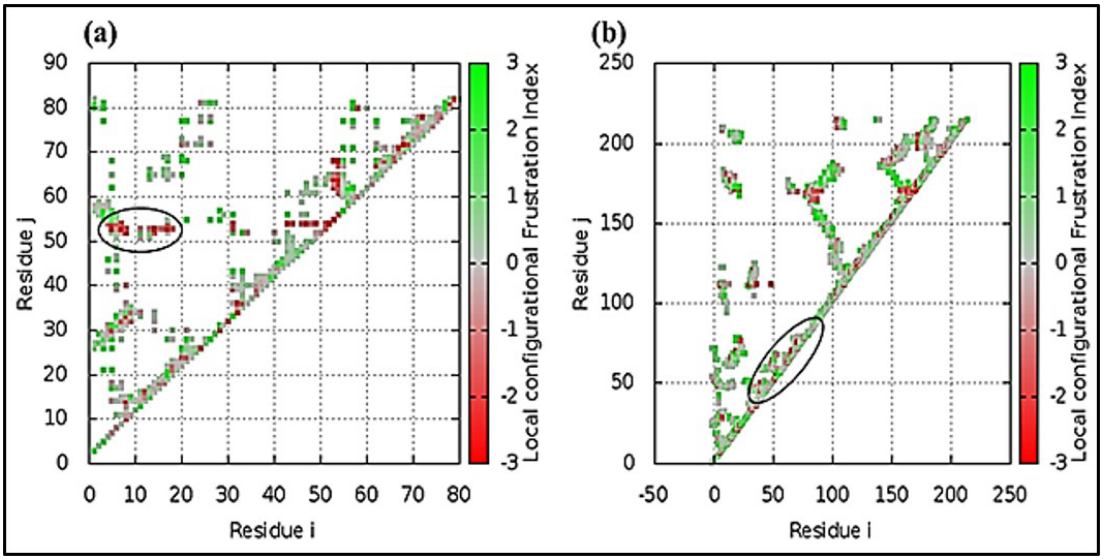


**Figure 8.** Contact maps of (a) GRX-III-GSH complex in and (b) GRX-II-GSH complex. Minimally frustrated and highly frustrated contacts are shown in green and red respectively. The distinct contacts in the oxidized GRX-I are compared with reduced GRX-I are circled black.

Upon visualization of frustration distribution throughout the tertiary structure for GRX-III-GSH, we came to know that from core of protein to the surface of one side of the protein was surrounded by minimally frustrated contacts and similarly half of the core and another side of protein occupied by highly frustrated contacts (Figure 6C).

In contrast, the distribution of frustrated contacts in GRX-II-GSH was crooked where minimally frustrated contacts occupied both core and some surface and on the contrary highly frustrated contacts reside at small part of the core and surface (Figure 6A). The center of origin of minimally frustrated contacts in GRX-III-GSH complex was Val 3 lied in β1-strand. In contrast, origin of highly frustrated contacts centered at Gln54 (β3-strand). It was difficult to find out one center of origin of minimally frustrated contacts in GRX-II-GSH as it was randomly distributed. The origination of highly frustrated contacts was at Thr40 and Arg41 and both are localized in the α3-helix.

The range value for frustration indices in GRX-III-GSH and GRX-II-GSH was -3 to +3. Frustration intensity measurement through chart was done only till the equal number of residues as GRX-II-GSH has much longer residue than the GRX-III-GSH. At residue number 85 in GRX-II-GSH the intensity and peak of highly frustrated contacts were much higher than in GRX-III-GSH. Similarly, at position from 48 to 58, the peak of highly frustrated interaction was higher in GRX-II-GSH.

We checked single residual frustration at amino acid level for GRX-III-GSH and GRX-II-GSH (Table S1). N-terminal and C-terminal of GRX-III-GSH was dominated by both minimally and highly frustrated residues, whereas middle portion of protein was abundant by neutral residues. The distribution of minimally and highly frustrated residues as well as neutral residues was random throughout the protein. α11-helix (192-205) was the only portion where both highly and minimally frustrated residues were absent and only non-frustrated residues (neutral) were present.

*3.5.8. Local frustration along the contact maps of GRX-III-GSH and GRX-II-GSH*

Calculating further for configurationally frustration indices at residue-residue contact level was difficult as the distribution of frustration energy is very different and asymmetric. In GRX-III-GSH, residue no. 53 showed highly frustrated contacts with residues from 5-18 and 42-53. One highly frustrated contact was also visible in the contacts map and that is residue no. 54 which interacts with residues from 60-68 (Figure 8). The distribution of minimally frustrated contacts was random throughout the map. The contacts map of GRX-II-GSH was also calculated using frustratometer server for configurational indices at residue-residue level. The distribution of frustrated contacts was in patches throughout the map and it was very difficult to localize minimally and highly frustration contacts exact position on the map. Although it was visible that majority of the highly frustrated contacts (total 11%) accounted were lying after the residues no. 80.

## 4. Conclusions

In the current study, we sought to compare and contrast the energy landscapes of various GRX isoforms and states. The result of this study suggest that oxidation of GRX-I causes some changes in the frustration pattern (reduces down minimally frustrated contacts by means of 2%, but in contrast slightly increases highly frustrated contacts for about 1%) and distribution inside the GRXs. It causes an additional -helix, increasing the likelihood of highly frustrated interactions. Although the frustration difference in two states of same isoform was not much considerable, the differences between two isoforms were, such as between reduced GRX-I & II. Both minimally and highly frustrated contacts inside of reduced GRX-II was as much as 6% and 2% higher respectively, when compared to the reduced GRX-I.

Frustrated distribution was also random in reduced GRX-II. Comparisons between oxidized forms of GRX-I and GRX-III revealed the overall difference of 8% for minimally frustrated contacts and 1% for highly frustrated interactions.

Further research shows that the binding of GSH reduces the number of minimally frustrated connections by 5% and increases the number of highly frustrated interactions by 3% in oxidized GRX-III (bound state) with GRX-III-GSH complex (unbound state). Moreover, GRX-II-GSH complex have more minimally frustrated contacts (by means of 3%) and less highly frustrated contacts i.e. 1% than GRX-III-GSH complex. Future screening and identification of residue that may be involved in regulating the activation of GRX-I and other GRXs may be aided by the hypothesized role of locally frustrated residue in activation (oxidation) and the energy landscape analysis of GRXs.

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## Supplementary Material

Supplementary material accompanies this paper.

Table S1. Comparison of High frustration index value and low frustration index value of every amino acid sequence (Highly frustration values highlighted in red and low frustration value highlighted in green).

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